Alcohol Dose-Dependently Enhances 3α-Androstanediol Formation in Frontal Cortex of Male Rats Concomitant with Aggression

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Abstract: Alcohol (EtOH) can enhance aggression in people and animal models. These effects are more salient in males than in females. Androgens, such as testosterone (T), may promote aggressive responding to EtOH. Effects of EtOH on androgens and aggression were examined in a resident-intruder paradigm. Gonadally-intact, or castrated (GDX), male rats were socially-isolated for 3 weeks and administered an acute dose of EtOH (2.0 g/kg) or saline (Experiment 1) or administered a dose-response regimen of EtOH (0.0, 0.5, 1.0, and 2.0 g/kg; Experiment 2). Cortical tissues were assessed for androgen concentrations and/or muscimol binding after receiving saline or EtOH. Compared to saline, acute EtOH administration in Experiment 1, enhanced aggression among intact rats and concomitantly enhanced formation of the T metabolite and neurosteroid, 3α-androstanediol, (3α-diol) in frontal cortex. T was also enhanced in cortex and circulation of intact but not GDX rats. Repeat testing in Experiment 2 revealed dose-dependent effects of EtOH to enhance aggression and cortical 3α-diol among both intact and GDX rats. EC_{50} for muscimol binding was lower for intact and EtOH-administered rats than for GDX or vehicle-administered rats, indicating that less GABA was needed to achieve half-maximal binding. Together, these data suggest that 3α-diol enhancement may partly underlie EtOH's effects on aggression, possibly *via* actions at $GABA_A$ receptors.

Keywords: 5α-reductase, aromatase, biosynthesis, ethanol, estrogen, $GABA_A$, neurosteroid,

INTRODUCTION

Investigations of social behavior suggest that androgens influence the propensity to engage in aggression. In support, males typically have greater endogenous androgen concentrations than do females and are typically more aggressive [1, 2]. Among adults, endogenous T levels are positively correlated with self-reported aggression among healthy men [3-5], verbal aggression and physical violence in spouse-abusing men [6, 7], and among prisoners, which demonstrate atypical violence [8-10]. As well, T levels also positively correlate with self-reported aggression among healthy women [11] and violent female prison inmates [12]. In double-blind studies, administration of T cyprionate or methyl-T can increase aggression in people [13, 14]. Indeed, studies utilizing animal models, including rats and monkeys, have recapitulated these effects [15, 16]. Together, these findings support the notion that T may have effects to enhance aggression.

Some of T's effects on aggression may be due in part to actions of its metabolites. T is metabolized by actions of the 5α-reductase enzyme to dihydrotestosterone (DHT). Among men, DHT levels positively correlate with spontaneous aggression and dominance [3], physical violence in Australian Bushmen [17], and delinquent and aggressive behavior of male adolescents [18]. Similarly, among women, DHT levels correlate significantly with feelings of anger [11]. DHT is metabolized by the 3α-hydroxysteroid dehydrogenase enzyme to form 3α-androstanediol (3α-diol), which may also influence aggression. In support, 3α-diol or T administration similarly increase the number of aggressive acts made by gonadectomized (GDX) C21 mice over that of vehicle-administered controls [19]. Moreover, in 5α-reductase knockout mice, that lack the ability to convert T to DHT or 3α-diol, T-enhanced aggression is significantly-reduced compared to that of wildtype mice that can readily metabolize T, and is not different from vehicle administration to wildtype mice [19]. These data suggest that some of T's effects to promote aggression may be due to actions of its 5α-reduced metabolites. However, T can also be converted to estradiol (E_2) by the aromatase enzyme. In contrast to T, human studies on E_2 and aggression have been sparse [20, 21]. However, in some animal species, estrogens have been found to enhance aggression [22-24]. Mice, that have transforming growth factor α knocked out, have high serum E_2 levels and demonstrate more aggression, which can be maintained by ethanol (EtOH) administration [25]. E_2 administration to mice can also increase aggression [25-30]. Thus, some of the variability in reports of T's effects on aggression may be related differences in capacity to metabolize T, and/or the ratio of 5α-reduced and aromatized metabolites.

Some evidence suggests that EtOH may also increase aggression. EtOH is associated with violent acts [22, 31, 32] and the majority of violent crimes are reported to involve EtOH use [31, 32]. EtOH use often precedes, precipitates, and/or accompanies domestic violence [33-37]. However, many individuals consume EtOH without untoward effects [35]. In support, meta analyses find that EtOH has a moderate effect on aggression (d = .49-.61) overall [33, 38, 39].

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Individuals who are predisposed to acting aggressively may be most vulnerable to the aggression-enhancing effects of EtOH [40, 41]. For example, aggressive play behavior is common among adolescent rats; however, perinatal exposure to EtOH increases aggressive play [42]. As such, determining the predisposing factors associated with EtOH-enhanced aggression is important.

