

## *Hypothalamic-pituitary-adrenal axis modulation of GABAergic neuroactive steroids influences ethanol sensitivity and drinking behavior*

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**N**euroactive steroids are endogenous neuromodulators that can be synthesized de novo in the brain as well as in the adrenal glands, ovaries, and testes (for review see ref 1). The biosynthetic pathway for these steroids is shown in *Figure 1*. Among these compounds, the metabolites of deoxycorticosterone (DOC) and progesterone,  $3\alpha,21$ -dihydroxy- $5\alpha$ -pregnan-20-one ( $3\alpha,5\alpha$ -THDOC or allotetrahydrodeoxycorticosterone) and  $3\alpha$ -hydroxy- $5\alpha$ -pregnan-20-one ( $3\alpha,5\alpha$ -THP or allopregnanolone) are the most potent positive modulators of  $\gamma$ -aminobutyric acid type A ( $GABA_A$ ) receptors.<sup>2,3</sup> Systemic administration of  $3\alpha,5\alpha$ -THDOC and  $3\alpha,5\alpha$ -THP induces anxiolytic, anticonvulsant, and sedative-hypnotic effects, similar to those induced by other  $GABA_A$

*Activation of the hypothalamic-pituitary-adrenal (HPA) axis leads to elevations in  $\gamma$ -aminobutyric acid (GABA)-ergic neuroactive steroids that enhance GABA neurotransmission and restore homeostasis following stress. This regulation of the HPA axis maintains healthy brain function and protects against neuropsychiatric disease. Ethanol sensitivity is influenced by elevations in neuroactive steroids that enhance the GABAergic effects of ethanol, and may prevent excessive drinking in rodents and humans. Low ethanol sensitivity is associated with greater alcohol consumption and increased risk of alcoholism. Indeed, ethanol-dependent rats show blunted neurosteroid responses to ethanol administration that may contribute to ethanol tolerance and the propensity to drink greater amounts of ethanol. The review presents evidence to support the hypothesis that neurosteroids contribute to ethanol actions and prevent excessive drinking, while the lack of neurosteroid responses to ethanol may underlie innate or chronic tolerance and increased risk of excessive drinking. Neurosteroids may have therapeutic use in alcohol withdrawal or for relapse prevention.*

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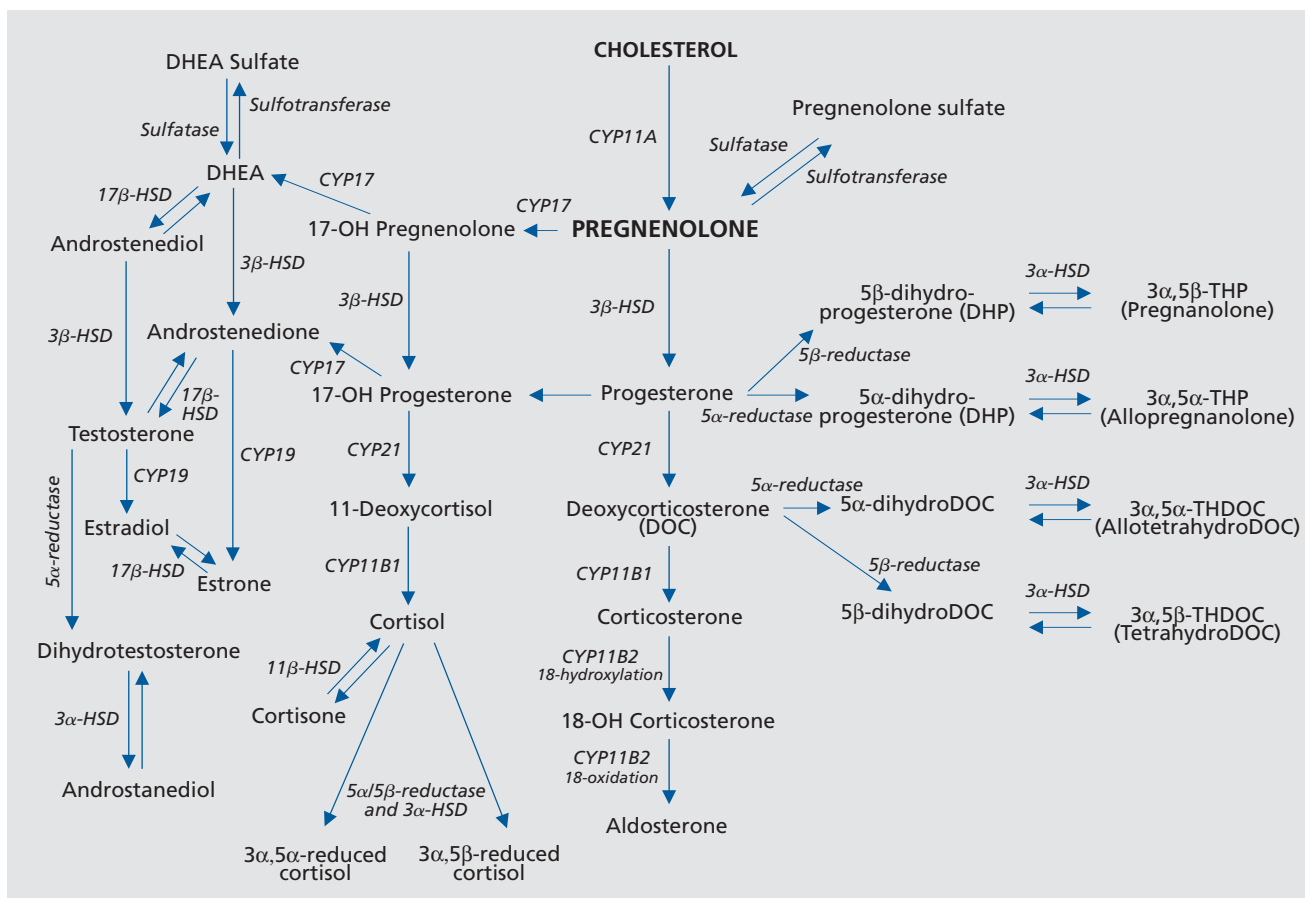
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## Selected abbreviations and acronyms

