Prenatal androgen receptor activation determines adult alcohol and water drinking in a sex-specific way

Sabine E. Huber[1], Iulia Zoicas[1], Martin Reichel[1,3], Christiane Mühle[1], Christian Büttner[2], Arif B. Ekici[2], Volker Eulenburg[4], Bernd Lenz[1†], Johannes Kornhuber[1†] & Christian P. Müller[1,†]

Department of Psychiatry and Psychotherapy, University Clinic, Friedrich-Alexander-University Erlangen-Nuremberg, Germany[1], Institute of Human Genetics, Friedrich-Alexander-University Erlangen-Nuremberg, Germany[2], Department of Nephrology and Hypertension, Friedrich-Alexander-University Erlangen-Nuremberg, Germany[3] and Institute of Biochemistry, Friedrich-Alexander-University Erlangen-Nuremberg, Germany[4]

ABSTRACT

Alcohol use disorders are major psychiatric disorders. Correlational studies in humans suggested organizational hormonal effects during embryonic development as a risk factor for adult alcohol dependence. Permanent changes can be induced by the activity of sex hormones, like testosterone. Here, we demonstrate a relationship between prenatal androgen receptor (AR)-activation and adult alcohol as well as water drinking in mice in a sex-dependent fashion. Prenatal AR inhibition using the antagonist flutamide decreased adult male alcohol consumption. In contrast, prenatal AR activation by dihydrotestosterone (DHT) led to an increase in adult alcohol consumption in females. These effects were different in adult water drinking, flutamide increased water consumption in females and DHT increased water consumption in males. Prenatal flutamide reduced locomotion and anxiety in adult males but was ineffective in females. We found that prenatal AR activation controls adult levels of monoaminergic modulatory transmitters in the brain and blood hormone levels in a sex-specific way. RNA-Seq analysis confirmed a prenatal AR mediated control of adult expression of alcohol drinking-related genes like *Bdnf* and *Per2*. These findings demonstrate that prenatal androgen activity is a risk factor for the establishment of alcohol consumption in adults by its organizational effects.

Keywords Alcohol, prenatal treatment, risk factor, sex.

Correspondence to: Christian P. Müller, Section of Addiction Medicine, Department of Psychiatry and Psychotherapy, University Clinic, Friedrich-Alexander-University Erlangen-Nuremberg, Schwabachanlage 6, Erlangen 91054, Germany. E-mail: christian.mueller@uk-erlangen.de
† These authors contributed equally.

INTRODUCTION

Alcohol is a widely consumed psychoactive drug which can lead to alcohol use disorders. There is a gender bias in alcohol consumption with 63 percent of males and 58 percent of females regularly drinking alcohol. The rate for alcohol dependence is about twice as high for males (~10.4 percent) than for females (~5.7 percent; SAMSHA, 2011, EMCDDA, 2012). Gender is a known risk factor for alcohol consumption and addiction. Another risk factor can be the prenatal environment, which is crucially dependent on the hormonal exposure during the embryonic development. It has been shown that the prenatal hormonal milieu has organizational effects on the brain (Phoenix *et al.* 1959; Arnold & Breedlove 1985; Lenz *et al.* 2012). Organizational effects are permanent effects that occur early in the development and are exerted by sex hormones, like testosterone or estrogen (Arnold & Breedlove 1985; Lenz *et al.* 2012). These permanent effects are the base for sex-specific behaviors at adult age including substance use and abuse (Lenz & McCarthy 2010; Lenz *et al.* 2012).

A recent study in mice manipulated the embryonic environment during the time of digit cartilage development by administering either the hormone dihydrotestosterone (DHT) or an androgen receptor (AR) antagonist. This led to changes in the second-to-fourth finger (2D:4D) ratio (Zheng & Cohn 2011). This 2D:4D ratio is frequently used as indicator of prenatal sex-hormone exposure (Kornhuber *et al.* 2011) and is considered to reflect androgen levels *in utero*. Correlation studies in humans suggest that a lower 2D:4D ratio, and, thus, a higher prenatal testosterone exposure, predicts adult physiological and behavioral phenotypes, like aggressive

behavior (Bailey & Hurd 2005), alcohol-abuse (Kornhuber *et al.* 2011; Han *et al.* 2016) and laterality (Manning *et al.* 2000). In humans, it has also been shown that maternal stress, alcohol consumption and smoking behavior during pregnancy increase the offspring's intrauterine androgen load (Lenz *et al.* 2017). However, a causal relationship between prenatal AR activity and adult alcohol drinking behavior has not been shown. Here, we asked whether prenatal AR activation would determine adult alcohol drinking and related behaviors, such as emotional and social behavior, which predispose for high alcohol consumption. We also investigated by which organizational effects in the brain and on hormonal control, behavioral effects could be mediated in the adult organism. Given the strong sex bias in alcohol drinking, we hypothesized that these effects would differ between males and females.

MATERIAL AND METHODS

Animals

Polygamous breeding pairs (2♀:1♂) were created with 10-week-old male and 8-week-old female CD 1 mice (Charles River Sulzfeld, Germany). Male and female offspring were group-housed in standard macrolon cages with food and water available *ad libitum*, until the start of the drinking study. During the drinking study, they were single housed, but with olfactory and visual contact to neighboring mice. Mice were kept under standard laboratory conditions (12:12 hour light/dark cycle, lights on at 7 am, room temperature: 19–22°C, humidity 55 ± 10 percent). Experiments were performed during the light phase, between 9 am and 4 pm, in accordance with the National Institutes of Health guidelines for the humane treatment of animals and the European Communities Council Directive (86/609/EEC) and were approved by the local governmental commission for animal health.

Prenatal treatment

Dams were checked daily for a vaginal plug. The occurrence of a plug was considered as embryonic day 0.5 (E0.5). The prenatal treatment was administered once daily to pregnant females on the days E12.5 – E15.5, when they have a higher sensitivity to the actions of sex hormones (Zheng & Cohn 2011), as two intraperitoneal (i.p.) injections spaced 30 minutes apart. Male and female offspring were prenatally treated with four different treatments: (1) vehicle (corn oil (Sigma Aldrich) with 1 percent ethanol)—vehicle; (2) flutamide (120 mg/kg, Sigma Aldrich)—DHT (abbreviation used in treatment description; 2 mg/kg, Sigma Aldrich); (3) vehicle—DHT; and (4) flutamide—vehicle. The AR antagonist flutamide (Neri *et al.* 1972) and the active metabolite of testosterone, DHT, were dissolved in vehicle. Both, testosterone and DHT, bind to the AR, but DHT has a higher potency. Based on the study of Zheng & Cohn (2011), we used DHT for prenatal AR activation and CD1 mice.

Alcohol drinking

A water drinking baseline was established in adult, alcohol-naïve prenatally treated mice. Following that, alcohol drinking was tested in these mice using a two-bottle free-choice drinking paradigm with increasing alcohol concentration (n = 10–15/group). Following an alcohol abstinence period, a taste preference test was performed using the two-bottle free-choice test (Stacey *et al.* 2012; Easton *et al.* 2013; see also: Supplementary information, SI).

Emotional and social behavior and memory

Naïve prenatally treated mice were tested at adult age for their emotional behavior using the open field (OF) and elevated plus maze (EPM) test (n = 9–15/group) as previously described (Easton *et al.* 2011; Süß *et al.* 2015). Mice were also tested in the social approach and novel object recognition test (n = 6–10/group) as previously described (Zoicas *et al.* 2016; for details see: SI).

ELISA

In order to test whether prenatal AR activity exerted its effect on adult behavior by organizational effects on hormone levels, we measured dihydrotestosterone (spelled out when measured) and corticosterone in adults. Blood of adult alcohol naïve (n = 8–19/group, group-housed) and alcohol-experienced mice (n = 9–15/group; from drinking study above, single-housed) was collected, centrifuged (10 minutes, 2000 *g*, 4°C) and stored at −80°C. Total dihydrotestosterone levels were quantified in duplicates with the following kit according to the manufacturers' instructions: 5alpha-dihydrotestosterone (DHT) ELISA DB52021 (2 µl male and 20 µl female mice serum, intra-assay-coefficients of variation (cv) 10 percent, inter-assay-cv 10 percent, IBL International, Hamburg, Germany). Total corticosterone levels were quantified in duplicates with the following kit according to the manufacturer's instructions: Corticosterone ELISA RE52211 (3 µl male and 3 µl female mice serum, intra-assay-coefficients of variation (cv) 7 percent, inter-assay-cv 13 percent, IBL International, Hamburg, Germany).

Post mortem neurochemistry

In order to test whether prenatal AR activity exerts its effects via organizational effects on monoamine systems that are involved in alcohol dependence (Spanagel 2009), we measured monoamine tissue levels in the

brain of alcohol naïve (group-housed) and alcohol drinking (single housed; from drinking study above) mice. For estimation of brain tissue monoamine levels, mice were sacrificed after free-choice alcohol consumption (n = 9–15/group) or water drinking (n = 7–21/group), quickly decapitated and the brain was harvested and snap frozen with dry ice. For neurochemical analysis, three brain areas known to be involved in the rewarding and reinforcing effects of drugs (McBride, Murphy & Ikemoto 1999) were dissected: the prefrontal cortex (PFC), hypothalamus (HYP) and ventral striatum (VS; Fig. S1). The tissue was homogenized and analyzed by HPLC-EC as previously described (Pum *et al.* 2008; Nuber *et al.* 2013; Deusser *et al.* 2015).

