Corticosteroid modulation and testosterone changes during alcohol intoxication affects voluntary alcohol drinking

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ABSTRACT

A number of studies have shown that stress and an activated hypothalamic-pituitary-adrenal (HPA) axis are associated with increased voluntary alcohol drinking. Recently, associations have been found between activated HPA and hypothalamic-pituitary-gonadal (HPG) axes in alcohol-preferring AA and non-preferring ANA, F2 (crossbred second generation from original AA and ANA), and Wistar rats. The aim of the present study has been to determine the role of corticosterone and alcohol-related testosterone-effects in subsequent alcohol drinking in AA, ANA, F2 and Wistar rats. The present study comprises of four substudies presenting new analyses of existing data, by which correlations between basal corticosterone levels, changes in testosterone levels during alcohol intoxications and subsequent voluntary alcohol consumption are investigated. The results displayed positive correlations between basal corticosterone levels and subsequent alcohol-mediated testosterone elevations, which was positively associated with voluntary alcohol consumption. The results also showed a negative correlation between basal corticosterone levels and alcohol-mediated testosterone decreases, which was negatively associated with alcohol consumption. In conclusion, the

present study displays novel results, according to which the HPA axis, one hand, relates to testosterone elevation (potentially causing and/or strengthening reinforcement) during alcohol intoxication, which in turn may relate to higher voluntary alcohol consumption (AA rats). Vice versa, the HPA axis may also relate to alcohol-mediated testosterone decrease (causing testosterone reduction and disinforcement) and low-alcohol drinking (ANA, F2 and Wistar rats). In addition, the present results showed that alcohol-mediated testosterone changes may also, independently of the HPA axis, correlate with voluntary alcohol drinking, which indicate the impact of genetic factors. Thus, the role of the HPA-axis may be more related to situational stress than to intrinsic factors. In further studies, it should be investigated, whether the present results also apply to stress and human alcohol drinking.

Keywords: Stress, voluntary alcohol consumption, high and low alcohol drinking rats, corticosterone, testosterone, HPA and HPG axes

1. Introduction

Stress and activation of the hypothalamic-pituitary-adrenal (HPA) axis have been associated with increased alcohol drinking and dependence in both experimental animals and in humans (Ciccocioppo et al., 2006; Fahlke et al., 1994; Gianoulakis, 1998; Koob, 2013; Pohorecky, 1990, 1991; Roman and Nylander, 2005; Zorilla et al., 2014). Tension reduction and stress response dampening have been suggested as primary explanatory factors on the behavioral level (Pohorecky, 1991). On a neurobiological level, the activation of the HPA-axis seems to be the primary etiological factor (Spanagel et al., 2014; Stephens and Wand,

2012; Zhou and Kreek, 2014). With this regard, emphasis has been put on the role of the corticotropin-releasing factor (CRF) (Phillips et al., 2015; Zorilla et al., 2014).

Recent results indicate that stress-related increased alcohol drinking may not only be caused by the HPA-axis. Within a broader context, it seems, that stress-related increased alcohol drinking and the development of alcohol dependence are caused, at least to some extent, by the hypothalamic-pituitary-adrenal-gonadal (HPAG) axes, i.e., the combined effects by the HPA and HPG axes (Apter and Eriksson, 2003, 2006; Etelälahti et al., 2011; Etelälahti and Eriksson, 2013, 2014). Here, the crucial factor may be the effect of alcohol on testosterone levels. Our earlier results indicate that an alcohol-mediated testosterone elevation may promote reinforcement and excessive voluntary alcohol drinking in a stress-related situation. On the other hand, in some situations, alcohol-mediated testosterone attenuation may cause disinforcement (this term has been used as an antonym of reinforcement by Harzem and Miles, 1978) and reduce the voluntary alcohol consumption (Apter and Eriksson 2006; Etelälahti et al., 2011; Etelälahti and Eriksson, 2013).

The present correlational study is based on new data from 4 original independent studies (Apter and Eriksson 2006; Etelälahti et al. 2011; Etelälahti and Eriksson 2013, 2014) comprising 4 substudies, respectively. The line of arguments regarding the original studies was started by Apter and Eriksson (2003, 2006). Hence the aim was to investigate the hypothesis of a link between the HPA and HPG axes and subsequent alcohol-mediated testosterone change in high-alcohol drinking AA and low-alcohol drinking ANA rats. The next aim was to verify the hypothesis with outbred high- and low-drinking F2 populations (Etelälahti et al., 2011). In the following study (Etelälahti and Eriksson 2013) the idea was to mimic the study by Johansson et al. (2000), in which Nandrolone Decanoate (ND) treatment increased voluntary alcohol drinking in low-alcohol drinking Wistar rats. Contrary to our hypothesis, ND treatment decreased voluntary alcohol consumption and alcohol-mediated

testosterone elevation in both AA and Wistar rats (Etelälahti and Eriksson 2013). The difference between the two studies turned out to be, that we used pure ND in oil, whereas in the previous study (Johansson et al., 2000) the ND product Deca-Durabolin containing Benzyl Alcohol (BA) was used. Thus, in the latest original study (Etelälahti and Eriksson 2014) we tested the effect of subchronic BA on voluntary alcohol drinking and testosterone change in AA and Wistar rats. The result was increased alcohol drinking in the AA and Wistar rats, which probably explained at least part, if not all, of the difference between our study (Etelälahti and Eriksson 2014) and the study by Johansson et al. (2000).