<b>3<math>\alpha</math>-HSD</b>	<i>3<math>\alpha</math> hydroxysteroid dehydrogenase</i>
<b>3<math>\alpha</math>,5<math>\alpha</math>-THDOC</b>	<i>3<math>\alpha</math>,21-dihydroxy-5<math>\alpha</math>-pregnan-20-one</i>
<b>3<math>\alpha</math>,5<math>\alpha</math>-THP</b>	<i>3<math>\alpha</math>-hydroxy-5<math>\alpha</math>-pregnan-20-one</i>
<b>ACTH</b>	<i>adrenocorticotrophic hormone</i>
<b>CRF</b>	<i>corticotropin-releasing factor</i>
<b>DOC</b>	<i>deoxycorticosterone</i>
<b>GABA</b>	<i><math>\gamma</math>-aminobutyric acid</i>
<b>HPA</b>	<i>hypothalamic-pituitary-adrenal</i>
<b>PMDD</b>	<i>premenstrual dysphoric disorder</i>

receptor positive modulators and ethanol (for review see ref 4). Neuroactive steroids interact with GABA<sub>A</sub> receptors via specific binding sites on  $\alpha$  subunits<sup>5</sup> that allosterically modulate binding to GABA and benzodiazepine recognition sites.<sup>6</sup> In addition, neuroactive steroids compete for [<sup>35</sup>S] t-butylbicyclophosphorothionate (TBPS) binding sites.<sup>6</sup> These steroids alter GABA<sub>A</sub> receptor func-

tion by enhancing GABA-mediated Cl<sup>-</sup> conductance and directly stimulating Cl<sup>-</sup> conductance in voltage clamp studies and [<sup>36</sup>Cl] flux studies.<sup>2,3,7</sup> Neuroactive steroids appear to interact with multiple neurosteroid recognition sites,<sup>8,9</sup> and these sites may differentiate direct gating of Cl<sup>-</sup> vs allosteric modulation of GABA-mediated conductance<sup>9</sup> or represent different properties of recognition sites on distinct GABA<sub>A</sub> receptor subtypes.<sup>10,11</sup> Studies of the structural requirements for neurosteroid activity at GABA<sub>A</sub> receptors include 3 $\alpha$  reduction and 5 $\alpha$ /5 $\beta$  reduction of the A ring, as well as hydroxylation of C21.<sup>12</sup> The 5 $\beta$ -reduced metabolites of DOC and progesterone, 3 $\alpha$ ,5 $\beta$ -THDOC and 3 $\alpha$ ,5 $\beta$ -THP are equipotent modulators of GABAergic transmission.<sup>8,13,14</sup> Humans synthesize these 5 $\beta$ -reduced neuroactive steroids; moreover, the concentrations of 3 $\alpha$ ,5 $\beta$ -THP are physiologically relevant and comparable to those of 3 $\alpha$ ,5 $\alpha$ -THP in human plasma and cerebrospinal fluid.<sup>15,16</sup> In addition, 3 $\alpha$ ,5 $\alpha$ - and 3 $\alpha$ ,5 $\beta$ -reduced



**Figure 1.** Biosynthetic pathway for neuroactive steroids. DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone

cortisol have antagonist properties at both GABA and neurosteroid recognition sites of GABA<sub>A</sub> receptors, and these compounds are the most abundant metabolites of cortisol in human urine.<sup>17</sup> However, to our knowledge, there is no data in the literature on the presence of these metabolites in human brain.

### Stress increases plasma and brain levels of GABAergic neuroactive steroids

The brain and plasma concentrations of GABA agonist-like neuroactive steroids are increased by acute stress and ethanol administration in rodents.<sup>18-21</sup> The increase in 3 $\alpha$ ,5 $\alpha$ -THP reaches pharmacologically significant concentrations in brain between 50 and 100 nM that is sufficient to enhance GABA<sub>A</sub> receptor activity and produce behavioral effects. Similarly, both stress and acute ethanol administration elevate levels of 3 $\alpha$ ,5 $\alpha$ -THP in human plasma,<sup>22-25</sup> although effects of ethanol in humans are controversial.<sup>26,27</sup> In addition, corticotropin-releasing factor (CRF) infusion increases 3 $\alpha$ ,5 $\alpha$ -THP levels in human plasma.<sup>28</sup> The levels detected in human plasma are lower than rodent plasma and brain. However, 3 $\alpha$ ,5 $\alpha$ -THP levels in post-mortem human brain are similar to rat brain and sufficient to have GABAergic activity.<sup>29</sup> Table 1 summarizes the effects of acute stress on neuroactive steroid levels in rodents, monkeys, and humans.

The increase in neuroactive steroid levels elicited by stressful stimuli, including ethanol administration, appears to be mediated by activation of the hypothalamic-pituitary-adrenal (HPA) axis, since it is no longer apparent in adrenalectomized animals.<sup>18,30,31</sup> Adrenalectomized animals

exhibit no circulating concentrations of 3 $\alpha$ ,5 $\alpha$ -THP and 3 $\alpha$ ,5 $\alpha$ -THDOC, but brain levels are still detectable,<sup>18</sup> suggesting that brain synthesis plays an important role in neurosteroid actions. Indeed, brain synthesis of 3 $\alpha$ ,5 $\alpha$ -THP can be increased by ethanol in adrenalectomized immature animals allowed sufficient time for adaptation,<sup>32</sup> suggesting that brain synthesis of neurosteroids may exhibit plasticity in response to physiological challenges.

### Neuroactive steroids and the HPA axis

The activation of the HPA axis in response to acute stress increases the release of CRF from the hypothalamus, which stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary; this, in turn, stimulates the adrenal cortex to release glucocorticoids, neuroactive steroid precursors, and GABAergic neuroactive steroids. Glucocorticoids, mainly cortisol in humans and nonhuman primates, and corticosterone in rodents, provide negative feedback on the hypothalamus and pituitary. Likewise, GABAergic neuroactive steroids inhibit CRF production and release, ACTH release, and subsequent corticosterone levels in rodents.<sup>33-35</sup> The ability of neuroactive steroids to reduce HPA axis activation may play an important role in returning the animal to homeostasis following stressful events. This physiological coping response appears to be critical for mental health, since it is dysregulated in various mood disorders, including depression, post-traumatic stress disorder, and premenstrual dysphoric disorder (PMDD).

Neuroactive steroid concentrations are altered in various pathophysiological conditions that involve dysfunction of

	Pregnenolone	Progesterone	3 $\alpha$ ,5 $\alpha$ -THP	DOC	3 $\alpha$ ,5 $\alpha$ -THDOC
<b>Rats</b>					
Acute ethanol	↑	↑	↑	↑	↑
Acute stress	↑	↑	↑	NA	↑
<b>Monkeys</b>					
Acute ethanol	--	NA	NA	--	NA
Acute stress/HPA axis	↑ / --	-- / ↓	NA	↑	NA
<b>Humans</b>					
Acute ethanol	↑	↑ / ↓	↑ / -- / ↓	NA	NA
Acute stress/HPA axis	↑	↑	↑	↑	NA

**Table 1.** Summary of the changes in neuroactive steroids and their precursors in rats, monkeys, and healthy human subjects induced by acute ethanol administration or by acute stress or HPA stimulation. These effects are described and referenced in the text. ↑ = increase; ↓ = decrease; -- = unchanged; na = not assayed; HPA axis: activation by naloxone, CRF, or ACTH; 3 $\alpha$ ,5 $\alpha$ -THDOC, 3 $\alpha$ ,21-dihydroxy-5 $\alpha$ -pregnan-20-one; 3 $\alpha$ ,5 $\alpha$ -THP, 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one; DOC, deoxycorticosterone; HPA, hypothalamic-pituitary-adrenal; CRF, corticotropin-releasing factor; ACTH, adrenocorticotrophic hormone