RNA isolation and RNA-Seq

We found that prenatal AR modulation had a strong effect on adult male and female alcohol and water drinking behavior. To further elucidate the organizational mechanisms underlying this relationship, we measured mRNA expression using RNA-Seq in the VS, a brain area important for alcohol and homeostatic drinking (Spanagel 2009). Prenatally treated alcohol-naïve mice were tested (Veh-Veh ♂ (n = 5) versus Flu-Veh ♂ (n = 5) and Veh-Veh ♀ (n = 5) versus Veh-DHT ♀ (n = 5)).

Areas of interest were dissected from 1-mm coronal sections according to anatomical coordinates taken from the Franklin & Paxinos (1997) mouse brain atlas. Total RNA from VS was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturers' instructions. Sequencing libraries were prepared, sequenced and aligned. Absolute read counts were determined, and differential expression analysis was performed (for details see: SI). *P* values were corrected for false discovery rate (FDR) using the Benjamini–Hochberg procedure.

Statistics

All quantitative data were expressed as mean ± SEM. Data were analyzed using two-way ANOVAs followed by preplanned comparisons (Ramsey 1993) using Fishers's LSD tests with Bonferroni-correction when appropriate. We compared prenatal treatment groups against the Veh-Veh control group. For the drinking study, every single day was considered, and ANOVA is based on single day values. Treatment combination was analyzed as between-subject and alcohol concentration as within-subject factor. A p value of <0.05 was considered statistically significant. Data were analyzed using IBM SPSS statistics Version 21 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Prenatal AR modulation increases baseline water drinking differently in males and females

We found that water intake in prenatally DHT-treated males was elevated compared to control group ($p < 0.05$; Fig. 1a) while it remained the same in the other treatment groups. In the females, however, changing the prenatal hormonal milieu increased water intake in all groups compared to control ($p < 0.05$; Fig. 1b).

Prenatal androgen receptor antagonism inhibits adult alcohol drinking in males

Adult male mice treated prenatally with the AR antagonist, flutamide, drank significantly less alcohol than vehicle controls ($p < 0.001$, Fig. 2a). They also developed a significantly reduced alcohol preference over water ($p < 0.05$, Fig. 2c). The effects on alcohol consumption as well as preference could be partially reversed by DHT, which had no effect alone ($p > 0.05$). These findings

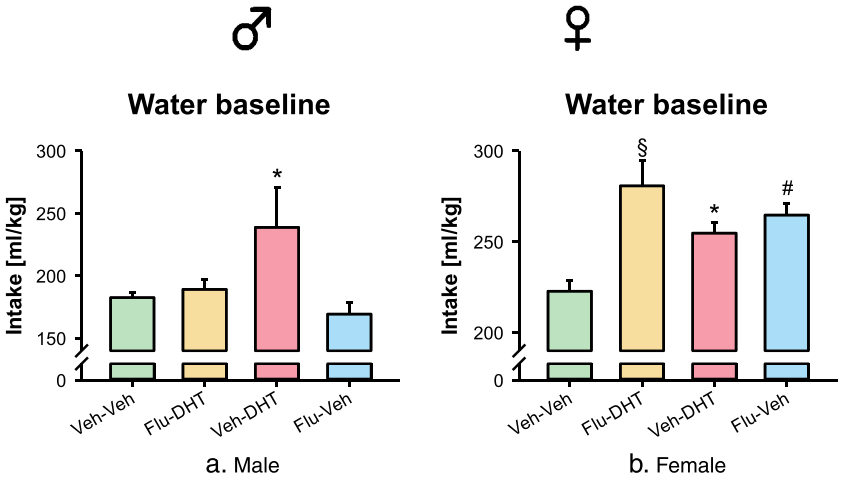

Figure 1 Effects of prenatal androgen receptor (AR) manipulation on adult water drinking in male and female mice in a two-bottle free-choice paradigm when both bottles contained water. (a) Water consumption of males treated prenatally with DHT is increased compared to the control group (Treatment $F_{3,158} = 4.148$, $p = 0.007$). (b) Baseline water drinking in females is increased in all treatment groups compared to control (Treatment $F_{3,158} = 7.890$, $p < 0.001$). Error bars show mean ± SEM. * $p < 0.05$, # $p < 0.01$, § $p < 0.001$ versus control

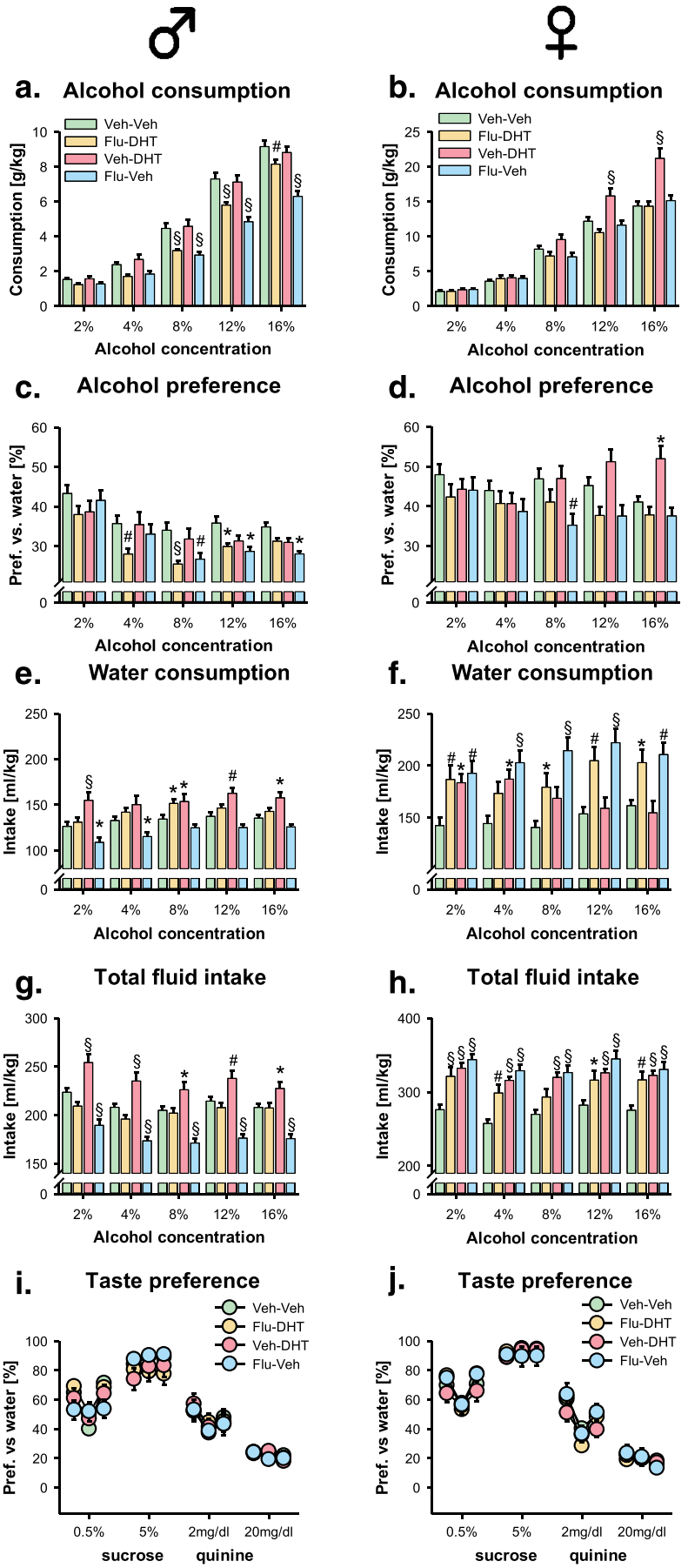

Figure 2 Effects of prenatal androgen receptor (AR) activation or inhibition on adult alcohol and water drinking behavior in male and female mice. (a) Alcohol consumption is reduced in flutamide-treated males (Treatment: $F_{3,1060} = 49.614$, $p < 0.001$; dose: $F_{4,1060} = 549.108$, $p < 0.001$; interaction: $F_{12,1060} = 5.158$, $p < 0.001$). (b) Increased alcohol consumption in dihydrotestosterone (DHT)-treated females ($p < 0.001$; treatment: $F_{3,1045} = 25.016$, $p < 0.001$; dose: $F_{4,1045} = 378.429$, $p < 0.001$; interaction: $F_{12,1045} = 5.575$, $p < 0.001$). (c) Reduced preference in the flutamide-treated males (treatment: $F_{3,1060} = 2.684$, $p < 0.001$; dose: $F_{4,1060} = 21.660$, $p < 0.001$; interaction: $F_{12,1060} = 1.182$, $p = 0.290$). (d) Increased preference in the DHT-treated females (treatment: $F_{3,1045} = 10.725$, $p < 0.001$; dose: $F_{4,1045} = 0.959$, $p = 0.429$; interaction: $F_{12,1045} = 1.626$, $p = 0.079$). (e) Prenatal treatment with DHT increased water consumption in male mice (treatment: $F_{3,1060} = 39.269$, $p < 0.001$; dose: $F_{4,1060} = 3.996$, $p = 0.003$; interaction: $F_{12,1060} = 0.547$, $p = 0.885$). (f) Prenatal flutamide-treatment increased water consumption in females (treatment: $F_{3,1045} = 28.617$, $p < 0.001$; dose: $F_{4,1045} = 0.590$, $p = 0.670$; interaction: $F_{12,1045} = 1.511$, $p = 0.114$). (g) Total fluid intake was increased with DHT- and decreased with flutamide-treatment (treatment: $F_{3,1060} = 99.558$, $p < 0.001$; dose: $F_{4,1060} = 7.598$, $p < 0.001$; interaction: $F_{12,1060} = 0.667$, $p = 0.785$). (h) Total fluid intake was increased in all treatment groups compared to control (treatment: $F_{3,1045} = 50.464$, $p < 0.001$; dose: $F_{4,1045} = 3.842$, $p < 0.01$; interaction: $F_{12,1045} = 0.353$, $p = 0.979$). (i) Prenatal AR activity had no effect on adult preference of sweet (0.5 and 5 percent sucrose) and avoidance of bitter (2 and 20 mg/dl quinine) solutions in males or (j) females ($p > 0.05$). Error bars show mean ± SEM. * $p < 0.05$, # $p < 0.01$, § $p < 0.001$ versus control

suggest that reduced prenatal AR activation protects males from adult alcohol drinking.