Based on the earlier original studies there seems to be indications on a coupling between the HPA and HPG-axes and high- and low-alcohol drinking. Thus the aims of the present study is to determine the overall correlational role of corticosterone in alcohol-induced effects on testosterone levels and subsequent alcohol drinking in rats.

2. Materials and methods

2.1 Animals

The present study comprise new correlational analyses of existing data from the rats that already were investigated in our earlier studies: male high alcohol-drinking AA and low-drinking ANA populations of generation F80 (substudy 1: n = 24 and n = 22 for the AA and ANA rats, respectively; Apter and Eriksson, 2003, 2006), crossbred F2 populations (substudy 2, n = 40 for low drinking and 40 for high drinking) of original AA and ANA rats of generation F89 (Etelälahti et al., 2011), AA (F > 90) and low-drinking Wistar populations (substudy 3, n = 40 for each population) (Etelälahti and Eriksson, 2013) and AA (F > 90)

and Wistar populations (substudy 4, n = 20 for each population) (Etelälahti and Eriksson, 2014). The breeding history of the outbred AA and ANA lines and F2 populations are described in original publications (Eriksson 1968; Hilakivi et al., 1984; Etelälahti et al., 2011). The Wistar Unilever (HsdCpb:Wu) rats for substudies 3 and 4 represent an outbred strain obtained from Harlan (now Envigo), Horst (The Netherlands). All rats were 2.0 – 3.5 months old at the beginning of the experiments (for closer details, see original publications). In substudy 3, the preference of higher than 50 % (approximately 2.5 – 3 g/kg/day) and lower than 2.5 g/kg/day were taken as norms for high-drinking and low-drinking rats, respectively. In this substudy, cutoffs for outliers, based on more than 2 standard deviations from the overall mean (3 AA rats with alcohol drinking less than 0.5 g/kg/day and 2 Wistars drinking more than 2.5 g/kg/day), were used for the correlation between alcohol consumption and testosterone changes. In addition, the lack of steroid hormone determination success in some cases further reduced the number of data points in substudies 1 – 3 (the corrected numbers are expressed in the result section).

In substudy 1 half of the rats were single housed and the other half group housed throughout the experiments (Apter and Eriksson 2006). In substudy 2 all animals were single housed throughout the experimental time (Etelälahti et al., 2011). In substudies 3 and 4 all rats were group housed during drug treatments and single housed during the voluntary alcohol consumption (Etelälahti and Eriksson 2013, 2014). In all substudies animal facilities were air-conditioned, with temperature 20-21 ° C, humidity at 47.6 % and a 12 h / 12 h light/dark cycle with lights on at 6 a.m., except for the experiment with reversed light cycle (experiment 2 of substudy 3), where lights went on at 6 p.m. The rats had free access to water and standard laboratory pellets (SDS RM1, Witham, Essex, England).

All substudies were approved by the County Administrative Board of Southern Finland and the ethical committee of the National Public Health Institute. The experimental

125 animal procedures were approved by the Institutional Animal Care and Use Committee at the
126 National Public Health Institute
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129 *2.2 Drug administrations*
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131 In substudies 1-4, alcohol doses (0.75 g/kg, substudy 1; 1.5 g/kg, substudies 1-4; 2
132 g/kg, substudy 2) were administered intraperitoneally (i.p.). In all substudies alcohol was
133 administered i.p. as a 10 % ethanol (wt/vol diluted in 0.9 % NaCl).
134 Nandrolone decanoate (ND) (Organon, Oss, the Netherlands) used in substudy 3 was
135 dissolved (50 mg/ml) in sterile oil (Arachidis oleum, Yliopiston Apteekki/ University
136 Pharmacy, Finland) and administered by subcutaneous injection (s.c.) (15 mg/kg). It was
137 considered essential to use pure ND, because the commonly used commercial ND product
138 (Deca-Durabolin®, N. V. Organon, Oss, the Netherlands) contains Benzyl Alcohol (10 %
139 v/v) as a preservative, which might cause unwanted effects of its own (Nair, 2001).
140 Benzyl alcohol (BA) (Yliopiston apteekki/ University Pharmacy, Finland) used in
141 substudy 4 was diluted (100 mg/ml) in sterile oil (Arachidis oleum, Yliopiston apteekki,
142 Finland), which was a dose corresponding to that in the Deca-Durabolin® used by Johansson
143 et al. (2000). The BA solution was administered by s.c. injection (30 mg/kg).
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146 *2.3 Blood sampling and analytical methods*
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148 Blood samples (200 µl) were taken at 0, 1, 2 and 3 hours (substudy 1) and 0, 1 and 2
149 hours (substudies 2-4) by puncture from the tip of the tail and immediately diluted with 500

µl saline and centrifuged after coagulation. Serum samples were frozen and kept at -70 °C until the analyses were carried out. Possible consecutive blood samples were taken from the same puncture after removing the coagulated blood plate to minimize handling stress.