Androgen milieu may be a factor influencing vulnerability to aggression-enhancing effects of EtOH. In humans, EtOH-induced aggression is more prevalent among men than women [43]. Men are more likely to display aggression when intoxicated [44, 45] and may also respond more aggressively to provocation when drinking than do women [46]. Likewise, aggressive personality traits in men can predict aggressive responding after EtOH ingestion [44, 47, 48]. Among rodents, GDX mice implanted with larger silastic T pellets demonstrated greater aggression when administered EtOH than did GDX conspecifics implanted with smaller T pellets [49]. Mice that had larger T implants exhibited a higher frequency of attacks at lower concentrations of EtOH (0.1-1.7 mg/kg) than did controls or those with smaller T implants (which did not differ). Likewise, aggression-enhancing effects of EtOH administration to squirrel monkeys have been observed during the mating season, when endogenous levels of T are high, but not during the non-mating season when T levels are low [50]. Furthermore, there is evidence to suggest that androgen milieu may enhance EtOH consumption, and thereby alter aggression. Among aggressive adolescent male hamsters, EtOH drinking is increased around post-natal day 35, when androgen secretion typically commences [51]. Testosterone administration can also stimulate the development of EtOH preference among GDX rats [52]. Thus, androgens may amplify effects of EtOH to promote aggression.

While both acute and chronic, heavy EtOH intake can suppress circulating T levels of men [53-57], and this has likewise been observed in some rodent models as well [58-61], EtOH has been demonstrated to enhance formation of pregnane neurosteroids [62] in brain independent of peripheral sources, yet androstane neurosteroids have been understudied in this phenomenon. The androstane neurosteroid, 3α-diol, which we have observed is necessary for normative aggression in some mice [19], can be formed *de novo* in brain and binds preferentially to $GABA_A$ receptors [63, 64]. GABAergic activation is associated with aggression in animal models [65] and may act as a mechanism by which 3α-diol alters aggression. The influence this androstane mechanism may have on EtOH-mediated social behavior and the impact that social responding may have on endocrine response to EtOH are not well-understood. As such, we conducted three experiments to assess the androgen response to EtOH in brain and plasma, the influence of androgens on EtOH-mediated social behavior, and to assess GABAergic activation as a target. First we administered an acute dose of saline vehicle or EtOH (2.0 g/kg) to gonadally-intact or GDX Long-Evans rats that did, or did not, undergo behavioral testing in a resident-intruder paradigm. Second, we assessed effects of a dose-response regimen of EtOH (0.0, 0.5, 1.0, 2.0 g/kg) on central and peripheral androgens as well as resident-intruder behavior of intact or GDX rats. Lastly, we assessed muscimol binding in cortical tissues of some intact or GDX rats administered saline or EtOH (2.0 g/kg). We anticipated that EtOH would enhance T and/or its 5α-reduced metabolites concomitant with aggression and that these effects may be due, in part, to actions of androgens synthesized *de novo* in brain.

MATERIALS AND METHODOLOGY

These methods were pre-approved by the Institutional Animal Care and Use Committee at the University at Albany - SUNY.

Subjects and Housing

Subjects were male, Long-Evans rats (n = 80) between 60 and 70 days of age that were bred in our colony at The University at Albany - SUNY (original stock from Taconic Farms). Subject age was not significantly correlated with any behavioral or neuroendocrine endpoint examined in the present investigation. All rats were experimentally- and sexually-naïve. Rats were single-housed for 21 days prior to the start of testing and had *ad libitum* access to food and water on a reversed 12-hour light cycle (lights off at 0800 h).

Surgery

At approximately 42 days of age, rats were anesthetized with a Rompun (12 mg/kg) and Ketaset (80 mg/kg) cocktail and received either GDX (n = 40) or sham GDX (n = 40) surgery. One GDX rat did not recover from surgery yielding 39 GDX rats.

Resident Intruder Paradigm

Social responding was assessed using a resident-intruder test. This test consisted of introducing a smaller, gonadally-intact conspecific (intruder) into the homecage of a residing experimental animal (resident). Resident rats had been socially-isolated for 21 days to promote territorial aggression (which is typically minimal among rats [66]) and as such, were not paired with same- or opposite-sex mates. Intruders were made anosmic by administering 0.05 ml of 10% zinc sulfate solution into each nares, so as to minimize offensive aggression. Intruder anosmia was verified by an inability to find a hidden fruit loop in the homecage for 24 h compared to control animals that had consumed the fruit loop by 24 h. The test lasted 5 minutes after the first aggressive interaction or a total of 5 minutes if no aggressive interactions occurred and was terminated if the intruder received 20 attack bites from the resident per methods of Miczek [67]. Subjects were tested no more than once every three days to prevent injury and fatigue.

During the test, the experimenter recorded the resident's latency to, and frequency of pro-social (sniffing, grooming, following with contact) and aggressive behaviors (attack bites, lateral threats, aggressive grooming, pins, pushes) and frequency of rearing as a general motor behavior. No differences were observed in pro-social or rearing behavior between any groups.

Procedure

For acute testing in Experiment 1, rats that were inact (n = 24) or GDX (n = 23) were administered an intraperitoneal (IP) injection of vehicle saline or EtOH (2.0 g/kg) and were either tested in the resident-intruder paradigm (intact+saline, n = 6; intact+EtOH, n = 6; GDX+saline, n = 5; GDX+EtOH, n = 6) or not (n = 6/group) 30 minutes later. A

30-minute latency prior to testing was utilized because this is the typical interval for which aggressive effects of EtOH are observed [68] and an adequate amount of time for biosynthesis of T and/or its metabolites to occur [28, 69]. Immediately following testing, or after 35 minutes for non-tested rats, brain and plasma were collected *via* rapid decapitation.