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the HPA axis. The HPA axis plays an important role in the pathophysiology of depression: patients with major depression have elevated cortisol levels, a consequence of hypersecretion of CRF due to lowered feedback mechanisms,<sup>36</sup> which also contributes to a blunted dexamethasone response.<sup>37</sup> Some neuroactive steroid concentrations are decreased in patients with major depression as well as in animal models of depression,<sup>15,16,38,39</sup> and administration of antidepressant drugs increases these neuroactive steroids in patients and in rodent brain and plasma.<sup>40-44</sup> This decrease in neuroactive steroids might play a role in the hyperactivity of CRF, since neurosteroids negatively regulate CRF expression and release from the hypothalamus. This increase might be mediated by the HPA axis via an increased serotonin neurotransmission that stimulates the release of CRF (for review see ref 45). While acute fluoxetine administration increases brain levels of  $3\alpha,5\alpha$ -THP, chronic administration of fluoxetine decreases  $3\alpha,5\alpha$ -THP and  $3\alpha,5\alpha$ -THDOC in rat brain and plasma,<sup>43</sup> probably as a consequence of a reduced basal HPA axis activity induced by antidepressant treatments.<sup>36</sup>

Neuroactive steroids are also altered in PMDD, although the literature is controversial, reporting either decrease, no change, or increase in  $3\alpha,5\alpha$ -THP plasma levels.<sup>22,46-53</sup> Differences in analytic methods, diagnostic criteria, or presence of other comorbid psychiatric disorders might account for these discrepancies. Furthermore, PMDD patients had a blunted  $3\alpha,5\alpha$ -THP response to stress<sup>22</sup> and to HPA axis challenges.<sup>53</sup> Women with a history of depression, regardless of PMDD symptoms, also had a blunted  $3\alpha,5\alpha$ -THP response to stress.<sup>54</sup> An altered neuroactive steroid response to stress and acute ethanol administration has been shown in socially isolated animals,<sup>38,55</sup> and this is accompanied by altered HPA axis responsiveness.<sup>56</sup> All this experimental evidence emphasizes the important link between HPA axis function and neuroactive steroid levels in the maintenance of homeostasis and healthy brain function.

## Neuroactive steroids have ethanol-like discriminative stimulus properties in rodents and nonhuman primates

The discriminative stimulus paradigm can be used as an *in vivo* assay of receptor-mediated activity, and may help define the neurotransmitter systems that underlie the behavioral effects of a given dose and class of drug.<sup>57</sup> In addition, drug discrimination can be used as an assay of

subjective effects for cross-species comparisons.<sup>58</sup> The relation between subjective effects of a drug and its reinforcing effects is largely asymmetrical: reinforcing effects are discriminable, but not all discriminable effects are reinforcing.<sup>58</sup> For example, ethanol can make a person feel simultaneously drowsy, euphoric, and calm, but only some of these subjective effects will be associated with increased drinking of ethanol.

Neurosteroids such as  $3\alpha,5\alpha$ -THP,  $3\alpha,5\beta$ -THP,  $3\beta,5\beta$ -THP, and  $3\alpha,5\alpha$ -THDOC have been characterized in drug discrimination procedures as similar to other GABA<sub>A</sub> receptor positive modulators, including benzodiazepines, barbiturates, and ethanol in rats and mice (reviewed in ref 59). Further, neurosteroids that are negative modulators of GABA<sub>A</sub> receptor function, such as pregnenolone sulfate and dehydroepiandrosterone sulfate, do not substitute for the discriminative stimulus effects of ethanol.<sup>60</sup> However, in male rats, the basis for the  $3\alpha,5\beta$ -THP discrimination also appears to be composed of *N*-methyl-D-aspartate (NMDA) receptor antagonism and serotonin-3 (5-HT<sub>3</sub>) receptor agonist activity,<sup>61</sup> an effect not found in mice.<sup>59</sup> These results suggest a species difference in the neurotransmitter systems underlying the  $3\alpha,5\beta$ -THP stimulus cues.

In the macaque monkey,  $3\alpha,5\alpha$ -THP produces a discriminative stimulus effect that is similar to that of ethanol, and sensitivity to these effects is dependent upon the phase of the menstrual cycle, with higher circulating progesterone in the menstrual cycle producing increased sensitivity to ethanol.<sup>62</sup> Furthermore, in male and female monkeys,  $3\alpha,5\alpha$ -THP can produce stimulus effects similar to both a relatively low (1.0 g/kg) and higher (2.0 g/kg) dose of ethanol.<sup>63</sup> The common element in all three species tested (mice, rats, and monkeys) appears to be positive GABA<sub>A</sub> receptor modulation.

The neurosteroid  $3\alpha,5\beta$ -THP substitution for ethanol shows wide individual differences in rats, mice, and monkeys.<sup>59,60,62</sup> This is an unusual finding, because there is extensive training involved in establishing the discrimination, and such overtraining dampens variance across individuals. It has been speculated that the source of such individual variance in sensitivity to neurosteroids is due to the additive effect of experimenter-administered neurosteroids with circulating levels in neurosteroids that differ due to individual variations of HPA axis function.<sup>60</sup> Monkeys also show a wide individual variation in the amount of ethanol they will self-administer, from an average of 1 to 2 drinks/day to an average of over 12

drinks/day. The relationship between sensitivity to ethanol-like effects of neurosteroids and propensity to self-administer ethanol has not been directly tested. However, the suggestion from data showing lower sensitivity to the discriminative stimulus effects of ethanol in the follicular phase of the menstrual cycle (when progesterone and DOC levels are low) and increased alcohol consumption in women during the follicular phase is intriguing.<sup>64</sup> In addition, it has been documented in women who drink heavily and monkeys who self-administer high daily doses of ethanol that their menstrual cycles are disrupted and progesterone levels are very low.<sup>65,66</sup> It will be of interest to first determine sensitivity to the discriminative stimulus effects of ethanol and then allow monkeys to self-administer ethanol to more directly correlate aspects of discriminative stimuli (subjective effects) with risk for heavy drinking.

### Neuroactive steroids mediate specific ethanol actions following acute administration in rodents

Systemic administration of moderate doses (1 to 2.5 g/kg) of ethanol increases both plasma and brain levels of  $3\alpha,5\alpha$ -THP and  $3\alpha,5\alpha$ -THDOC.<sup>19,21,31,67,68</sup> Ethanol-induced elevations in neuroactive steroids reach physiologically relevant concentrations that are capable of enhancing GABAergic transmission. The effect of ethanol on neuroactive steroid levels is dose- and time-dependent, and correlates with the time course of some, but not all, effects of ethanol. For example, the motor incoordinating effects of ethanol appear prior to elevations in neuroactive steroids,<sup>69</sup> whereas the anticonvulsant effects of ethanol appear in congruence with elevations of these steroids.<sup>68</sup> A large body of evidence from multiple laboratories suggests that ethanol-induced elevations of GABAergic neuroactive steroids contribute to many behavioral effects of ethanol in rodents. Neuroactive steroids have been shown to modulate ethanol's anticonvulsant effects,<sup>68</sup> sedation,<sup>30</sup> impairment of spatial memory,<sup>4,70</sup> anxiolytic-like,<sup>71</sup> and antidepressant-like<sup>72</sup> actions. Each of these behavioral responses is prevented by pretreatment with the biosynthesis inhibitor finasteride and/or by prior adrenalectomy. The hypnotic effect of ethanol is partially blocked by adrenalectomy. Importantly, administration of the immediate precursor of  $3\alpha,5\alpha$ -THP restores effects of ethanol in adrenalectomized animals, showing that brain synthesis of neu-

roactive steroids modulates effects of ethanol.<sup>30</sup> However, neuroactive steroids do not appear to influence the motor incoordinating effects of ethanol, since neither finasteride administration or adrenalectomy diminish these actions.<sup>69</sup> Taken together, these studies suggest that elevations in neuroactive steroids influence many of the GABAergic effects of ethanol in vivo and the effects of neuroactive steroids may determine sensitivity to many behavioral effects of ethanol.