Testosterone concentrations were measured from serum using the testosterone radioimmunoassay kit (Orion Diagnostica, Espoo, Finland). The minimum detectable concentration was 0.1 nmol/L. The intra-assay coefficient of variation (CV) was 9.1 % at a testosterone concentration of 4.8 nmol/L, and the inter-assay CV was 8.3 % at a testosterone concentration of 18.8 nmol/L.

Corticosterone concentrations were determined from serum using an ImmuChem Double Antibody Corticosterone RIA Kit (MP Biomedicals, Orangeburg, NY). The inter-assay CV was 7.2 % and the intra-assay CV was 4.9 % at corticosterone levels of 100-200 ng/mL.

The radioimmunoassay was quantified by a Wallac Wizard 1470 automatic gamma counter (GMI, Inc., Ramsey, MN).

2.4 Experimental design

In contrast to substudies 2-4, in substudy 1 the AA and ANA rats were tested in both group- and single-cages. The treatment conditions involved alcohol administration randomly with doses 0.75 and 1.5 g/kg (at least 1 week between the treatments) with blood sampling at 0, 1, 2 and 3 hours post alcohol/saline injection. Voluntary alcohol consumption was not tested in substudy 1. (Apter and Eriksson 2006)

In substudy 2 (crossbred F2 populations) all animals were challenged with a priming alcohol i.p. dose, 2 g/kg. This was followed by a 3-week voluntary alcohol-drinking period

with a two-bottle choice between tap water and alcohol solution in water. The average amount of alcohol drinking for the higher drinking rats on the third week was 1.5 ± 0.1 g/kg per day (range: 1.0-3.5 g/kg per day) and 0.6 ± 0.01 g/kg per day for the low-drinking (range: 0.34-0.64 g/kg per day). After a washout period of 1 week, about half of the highest and lowest alcohol drinkers were challenged with a second dose (1.5 g/kg) of alcohol. The rest of the animals (matched for same alcohol intake) got an i.p. control injection of saline, same final volume as in the corresponding alcohol test. (Etelälahti et al., 2011)

In substudy 3 AA and Wistar rats were randomly divided into control and treatment groups after which the rats received daily s.c. injections of ND for 14 days. Correspondingly, control rats were given daily injections of vehicle oil (Arachidis oleum). In this substudy (3) two experiments with identical designs, except for reversed day/night cycle, were conducted with both populations. The first treatment periods were followed by one-week washout periods. After washout, and subsequent alcohol administration (1.5 g/kg) and blood tests, all rats were placed into single cages for the 3-week voluntary drinking period. (Etelälahti and Eriksson 2013)

Substudy 4 was conducted as substudy 3, except for that BA instead of ND was used and that only day/night cycle with lights on at 6 a.m. was applied. (Etelälahti and Eriksson 2014)

During the voluntary alcohol consumption periods in substudies 2-4, the animals had free access to two 100 ml bottles, one with tap water and the other with 10 % (wt/vol) ethanol (Berner Oy, Helsinki, Finland) in tap water. All injections (alcohol, ND, BA, vehicle oil and saline) were administered in the mornings at about 7.30-9.00 a.m. in all substudies. Alcohol injections were given randomly in substudy 1, before alcohol drinking and one week after drinking in substudy 2, and one week after ND or BA treatment before alcohol drinking in substudies 3 and 4. For closer details on experimental conditions, see original publications.

2.5 Statistical analyses

Data were analyzed using SPSS version 22 (SPSS Inc., Chicago, IL). Correlational comparisons (both Pearson's r and Spearman's rho) were assessed by Fisher's Z-test. Nonparametric correlation analyses were used when data did not fulfill the requirements of parametric tests, such as normal distribution. Normality was tested with the Kolmogorov-Smirnov and Shapiro-Wilk tests. The combined significance (two p-values combined) was derived by the Fisher's combined probability test. Significance was assessed at two main confidence levels: 95 % ($\alpha = 1 - 0.95 = 0.05$) and 90 %, suggesting trends, ($\alpha = 1 - 0.90 = 0.10$). All lower levels of confidence were considered non-significant ($p > 0.05$) or not even regarded as trends ($p > 0.10$).

3. Results

3. 1 AA versus ANA rats and F2 populations (substudy 1 and 2)

According to substudy 1, the high-drinking AA rats display a trend towards a positive correlation (rho = 0.373, p = 0.073, n = 24) between testosterone elevations during the first hour post alcohol intoxication (after a dose of 0.75 g/kg) and basal corticosterone levels (Fig. 1). In contrast, at the same time point, a negative correlation was seen in the non-drinking ANA rats (rho = -0.362, p = 0.107, n = 21) between decreasing testosterone levels and basal corticosterone levels. Altogether, the correlation difference between the lines was significant

(Z = 2.401, p = 0.016). In addition, a significant negative correlation (rho = -0.466, p = 0.029, n = 21) was displayed in ANA rats 2 hours after alcohol injection. No other, nor trends for, correlation differences were found.