For repeated testing in Experiment 2, rats that were intact (n = 16) or GDX (n = 16) received an injection of saline or EtOH (0.5, 1.0, or 2.0 g/kg, IP), 30 minutes prior to testing. Each subject was tested four times over a period of ten consecutive days until they had received each of the four EtOH doses in a counterbalanced, Latin square design. Rats were tested once every three days to prevent confounding injury or fatigue. Thus, intact or GDX rats were tested on days 1, 4, 7, and 10. On day 1, intact or GDX rats received 0.0 g/kg (n = 4), 0.5 g/kg (n = 4), 1.0 g/kg (n = 4), or 2.0 g/kg EtOH (n = 4). Rats then progressed through the dose-response regimen as depicted (Table **1**).

Table 1. Design for Experiment 2

		EtOH Dose (g/kg)			
		Day1	**Day 4**	**Day 7**	**Day 10**
Intact	Group 1 (n = 4)	saline	0.5	1.0	2.0
	Group 2 (n = 4)	2.0	saline	0.5	1.0
	Group 3 (n = 4)	1.0	2.0	saline	0.5
	Group 4 (n = 4)	0.5	1.0	2.0	saline
GDX	Group 1 (n = 4)	saline	0.5	1.0	2.0
	Group 2 (n = 4)	2.0	saline	0.5	1.0
	Group 3 (n = 4)	1.0	2.0	saline	0.5
	Group 4 (n = 4)	0.5	1.0	2.0	saline

This table depicts course of testing for rats in Experiment 2. Intact (n = 16) and gonadectomized (GDX; n = 16) rats were administered 0.0, 0.5, 1.0, and 2.0 g/kg EtOH over the course of ten days and assessed for resident-intruder aggression 30 mins later.

The range of the dosages was chosen because 0.5 g/kg is among the lowest effective doses consistently reported to elicit aggression towards an intruder [70] whereas 2.0 g/kg is the highest EtOH dose that has been reported to increase T biosynthesis [69]. Higher EtOH dosages can decrease plasma T levels [60, 71, 72]. Immediately following the final test occasion, experimental rats were killed by rapid decapitation and whole brain and trunk blood were collected.

Assessment of Steroid Concentrations

Following tissue collection, some brains were flash frozen on dry ice, blood was centrifuged and tissues were stored at -80 ^{o}C for later analysis. Frontal cortex was later grossly dissected on ice and assessed for T, DHT, 3α-diol, and E_2 concentrations along with plasma *via* radioimmunoassay as previously described [73-75]. Briefly, the T antibody (T3-125; Endocrine Sciences, Calabasas Hills, CA) was diluted 1:20, 000 and binds between 60% and 65% of [^{3}H] T (NET-387: specific activity = 51.0 ci/mmol). The DHT antibody (DT3-351; Endocrine Sciences) was diluted 1:10, 000 and binds between 60% and 65% of [^{3}H] DHT (NET-302: specific activity = 43.5 ci/mmol). The 3α-diol antibody (X-144; Dr. P.N. Rao, Southwest Foundation for Biomedical Research, San Antonio, TX) was diluted 1:20, 000 and binds approximately 96% of [^{3}H] 3α-diol (NET-806: specific activity = 41.00 ci/mmol). The E_2 antibody (Dr. Niswender, #244, Colorado State University, Fort Collins, CO) was diluted 1:30, 000 and binds approximately 90% of [^{3}H] E2 (NET-317: specific activity = 51.3 ci/mmol). Standard curves for all steroids were run in duplicate and ranged from 50 - 2000 pg in concentration. Standards were added to BSA assay buffer, followed by addition of the appropriate antibody and [^{3}H] steroid. T and DHT assays were incubated overnight at 4 ^{o}C whereas 3α-diol was incubated overnight at room temperature. E_2 was incubated at room temperature for 50 min. Separation of bound and free steroid was accomplished by the rapid addition of dextran-coated charcoal. Samples were centrifuged at 3000 X *g* for 10 min, following incubation with charcoal. Supernatant was decanted into 5 ml scintillation cocktail. The intra- and interassay coefficients of variance were: T = 4% and 4%, DHT = 5% and 9%, 3α-diol = 9% and 11%, E2 = 7% and 9%.

Assessment of Muscimol Binding

Following tissue collection, some cortical tissues from representative intact and GDX groups in Experiment 2 (n = 3/group) were used without freezing in order to assess for muscimol binding as previously described [76-77]. Briefly, tissues were homogenized in 10 ml of 0.32 M sucrose and centrifuged at 1000 X *g* for 10 min. The pellet was discarded and the supernatant was centrifuged for 20 min at 17, 000 X *g*. The crude mitochondrial pellet was resuspended in 10 ml ice-cold distilled water and incubated at 4 °C for 10 min. The homogenate was then centrifuged at 40, 000 X *g* for 20 min. The membrane pellet obtained was washed three times with distilled water and incubated in 0.05 M Tris-HCL buffer for 30 min at 36 °C. Membranes were thawed and resuspended in 0.05 M Tris-HCL buffer. All tissues were preincubated at 36 °C for 30 min and run in triplicate. Following preincubation, [^{3}H]-muscimol (NET-574: specific activity = 14.72 Ci/mmol; New England Nuclear, Boston, MA, USA) 10-100 nM concentrations were added and incubation was continued at 4 °C for 30 minutes. Non-specific binding was determined by addition of 1 mM cold GABA as a displacer of bound [^{3}H]-muscimol. The bound and free fractions were separated by vacuum filtration through GF/C glass filters and quickly washed twice with 0.05 M Tris buffer. Filters were dried overnight and 3ml scintillation cocktail was added for scintillatiuon counting.