### Neuroactive steroid precursors are increased by acute ethanol administration in rodents

While several studies have demonstrated that acute ethanol challenges can result in significant increases in neuroactive steroids in plasma and brain, fewer studies have examined in detail the importance of ethanol's effect on their precursors. As early as the 1940s, it was found that DOC acetate and progesterone induced anesthetic effects in rats<sup>73</sup> and both DOC and progesterone had antiseizure effects,<sup>74</sup> probably due to their  $3\alpha$ -reduced metabolites.<sup>75,76</sup> DOC, the precursor of  $3\alpha,5\alpha$ -THDOC, and progesterone, the precursor of  $3\alpha,5\alpha$ -THP, can readily cross the blood-brain barrier and distribute throughout the brain. These precursors of GABAergic neuroactive steroids are synthesized in the adrenals, beginning with cholesterol's metabolism to pregnenolone (*Figure 1*). While small amounts of these steroids may be formed *de novo* in the brain, ethanol-induced increases in neuroactive steroids are predominantly formed from adrenal precursors.<sup>77</sup> Plasma and brain concentrations of pregnenolone and progesterone are increased more rapidly than  $3\alpha,5\alpha$ -THP after acute ethanol administration.<sup>31,78</sup> Other studies have also shown increases in both plasma and brain DOC after acute ethanol administration. DOC levels were increased in cerebral cortex, cerebellum, hippocampus, hypothalamus, and olfactory bulb and tubercle, ranging from 28-fold increases in the cerebellum to 38-fold increases in the hypothalamus.<sup>79</sup> A significant increase in DOC levels across many brain regions has also been reported by Kraulis et al following intravenous injections of [ $1,2$ - $^3$ H]-DOC.<sup>80</sup> A strong correlation exists between plasma and brain levels of DOC. The temporal and regional associations found in these studies suggest that the steroids originate in the adrenals and are transported to the brain. Upon entering the brain the steroids are metabolized by  $5\alpha$ -reductase and  $3\alpha$ -dehydrogenase enzymes. These enzymes display brain

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region and cell specific expression<sup>81</sup> that may be responsible for the regional distribution of steroid levels following acute ethanol administration. Furthermore, DOC levels measured in the Khisti et al study are comparable to  $3\alpha,5\alpha$ -THDOC levels measured in plasma and brain,<sup>21</sup> suggesting that DOC formed after acute ethanol administration may be largely converted to the GABAergic neurosteroid  $3\alpha,5\alpha$ -THDOC. Studies of ethanol's effects on neurosteroid precursors are important not only to determine the sources and synthesis of potent metabolites, but also to establish their role in physiological functions.

## Effects of neuroactive steroids on drinking behavior in rodents

The GABAergic system is important in regulating ethanol consumption, and neurosteroids can also alter drinking behavior through their actions on GABA<sub>A</sub> receptors.  $3\alpha,5\alpha$ -THP dose-dependently increased ethanol self-administration in nondependent ethanol-preferring P rats, while decreasing ethanol administration in ethanol-dependent P rats.<sup>4</sup> This suggests a complex relationship whereby neurosteroids may promote drinking in nondependent animals consuming small amounts of ethanol, while protecting against excessive drinking in dependent animals. This possibility is supported by data in male C57BL/6J mice where  $3\alpha,5\alpha$ -THP dose-dependently modulated ethanol intake in a 2-hour session, with low doses (3.2 mg/kg) increasing ethanol consumption and high doses (24 mg/kg) decreasing ethanol consumption.<sup>82</sup> In addition, at doses of 10 and 17 mg/kg,  $3\alpha,5\alpha$ -THP has been shown to have rewarding properties in mice.<sup>83</sup> However, other studies in nondependent rats have shown that pretreatment with a 3 mg/kg dose of  $3\alpha,5\alpha$ -THP, but not a 1- or 10-mg/kg dose, increases oral self-administration of ethanol.<sup>84</sup> This result suggests that  $3\alpha,5\alpha$ -THP dose-dependently mediates some of the reinforcing effects of ethanol, and its concentration in brain may have an important influence on drinking behavior. Indeed, Sardinian alcohol-preferring rats have larger  $3\alpha,5\alpha$ -THP and  $3\alpha,5\alpha$ -THDOC elevations after ethanol administration than their non-alcohol-preferring counterparts.<sup>21</sup> Other studies have shown that increased ethanol intake after  $3\alpha,5\alpha$ -THP administration is selective for ethanol-reinforced responding, and cannot be attributed to palatability or increased motor activity during the experimental sessions.<sup>85</sup> Furthermore, the ethanol

enhanced responses following  $3\alpha,5\alpha$ -THP administration produces the opposite effect of other GABA<sub>A</sub> receptor agonists, such as muscimol and barbiturates,<sup>85</sup> suggesting a unique role for GABA<sub>A</sub> receptor neurosteroid binding sites in regulating ethanol consumption. Interestingly, ethanol-dependent rats develop tolerance to ethanol-induced increases in neurosteroid levels,<sup>4,79</sup> which may influence the excessive drinking that is observed in ethanol-dependent rats.<sup>86</sup> Together, these data suggest a strong relationship between neurosteroid levels and ethanol consumption that may involve both genetic and environmental factors.

## Mechanisms of ethanol-induced elevations of neuroactive steroids in plasma and brain

Ethanol-induced elevations in neuroactive steroids appear to involve activation of the HPA axis to increase circulating levels of neuroactive steroids and their precursors, as well as direct effects of ethanol on brain synthesis. Adrenalectomy completely blocks the effects of ethanol on cerebral cortical  $3\alpha,5\alpha$ -THP concentrations; however, the effect of ethanol on cerebral cortical levels of  $3\alpha,5\alpha$ -THP can be restored by administration of its precursor,  $5\alpha$ -dihydroprogesterone ( $5\alpha$ -DHP), to adrenalectomized rats.<sup>30</sup> Since the steroid biosynthetic enzymes are present across brain,<sup>87</sup> it is likely that ethanol-induced increases in brain levels of neuroactive steroids involve brain synthesis that may contribute to effects of ethanol. The first step in steroid synthesis is the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where P450<sub>scc</sub> converts it to pregnenolone. This step is mediated by steroidogenic acute regulatory protein (StAR) and/or the peripheral benzodiazepine receptor. Ethanol rapidly increases the synthesis and translocation of StAR protein from the cytosol to the mitochondria in the adrenal gland.<sup>30</sup> Hence, it is likely that increases in GABAergic neuroactive steroids in adrenals are secondary to ethanol-induced increases in all steroid synthesis initiated by StAR activity.