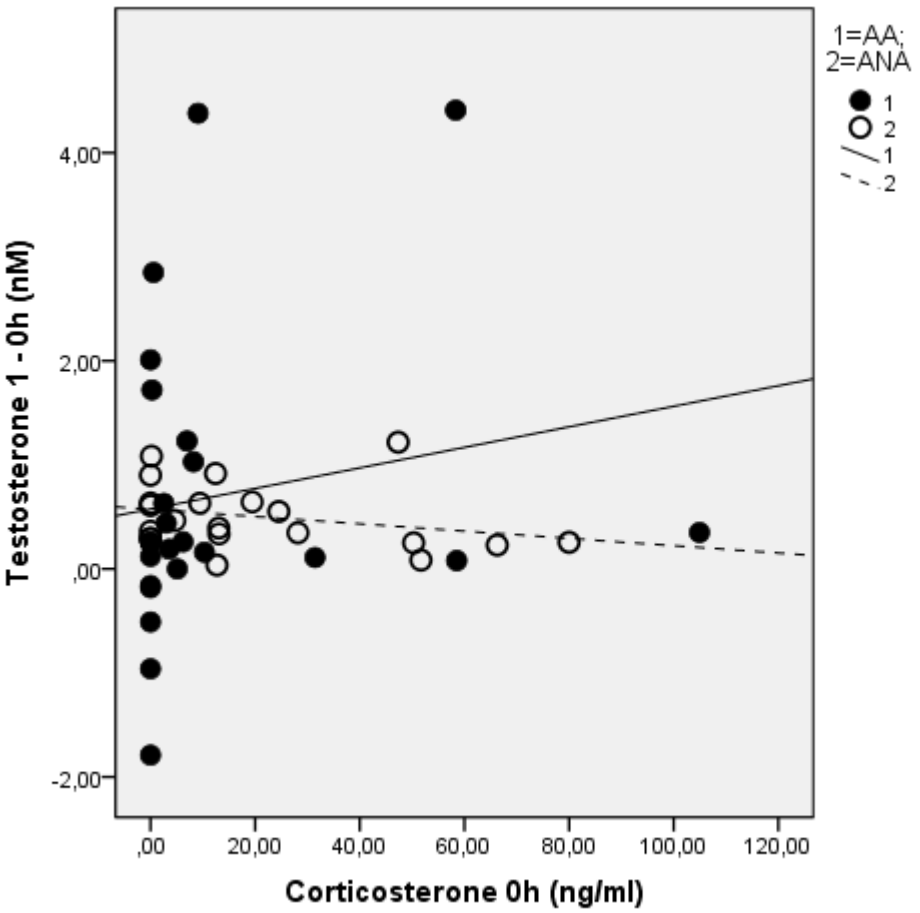

Fig. 1. Correlations between changes in testosterone concentrations at 1 hour post alcohol injection (dose = 0.75 g/kg) and starting corticosterone levels (0h) for AA and ANA rats. Correlations and p-values for AA: rho = 0.373, p = 0.073 and for ANA: rho = -0.362, p = 0.107 (correlational comparison Z = 2.401, p = 0.016).

The AA and ANA F2 high and low consumption populations did not show significant differences between basal corticosterone levels and subsequent alcohol-mediated testosterone changes neither before voluntary alcohol drinking nor after drinking (substudy 2). However, a significant negative correlation appeared in the low-drinking population between the mean alcohol drinking on the third week and the testosterone change at two hours post priming with the 2 g/kg alcohol injection ($r = -0.386$, $p = 0.018$, $n = 37$) (Fig.2). At one hour after alcohol injection the correlation coefficient was not significant ($r = -0.177$, $p = 0.288$, $n = 37$). Also, in the higher drinking population the correlation coefficients were not significant (at one hour $r = -0.189$, $p = 0.262$ and at 2 hours $r = -0.083$, $p = 0.627$, $n = 37$). In fact, the high drinkers were very few (5 out of 37) and thus this group is a mix, which excludes the use of Fisher Z correlational comparison.