Assessment of Blood EtOH Levels

In order to assess whether alterations in EtOH metabolism could underlie observed behavioral and/or neuroendocrine effects, blood EtOH concentrations were assessed in the first Experiment. Blood EtOH was determined using an EnzyChrom EtOH assay kit (ECET-100; BioAssay Systems, Hayward, CA). Assays were carried out per developer instructions. Briefly, serum was added to a formazan/phenazine methosulfate-containing solution, followed by alcohol dehydrogenase. The catalyzed NADH couples with the reagent and optical density was determined (565 nm) at 0 mins and 5 mins *via* an EL_X800 universal microplate plate reader (BioTek Instruments, Inc., Winooski, VT).

Statistical Analyses

In Experiment 1, two-way Analyses of Variance (ANOVAs) were conducted with hormonal milieu (intact or GDX) and EtOH condition (saline or 2.0 g/kg) as independent variables to assess differences in behavioral and endocrine endpoints. Significant effects were followed up with Fisher's Protected Least Significant Differences (PLSD) *post-hoc* tests to determine group differences. In Experiment 2, repeated measures ANOVAs were utilized to determine behavioral effects of hormonal milieu (intact or GDX) and EtOH dose (saline, 0.5, 1.0, or 2.0 g/kg) on dependent measures. Two-way ANOVAs (hormonal milieu X EtOH dose) followed by Fisher's PLSD were used to assess endocrine and blood EtOH differences at the time of last testing. Results of muscimol binding were assessed with a repeated measures ANOVA with hormonal milieu (intact or GDX), EtOH dose (saline or 2.0 g/kg), and GABA concentration (10, 30, 60, 80, or 100 nM) as independent variables. Significant main effects of repeated measures ANOVAs were followed up with one-way ANOVAs with α corrected for multiple comparisons. The α-level for statistical significance was $P \leq 0.05$ and trends towards significance were noted in text when $P < 0.10$.

RESULTS

Experiment 1 - Acute Social Responding to EtOH Among Intact or GDX Rats

Behavioral Endpoints

Androgen milieu altered aggressive responding to EtOH. Among tested rats, hormonal milieu and EtOH condition significantly interacted such that, compared to saline, acutely-administered EtOH (2.0 g/kg) reduced latency to [$F(1, 19) = 6.20$, $P < 0.05$] (Fig. **1**, top) and increased frequency of [$F(1, 19) = 5.31$, $P < 0.05$] (Fig. **1**, middle) aggressive behavior among intact rats, whereas the opposite pattern was observed among GDX rats.

Endocrine Endpoints

Androgen concentrations were altered by EtOH administration and testing. Among rats tested in the resident-intruder paradigm, there was a significant interaction for EtOH (2.0 g/kg) to enhance 3α-diol concentrations in frontal cortex of intact rats, but not in GDX rats, compared to saline [$F(1, 19) = 4.47$, $P < 0.05$] (Fig. **1**, bottom). Notably, the opposite interaction was observed for E_2 wherein EtOH (2.0 g/kg), compared to saline, decreased E_2 in frontal cortex of inact rats but increased E_2 in cortex of GDX rats [$F(1, 19) = 4.29$, $P = 0.05$] (Table **2**, top). While, there was an apparent effect for EtOH (2.0 g/kg) to increase cortical T among intact rats, this was not significant (Table 2, top). In plasma, T levels were significantly reduced among GDX, compared to intact rats [$F(1, 19) = 8.34$, $P < 0.05$] (Table **2**, bottom). Among tested rats, no changes were observed in DHT or plasma levels of 3α-diol or E_2.

Among non-tested rats, there were significant interactions for EtOH (2.0 g/kg), compared to saline, to decrease cortical 3α-diol [$F(1, 20) = 10.13$, $P < 0.05$] and increase cortical E_2 [$F(1, 20) = 8.12$, $P < 0.05$] among intact rats, and to have the opposite effect among GDX rats (Table **3**, top).