Prenatal androgen receptor activation boosts adult alcohol drinking in females

In female offspring, AR activation by DHT significantly enhanced adult alcohol drinking and alcohol preference versus water ($p < 0.01$, Fig. 2b,d). Pre-treatment with flutamide abolished these effects. Treatment with flutamide alone decreased alcohol preference versus water at a medium dose of alcohol (8 vol. percent; $p < 0.05$, Fig. 2d). These findings suggest that enhanced prenatal AR activation boosts adult alcohol drinking in females.

Prenatal androgen receptor activation enhances adult water consumption in males

Adult water consumption was regulated by prenatal AR modulation in a different way than alcohol drinking. In males, prenatal AR activation by DHT significantly enhanced water consumption ($p < 0.05$, Fig. 2e). Males treated prenatally with flutamide alone showed a decreased total fluid intake ($p < 0.001$), while DHT-treated males showed an increase in total fluid intake ($p < 0.05$, Fig. 2g). These findings suggest a prenatal AR control on adult water drinking in males.

Prenatal androgen receptor antagonism boosts adult water consumption in females

In females, prenatal AR antagonism by flutamide significantly enhanced adult water consumption ($p < 0.05$, Fig. 2f). Additionally, we found an increase in all treatment conditions versus the control group in their total fluid intake ($p < 0.05$, Fig. 2h). These findings suggest a prenatal control of adult water drinking in females that dissociates from males.

Prenatal androgen receptor activity and adult taste preference

We found no difference between treatments and sex in the preference of sucrose or the avoidance of quinine at adult age ($p > 0.05$; Fig. 2i,j). These findings suggest that prenatal AR modulation has no influence on adult preference of sweet taste or avoidance of bitter taste. Thus, altered taste preference cannot account for the observed effects on alcohol-drinking behavior.

Prenatal androgen receptor activity has no effect on adult liver alcohol dehydrogenase activity

The activity of the liver alcohol dehydrogenases was not significantly different between groups (Fig. S2A–B), indicating that alcohol metabolism was not affected by prenatal AR modulation.

Prenatal dihydrotestosterone and flutamide treatment alter locomotion and anxiety in males

We found that prenatal AR antagonism led to a reduced locomotion in males at adult age. This was observed in the OF test for locomotion in the whole arena ($p < 0.01$, Fig. 3a) as well as for locomotion in the center zone ($p < 0.05$, Fig. 3e). There was also a lower number of entries into the center zone of the OF ($p < 0.05$, Fig. 3c). This effect was canceled out by DHT. The flutamide-DHT-treated males showed no changes in locomotor activity. Interestingly, activation of prenatal AR also led to a reduced locomotion of the males in the OF ($p < 0.01$, Fig. 3a). We observed a general attenuation of the locomotor activity, which might explain the observed effects. The time spent in the center of the OF (data not shown) was not affected by prenatal treatment, suggesting that emotional behavior is not affected.

In the EPM, we also found a reduced locomotor activity in the flutamide-treated males ($p < 0.05$, Fig. 3i). There was an increase in time spent on the open arms in the flutamide group ($p < 0.05$) and a trend in the same direction in the DHT treated males (Fig. 3g). These findings suggest that prenatal AR antagonism has a tendency for anxiolytic effects in males and seems to interfere with locomotor activity.

Prenatal treatment had no effect on locomotion or anxiety in females

There were no significant differences in the OF test in either locomotion or center entries in the females at adult age ($p > 0.05$, Fig. 3b,d,f). Also in the EPM, prenatal treatment had no effect in females at adult age ($p > 0.05$, Fig. 3h,j). These findings suggest that neither emotional behavior nor locomotor activity in females is influenced by prenatal AR activity. No effect on social preference and social recognition was found in adult males and females (Fig. S3A–D).

Impaired object recognition in adult females, but not in males

Males showed a higher investigation of the *novel* versus the *same* object in the novel object recognition test, independent of prenatal treatment, reflecting object recognition and preference for novelty ($p < 0.05$, Fig. S3E). Whereas females of the control and Flu-DHT-treated group showed intact object recognition, DHT- as well as flutamide-treated females showed impaired object recognition, reflected by similar investigation of the *same* and the *novel* object ($p < 0.05$, Fig. S3F).