To determine if ethanol could alter other steroidogenic enzyme activity in rat brain and adrenal minces, Morrow and colleagues investigated the effects of ethanol on  $5\alpha$ -reductase and  $3\alpha$ -hydroxysteroid dehydrogenase ( $3\alpha$ -HSD) enzyme activity (unpublished data). Ethanol (10 to 100 mM) did not alter  $5\alpha$ -reductase activity, measured by the conversion of [<sup>14</sup>C]progesterone to [<sup>14</sup>C] $5\alpha$ -DHP in tis-

sue minces. In contrast, ethanol (30 to 100 mM) increased the conversion of [ $^{14}\text{C}$ ]5 $\alpha$ -DHP to [ $^{14}\text{C}$ ]3 $\alpha$ ,5 $\alpha$ -THP by a maximum of  $30 \pm 3.6\%$  in the olfactory bulb and tubercle, but had no effect in the adrenal gland. Ethanol did not alter nicotinamide adenine dinucleotide phosphate (NADPH) effects on enzyme activity. Fluoxetine was tested as a positive control since previous studies showed that fluoxetine decreased the  $K_m$  of a recombinant 3 $\alpha$ -HSD enzyme.<sup>88</sup> Fluoxetine increased the activity of 3 $\alpha$ -HSD enzyme in the olfactory bulb and tubercle and adrenal gland and this effect was blocked by the 3 $\alpha$ -HSD inhibitor indomethacin. Since the 3 $\alpha$ -HSD enzyme possesses bidirectional activity, the effect of ethanol on the oxidative activity of 3 $\alpha$ -HSD was determined. Ethanol did not alter the conversion of [ $^3\text{H}$ ]3 $\alpha$ ,5 $\alpha$ -THP to [ $^3\text{H}$ ]5 $\alpha$ -DHP in rat olfactory bulb and tubercle or adrenal gland. An increase in the reductive activity of the 3 $\alpha$ -HSD with no change in the oxidative direction would cause a greater conversion of 5 $\alpha$ -DHP to 3 $\alpha$ ,5 $\alpha$ -THP. This effect could contribute to ethanol-induced increases in brain 3 $\alpha$ ,5 $\alpha$ -THP levels. Indeed, the increased reductive activity of 3 $\alpha$ -HSD would be predicted to increase brain levels of both 3 $\alpha$ ,5 $\alpha$ -THP and other 3 $\alpha$ ,5 $\alpha$ -reduced neuroactive steroids such as 3 $\alpha$ ,5 $\alpha$ -THDOC.

### Suppression of neuroactive steroid responses following chronic ethanol exposure in rats

It is well known that chronic stress results in adaptation of the HPA axis, leading to decreased levels of corticosterone in rats.<sup>89</sup> Repeated exposure to alcohol also blunts the response of the HPA axis to a second ethanol challenge.<sup>90</sup> This blunting of the HPA axis is associated with reduction in CRF and ACTH elevations following ethanol challenge.<sup>91</sup> In line with these observations, chronic ethanol consumption in rats results in blunted elevation of cerebral cortical 3 $\alpha$ ,5 $\alpha$ -THP<sup>4</sup> and plasma and brain DOC levels following acute ethanol challenge,<sup>79</sup> compared with pair-fed control rats. These findings suggest that there is tolerance to ethanol-induced increases in neuroactive steroid levels. Since decreases in brain neurosteroid levels were concomitant with decreases in plasma neurosteroid levels, it is likely that the observed decreases in 3 $\alpha$ ,5 $\alpha$ -THP and DOC levels were dependent on blunted HPA axis activity. Thus, adaptations of the HPA axis may contribute to tolerance to effects of ethanol that are mediated by the GABAergic neuroactive steroids.

Chronic ethanol administration to rodents and humans produces tolerance to ethanol and cross-tolerance to benzodiazepines and barbiturates. In contrast, ethanol-dependent rats are sensitized to the anticonvulsant effects of both 3 $\alpha$ ,5 $\alpha$ -THP and 3 $\alpha$ ,5 $\alpha$ -THDOC.<sup>92-94</sup> These studies also show that GABA<sub>A</sub> receptor sensitivity to 3 $\alpha$ ,5 $\alpha$ -THP and 3 $\alpha$ ,5 $\alpha$ -THDOC is enhanced in ethanol-dependent rats, likely due to the reduction of ethanol-induced levels in these animals described above. Since ethanol-dependent rats are sensitized to anticonvulsant actions of neuroactive steroids, this class of compounds may be therapeutic during ethanol withdrawal. Indeed, neurosteroid therapy may have advantages over benzodiazepine therapy since benzodiazepines exhibit cross-tolerance with ethanol. Further studies are needed to explore this possibility.

### Effects of ethanol on neuroactive steroids in humans

The potential role of neuroactive steroids in alcohol action in humans is relatively unexplored and inconsistent. Recent human studies show that male and female adolescents seen in the emergency room for alcohol intoxication had elevated plasma levels of the neuroactive steroid 3 $\alpha$ ,5 $\alpha$ -THP.<sup>24,25</sup> Furthermore, various subjective effects of ethanol during the rising phase of the blood alcohol curve are diminished by prior administration of the neurosteroid biosynthesis inhibitor finasteride.<sup>95</sup> Finasteride reduces the formation of both 3 $\alpha$ ,5 $\alpha$ -THP and 3 $\alpha$ ,5 $\alpha$ -THDOC by inhibiting the reduction of progesterone and DOC to intermediate precursors. Indeed, finasteride pretreatment blocked subjective effects of alcohol using three different scales to measure the activating, sedating, anesthetic, and peripheral dynamic aspects of alcohol actions. The ability of finasteride to reduce the subjective effects of alcohol was not observed in individuals carrying the GABA<sub>A</sub>  $\alpha 2$  subunit polymorphism associated with alcoholism, suggesting that individuals carrying this polymorphism have reduced sensitivity to both alcohol and finasteride.<sup>95</sup> Other studies show that 3 $\alpha$ ,5 $\alpha$ -THP levels are decreased during the peak of alcohol withdrawal and return to normal levels upon recovery.<sup>96,97</sup> Likewise, abstinent alcoholics exhibit diminished progesterone levels as well as a lowered ratio of progesterone to pregnenolone.<sup>98</sup> In contrast, laboratory administration of low or moderate doses of ethanol appears to have no effect on plasma 3 $\alpha$ ,5 $\alpha$ -THP levels<sup>26</sup>

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or to decrease  $3\alpha,5\alpha$ -THP levels.<sup>27,99</sup> The basis of these conflicting results is unknown, but may involve pharmacologically different ethanol doses, different analytic methods to measure neurosteroids, or environmental factors that influence neurosteroid synthesis in humans. Alternatively, different neuroactive steroids may be elevated in humans vs rodents, or the effects of ethanol on neuroactive steroid levels in humans may be restricted to brain. *Table 1* summarizes the different effects of ethanol on neuroactive steroid levels in rodents, monkeys, and humans.