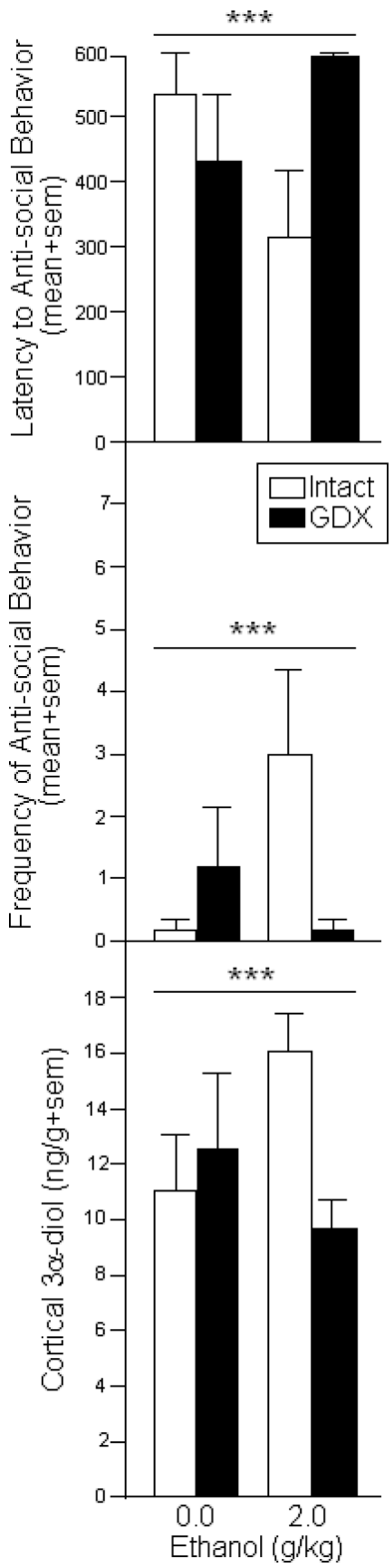

Fig. (1). Compared to saline, EtOH (2.0 g/kg) administration to intact, but not GDX, rats reduces the latency (seconds+sem) to engage in aggression (top) and increases the number (middle) of aggressive behaviors that resident rats direct towards intruders. Frontal cortex levels of 3α-androstanediol (3α-diol) are elevated concomitant with aggressive behavior among rats (bottom). *** indicates significant interaction between hormonal milieu and EtOH treatment ($P < 0.05$).

Both cortical [$F(1, 20) = 7.48$, $P < 0.05$] (Table 3, top) and circulatory [$F(1, 20) = 18.05$, $P < 0.05$] (Table **3**, bottom) T levels were significantly lower among GDX, compared to intact, rats. Among non-tested rats, no differences were observed in concentrations of DHT or plasma levels of 3α-diol or E_2.

Table 2. Androgen Levels Among Rats Acutely-Administered EtOH

Frontal Cortex (ng/g)	Intact		GDX	
	EtOH Dose (g/kg)		EtOH Dose (g/kg)	
	0.0 (n = 6)	2.0 (n = 6)	0.0 (n = 5)	2.0 (n = 6)
T	2.7±0.8	4.2±1.3	2.6±0.3	2.3±0.5
DHT	10.8±1.9	7.4±1.7	6.5±1.8	6.7±1.0
E_2***	1.3±0.2	1.1±0.1	0.8±0.1	1.1±0.1
Plasma (ng/ml)	**Intact**		**GDX**	
	EtOH Dose (g/kg)		**EtOH Dose (g/kg)**	
	0.0 (n = 6)	**2.0 (n = 6)**	**0.0 (n = 6)**	**2.0 (n = 6)**
T	11.3±1.5	18.9±4.4	7.7±3.1*	6.4±0.3*
DHT	2.4±0.4	1.9±0.4	1.6±0.4	1.9±0.4
3α-diol	2.1±0.4	1.7±0.2	2.7±0.3	1.9±0.1
E_2	2.7±0.1	2.6±0.4	2.6±0.4	2.5±0.5

This table depicts testosterone (T) and its metabolites, dihydrotestosterone (DHT), 3α-androstanediol (3α-diol), and estradiol (E_2) in frontal cortex and/or plasma among acutely-tested, intact or gonadectomized (GDX) rats administered saline or EtOH (2.0 g/kg). * indicates significant reduction in GDX, compared to intact, rats ($P < 0.05$). *** indicates significant interaction between hormonal milieu and EtOH administration ($P = 0.05$).

Table 3. Androgen Levels Among Non-Tested Rats Acutely-Administered EtOH

Frontal Cortex (ng/g)	Intact		GDX	
	EtOH Dose (g/kg)		EtOH Dose (g/kg)	
	0.0	2.0	0.0	2.0
T	4.7±0.8	3.4±0.7	2.3±0.3*	2.4±0.5*
DHT	14.0±2.8	12.0±2.3	11.7±2.9	10.2±2.1
3α-diol***	1.2±0.2	0.7±0.2	0.6±0.1	1.0±0.1
E_2***	1.2±0.1	1.5±0.1	1.7±0.1	1.3±0.1
Plasma (ng/ml)	**Intact**		**GDX**	
	EtOH Dose (g/kg)		**EtOH Dose (g/kg)**	
	0.0	**2.0**	**0.0**	**2.0**
T	11.2±2.5	9.9±2.0	3.9±0.5*	3.3±0.4*
DHT	18.3±4.0	18.1±5.0	19.1±3.7	11.2±3.4
3α-diol	1.6±0.3	2.0±0.2	2.2±0.3	1.7±0.2
E_2	2.1±0.5	2.6±0.2	2.2±0.5	2.4±0.5

This table depicts testosterone (T) and its metabolites, dihydrotestosterone (DHT), 3α-androstanediol (3α-diol), and estradiol (E_2) in frontal cortex and/or plasma among non-tested, intact or gonadectomized (GDX) rats acutely administered saline or EtOH (2.0 g/kg; n = 6/group). * indicates significant reduction in GDX, compared to intact, rats ($P < 0.05$). *** indicates significant interaction between hormonal milieu and EtOH administration ($P < 0.05$).

Blood EtOH levels did not account for differences in behavioral or neuroendocrine status. Blood EtOH levels (mg/dL) were greater among intact (Tested: 426.3 ± 66.8; Non-tested: 366.6 ± 27.4) and GDX (Tested: 393.6 ± 17.9; Non-tested: 366.4 ± 12.2) rats administered EtOH compared to vehicle-administered rats which were below detectable limits in all conditions.

Experiment 2 - Social Responding to Repeated EtOH-Testing Among Intact or GDX Rats

Behavioral Endpoints

Androgen milieu and EtOH dosing altered aggressive responding towards an intruder. There were significant main effects for intact rats to demonstrate shorter latencies to engage in aggressive behavior, compared to GDX rats [$F(1, 30) = 4.52$, $P < 0.05$], and for the 2.0 g/kg EtOH dose to reduce latency to aggress among all rats, compared to saline [$F(3, 90) = 2.59$, $P = 0.05$] (Fig. **2**, top). There was also a non-significant trend for EtOH (2.0 g/kg) to enhance the frequency of aggressive behavior compared to saline [$F(3, 90) = 2.22$, $P < 0.10$] (Fig. **2**, middle).