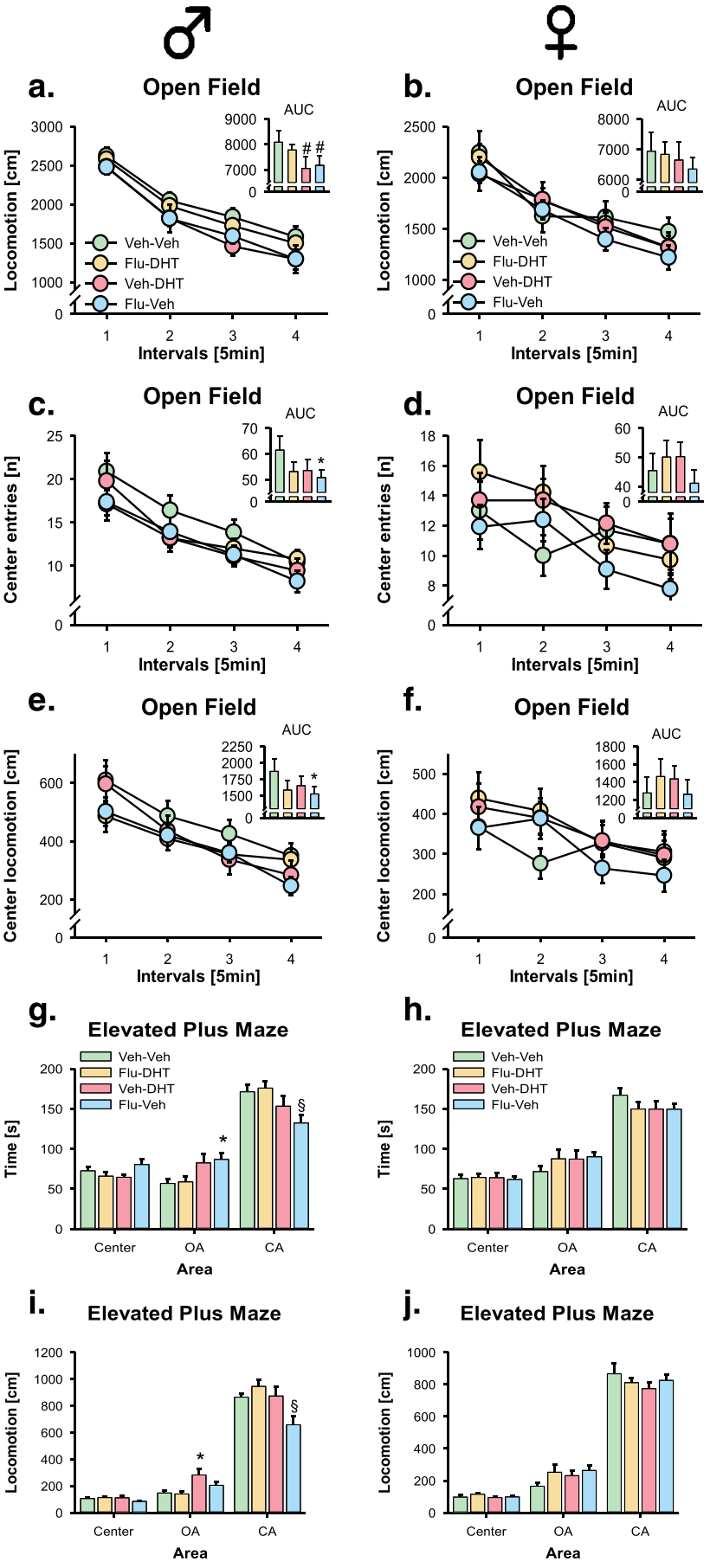

Figure 3 Changes in adult locomotion and anxiety in males after prenatal androgen receptor (AR) treatment, but not in females. (a) Locomotion in the open field (OF) is reduced (treatment: $F_{3,196} = 4.898$, $p = 0.003$; block: $F_{(3,196)} = 76.079$, $p < 0.001$; interaction: $F_{(9,196)} = 0.158$, $p = 0.998$) in the dihydrotestosterone (DHT)- and flutamide-treated males. (b) Locomotion in the maze is not altered in females (treatment: $F_{3,200} = 0.816$, $p = 0.486$; block: $F_{3,200} = 23.099$, $p < 0.001$; interaction: $F_{9,200} = 0.307$, $p = 0.972$). (c) The prenatal AR antagonism in males reduced the number of entries into the center (treatment: $F_{3,196} = 2.864$, $p = 0.038$; block: $F_{3,196} = 26.980$, $p < 0.001$; interaction: $F_{9,196} = 0.481$, $p = 0.886$). (d) In females, prenatal treatment did not affect the number of entries (treatment: $F_{3,200} = 1.782$, $p = 0.152$; block: $F_{3,200} = 4.373$, $p = 0.005$; interaction: $F_{9,200} = 0.655$, $p = 0.749$). (e) Center locomotion was reduced in the OF of flutamide-treated males (treatment: $F_{3,196} = 2.751$, $p = 0.044$; block: $F_{3,196} = 18.354$, $p < 0.001$; interaction: $F_{9,196} = 0.462$, $p = 0.899$). (f) Center locomotion was not influenced by prenatal AR activity in females (treatment: $F_{3,200} = 1.059$, $p = 0.368$; block: $F_{3,200} = 4.033$, $p = 0.008$; interaction: $F_{9,200} = 0.534$, $p = 0.848$). (g) The time spent on the open arm is enhanced in flutamide males (treatment: $F_{3,147} = 0.001$, $p = 1.000$; area: $F_{2,147} = 162.492$, $p < 0.001$; interaction: $F_{6,147} = 6.056$, $p < 0.001$), while it is unaffected by prenatal treatment in females (h; treatment: $F_{3,150} = 0.000$, $p = 1.000$; area: $F_{2,150} = 152.319$, $p < 0.001$; interaction: $F_{6,150} = 1.281$, $p = 0.269$). (i) The locomotion on the open arm is enhanced in DHT males and reduced in flutamide males in the closed arm (treatment: $F_{3,147} = 5.214$, $p = 0.002$; area: $F_{2,147} = 538.019$, $p < 0.001$; interaction: $F_{6,147} = 6.251$, $p < 0.001$), while it is unaffected by prenatal treatment in females (j; treatment: $F_{3,150} = 0.542$, $p = 0.654$; area: $F_{2,150} = 561.288$, $p < 0.001$; interaction: $F_{6,150} = 1.420$, $p = 0.210$). Error bars show mean ± SEM. * $p < 0.05$, # $p < 0.01$, § $p < 0.001$ versus control

Adult dihydrotestosterone and corticosterone serum levels in males

Neither prenatal AR activation nor inhibition had an effect on adult dihydrotestosterone serum levels in males drinking water (Fig. 4a). Alcohol consumption did not change dihydrotestosterone serum levels in the control group. However, alcohol intake reduced adult dihydrotestosterone levels in males treated prenatally with DHT ($p < 0.05$) or flutamide ($p < 0.001$).

In alcohol-naïve males, prenatal treatment reduced adult corticosterone levels compared to control ($p < 0.05$, Fig. 4c). Alcohol drinking had no effect on serum corticosterone in the control group. However, alcohol-experienced flutamide-treated males showed an increase in corticosterone level in comparison to alcohol-naïve flutamide-treated males ($p < 0.05$).

Adult dihydrotestosterone and corticosterone serum levels in females

In water-drinking females, prenatal AR activity had no effect on adult dihydrotestosterone serum levels (Fig. 4b). Alcohol consumption did not change dihydrotestosterone serum levels in the control group. However, alcohol

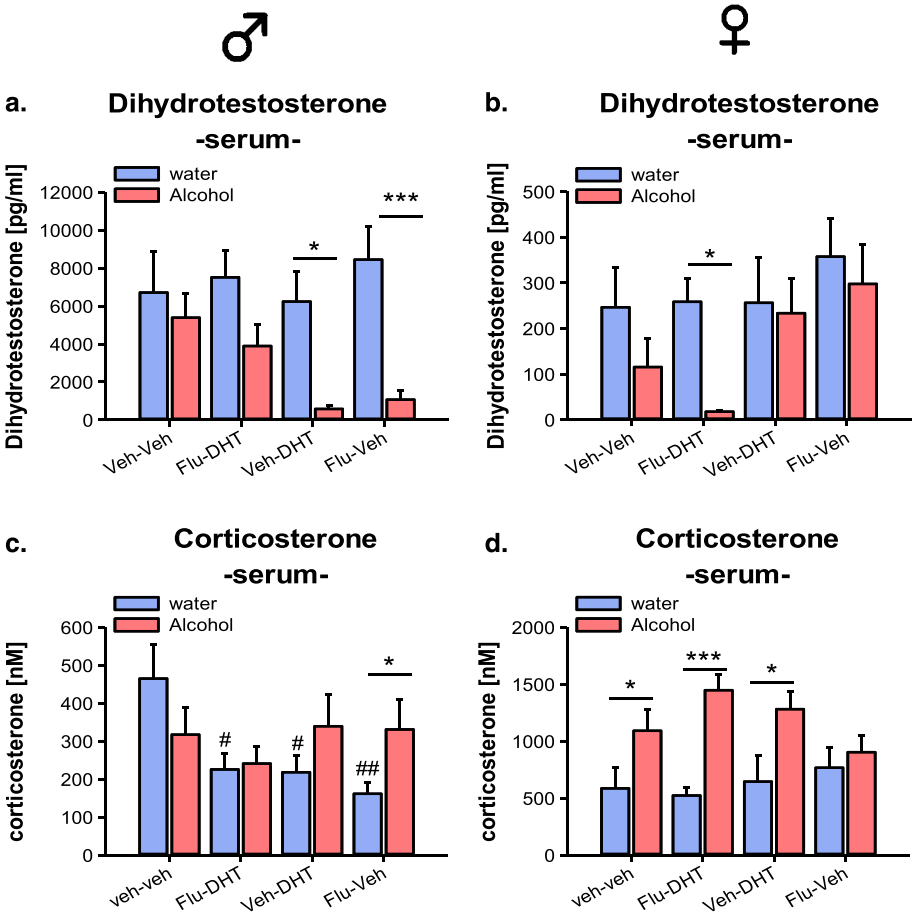

Figure 4 Effects of prenatal androgen receptor (AR) activation or inhibition on adult serum dihydrotestosterone and corticosterone levels before and after alcohol consumption. (a) Prenatal treatment did not influence adult dihydrotestosterone levels ($F_{3,103} = 1.078$, $p = 0.362$), but dihydrotestosterone response to alcohol ($F_{1,103} = 17.285$, $p < 0.001$; interaction $F_{3,103} = 1.450$, $p = 0.233$). dihydrotestosterone (DHT)- and flutamide-treated males had reduced dihydrotestosterone levels after alcohol consumption. (b) ANOVA showed prenatal treatment was significantly different in females ($F_{3,88} = 2.791$, $p = 0.045$), but we only saw an effect of alcohol ($F_{1,88} = 4.756$, $p = 0.032$) reducing dihydrotestosterone levels in the Flu-DHT-treated group (Interaction: $F_{3,88} = 0.906$, $p = 0.442$). (c) Serum corticosterone levels were affected by treatment ($F_{3,102} = 2.603$, $p = 0.056$), and preplanned comparisons revealed all groups to have reduced corticosterone levels compared to control. ANOVA revealed no alcohol effect on corticosterone ($F_{1,102} = 0.872$, $p = 0.353$; interaction $F_{3,102} = 2.514$, $p = 0.063$), but preplanned comparison revealed an increase in corticosterone levels after alcohol in the flutamide males. (d) Prenatal androgen receptor (AR) activity did not affect corticosterone levels in females ($F_{3,88} = 0.465$, $p = 0.708$), but alcohol consumption had an effect ($F_{1,88} = 21.516$, $p < 0.001$; interaction: $F_{3,88} = 2.109$, $p = 0.105$). Corticosterone levels were raised in all groups except the flutamide-treated females. Error bars show mean ± SEM. # $p < 0.05$, ## $p < 0.01$ versus control; * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ water versus alcohol

drinking reduced serum dihydrotestosterone levels in the Flu-DHT-treated females ($p < 0.05$).

Prenatal treatment did not change adult serum corticosterone levels in alcohol-naïve females (Fig. 4d). Alcohol drinking increased corticosterone levels in the control group, but not in the Flu-treated animals. Corticosterone levels were also increased following alcohol consumption in the DHT- and in the Flu-DHT-treated females ($p < 0.05$).

Prenatal androgen receptor activation changes monoamine levels in males

Here, we asked how a manipulation of the prenatal hormonal milieu would change monoamine homeostasis and how alcohol drinking would modulate this effect. We established a stable consumption of water or 16 vol. percent alcohol in a free-choice alcohol drinking paradigm. Animals were sacrificed, and tissue levels of dopamine (DA), serotonin (5-HT) and noradrenaline (NA) were measured in the PFC, HYP and VS. We found that the prenatal treatment had differential effects on adult monoamine levels depending on sex.

In males, prenatal treatment did not change DA levels of alcohol-naïve mice in PFC. Alcohol consumption in the control group decreased DA level ($p < 0.05$, Fig. 5a). In contrast, alcohol drinking resulted in an increased amount of tissue DA in flutamide-treated males ($p < 0.001$). 5-HT levels in the PFC of alcohol-naïve prenatally treated males were not different from vehicle-treated controls. Alcohol drinking did not change 5-HT levels significantly in the PFC of control animals. In prenatally treated males, no significant difference in 5-HT levels was observed following alcohol consumption compared to veh control (Fig. 5b). The prenatal treatment did not alter NA levels in PFC in alcohol-naïve males. However, the amount of NA was decreased following alcohol drinking in the control group ($p < 0.05$, Fig. 5c). Alcohol drinking did not affect adult NA levels in prenatally treated males.