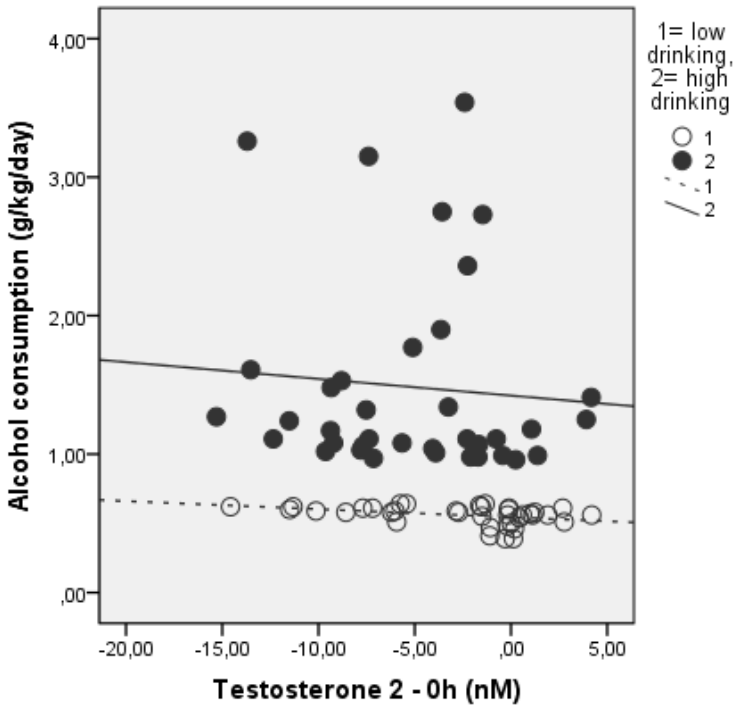

Fig. 2. Correlations between voluntary alcohol consumption and the testosterone change from 0 to 2 hours post alcohol injection in high and low drinking F2 rats (dose = 2 g/kg). Correlation coefficient is not significant for higher drinkers but significant (r = -0.386, p = 0.018) for the low drinkers.

3.2 AA versus Wistar rat and the effect of Nandrolone Decanoate (substudy 3)

In experiment 1 no significant correlational comparisons and correlations between basal corticosterone levels and testosterone changes during 1 and 2 hours were observed in the AA and Wistar rats. However, in experiment 2 the corresponding correlational comparisons displayed a tendency for difference (Z = 1.818, p = 0.069) between the positive correlation in AA rats (r = 0.606, p = 0.149, n = 7) and negative correlation in Wistars (r = -0.439, p = 0.237 = 9). No significance nor tendencies were seen within 1 hour.

The alcohol drinking average of the third week of drinking and testosterone elevation (2 hours after alcohol injection) displayed a positive correlation in experiment 1 (lights on at 6 a.m., r = 0.469, p = 0.288, n = 7) for AA and negative correlation (r = -0.553, p = 0.155, n = 8) for Wistars rats (correlational comparison Z = 1.687, p = 0.092) (Fig.3). Also, in experiment 2, corresponding correlations emerged with a positive r = 0.480, p = 0.276, n = 7 in AA and negative r = -.542, p = 0.132, n = 9 in Wistar rats (correlational comparison Z = 1.751, p = 0.080, trend). The combined correlational significance is $p < 0.05$ for AA compared with Wistar, regarding 3rd drinking week compared with testosterone change 2 hours after alcohol injection (Fig. 3).

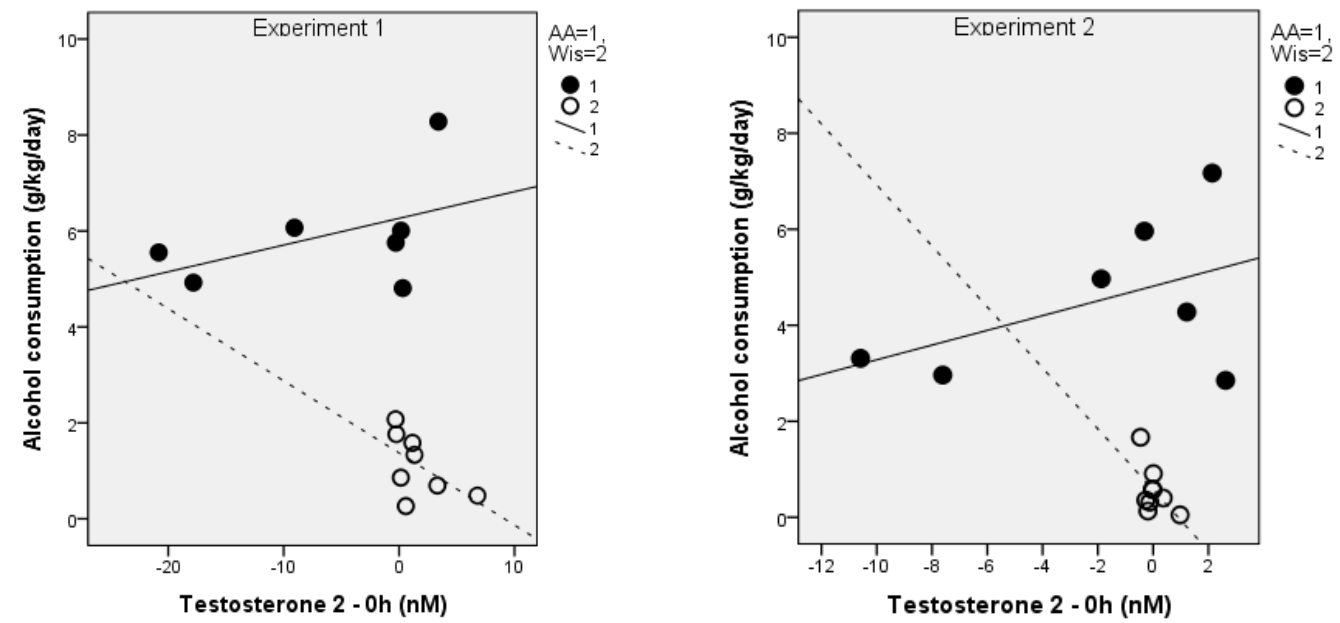

Fig. 3. AA and Wistar rat correlations between voluntary alcohol consumption and testosterone change from 0 to 2 hours of alcohol intoxication (by a dose of 1.5 g/kg). Experiment 1 was with a 12 h / 12 h light/dark cycle with lights on at 6 a.m. ($r = 0.469$, $p = 0.288$ and $r = -0.553$, $p = 0.155$ for AA and Wistars, respectively; correlational comparison $Z = 1.687$, $p = 0.092$) and experiment 2 with reversed light cycle, where lights went on at 6 p.m. ($r = 0.480$, $p = 0.276$ and $r = -.542$, $p = 0.132$ for AA and Wistars, respectively; correlational comparison $Z = 1.751$, $p = 0.080$). Overall combined correlational significance is $p < 0.05$ for AA compared with Wistars.