Endocrine Endpoints

Androgen levels were altered with hormone manipulation and EtOH administration. Iintact rats had significantly greater concentrations of 3α-diol in frontal cortex compared to GDX rats [$F(1, 24) = 3.99$, $P = 0.05$]. However, there was a general dose-dependent increase in cortical 3α-diol with EtOH, such that 2.0 g/kg EtOH significantly enhanced cortical 3α-diol over saline [$F(3, 24) = 3.56$, $P < 0.05$] (Fig. **2**, bottom). In plasma, GDX rats had significantly lower concentrations of T [$F(1, 24) = 11.07$, $P < 0.05$], DHT [$F(1, 24) = 7.40$, $P < 0.05$], and 3α-diol [$F(1, 24) = 22.01$, $P < 0.05$] compared to intact rats (Table **4**, bottom). Among repeat-tested rats, no significant differences were observed in E_2 or cortical T or DHT.

Androgens and EtOH may act agonistically at GABAergic substrates. As anticipated, GABA-promoted muscimol binding reached half maximal efficacy at lower concentrations of GABA among intact rats administered 2.0 g/kg EtOH (56.7±14.5 nM) compared to GDX rats administered 2.0 g/kg EtOH (86.7±6.7 nM) or vehicle-administered rats (Intact-EC_{50}: 73.3±6.7 nM; GDX-EC_{50}: 73.3±6.7nM).

DISCUSSION

The present investigation supported the hypothesis that central androgens may partly underlie aggressive responding to EtOH among rats. First, compared to saline, acute EtOH (2.0 g/kg) administration to intact rats concomitantly enhanced 3α-diol concentrations in brain and aggression towards an intruder. GDX rats did not show increased aggression or 3α-diol formation when EtOH (2.0 g/kg) was administered acutely. Among, rats that did not engage in aggression-testing, the opposite pattern was observed for cortical 3α-diol. Second, in the repeated measures experiment, intact rats demonstrated dose-dependent enhancement in cortical 3α-diol concomitant with dose-dependent elevations in aggression. Among GDX rats, the 2.0 g/kg EtOH dose was the only dose to enhance 3α-diol and it was also observed to increase

aggressive responding towards an intruder. Notably, rats that underwent repeated EtOH administration and repeated