In the HYP, prenatal treatment did not alter adult DA levels of alcohol-naïve males. Alcohol reduced levels of DA in the control group (Fig. 5d). However, this effect did not reach statistical significance for pairwise comparison. There were no differences in DA levels between the alcohol-experienced prenatally treated males. 5-HT levels in HYP of alcohol-naïve prenatally treated males did not differ. In the control group, 5-HT levels increased after alcohol drinking ($p < 0.05$, Fig. 5e). Levels of 5-HT were reduced in the alcohol-experienced males compared to control group ($p < 0.05$). NA levels in the HYP of DHT-treated alcohol-naïve males were increased ($p < 0.05$, Fig. 5f). NA levels stayed the same after alcohol drinking in control group. However, alcohol-experienced flutamide males had reduced NA levels compared to control males ($p < 0.05$).

In the male VS, DA levels were not significantly changed by prenatal treatment. While it seems that DA level was reduced in the control group following alcohol consumption, this was not significant. Visual inspection of the data suggests a decline in DA level in flutamide-treated males compared to control group (Fig. 5g); however, this difference did not reach statistical significance. DA levels in prenatally treated males were not different from control group following alcohol consumption (Fig. 5g). 5-HT levels in VS of alcohol-naïve prenatally treated males did not differ between treatment groups. Alcohol drinking had no effect on 5-HT levels in the veh-treated group. However, 5-HT levels of prenatally DHT- as well as flutamide-treated males were reduced following alcohol consumption ($p < 0.01$, Fig. 5h). NA levels in male VS were not altered by prenatal treatment nor by alcohol consumption ($p > 0.05$; Fig. 5i).

Prenatal androgen receptor activation changes monoamine levels in females

In adult females, prenatal treatment had no effect on DA levels in PFC of alcohol-naïve mice. Alcohol consumption in the control or prenatally treated females did not affect adult DA levels in the PFC (Fig. 6a). 5-HT levels in PFC were reduced in alcohol-naïve prenatally DHT- and flutamide-treated females. Alcohol drinking in the control females reduced 5-HT levels ($p < 0.001$, Fig. 6b). In the alcohol-experienced females, 5-HT levels were reduced in all prenatal treatment groups compared to control ($p < 0.05$). Prenatal treatment reduced NA levels in the PFC of alcohol-naïve females. The amount of NA was decreased following alcohol drinking in the control group ($p < 0.001$, Fig. 6c). DHT- and flutamide-treated females had lower NA levels after alcohol drinking ($p < 0.05$).

In the HYP, DA levels of alcohol-naïve females were unaffected by prenatal treatment. Overall, alcohol consumption reduced DA levels, but pre-planned comparisons revealed no significant differences (Fig. 6d). 5-HT levels in HYP were reduced in alcohol-naïve prenatally DHT- and flutamide-treated females. Alcohol drinking in the control females did not change amount of 5-HT. In the alcohol-experienced females, 5-HT levels were reduced in all prenatal treatment groups compared to control ($p < 0.001$, Fig. 6e). Prenatal treatment did not alter NA levels in female HYP. Adult NA was not changed by alcohol drinking in the control group. However, reduced NA levels were found in the flutamide-treated females after alcohol drinking ($p < 0.001$, Fig. 6f).

In females, VS levels of DA were reduced in the alcohol-naïve groups prenatally treated with either

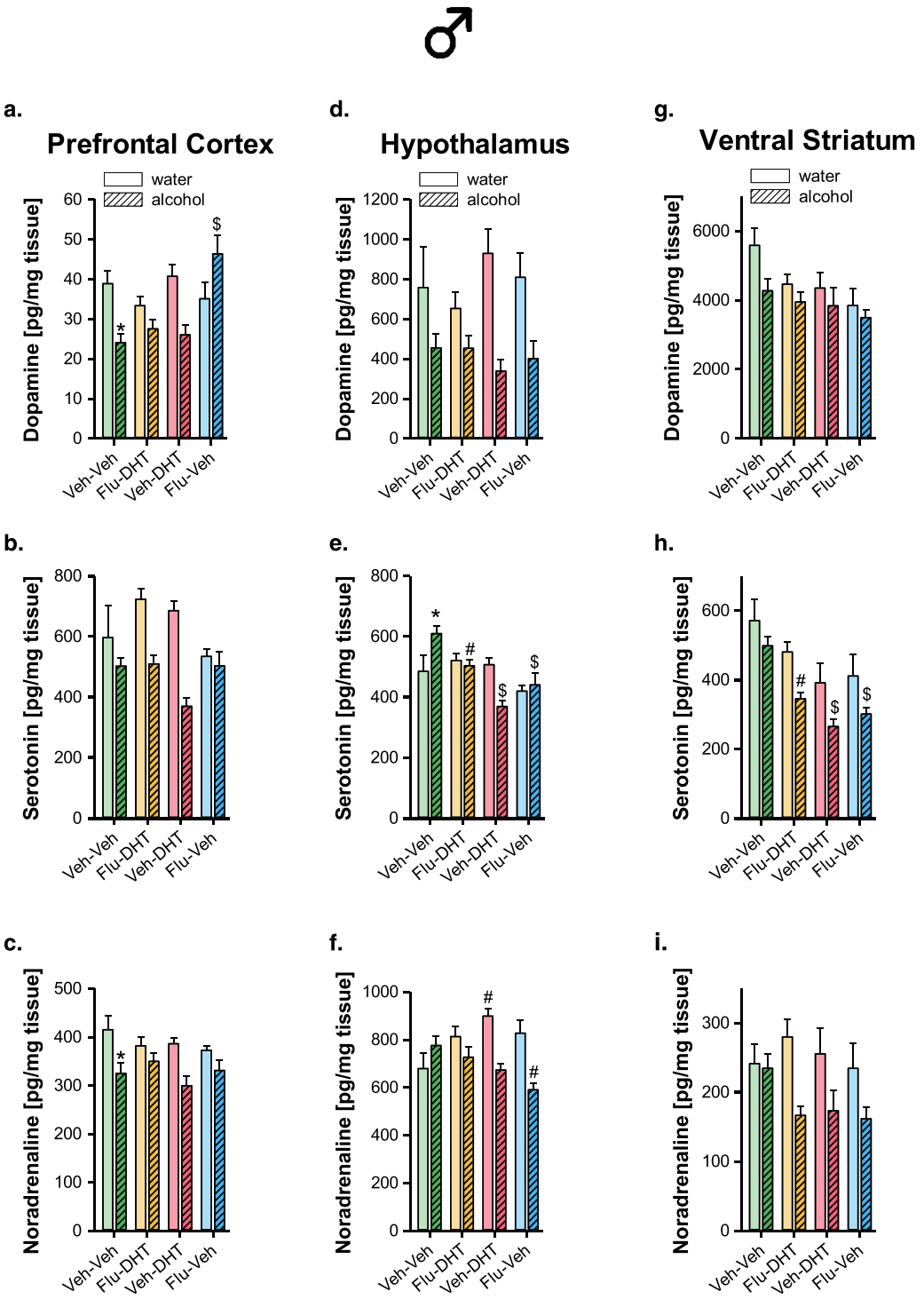

Figure 5 Adult monoamine levels are changed after prenatal androgen receptor (AR) activation or inhibition dependent on brain area and gender. We found a main effect of alcohol in the prefrontal cortex (PFC) for dopamine (DA, (a) $F_{1,100} = 6.402$, $p = 0.013$), serotonin (5-HT, (b) $F_{1,105} = 33.843$, $p < 0.001$) and noradrenaline (NA, (c) $F_{1,104} = 22.240$, $p < 0.001$) in males. Treatment had an effect in male PFC DA levels ($F_{3,100} = 4.466$, $p < 0.005$) and in 5-HT levels ($F_{3,105} = 3.054$, $p = 0.032$). An interaction effect was found in male PFC DA ($F_{3,100} = 6.963$, $p < 0.001$) and 5-HT ($F_{3,105} = 5.113$, $p = 0.002$) levels. Prenatal AR activity affected only the 5-HT hypothalamus (HYP) tissue levels in males (e; $F_{3,104} = 7.861$, $p < 0.001$), but alcohol had no effect on these levels ($F_{1,104} = 0.015$, $p = 0.903$). Alcohol consumption altered tissue levels of DA (d, $F_{1,101} = 24.532$, $p < 0.001$) and NA (f, $F_{1,106} = 11.519$, $p = 0.001$) in male HYP. We found a main effect of treatment in male ventral striatum (VS) for 5-HT (h; $F_{3,95} = 4.854$, $p = 0.003$) only. Alcohol consumption altered tissue levels of 5-HT ($F_{1,95} = 10.141$, $p = 0.002$) and NA (i; $F_{1,94} = 7.359$, $p = 0.008$) in male VS. A trend was observed for alcohol effects in DA levels (g; $F_{1,95} = 3.670$, $p = 0.058$). Error bars show mean ± SEM. * $p < 0.05$, and $p < 0.001$ alcohol versus water; # $p < 0.05$, § $p < 0.01$, $ $p < 0.001$ versus Veh-Veh control