With regards to the effect of nandrolone, no significant correlational comparisons and correlations between basal corticosterone levels and testosterone changes during 1 and 2 hours were observed in the AA and Wistar rats. Neither were there any significant correlational comparisons and correlations between voluntary alcohol consumption and testosterone change at 2 hours post injection. However, the alcohol drinking average of the

third week of drinking and testosterone elevation, 1 hour after alcohol injection, displayed a positive correlation in experiment 1 ($r = 0.624$, $p = 0.134$, $n = 7$) for AA and negative correlation ($r = -0.481$, $p = 0.159$, $n = 10$) for Wistars rats (correlational comparison $Z = 2.004$, $p = 0.045$). Also, in experiment 2, corresponding correlations emerged with positive $r = 0.594$ ($p = 0.160$, $n = 7$) in AA and negative $r = -.149$ ($p = 0.681$, $n = 10$) in Wistar rats (correlational comparison $Z = 1.331$, $p = 0.183$). Thus, the combined significance (experiments 1 and 2) of the correlational differences between positive and negative correlations display $p < 0.05$ for AA controls compared with the AA-ND group.

3.3 AA versus Wistar rats and the effect of Benzyl Alcohol (substudy 4)

In substudy 4 during control situations (no BA), the high-drinking AA rats displayed a significant positive correlation ($r = 0.749$, $p = 0.013$, $n = 10$) between testosterone elevations at 2 hours post alcohol injection (after a dose of 1.5 g/kg) and the basal corticosterone levels (Fig. 4). The low-drinking Wistar rats, on the other hand, displayed a negative correlation ($r = -0.401$, $p = 0.284$, $n = 9$) between decreasing testosterone levels and basal corticosterone level during the 2 hours at control situations. A significant correlation difference between the lines was observed ($Z = 2.508$, $p = 0.012$). However, no significant corresponding correlations at 1 hour post alcohol injection were observed. Also, no significant effects by BA for a correlational difference, regarding testosterone elevation and corticosterone, were observed in, or between, AA and Wistar rats.

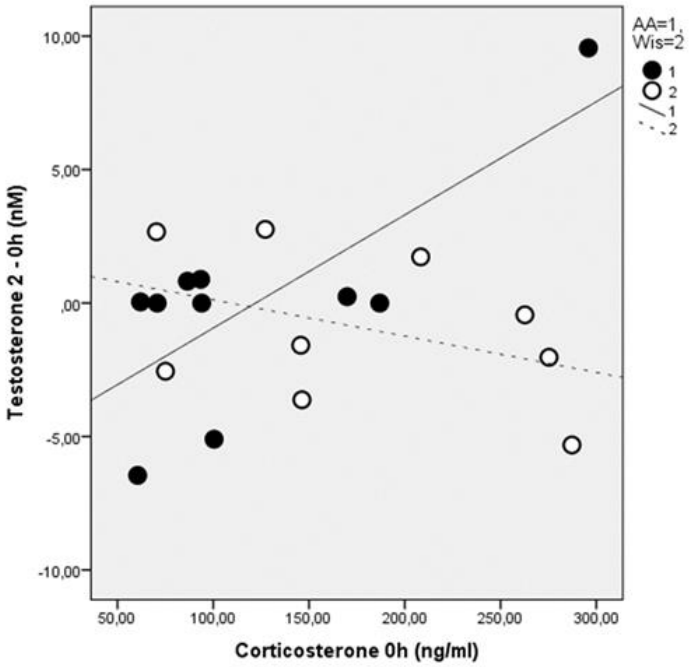

Fig. 4. Correlations between change in testosterone concentrations at 2 hours post alcohol injection (by a dose of 1.5 g/kg) and basal corticosterone levels (0h) in AA and Wistar rats ($r = 0.749$, $p = 0.013$ and $r = -0.401$, $p = 0.284$ for AA and Wistars, respectively; correlational comparison $Z = 2.508$, $p = 0.012$).

During the control situations (no BA) a positive correlation ($r = 0.433$, $p = 0.212$, $n = 10$ for AA and negative correlation $r = -0.539$, $p = 0.155$, $n = 9$ for Wistar rats; correlational comparison $Z = 1.917$, $p = 0.055$) was found between the alcohol drinking average of the third week of voluntary alcohol drinking and testosterone elevation at 2 hours after alcohol injection. At 1 hour post alcohol injection the positive significant correlation was $r = 0.638$, $p = 0.047$, $n = 10$ for AA and the negative correlation was $r = -0.206$, $p = 0.600$, $n = 9$ for Wistars (correlational comparison $Z = 1.727$, $p = 0.084$).