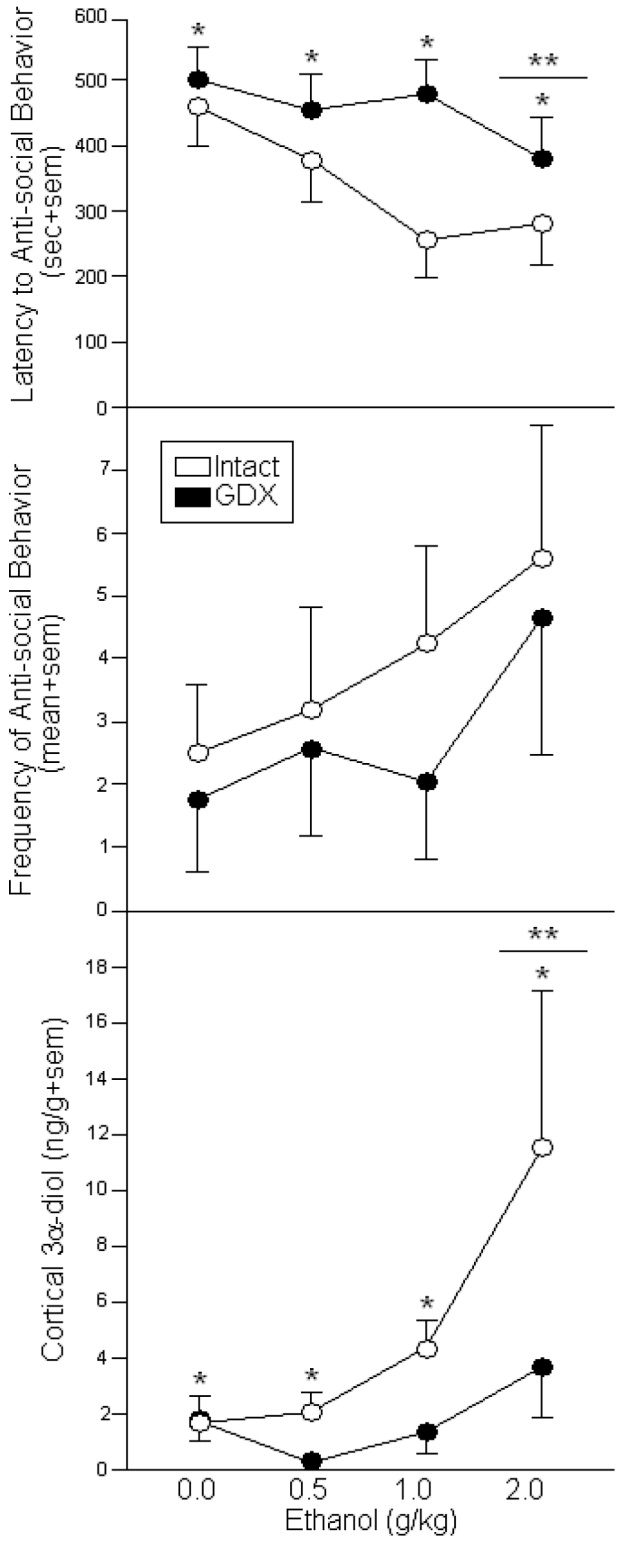

Fig. (2). EtOH administration dose-dependently reduces latency (seconds+sem) to aggression to a greater degree among intact, than GDX, rats (top). Number of aggressive behaviors directed by residents towards intruders is non-significantly elevated (middle) and cortical 3α-diol is dose-dependently enhanced (bottom) with increasing EtOH concentration to a greater degree among intact, than GDX, rats. * indicates intact males are significantly different from GDX males ($P \leq 0.05$). ** indicates 2.0 g/kg EtOH is significantly different from saline administration ($P \leq 0.05$).

aggression testing in Experiment 2 demonstrated greater aggression overall and more selective 3α-diol enhancement to EtOH (2.0 g/kg) than did acutely-tested rats in Experiment 1. Differences in these testing paradigms preclude consideration of the vehicle-administered groups as being commensurate. An acute stressor, as is the case in Experiment 1, is known to elicit a greater neuroendocrine response than one that the rodent has habituated to. As well, repeated aggression testing, as is the case in Experiment 2, has been demonstrated to be rewarding such that rodents are more likely to engage in aggression when given future opportunities [78]. Lastly, findings of the present study support the notion that EtOH's ability to promote aggression may involve actions at $GABA_A$ receptors. While, not significant, it was observed that frontal cortex of intact rats administered 2.0 g/kg EtOH at the time of tissue collection had lower half-maximal threshold for muscimol binding compared to GDX rats administered EtOH (2.0 g/kg) or rats administered vehicle. These data suggest that intact rats, who had the greatest increase in 3α-diol, may have greater activation at GABAergic receptor sites. Together, these data support previous findings and extend them in several important ways.

Past investigations have reported on androgens' effects to amplify EtOH-enhanced aggression in other species, including mice [49], hamsters [51], and squirrel monkeys [50]. Although few studies have addressed whether androgens and EtOH may have amplifying effects on aggression, in part due to biosynthesis of androgens, there is some support for this notion. In humans, elevated T levels have been found in violent, antisocial, alcoholic offenders [8, 79]. Golden hamsters allowed to self-administer EtOH in adolescence were found to demonstrate greater aggression and had two-fold increases in endogenous T compared to did hamsters that were not exposed to EtOH [51]. Interestingly, more recent studies, which are not hindered by potential confounds of behavioral testing, demonstrate that EtOH administration can alter central androgen levels. Alomary and colleagues [69] report that among male Wistar rats, a single dose of 2.0 g/kg EtOH (IP) resulted in a 4-fold increase in total T in frontal cortex and a 3-fold increase in plasma T concentrations 30 min after injection compared to saline-administered controls. This effect of EtOH to enhance steroidogenesis was influenced by secretion from the peripheral glands, such that EtOH-induced T production was attenuated among rats that were GDX and adrenalectomized. We, too, find that GDX alone attenuates the EtOH-induced increase in central T compared to that of intact rats. The present study extends these observations of EtOH-induced increases in T to its metabolite, 3α-diol. Other investigations, unrelated to aggression research, have noted that blocking DHT's conversion to 3α-diol *via* administration of the COX-2 inhibitor, indomethacin, increases EtOH clearance in mice [80]. Moreover, indomethacin administration can also attenuate EtOH's soporific effects in mice [80-82]. Thus, 3α-hydroxylated metabolites of T may amplify EtOH's agonistic effects.

Although, enhancement of androstane neurosteroids are understudied in this area, these findings are not surprising in light of reports that the pregnane neurosteroid, 5α-pregnan-3α-ol-20-one (3α,5α-THP, also called allopregnanolone), is

Table 4. Androgen Levels Among Repeatedly-Tested Rats

Frontal Cortex (ng/g)	Intact				GDX			
	EtOH Dose (g/kg)				EtOH Dose (g/kg)			
	0.0	0.5	1.0	2.0	0.0	0.5	1.0	2.0
T	4.8±1.8	4.2±0.4	4.1±1.2	2.8±1.6	3.3±0.4	2.2±0.8	2.2±0.8	3.3±0.2
DHT	3.1±1.0	1.0±0.7	2.4±1.1	2.0±1.2	1.3±0.8	1.2±0.5	1.3±0.9	0.9±0.7
E_2	0.6±0.2	0.5±0.3	0.7±0.4	0.3±0.2	1.0±0.4	0.5±0.3	1.0±0.4	0.7±0.4
Plasma(ng/ml)	**Intact**				**GDX**			
	EtOH Dose (g/kg)				EtOH Dose (g/kg)			
	0.0	0.5	1.0	2.0	0.0	0.5	1.0	2.0
T	22.7±7.3	25.3±1.9	15.1±5.3	39.9±13.8	12.6±7.3*	11.2±7.8*	10.1±5.9*	2.6±1.5*
DHT	15.1±3.5	4.9±1.9	9.4±1.8	10.7±3.5	7.1±2.5*	7.2±1.8*	5.9±1.6*	2.0±0.7*
3α-diol	6.5±1.1	7.5±3.5	11.3±3.1	3.4±1.0	1.7±1.2*	1.3±0.4*	1.1±0.7*	0.5±0.3*
E_2	15.4±2.7	6.9±0.6	11.6±2.5	9.4±3.9	14.6±3.0	10.7±3.5	3.2±2.1	9.4±3.3