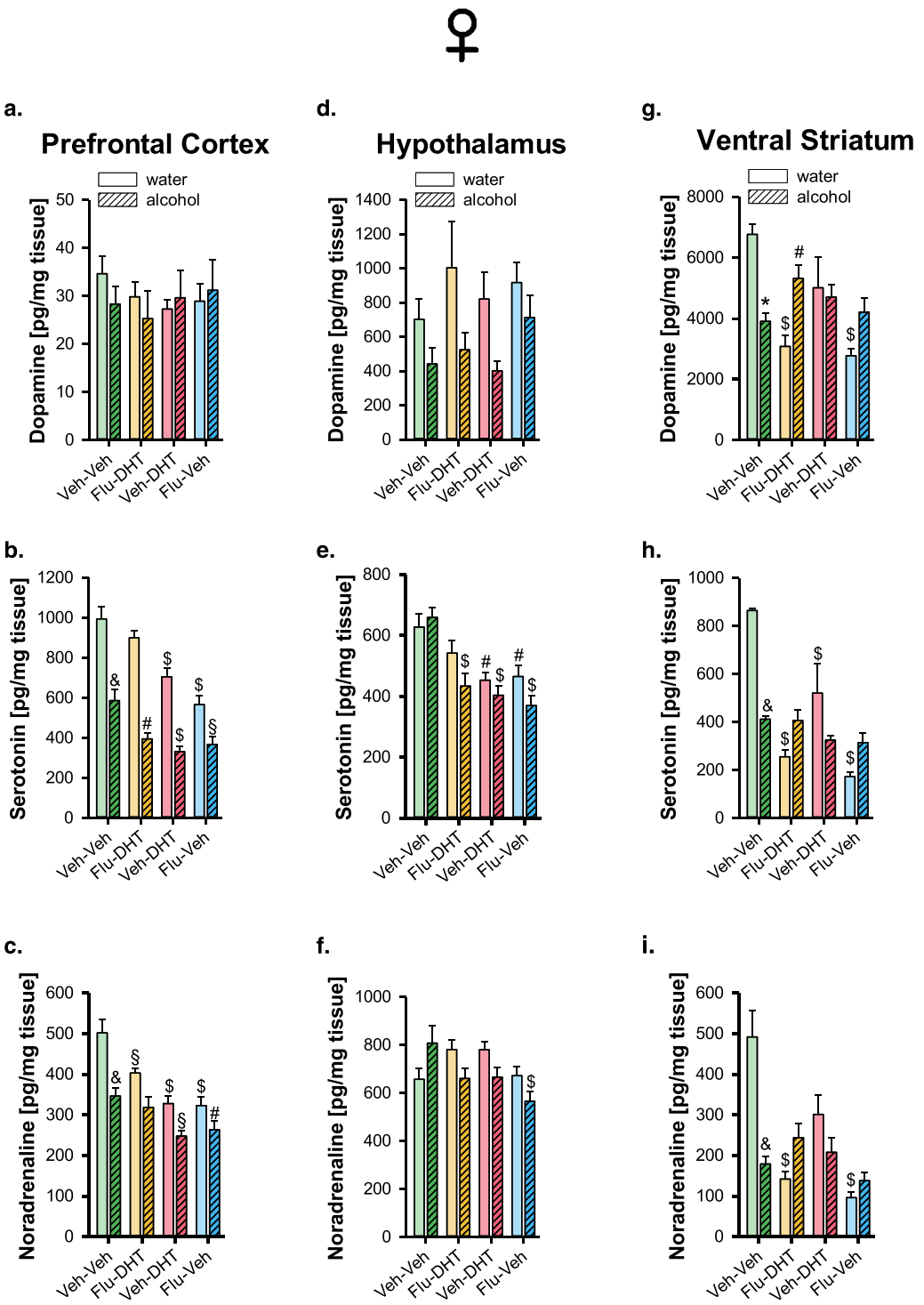

Figure 6 Adult monoamine levels are changed after prenatal androgen receptor (AR) activation or inhibition dependent on brain area and gender. In female prefrontal cortex (PFC), we found an effect of treatment on monoamine tissue levels for serotonin (5-HT, b; $F_{3,76} = 21.551$, $p < 0.001$) and noradrenaline (NA, c; $F_{3,75} = 18.595$, $p < 0.001$). We also found an alcohol effect in 5-HT ($F_{1,76} = 142.911$, $p < 0.001$) and NA ($F_{1,75} = 41.525$, $p < 0.001$). We found, however, no significant differences for dopamine (DA) levels (a; treatment: $F_{3,75} = 0.292$, $p = 0.831$, alcohol: $F_{1,75} = 0.245$, $p = 0.622$). In females hypothalamus (HYP) prenatal treatment altered tissue levels in 5-HT (e; $F_{3,75} = 14.757$ $p < 0.001$) and NA (f; $F_{3,75} = 2.882$, $p = 0.041$). Exposure to alcohol changed tissue levels in DA as revealed by ANOVA (d; $F_{1,74} = 8.610$, $p = 0.004$) and 5-HT ($F_{1,75} = 4.481$, $p = 0.038$). In female ventral striatum (VS), we found a main effect of treatment on monoamine tissue levels for DA (g; $F_{3,65} = 5.475$, $p = 0.002$), 5-HT (h; $F_{3,64} = 28.404$, $p < 0.001$) and NA (i; $F_{3,66} = 15.129$, $p < 0.001$). We also found an alcohol effect in 5-HT ($F_{1,64} = 8.960$, $p = 0.004$) and NA ($F_{1,66} = 7.482$, $p = 0.008$) but not for DA ($F_{1,65} = 0.129$, $p = 0.721$). Error bars show mean ± SEM. * $p < 0.05$, and $p < 0.001$ alcohol versus water; # $p < 0.05$, § $p < 0.01$, $ $p < 0.001$ versus Veh-Veh control

flutamide or Flu-DHT ($p < 0.001$, Fig. 6g). Alcohol drinking in the control females reduced DA levels ($p < 0.05$). In the alcohol-drinking Flu-DHT-treated females, DA levels were increased compared to control ($p < 0.01$). Prenatal treatment with DHT as well as flutamide reduced 5-HT levels in the VS of alcohol-naïve females ($p < 0.001$; Fig. 6h). Alcohol drinking decreased 5-HT levels in the veh-treated control group ($p < 0.001$). However, alcohol consumption in the prenatally DHT- or flutamide-treated females did not significantly affect adult 5-HT levels in the VS ($p > 0.05$). NA levels in the VS were reduced in the flutamide and Flu-DHT-treated alcohol-naïve females ($p < 0.001$; Fig. 6i). Alcohol drinking reduced NA level in control group ($p < 0.001$); however, it did not change NA levels of prenatally treated females.

RNA-Seq analysis in males predisposed to drinking less alcohol

To investigate the possibility of the prenatal treatment having an organizational effect on brain gene expression in adult animals influencing the drinking phenotype, we performed an RNA-Seq analysis in the VS. We compared the flutamide-treated males, which are predisposed to decreased alcohol drinking, with vehicle-treated controls. Animals were prenatally treated, but were tested before drinking would commence. After correction for multiple testing (FDR < 0.05), we identified 211 differentially expressed genes (DEGs) compared to the control (Table S1). The total number of genes that are either up- or downregulated are summarized in Fig. 7a.

Some of the DEGs identified were already known to be involved in alcohol consumption or addiction, such as the brain derived neurotrophic factor (*Bdnf*) and opioid receptor mu 1 (*Oprm1*). In order to analyze the biological relevance of the gene expression changes, we performed an ingenuity pathway analysis (IPA, Ingenuity® Systems, www.ingenuity.com). We found several networks that were differentially expressed in males (Table S5). The top networks included networks for cellular development, cellular growth and proliferation. Prenatal flutamide treatment has organizational effects on a large number of functions (Table S3). Using IPA causal network analysis, we identified the dopamine (Fig. 7b) and corticosterone (Table S7) regulatory networks as potentially responsible for differential gene expression.

RNA-Seq analysis in females predisposed to drinking more alcohol

We compared the DHT-treated females, which are predisposed to increased alcohol drinking, with vehicle control females. Animals were prenatally treated, but were tested before drinking would commence. After correction for multiple testing (FDR < 0.05), we identified 231 DEGs compared to the control (Table S2).

Some of the DEGs identified were already known to be involved in alcohol consumption or addiction, such as the period circadian clock 2 (*Per2*) gene (Spanagel *et al.* 2005). We found several networks that were involved in females (Table S6). The top hits included networks for cellular development, cellular growth and proliferation. We found a large number of functions changed in the prenatally DHT-treated females (Table S4). IPA causal network analysis suggested upstream effectors responsible for DEGs such as, e.g. beta-estradiol (Fig. 7c) and *Park2* (Parkinson disease 2, parkin; Table S8).

DISCUSSION

Several correlational studies suggested a relationship between high androgen levels in the prenatal environment and alcohol dependence in later adulthood (Kornhuber *et al.* 2011; Han *et al.* 2016). However, a causal relationship has not been demonstrated. Here, we show in a mouse model that prenatal hormonal activity can strongly influence the adult alcohol and water drinking phenotype. We report that modulating the hormonal milieu prenatally by AR activation or AR blockade can influence later adult drinking. In this study, we used DHT because (1) it has a higher affinity to the AR and (2) it cannot be aromatized to estradiol, like testosterone, and thus the effects are AR driven and not dependent on estrogen receptor. These AR effects are highly sex dependent. We show that the same treatment in females was ineffective or even resulted in an opposite effect compared to male littermates. Males experiencing enhanced AR activation during embryonic development showed no changes in either alcohol consumption or preference, while female littermates increased their adult alcohol consumption as well as their alcohol preference drastically. In contrast, reduced prenatal AR activity had the opposite effect: adult females displayed no differences in alcohol consumption, while males showed reduced alcohol intake and preference. Interestingly, prenatal effects on adult alcohol drinking differ from regulation of normal water drinking in both sexes. We found that water consumption during the baseline as well as during alcohol drinking and the total amount of fluid consumed, were modified when the prenatal hormonal milieu was changed. This suggests a double dissociation in the sex-dependent effects of prenatal AR activity on adult alcohol versus water drinking. Previous studies have shown that blood alcohol concentrations are below human intoxication levels. As such, our paradigm reflects rather controlled than addicted alcohol consumption. The activity of the liver alcohol dehydrogenases was not significantly different between

a.