No significant effects by BA for a correlational difference between the control and

BA groups, regarding alcohol drinking and testosterone elevation, were observed in AA and Wistar rats.

3.4 Summary of main results

Tendencies and significance for positive correlations between basal corticosterone and subsequent alcohol-mediated testosterone increase were displayed by the high-drinking AA rats in substudy 1 ($p = 0.073$) and substudy 4 ($p = 0.013$) (Table 1). In contrast, the low-drinking ANA rats displayed a significant negative correlation in substudy 1 ($p = 0.029$). Tendency and significance for correlational differences (Z) were displayed in substudy 1 ($p = 0.016$), substudy 3, experiment 2 ($p = 0.069$) and substudy 4 ($p = 0.012$). Basal corticosterone levels did not significantly differ between high and low alcohol drinking rats in the different substudies. There were also no significant correlations between basal corticosterone and subsequent voluntary alcohol consumption. The high-drinking F2 population could not be included in the calculations because this population was a mixture of both high- and low-drinking rats.

Regarding the association between alcohol-mediated testosterone changes and subsequent voluntary alcohol drinking, the low-drinking F2 rats displayed significant negative correlation in substudy 2 ($p = 0.018$). In addition, tendencies for correlational differences (Z) were displayed in substudy 3, experiment 1 ($p = 0.092$) and experiment 2 ($p = 0.080$), and substudy 4 ($p = 0.055$). Already the fact that all 3 positive correlations were related to high drinking and that all 4 negative correlations were related to low alcohol drinking shows that the overall correlational significance is $p = 0.0078$ (seven independent correlations in the expected directions).

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Table 1. Summary of correlational results

Substudies	$T_{2\text{-}0h}$/Corticosterone$_{0h}$	$T_{2\text{-}0h}$/Alcohol drinking
1		
(0.75g/kg, 1h)		
AA (N=24)	rho = .373, p = .073	
ANA (N=21)	rho = -.362, p = .107	
Significance	Z = 2.401, p = .016	
(1.5g/kg, 2h)		
AA (N=24)	rho = NS	
ANA (N=21)	rho = -.466, p = .029	
Significance	Z = NS	
2		
(2.0g/kg, 2h)		
F2 low (N=37)	r = .082, p = .630, NS	r = -.386, p = .018
3, Experiment 1		
(1.5g/kg, 2h)		
AA (N=7)	r = NS	r = .469, p = .288, NS
Wistar (N=8)	r = NS	r = -.553, p = .155, NS
Significance	Z = NS	Z = 1.687, p = .092
3, Experiment 2		
(1.5g/kg, 2h)		
AA (N=7)	r = .606, p = .149, NS	r = .480, p = .276, NS
Wistar (N=9)	r = -.439, p = .237, NS	r = -. 542, p = .132
Significance	Z = 1.818, p = .069	Z = 1.751, p = .080
4		
(1.5g/kg, 2h)		
AA (N=10)	r = .749, p = .013	r = .433, p = .212, NS
Wistar (N=9)	r = -.401, p = .284, NS	r = -.539, p = .155
Significance	Z = 2.508, p = .012	Z = 1.917, p = .055

$T_{2\text{-}0}$ = alcohol-mediated testosterone increase during 2 hours (except for substudy 1, also with 1 hour's elevation). Tendency ($p \leq 0.10$) and significance ($p \leq 0.05$) for the different substudies are displayed. NS = no significance nor tendency ($p > 0.10$). Z = correlational difference between high (AA) and low (ANA, F2, Wistar) voluntary alcohol consumption.

Altogether, significance and tendencies in both testosterone change vs basal corticosterone as well as testosterone change vs voluntary alcohol drinking were only displayed in the expected directions.

Although, basal testosterone levels in general were higher in AA rats compared with ANA (substudy 1, Apter and Eriksson 2003) and Wistars (substudy 4, Etelälahti and Eriksson, 2014), no correlational evidence was observed between basal testosterone levels and subsequent voluntary alcohol consumption.

4. Discussion

A number of studies have shown an association between stress, activated HPA-axis and excessive alcohol consumption (Ciccocioppo et al., 2006; Fahlke et al., 1994; Gianoulakis, 1998; Koob, 2013; Pohorecky, 1990, 1991; Roman and Nylander, 2005; Spanagel et al., 2014; Stephens and Wand, 2012; Zhou and Kreek, 2014; Zorilla et al., 2014). Our earlier studies have indicated that stress and an activated HPA-axis may be the initial step, which linked to the HPG-axis, could be the crucial step on the pathway towards alcohol dependence (Apter and Eriksson, 2003, 2006). The results of our most recent studies (Etelälahti et al., 2011; Etelälahti and Eriksson, 2013, 2014), confirm our earlier findings, according to which alcohol-mediated testosterone elevation is associated with increased alcohol drinking or, vice versa, that an alcohol-mediated testosterone decrease is associated with diminished drinking. Since the nature of these associations is still unclear, our aim in the present study was to more closely investigate and identify these associations.