This table depicts concentrations of testosterone (T) and its metabolites, dihydrotestosterone (DHT), 3α-androstanediol (3α-diol), and estradiol (E_2) in frontal cortex and/or plasma among repeat-tested, intact or gonadectomized (GDX) rats following their last testing session with saline or EtOH (0.5, 1.0 or 2.0 g/kg) administration (n = 4/group). * indicates significant reduction in GDX, compared to intact, rats ($P < 0.05$).

increased following administration of 2.0 g/kg EtOH [62]. Further, some evidence suggests that alteration in 3α,5α-THP may underlie some of the subjective effects of EtOH in people [83] as well as some of the aggressive responses among rodents [78, 84]. A likely component of both pregnane and androstane neurosteroidogenesis in response to EtOH, is activation of the hypothalamic-pituitary-adrenal (HPA) stress axis. EtOH (1.0 to 2.0 g/kg) has been demonstrated to alter HPA feedback within 20 minutes [85, 86] with effects peaking 40 and 80 mins later [85]. Notably, central formation of 3α,5α-THP and 3α-diol are rapidly enhanced in response to HPA activation [87, 88]. Further, when elevated, 3α,5α-THP and 3α-diol can act as homestatic modulators to dampen HPA activation, albeit, likely *via* different mechanisms. When elevated 3α,5α-THP can act at GABAergic sites in hypothalamus to reduce HPA-activating factors [89, 90] whereas 3α-diol (which also binds GABAergic membrane receptors, but with less affinity than 3α,5α-THP) can act at estrogen receptor β sites which can dampen HPA responding [91-94]. As such, activation of HPA may serve as part of the mechanism by which EtOH promotes neurosteroidogenesis.

Other important factors in considering EtOH-enhanced steroidogenesis are the demand characteristics of the testing situation. We included a group of non-tested rats, acutely administered saline or EtOH (2.0 g/kg), and found opposite effects for central 3α-diol in cortex. Administration of EtOH to non-tested intact rats decreased central 3α-diol, however, administration to rats engaging in territorial aggression-testing enhanced cortical 3α-diol. These data suggest that social interaction can alter the androgen response to EtOH. However, it must be noted that all rats in the current investigation were socially-isolated which can enhance steroidogenic effects of EtOH [86]. Social isolation alone has been found to decrease 3α,5α-THP in brain and plasma of rats [95] and increases expression of $GABA_A$ receptor subunits α_4 and δ in hippocampus [96]. It may be the case that 3α-diol production is enhanced in response to EtOH among isolated rats as well, considering 3α-diol's enhancement in response to stress [88]. Thus, social isolation likely contributed to observed effects. Future investigations on 3α-diol's role in EtOH-mediated aggression will need to utilize an animal model that more readily displays territorial aggression so as to remove social isolation as an additional variable. Examining co-variation of 3α-diol across the lifecycle concomitant with EtOH administration may elucidate normative and androgen-dependent effects of EtOH-promoted aggression.

EtOH has also been suggested to alter androgen levels *via* its effects on its aromatization to E_2 [28, 61, 97-99]. As well, circulating E_2 levels were significantly increased 30 min after 0.6 g/kg EtOH in male mice [28]. In the current investigation, rats acutely-administered EtOH had significantly altered E_2 levels in frontal cortex independent of changes in circulation. Among both intact and GDX rats E_2 levels were lower when 3α-diol levels were increased. These data support the notion that EtOH can enhance 3α-diol formation in cortex partly by enhancing 5α-reduction of T over aromatization. Indeed, investigations in people find that EtOH can alter peripheral androgen metabolism [56] and that circulating E_2 levels are negatively correlated with EtOH-induced aggression [20]. Although effects on chronic EtOH to decrease activity of these steroidogenic enzymes in testes have been demonstrated [100, 101], studies on effects of acute administration of EtOH and activity of these enzymes are needed. As such, altered T metabolism may be one mechanism by which EtOH can enhance central 3α-diol in our model.

CONCLUSION

These data support the notion that enhancement of 3α-diol in frontal cortex may partly underlie EtOH enhanced aggression among rats. Both acute and repeated EtOH administration enhanced aggression among rats at when administered at 2.0 g/kg. These effects were observed in GDX, as

well as gonadally-intact rats. As well, the HPA factor, 3α-diol, was enhanced in brain, but not plasma, concomitant with aggression and recent findings confirm HPA activation with acute EtOH exposure [102]. Together, these data suggest that neurosteroidogenesis may account for observed effects. These effects may be related to actions at GABAergic targets, however, there are many sites of action that need to be explored, particularly considering 3α-diol's effects at estrogenic targets. Future investigations should aim to utilize an animal model that readily displays territorial aggression and should manipulate 3α-diol directly to assess its role as a causative factor in EtOH-facilitated aggression.

ABBREVIATIONS

3α-diol	=	3α-androstanediol
3α,5α-THP	=	5α-pregnan-3α-ol-20-one
EtOH	=	Alcohol
ANOVA	=	Analyses of Variance
DHT	=	Dihydrotestosterone
E_2	=	Estradiol
GDX	=	Gonadectomy
HPA	=	Hypothalamic-Pituitary-Adrenal
IP	=	Intraperitoneal
PLSD	=	Protected Least Significant Differences
T	=	Testosterone

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