Expression change	P value	Flu-Veh vs. Veh-Veh males	Veh-DHT vs. Veh-Veh females
phenotype		reduced alcohol drinking	enhanced alcohol drinking
Up-regulated	FDR<0.05	117	124
Down-regulated	FDR<0.05	94	107

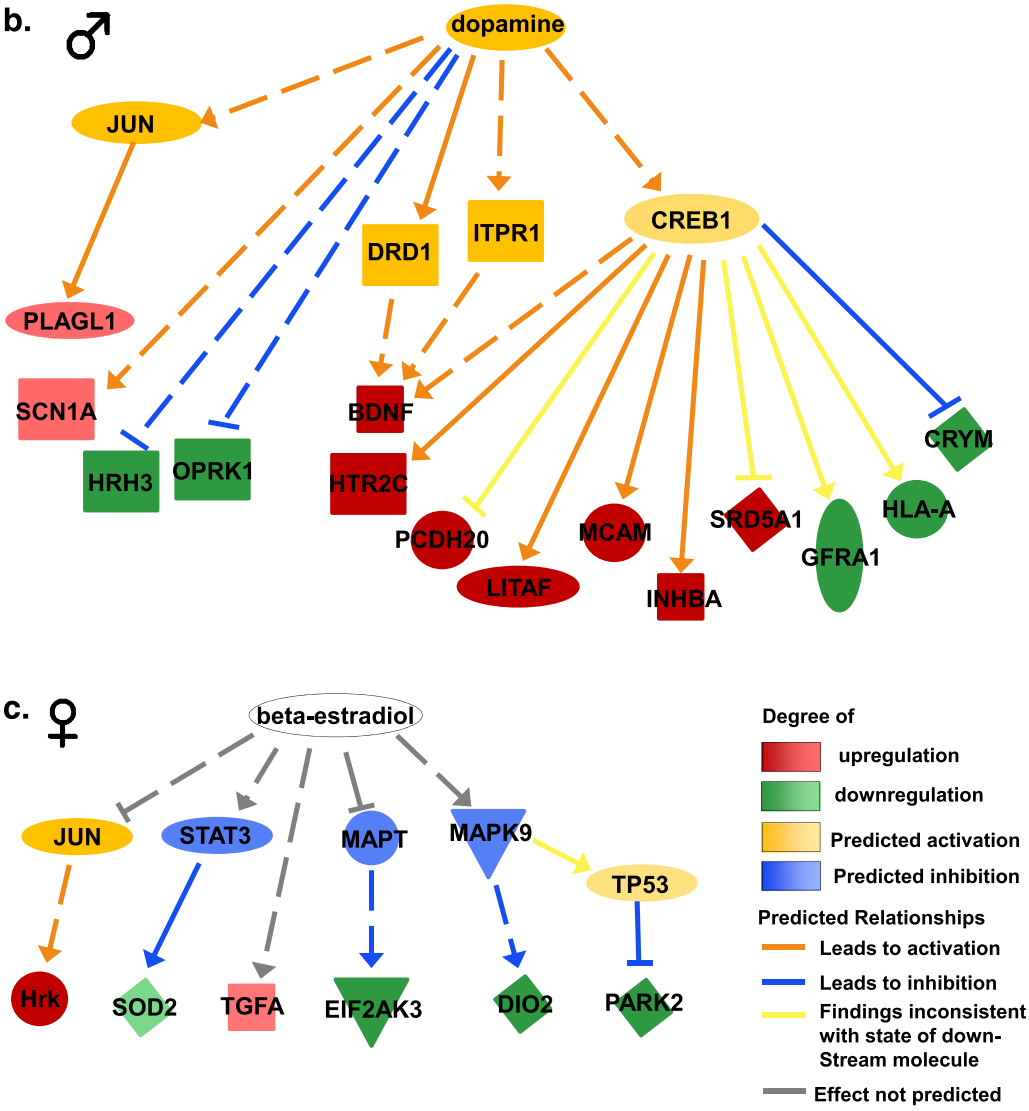

Figure 7 (a) Up- and downregulated genes in adulthood of prenatally flutamide-treated males and dihydrotestosterone (DHT)-treated females. (b) Causal network analysis (ingenuity pathway analysis, IPA) for dopamine in males. Mechanistic network shows predicted activation/inhibition status of the connected regulators. Solid line indicates direct interaction and dashed line an indirect interaction. Differentially expressed genes (DEGs) are: *Pcdh20* (protocadherin 20), *Htr2c* (serotonin receptor 2C), *Scn1a* (sodium channel voltage-gated type I alpha), *Hrh3* (histamine receptor H3), *Srd5a1* (steroid 5 alpha-reductase 1), *Plagl1* (pleiomorphic adenoma gene-like1), *Litaf* (LPS-induced TN factor), *Bdnf* (brain derived neurotrophic factor), *Mcam* (melanoma cell adhesion molecule), *Inhba* (inhibin beta-A), *Oprk1* (opioid receptor kappa 1), *Gfra1* (glial cell line derived neurotrophic factor family receptor alpha 1), *Hla-a* (major histocompatibility complex class I A) and *Crym* (crystalline mu). Predicted regulatory molecules are the following: *Jun* (jun proto-oncogene), *Drd1* (dopamine receptor D1), *Creb1* (cAMP responsive element binding protein 1) and *Itpr1* (inositol 1,4,5-trisphosphate receptor 1). (c) Causal network analysis (IPA) for beta-estradiol in females. Mechanistic network shows predicted activation/inhibition status of the connected regulators. Solid line indicates direct interaction and dashed line an indirect interaction. DEGs are: *Hrk* (hara-kiri BCL2 interacting protein), *Sod2* (superoxide dismutase 2, mitochondrial), *Tgfa* (transforming growth factor alpha), *Eif2ak3* (eukaryotic translation initiation factor 2 alpha kinase 3), *Dio2* (deiodinase iodothyronine type II) and *Park2* (parkinson disease 2, parkin). Predicted regulatory molecules are: *Jun* (jun proto-oncogene), *Stat3* (signal transducer and activator of transcription 3), *Mapt* (microtubule-associated protein tau), *Mapk9* (mitogen-activated protein kinase 9) and *Tp53* (tumor protein p53)

groups, indicating that alcohol metabolism was not affected by prenatal AR modulation.

Sensation seeking and low levels of trait anxiety predispose to high alcohol consumption and addiction (Zuckermann 1996; Mielenz *et al.* 2017). Anxiety-related behaviors are modulated by testosterone (Celec, Ostatníková & Hodosy 2015). It has been shown that acute administration of testosterone can reduce anxiety in the EPM test (Aikey *et al.* 2002). This anxiolytic effect is most likely mediated by 5-alpha reductase that reduces testosterone to DHT (Aikey *et al.* 2002; Celec *et al.* 2015). This anxiolytic effect could be blocked by administration of the AR-antagonist flutamide (Hodosy *et al.* 2012). In this study, we found an effect of prenatal flutamide exposure on the time spent in the open arm of the EPM in the males and a slight reduction in locomotion in the OF. However, we also saw that prenatal DHT-treatment increased locomotor activity in OA and showed a trend to spend more time in the OA. It seems that even though the AR was differentially modulated it exerts a similar effect on anxiety-related behavior. A possible explanation might be that the critical stage to influence anxiety-like behavior is later in development. In females, the prenatal treatment had no consequences on adult locomotion and anxiety. A previous study found anxiogenic effects in females and a trend for males after maternal treatment with testosterone. Both effects could be reversed by co-treatment with flutamide (Hu *et al.* 2015). Social behavior was not affected by prenatal AR activity in either sex. When examining for object recognition, we found that females treated with either DHT or flutamide were impaired. Overall, the prenatal AR treatment did not yield an adult phenotype which was previously associated with enhanced alcohol drinking risk.

We analyzed the adult levels of dihydrotestosterone and corticosterone to investigate the underlying organizational effect of prenatal AR treatment on adult behavior. There was no influence of prenatal AR treatment on dihydrotestosterone levels in serum of both sexes. This is in line with previous reports by Casto, Ward & Bartke (2003), who demonstrated that flutamide treatment during gestation had no effects on plasma testosterone levels in adult male rats. Previous findings showed that alcohol exposure reduces serum levels of testosterone in rats (Cicero & Badger 1977; Apter & Eriksson 2003). We found that serum dihydrotestosterone levels were indeed decreased after alcohol drinking, but only in prenatally DHT- and flutamide-treated males and Flu-DHT treated females. Dihydrotestosterone is metabolized from testosterone by 5-alpha reductase. Thus, we expected a reduction of dihydrotestosterone after alcohol exposure (Cicero & Badger 1977; Apter & Eriksson 2003). These findings might be due to a stable rate of metabolization in the control and Flu-DHT-treated groups even if testosterone levels are lower. Overall, adult dihydrotestosterone levels are not affected by prenatal AR activity.

In males, prenatal AR treatment reduced adult serum corticosterone levels compared to the vehicle control. This is in contrast to Shors & Miesegaes (2002), who found AR-antagonism *in utero* had no effects on corticosterone levels in adult rats. However, they used a different antagonist, cyproterone acetate, different route of application (s.c.) and injected for a longer period during pregnancy (day 10–19) than in this study, which might explain the different findings. In males treated prenatally with flutamide and females prenatally treated with vehicle, Flu-DHT or DHT, adult serum corticosterone levels were increased after alcohol consumption. This expands previous findings showing that acute alcohol administration increases corticosterone levels (Willey *et al.* 2012).