The novel results of the present substudies 1, 3 (experiment 2) and 4 indicate that basal corticosterone levels may significantly correlate positively or negatively with

subsequent alcohol-mediated testosterone elevations or reductions, respectively. The fact that basal corticosterone concentrations correlates with subsequent changes in testosterone eliminates the possibility that the direction of the correlation would be from testosterone change to corticosterone. Also, it seems improbable that the corticosterone-testosterone correlation could be independently caused by a third factor.

Another novel finding in the present substudies 2-4 is that alcohol-mediated changes in testosterone, elevations or reductions, significantly correlated with subsequent voluntary alcohol consumption, high and low respectively. As testosterone changes correlate with subsequent voluntary alcohol consumption the possibility that the direction of the correlation would be from alcohol drinking to testosterone change is eliminated. Again, it seems unlikely that the correlation between alcohol consumption and testosterone could independently be caused by a third factor. The fact, that the ND treatment caused a significant positive correlation between reduced voluntary alcohol consumption and reduced testosterone levels in AA rats, support the notion of a more universal effect, instead of just genetic rat strain differences.

The results of the present study raise some fundamental questions. Our hypothesis has been (Apter and Eriksson, 2003, 2006; Etelälahti et al., 2011; Etelälahti and Eriksson, 2013, 2014) that alcohol intake, by activating the HPA axis and subsequently causing testosterone elevation, is reinforcing because of β-Endorphin (BEP) elevation, as a consequence of the complex feedback system associated with the HPG homeostasis. This hypothesis is supported by earlier data on the reinforcing properties of testosterone and other androgens (Alexander et al., 1994; Arnedo et al., 2000; Dai et al., 2002; de Beun et al., 1992; Frye, 2007; Wood, 2004) and BEP elevations during alcohol intoxication (Adams and Cicero, 1991; Barret et al., 1987; Frias et al., 2000; Frias et al., 2002; Gianoulakis et al., 1989; Kornet et al., 1992; Patel and Pohorecky, 1989; Schulz et al., 1980; Thiagarajan et al., 1989; Zalewska-Kaszubska et al.,

2006). Our present data confirms the reinforcing role of the HPA-HPG axes. However, in addition to the role of the testosterone elevation, also other mechanisms should be considered. The most prominent players are stress and HPA activation with their own subsequent pathways. Also, the role of reinforcing testosterone may have other routes than the BEP pathway (direct or indirect), such as by the activities of metabolites of testosterone acting on GABA(A)/benzodiazepine receptor in connection to the dopamine pathway in nucleus accumbens (Frye, 2007). The roles and interactions of these different mechanisms, or other related mechanisms, are still to be resolved.

The second part of proposed key questions is related to the alcohol-mediated testosterone decrease, subsequently also decreasing voluntary alcohol consumption. It is easily conceived that a testosterone decrease may cause disinforcement (term introduced by Harzem and Miles, 1978), also including attenuated mood, stress, depression and other negative effects (Kaldewaij et al., 2016; Zitzmann and Nieschlag, 2001), which may decrease voluntary alcohol consumption. Our present data also confirms the disinforcing role of the HPA-HPG axes. The crucial question here is, what mechanisms explain our results, which show, on one hand that basal levels of corticosterone correlate positively with alcohol-mediated testosterone elevation and subsequent increased voluntary alcohol consumption. Yet, on the other hand, that basal corticosterone levels also correlate positively with an alcohol-mediated testosterone decrease and subsequent reduction in alcohol intake. Clearly it can be concluded that genetic and/or situational factors, which still remain to be elucidated, are likely to exist. However, although the present study is limited to only one rat line (AA) with high alcohol consumption, the effect of ND, decreasing testosterone and alcohol consumption in the AA rats, demonstrate the possible involvement of a situational factor.

A limitation of the present study is that the degree of stress has not been assessed. However, the fact that the original high-alcohol drinking AA rat populations are known to be

stressed by individual housing in contrast to the low-alcohol drinking ANA rats (Apter and Eriksson, 2006) may relate to the above addressed questions. An additional limitation of the present study is the low number of rat populations investigated (one high-drinking AA strain and three low-drinking ANA, F2 and Wistar strains).

In conclusion, the present study displays novel results, according to which a stress-activated HPA axis correlates positively with testosterone elevation during alcohol intoxication (causing reinforcement), which in turn correlates positively with subsequent increased voluntary alcohol consumption in AA rats. Vice versa, non-activated HPA axis seems to correlate negatively with alcohol-mediated testosterone elevation (causing testosterone reduction and disinforcement) and subsequent low-alcohol drinking in ANA, F2 and Wistar rats. In addition, the present results show that alcohol-mediated testosterone changes may also, independently of the HPA axis, correlate with voluntary alcohol drinking, which indicates the existence of a genetic factor. Thus, the impact of the HPA-axis may be more the result of a situational stress factor than a constitutional factor with or without genetic influence. In the future, stress-related studies should more often take into account both the HPA-and the HPG-axes. The relevance of the present results should also be investigated in a human setting.

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