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Increased steroid hormone dehydroepiandrosterone and pregnenolone levels in post-mortem brain samples of alcoholics

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ABSTRACT

Intra-tissue levels of steroid hormones (e.g., dehydroepiandrosterone [DHEA], pregnenolone [PREGN], and testosterone [T]) may influence the pathological changes seen in neurotransmitter systems of alcoholic brains. Our aim was to compare levels of these steroid hormones between the post-mortem brain samples of alcoholics and non-alcoholic controls. We studied steroid levels with quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) in post-mortem brain samples of alcoholics ($N = 14$) and non-alcoholic controls ($N = 10$). Significant differences were observed between study groups in DHEA and PREGN levels (p values 0.0056 and 0.019, respectively), but not in T levels. Differences between the study groups were most prominent in the nucleus accumbens (NAC), anterior cingulate cortex (ACC), and anterior insula (AINS). DHEA levels were increased in most alcoholic subjects compared to controls. However, only a subgroup of alcoholics showed increased PREGN levels. Negative Spearman correlations between tissue levels of PREGN and previous reports of [^{3}H]naloxone binding to μ-opioid receptors were observed in the AINS, ACC, NAC, and frontal cortex (R values between −0.6 and −0.8; p values ≤ 0.002), suggesting an association between the opioid system and brain PREGN levels. Although preliminary, and from relatively small diagnostic groups, these results show significantly increased levels of DHEA and PREGN in the brains of alcoholics, and could be associated with the pathology of alcoholism.

Introduction

Allostatic alterations in the reward and stress systems are considered to be a nexus for developing alcohol dependence (Koob, 2013). Steroid hormones affect both reward and stress processes. There are bidirectional interactions between alcohol consumption and steroid hormones. Neuroactive steroids are considered to be critical for modifying behavioral responses to alcohol (Helms, Rossi, & Grant, 2012), and consumption of alcohol can influence the activity of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes (Adinoff et al., 1990; Frias, Rodriguez, Torres, Ruiz, & Ortega, 2000; Mendelson, Mello, & Ellingboe, 1977; Välimäki, Härkönen, Eriksson, & Ylikahri, 1984). Furthermore, increased *de novo* steroidogenesis has also been reported in the rat brain after alcohol exposure (Sánchez, Castro, Torres, & Ortega, 2014; Sanna et al., 2004). However, considerable species differences exist in the way alcohol influences endogenous steroid levels (Porcu et al., 2010), and *de novo* steroidogenesis might not occur in the adult human brain (Steckelbroeck et al., 2010).

Testosterone (T) and glucocorticoid steroids have been studied widely and have been associated with the pathology of alcohol-use disorder (Edwards, Little, Richardson, & Vendruscolo, 2015; Lenz et al., 2012). However, other neuroactive steroids (e.g., dehydroepiandrosterone [DHEA] and pregnenolone [PREGN]) have been associated with alcohol consumption (Helms et al., 2012). In healthy non-alcoholic volunteers, alcohol consumption that leads to blood alcohol levels of ~0.06 mg/dL increases plasma DHEA and PREGN levels, which mediates some of the subjective effects of

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alcohol (Pierucci-Lagha et al., 2006). Furthermore, high saliva levels of DHEA have been associated with drinking to cope with stress in women (Wemm et al., 2013). Unconjugated DHEA has been associated with increases in catecholamine synthesis, decreases in monoamine oxidase (MAO) activity, enhanced activation of NMDA, and inhibition of GABA-A receptor function (Imamura & Prasad, 1998; Maninger, Wolkowitz, Reus, Epel, & Mellon, 2009; Pérez-Neri, Montes, & Ríos, 2009). Unconjugated PREGN has been associated with feedback control of endocannabinoid system functions (Vallée et al., 2014) and a reduction of acute alcohol self-administration in rodents (Besheer, Lindsay, O'Buckley, Hodge, & Morrow, 2010; Rezvani & Levin, 2014). Furthermore, neuroactive steroid levels are influenced by neurotransmitter systems that are important for alcohol-use disorder. For example, the μ-opioid receptor (MOR) antagonist naloxone increases plasma levels of PREGN in cynomolgus monkeys (Porcu, Rogers, Morrow, & Grant, 2006). However, plasma levels of steroids do not necessarily represent steroid levels in the brain (Alomary et al., 2003; Little et al., 2008), probably because the brain has *de novo* steroidogenesis and active steroid metabolism. Therefore, there is a need to also measure the levels of steroid hormones in the target tissue.

The aim of the present study was to measure differences in steroid levels in the nucleus accumbens (NAC), anterior insula (AINS), hippocampus (HIPP), frontal cortex (FC), amygdala (AMY), and anterior cingulate cortex (ACC) in post-mortem brain samples of alcoholics and non-alcoholic controls. Furthermore, T seems to play a role in psychological traits such as antisocial behavior (Yildirim & Derksen, 2012). In the present study, alcoholics were divided into two subgroups according to Cloninger's typology of alcoholism, where antisocial behavior is associated with early-onset Cloninger type 2 alcoholism, but not with the late-onset type 1 alcoholism (Cloninger, 1995). In these same study subjects, we have previously reported on differences in neurotransmitter systems associated with steroid function, including GABA-A (Laukkanen et al., 2013), NMDA receptor subunit 2B (Kupila et al., 2015), MOR binding (Laukkanen, Kärkkäinen, Kautiainen, Tiihonen, & Storvik, 2015), and brain tissue levels of endocannabinoids (Kärkkäinen et al., 2013; Lehtonen et al., 2010). The secondary aim of the present study was to calculate whether these previously published measurements are correlated with brain steroid levels. To our knowledge, steroid levels have not been previously studied in post-mortem brain samples of alcoholics.