Maladaptations in brain monoamine systems, especially in the dopamine (Spanagel 2009) and serotonin systems (Müller & Homberg 2015), have been implicated in alcohol addiction development. We asked whether the organizational effects are possibly mediated by alterations in monoaminergic function. We found that in the PFC, prenatal AR treatment reduced 5-HT and NA tissue levels in females, but not in males. In the HYP, NA levels in DHT-treated males were increased, while in females DHT as well as flutamide treatment reduced levels of 5-HT. In the VS, prenatal treatment had no effect in males, while it reduced DA, 5-HT and NA levels in females treated with flutamide and Flu-DHT. Alcohol reduced the levels of all monoamines in the PFC, independent of gender, and in the female VS. In the HYP alcohol reduced DA and NA levels in males, as well as DA and 5-HT levels in females. DA was neither in PFC nor in HYP directly affected by prenatal AR activity. The HYP plays an important role in regulation of thirst (McKinley & Johnson 2004). We found a dysregulation of 5-HT in the female HYP. Prenatally treated females, as shown here, have an increased water consumption. It should be noted, however, that a drawback of the present design is that we cannot completely dissociate the effects alcohol drinking has on hormone and monoamine levels from the effects of long-term social isolation on these measures. It was shown that housing conditions influence levels of testosterone (Sayegh *et al.* 1990), corticosterone, as well as monoamine levels (Ieraci *et al.* 2016; Miachon *et al.* 1993).

We performed a RNA-Seq study to analyze if prenatal AR-activation/inhibition results in organizational changes in mRNA expression that put the individual at higher risk for alcoholism as shown previously on single gene level (Stacey, Clarke & Schumann 2009, 2012, 2016; Easton *et al.* 2013). We found a large number of differentially expressed genes in mice with high versus low risk for alcohol consumption, which differed by the

sex. Some of these genes affected by the prenatal treatment were already implicated to play a role in addiction. BDNF was upregulated in the males predisposed for reduced alcohol drinking. This is in line with previous findings which showed that reduction in BDNF expression correlates with an increase in alcohol consumption (Hensler, Ladenheim & Lyons 2003; Janak *et al.* 2006). Also, activation of Glial cell line-derived neurotrophic factor (GDNF) leads to a decrease of alcohol self-administration (He *et al.* 2005; Janak *et al.* 2006). We found that *Gfra1*, coding for a receptor for GDNF, was downregulated in flutamide-treated males. Two different types of opioid receptors, *Oprk1* and *Oprm1*, were downregulated by prenatal flutamide treatment. Activation of the dynorphin receptor KOR (kappa opioid receptor, Oprk1) is required for the BDNF-mediated decrease in alcohol intake (Logrip, Janak & Ron 2008). In our data, *Oprk1* is downregulated while *Bdnf* is upregulated. The second μ-opioid receptor *Oprm1*, which we found downregulated in males, has also already been shown to play a role in alcohol self-administration. Mice lacking the μ-opioid receptor do not self-administer alcohol (Roberts *et al.* 2000). Males with lower alcohol drinking predisposition were found to express more of *Htr2c*, coding for the 5-HT_{2c}-receptor. Exposure to alcohol vapor for several days, which sensitizes animals to alcohol effects, also leads to an increase in expression of 5-HT_{2C}-receptor mRNA (Yoshimoto *et al.* 2012; Müller & Homberg 2015). In contrast, administration of a 5-HT_{2C}-receptor-agonist reduced alcohol self-administration in various drinking studies (Yoshimoto *et al.* 2012; Müller & Homberg 2015). Monoamine analysis yielded a visible trend for DA decline, which is in line with the genetic findings, indicating DA regulatory network as a driving force for differential gene expression.

In females, AR organizational effects on gene expression had a completely different shape. We found the clock gene Per2 downregulated in the prenatally DHT-treated animals, which are predisposed for high alcohol consumption. This is in accordance with the findings from Spanagel *et al.* (2005) who showed that $Per2^{Brdm1}$ mutant mice, which have a reduced expression of *mPer1* and *mPer2*, show higher voluntary alcohol consumption. In this study, the gene coding for corticotropin releasing hormone (*Crh*) was downregulated. Recent studies implicated its receptors to be involved in alcohol consumption. It was shown that CRF1 antagonism can reduce alcohol self-administration in rats (Lowery *et al.* 2010). We also found a number of genes dysregulated in prenatally treated males and females, which are closely related or belonging to the same family of genes that were previously shown to play a role in alcohol drinking behavior, such as *Camk1g* (Easton *et al.* 2013), *Efhd1* (Mielenz *et al.* 2017), *Fgf2* and *Fgf3* (Talukdar *et al.* 2016), *Rassf2* and *Rasgef1b* (Stacey *et al.* 2012, 2016).

Altogether, this study suggests that prenatal AR activity crucially determines adult alcohol as well as water drinking, thus, providing now a functional base for human correlational studies. Here, we show that these effects are highly sex dependent and relate to sex-specific organizational effects in homeostatic systems and alcoholism-related gene expression. These findings suggest the prenatal period and hormonal internal environment as a major susceptibility factor for adult alcohol use disorder and might open a new time window for early addiction prevention.

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Declaration of Interest

The authors declare no conflict of interest.

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Author Contributions

J.K., B.L. and C.P.M. initiated the study. S.E.H. performed experiments, and S.E.H. and C.P.M. analyzed the data. S. E.H. and I.Z. performed social behavioral experiments. S. E.H., V.E. and C.M. performed ELISA and ADH analysis. S.E.H, M.R., C.B. and A.B.E. performed the RNA-Seq study. S.E.H. and C.P.M. wrote the manuscript, and all authors discussed the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Regions for brain dissection for post mortem neurochemical analysis. (A) Prefrontal cortex, (B) ventral striatum and (C) hypothalamus redrawn from Franklin & Paxinos .

Figure S2 Liver ADH activity in prenatally treated (A) males and (B) females. Data were presented in Box and whisker plots with the box representing the ±25 percentile and the whisker min/max, respectively. No statistical significant differences was observed between the groups (adjusted P value >0.4, Kruskal–Wallis test).

Figure S3 Social preference, social novelty and object recognition in adult males and females with prenatal androgen receptor (AR) manipulation. (A) Social preference in male mice is not changed by prenatal treatment ($F_{3,50} = 0.000$, $p = 1.000$). They spent more time investigating the *same* mouse than the empty cage ($F_{1,50} = 152.187$, $p < 0.001$). (B) Female social preference is not changed after prenatal treatment ($F_{3,64} = 0.000$, $p = 1.000$). More time is spent investigating the *same* mouse than the empty cage ($F_{1,64} = 130.940$, $p < 0.001$). (C) Social recognition in males is unaffected by prenatal androgen receptor (AR) activity ($F_{3,50} = 0.000$, $p = 1.000$); they spent more time investigating the *novel* mouse than the *same* mouse ($F_{1,50} = 43.791$, $p < 0.001$). (D) Prenatal AR activation or inhibition did not alter social recognition in females ($F_{3,64} = 0.000$, $p = 1.000$). More time was spent investigating the *novel* mouse than the *same* mouse ($F_{1,64} = 78.277$, $p < 0.001$). (E) Male object recognition was not altered by prenatal treatment ($F_{3,50} = 0.000$, $p = 1.000$). Animals spent more time investigating the *novel* object ($F_{1,50} = 31.740$, $p < 0.001$). (F) No main effect of prenatal treatment ($F_{3,60} = 0.000$, $p = 1.000$) on object recognition was found. However, a main effect on investigation time was found ($F_{1,60} = 10.220$,

$p = 0.002$). While the control and the Flu-DHT-treated groups spent more time investigating the *novel* object, the dihydrotestosterone- and flutamide-treated females spent similar time investigating the *same* and the *novel* object indicating an impaired object recognition. Error bars show mean ± SEM. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Table S1 Differentially expressed genes in the prenatally flutamide versus vehicle-treated males. Flutamide-treated males were analyzed because of their lower alcohol drinking phenotype found in free-choice alcohol drinking during adulthood (FDR < 0.05).

Table S2 Differentially expressed genes in the prenatally dihydrotestosterone (DHT) versus vehicle-treated females. DHT-treated females were chosen for analysis because of their enhanced alcohol drinking phenotype during adulthood (FDR < 0.05).

Table S3 Gene ontology (GO) analysis revealed the top diseases and functions affected by prenatal fluatmide treatment (versus control) in males, generated by ingenuity pathway analysis (IPA). GO, *p*-values, and molecules involved in biological processes.

Table S4 Significant differences in biological processes involved in diseases and functions found between prenatally dihydrotestosterone (DHT) treated and control females, generated using ingenuity pathway analysis (IPA). Gene ontology (GO), *p*-values and molecules involved in biological processes.

Table S5 Functional networks created with molecules found in dataset involved in biological processes, which are affected by prenatal fluatmide treatment in males, generated using ingenuity pathway analysis (IPA). Table showing involved molecules and biological function that are affected.

Table S6 Functional networks created with molecules found in dataset involved in biological processes, which are affected by prenatal dihydrotestosterone (DHT) treatment in females, generated using ingenuity pathway analysis (IPA). Table showing involved molecules and biological function that are affected.

Table S7 Regulatory networks identified by causal network analysis (ingenuity pathway analysis) likely responsible for differential gene expression in prenatally flutamide- versus vehicle-treated males. Table displays predicted master regulators, involved regulators and targeted molecules detected in the dataset.

Table S8 Regulatory networks identified by causal network analysis (ingenuity pathway analysis) likely responsible for differential gene expression in prenatally dihydrotestosterone- versus vehicle-treated females. Table displays predicted master regulators, involved regulators and targeted molecules detected in dataset.