Humans, but not rodents, synthesize multiple  $5\beta$ -reduced neuroactive steroids including  $3\alpha,5\beta$ -THP and  $3\alpha,5\beta$ -THDOC.  $3\alpha,5\beta$ -THP levels are comparable to those of  $3\alpha,5\alpha$ -THP in human plasma and cerebrospinal fluid.<sup>15,16</sup> These neuroactive steroids also modulate GABAergic transmission,<sup>8,13,14</sup> but have not been measured in humans after ethanol administration. Additionally, the primary stress steroids in humans are cortisol and 11-deoxycortisol, while progesterone and corticosterone are the primary stress steroids in rodents.  $3\alpha,5\beta$ -reduced cortisol is a negative modulator of GABA<sub>A</sub> receptors,<sup>17</sup> and could contribute to the subjective effects of ethanol in humans. Thus, the combined effects of  $3\alpha,5\alpha$ - and  $3\alpha,5\beta$ -reduced neuroactive steroids may contribute to the effects of ethanol in humans and nonhuman primates. These steroids have never been measured following ethanol, stress, or HPA axis activation in humans or nonhuman primates.

Comprehensive studies of neuroactive steroid levels in humans are needed. While  $3\alpha,5\alpha$ -THP and  $3\alpha,5\alpha$ -THDOC are the primary neuroactive steroids in rodents, other neuroactive steroids may be more relevant in humans. For example, plasma progesterone of adrenal origin is present at much higher levels in rodents than humans, suggesting an explanation for higher levels of plasma  $3\alpha,5\alpha$ -THP in rodents vs humans. Other GABAergic  $3\alpha,5\alpha$ - and  $3\alpha,5\beta$ -reduced neuroactive steroids, derived from DOC, dehydroepiandrosterone (DHEA), and testosterone, are known GABAergic modulators<sup>100-102</sup> that may be elevated by HPA axis activation in humans. Unfortunately, simple inexpensive analytic methods to measure these steroids are not available. The ability of finasteride to block the subjective effects of ethanol in humans may be due to its ability to prevent the formation of any or all of these neuroactive steroids. Indeed, the combined effects of all steroids regulated by acute or chronic ethanol exposure may contribute to its actions in all species.

## Effects of ethanol on neuroactive steroid precursors in nonhuman primates and humans

We have recently shown that acute ethanol challenges in cynomolgus monkeys do not change plasma pregnenolone and DOC levels. Two doses of ethanol, 1.0 and 1.5 g/kg, were tested via intragastric administration, and neither was able to increase neuroactive steroid precursors or circulating cortisol levels despite an average blood ethanol level of 147 mg/dL.<sup>103,104</sup> In contrast, acute ethanol administration increases pregnenolone, progesterone, DOC, and their neuroactive metabolites in rat brain and plasma,<sup>4,31,79,105</sup> and this increase is also prevented by adrenalectomy/orchiectomy, consistent with ethanol activation of the HPA axis.<sup>31,105</sup> These results suggest that higher doses of ethanol might be necessary to stimulate the HPA axis and thus increase pregnenolone and DOC levels in nonhuman primates. Indeed, Williams and collaborators<sup>106</sup> have shown that intravenous administration of ethanol up to 1.9 g/kg failed to increase plasma ACTH levels in rhesus monkeys. Other studies using 2.0 g/kg ethanol have reported increased cortisol levels in monkeys under conditions where monkeys were restrained on a flat surface while receiving ethanol, which may contribute to HPA axis activation.<sup>107</sup> The possibility that pregnenolone, DOC, and their neuroactive metabolites might be differentially regulated in nonhuman primates compared with rodents cannot be ruled out; future studies will be necessary to further address this question.

The effects of ethanol on neuroactive steroid precursors in humans are inconsistent to date. Laboratory administration of moderate doses of ethanol (0.7 to 0.8 g/kg) has recently been reported to increase pregnenolone and DHEA levels and to decrease progesterone levels in healthy human subjects.<sup>27</sup> In contrast, Holdstock et al<sup>26</sup> reported that ethanol administration to healthy volunteers increased progesterone levels in women during the luteal phase, but had no effect during the follicular phase or in men. Low alcohol consumption in premenopausal women was associated with increased estradiol, androstenedione, and testosterone levels throughout the menstrual cycle, while progesterone levels were increased only in the luteal phase.<sup>108</sup> Moreover, abstinent alcoholic women had diminished progesterone levels and a lower progesterone to pregnenolone ratio during the luteal phase.<sup>98</sup> In contrast, others reported that chronic male alcoholics had higher basal progesterone compared with healthy controls.<sup>109</sup>



These variable data suggest that genetic and/or environmental factors may influence effects of ethanol on steroid precursors.

### HPA axis modulation in alcohol-dependent humans

Among the neuropsychiatric disorders that show alterations in HPA axis responsiveness is alcoholism. ACTH and cortisol secretion is increased during ethanol intoxication and acute alcohol withdrawal.<sup>110-117</sup> In contrast, an attenuated responsiveness of the HPA axis has been found in both drinking and abstinent alcohol-dependent patients. Alcohol-dependent patients have low cortisol and 11-deoxycortisol basal levels, show a greater suppression in cortisol and ACTH concentrations following dexamethasone test, and have a reduced cortisol response to exogenous ACTH administered after dexamethasone.<sup>118</sup> Moreover, they have attenuated ACTH and cortisol responses after pituitary stimulation by ovine or human CRF<sup>119-122</sup> and an altered ACTH response to naloxone.<sup>123</sup> An altered cortisol and ACTH response to ovine CRF and naloxone have also been found in sons of alcoholics.<sup>124-126</sup> These data are consistent with the idea that HPA axis dysregulation may contribute to altered neurosteroid responses in human alcoholism, though studies showing this consequence of alcoholism are not available to date.

### HPA axis modulation of DOC and pregnenolone in cynomolgus monkeys

While stimulation of the HPA axis by acute stress or ethanol administration plays a pivotal role in increasing GABAergic neuroactive steroids and their precursors in rodent brain and plasma, few data are available for non-human primates. We have recently demonstrated that plasma DOC and pregnenolone levels in ethanol-naïve cynomolgus monkeys are differentially regulated by various challenges to the HPA axis.<sup>103,104</sup> Plasma DOC levels are sensitive to hypothalamic and pituitary activation of the axis and to negative feedback mechanisms assessed by the dexamethasone test. Thus, administration of naloxone at the doses of 125 and 375 µg/kg increased plasma DOC levels up to 86% and 97%, respectively. This is consistent with data showing an activation of the HPA axis and increased cortisol and ACTH levels in humans and non-human primates.<sup>125,127,128</sup> CRF (1 µg/kg) increased plasma DOC levels up to 111%, and this increase was positively

correlated with the increase in cortisol levels in the same subject, dexamethasone (130 µg/kg) decreased DOC levels by 42%, in agreement with a suppression of HPA axis activity. In contrast, administration of ACTH (10 ng/kg) 4-6 hours after 0.5 mg/kg dexamethasone had no effect on plasma DOC levels, suggesting that DOC synthesis is independent of ACTH stimulation of the adrenals. Furthermore, changes in DOC levels were correlated with changes in cortisol levels only for some of these challenges, suggesting that other neuroendocrine factors could regulate DOC synthesis in nonhuman primates.<sup>103</sup>

Pregnenolone levels in the same cynomolgus monkey subjects were differentially regulated from DOC. Naloxone administration (125 and 375 µg/kg) increased plasma pregnenolone up to 222 and 216%, respectively. In contrast, CRF (1 µg/kg) and dexamethasone (130 µg/kg) had no effect on pregnenolone levels, while ACTH (10 ng/kg), 4 to 6 hours after 0.5 mg/kg dexamethasone, decreased plasma pregnenolone levels by 43%. CRF and ACTH administration decreased the ratio of plasma pregnenolone:DOC, suggesting increased metabolism of pregnenolone into DOC or other steroids.<sup>104</sup> Thus, circulating pregnenolone levels are subject to complex regulation involving factors other than direct HPA axis modulation. Naloxone could increase pregnenolone levels through mechanisms independent of HPA axis activation, given that exogenous CRF and ACTH had no effect on pregnenolone levels. Opioid receptors are present in peripheral tissue including the adrenals,<sup>129</sup> and a direct action of naloxone on these receptors cannot be ruled out. Opioidergic neurons regulate gonadotropin-releasing hormone (GnRH) secretion,<sup>130</sup> and it is possible that the increase in plasma pregnenolone levels induced by naloxone is due to increased gonadal steroidogenesis via opioid inhibition of GnRH. Furthermore, naloxone could have a direct action on the enzymes involved in steroid biosynthesis. Further studies are needed to investigate these possibilities.

### Are neuroactive steroid responses to HPA axis stimulation linked to alcohol drinking?

Neuroactive steroid responses to HPA axis challenges in ethanol-naïve animals may predict future alcohol consumption. Studies have so far focused on nonhuman primates. Dexamethasone suppresses DOC levels in monkey plasma and the degree of dexamethasone suppression measured in ethanol-naïve monkeys was predictive of sub-

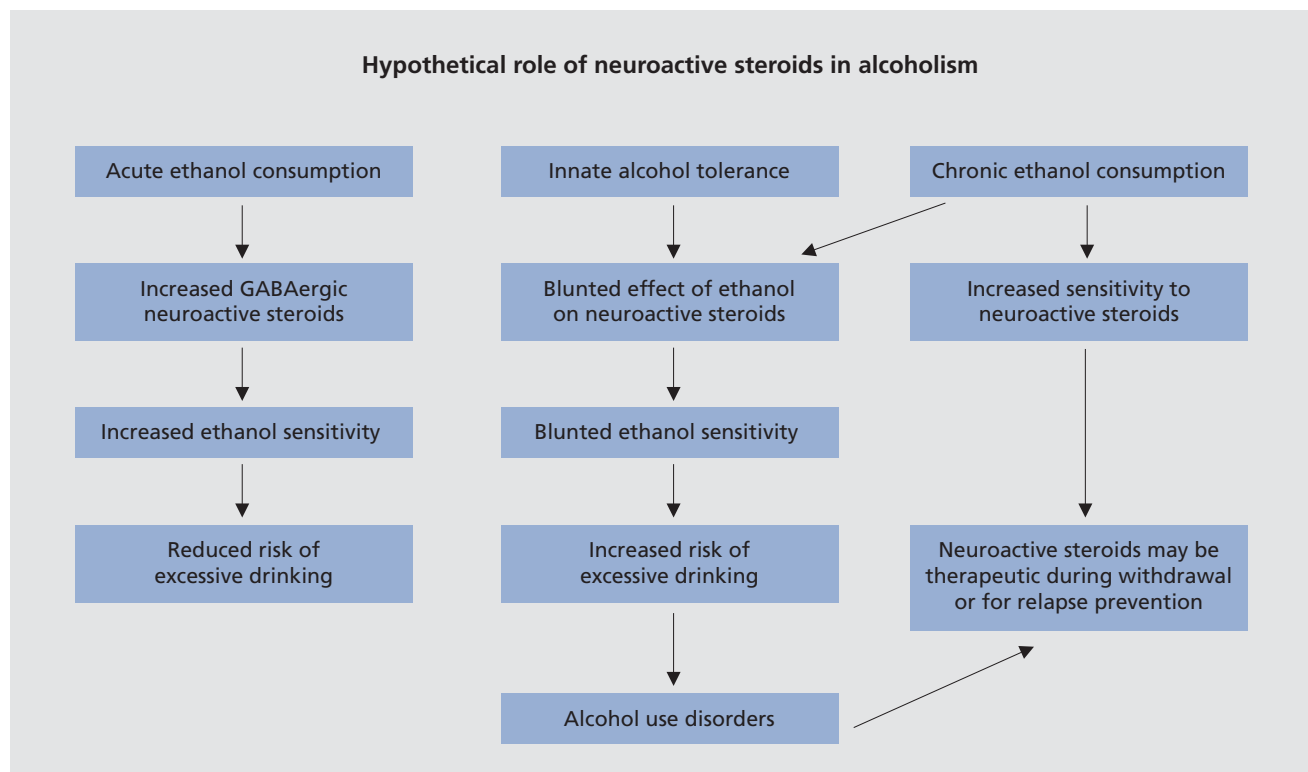
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sequent alcohol drinking in these monkeys. That is, the highest alcohol drinking was found in the monkeys that showed the lowest suppression of DOC levels in response to dexamethasone.<sup>103</sup> In this study, the monkeys with the lowest response to dexamethasone also developed a pattern of chronic binge drinking, drinking the equivalent of 16 or more drinks in 22 h in approximately 20% of their drinking sessions (Grant et al, submitted). This binge drinking pattern of high quantity of alcohol intake in short time periods persisted throughout 1 year of ethanol self-administration (Grant et al, unpublished). In contrast, no other DOC responses to HPA axis stimulation in ethanol-naïve monkeys were predictive of subsequent voluntary drinking or binge drinking. The effect of dexamethasone on plasma DOC levels in monkeys appears to be a trait marker of risk for high alcohol consumption. This trait marker also correlated with alcohol intakes in a small group (n=4) of rhesus monkeys (unpublished data collected in collaboration with David P. Friedman at Wake Forest University). These findings need to be replicated in other primate studies of ethanol self-administration,

including cohorts of humans that have not yet started drinking. This adaptation in precursor responses suggests there will also be adaptations in GABAergic neuroactive steroids derived from DOC.

## Potential role of neuroactive steroids in ethanol sensitivity and risk for alcoholism: a hypothesis

While the physiological significance is unknown, dysregulation of the HPA axis is associated with ethanol dependence in humans.<sup>118,122</sup> HPA axis suppression in alcohol dependence results in diminished elevations of GABAergic neuroactive steroids as described above. Diminished elevations of GABAergic neuroactive steroids following ethanol exposure would result in reduced sensitivity to the anxiolytic, sedative, anticonvulsant, cognition-impairing, and discriminative stimulus properties of ethanol. Reduced sensitivity to ethanol is associated with greater risk for the development of alcoholism in individuals with alcoholism in their family.<sup>131,132</sup>



**Figure 2.** Schematic representation of the hypothetical role of neuroactive steroids in ethanol sensitivity and risk for alcoholism. GABA,  $\gamma$ -aminobutyric acid

Moreover, individuals with the GABA<sub>A</sub> receptor  $\alpha 2$  subunit polymorphism that is associated with alcohol dependence exhibit substantially reduced sensitivity to the subjective effects of ethanol compared with individuals that lack this polymorphism.<sup>95</sup> Likewise, rats and mice with low sensitivity to various behavioral effects of alcohol tend to self-administer greater amounts of ethanol in laboratory settings. The BXD recombinant inbred strains of mice, PKC $\gamma$  and PKC $\epsilon$  knockout mice, alcohol-preferring P rats, and high-alcohol-drinking (HAD) rats are but a few examples. Taken together, these observations suggest that ethanol-induced elevations of GABAergic neuroactive steroids in brain may underlie important aspects of ethanol sensitivity that may serve to prevent excessive alcohol consumption (Figure 2). The loss of these responses may promote excessive alcohol consumption to achieve the desired effects of ethanol. A deficiency in neurosteroid responses to ethanol intake could result from suppression of the HPA axis or other genetic/environmental factors that inhibit neurosteroid synthesis in brain. Hence, the lack of neurosteroid elevations in response to ethanol could underlie innate ethanol tolerance or ethanol tolerance induced by long-term ethanol use. Indeed, the observation that finasteride did not alter the subjective effects of ethanol in subjects with the GABA<sub>A</sub> receptor  $\alpha 2$  subunit polymorphism associated with alcohol dependence<sup>95</sup> is consistent with the idea that neurosteroid responses contribute to ethanol sensitivity and risk for alcoholism. Both forms of tolerance may promote excessive alcohol consumption. Excessive alcohol consumption, particularly binge drinking, is a significant risk factor for all alcohol use disorders, including alcohol dependence and alcoholism. The restoration of ethanol sensitivity in ethanol-dependent patients may therefore have thera-

peutic utility. However, it is unclear at this time whether neuroactive steroid supplementation would reduce excessive alcohol consumption in humans. Indeed, as mentioned above, low doses of neuroactive steroids increased operant ethanol self-administration under some conditions,<sup>84</sup> while neuroactive steroids reduce ethanol consumption at high doses<sup>82</sup> or in ethanol-dependent rats.<sup>4</sup> The relationship between HPA axis response, GABAergic neuroactive steroids, and alcohol drinking deserves further studies in nonhuman primates and humans.

## Summary and conclusions

The effects of acute ethanol administration on neuroactive steroid levels found in rodents have not been found in monkeys or humans. Does this mean that neuroactive steroids do not have an important role in ethanol action in these species? We doubt this conclusion, since monkeys exhibit discriminative stimulus properties of ethanol and neuroactive steroids that are indistinguishable.<sup>62</sup> Furthermore, the steroid biosynthesis inhibitor finasteride blocks the subjective effects of ethanol in humans.<sup>95</sup> Primates may synthesize different GABAergic neuroactive steroids in response to ethanol challenge. These steroids may include  $3\alpha,5\alpha$ - and  $3\alpha,5\beta$ -reduced derivatives of progesterone, DOC, and testosterone, all of which have potent GABAergic activity. Further studies are needed to translate a large body of rodent research on GABAergic neuroactive steroids to better understand the role of endocrine factors in alcohol sensitivity and risk for alcoholism. □

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## **La modulación de los esteroides neuroactivos por el eje hipotálamo-hipofisis-suprarrenal, influye en la sensibilidad al etanol y en la conducta frente a la bebida**

La activación del eje hipotálamo-hipofisis-suprarrenal (HHS) determina una elevación de los esteroides neuroactivos GABA (ácido  $\gamma$ -aminobutírico)-érgicos que refuerzan la neurotransmisión de GABA y restablecen la homeostasia después del estrés. Esta regulación del eje HHS mantiene sana la función cerebral y la protege frente a las enfermedades neuropsiquiátricas. La sensibilidad al etanol depende de las elevaciones de esteroides neuroactivos que potencian los efectos GABAérgicos del etanol y pueden impedir el consumo excesivo de alcohol por los roedores y seres humanos. La sensibilidad baja al alcohol se asocia a un mayor consumo de éste, con el riesgo consiguiente de etilismo. De hecho, las ratas dependientes del etanol muestran una respuesta neuroesteroidea a la administración de etanol muy reducida, lo que puede contribuir a la tolerancia etanólica y a la propensión a beber mayores cantidades de alcohol. En esta revisión se ofrecen pruebas que respaldan la hipótesis de que los neuroesteroides contribuyen a las acciones del etanol e impiden un consumo excesivo, mientras que la falta de respuesta neuroesteroidea al etanol podría explicar la tolerancia innata o crónica y el mayor riesgo de excesos en la bebida. Los neuroesteroides podrían tener una utilidad terapéutica en la abstinencia del alcohol o en la evitación de las recaídas.

## **Le comportement alcoolique la sensibilité à l'éthanol dépendent de la modulation des stéroïdes neuroactifs GABAergiques au niveau de l'axe hypothalamo-hypophyso-surrénalien**

L'activation de l'axe hypothalamo-hypophyso-surrénalien (HHS) entraîne une élévation de la sécrétion des stéroïdes neuroactifs GABA-ergiques (acide  $\gamma$ -aminobutyrique) qui stimulent la neurotransmission GABA et restaurent l'homéostasie après le stress. Cette régulation de l'axe HHS maintient une fonction cérébrale saine et protège des maladies neuropsychiatriques. Les élévations des stéroïdes neuroactifs influent sur la sensibilité à l'éthanol en augmentant ses effets GABAergiques et peuvent ainsi prévenir les consommations alcooliques excessives chez les rongeurs et chez l'homme. Une faible sensibilité à l'éthanol est associée à une plus grande consommation d'alcool et à un risque d'alcoolisme plus important. Les réponses neurostéroïdes à l'administration d'éthanol chez des rats rendus alcoolodépendants sont donc diminuées, ce qui peut contribuer à une tolérance à l'éthanol et à une propension à en boire de plus grandes quantités. Cette revue de la littérature fournit des arguments en faveur de l'hypothèse d'une contribution des neurostéroïdes aux effets de l'éthanol et à la prévention de sa consommation excessive alors qu'un déficit des réponses neurostéroïdes peut être à la base d'une tolérance innée ou invétérée chronique et d'un risque augmenté de consommation excessive d'alcool. Les neurostéroïdes peuvent avoir une utilité thérapeutique dans le sevrage alcoolique ou dans la prévention d'une rechute.

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