Materials and methods

Study subjects and diagnostics

The selection and collection of these post-mortem human brains, psychological diagnostics, and sample preservation methods have been described in detail (Lehtonen et al., 2010; Mantere et al., 2002; Storvik, Häkkinen, Tupala, & Tiihonen, 2009). Briefly, left hemispheres were obtained during clinical necropsy at the Department of Forensic Medicine, University of Oulu, Finland, and the Department of Forensic Medicine, University of Eastern Finland, Finland. This portion of the study was approved by the Ethics Committees of the University of Oulu (27.12.1997; latest amendment Dnro 125/2009) and the National Board of Medicolegal Affairs, Helsinki, Finland (Dnro 3020/322/96 and 3141/32/200/98). The brains were removed, cleaned of the dura, and divided at the midsagittal plane. The left hemisphere was placed on a glass plate before freezing at −75 °C. None of the hemispheres exhibited damage or neuroanatomical abnormalities. Brain samples were cryo-sectioned into 100-μm cantomeatal slices that were allowed to air dry before storage at −25 °C with dehydrating agents until use.

The study groups consisted of Cloninger type 1 alcoholics ($N = 6$, four males and two females; age at the time of death [AGE] 57.5 ± 12.1 years [mean ± SD]; post-mortem interval [PMI] 13.9 ± 3.0 h; blood alcohol concentration [BAC] 2.7 ± 1.4 mass/mass ‰ [1 mass/mass ‰ is equivalent to approximately 106 mg of alcohol in 1 dL of blood]), type 2 alcoholics ($N = 8$, all male; AGE 34.6 ± 11.4 years; PMI 14.1 ± 3.2 h; BAC 1.8 ± 1.3‰), and a non-alcoholic control group ($N = 10$; eight males and two females; AGE 53.5 ± 10.1 years; PMI 14.8 ± 8.8 h; BAC 0.04 ± 0.12‰) (Table 1). Two physicians reviewed medical records and anamnestic data, which included extant criminal records. Alcoholism, determined by frequent medical appointments due to alcohol-related problems, was coded according to DSM-IV criteria (American Psychiatric Association, 1994) and further sub-classified as type 1 or type 2 alcoholism according to Cloninger's typology, which resembles Babor and Early/Late onset typologies of alcoholism (Cloninger, 1995; Leggio, Kenna, Fenton, Bonenfant, & Swift, 2009). The two main separating criteria for the present study were early onset of alcohol abuse (<25 years old) and a record of severe antisocial behavior for type 2 alcoholics. Subjects with psychotic disorders or any other neurological disease, those taking medication that could affect the CNS (such as neuroleptics or antidepressants), and subjects with severe inflammation as a cause of death (e.g., acute pancreatitis or pneumonia) were excluded. All type 1 and six type 2 alcoholics had ethanol in their blood at their time of death. One type 2 alcoholic had an abstinence period of 5 days and another had abstained for 3–7 days. One of the controls had a small amount of ethanol in his blood at the time of death (0.36‰ blood alcohol content). Two of the type 1 and three of the type 2 alcoholics had traces of benzodiazepines in their blood samples. Evaluations for the duration of heavy alcohol use, family histories of alcohol misuse, and tobacco smoking, based on medical records, were considered to be unreliable and thus not considered in the final analysis.

Table 1
The study subjects: age at time of death, post-mortem interval (PMI), blood alcohol concentration (BAC), and cause of death.

Group and subject	Sex	Age (years)	PMI (h)	BAC (‰)	Cause of death
Non-alcoholic controls					
1	Male	55	5.5	0.0	Acute myocardial infarction
2	Male	45	9.5	0.0	Acute myocardial infarction
3	Male	77	7.5	0.0	Acute myocardial infarction
4	Female	57	11.0	0.0	Acute myocardial infarction
5	Male	50	18.5	0.0	Acute myocardial infarction
6	Female	60	12.0	0.0	Acute myocardial infarction
7	Male	49	33.0	0.4	Acute myocardial infarction
8	Male	53	29.0	0.0	Acute myocardial infarction
9	Male	53	11.0	0.0	Acute myocardial infarction
10	Male	36	11.0	0.0	Dissection of aorta
Alcoholics					
Type 1					
1	Male	45	12.0	1.5	Suicide by hanging
2	Male	42	14.8	0.8	Acute myocardial infarction
3	Male	76	10.5	3.2	Acute myocardial infarction
4	Female	56	19.0	4.1	Ethanol intoxication
5	Male	69	16.0	4.7	Ethanol intoxication
6	Female	57	11.0	2.0	Right subdural hemorrhage
Type 2					
7	Male	49	12.0	1.7	Fibrotic degeneration of myocardium
8	Male	37	9.5	3.0	Gunshot wound
9	Male	47	15.5	3.0	Knife wound
10	Male	20	14.5	1.3	Knife wound
11	Male	46	18.0	0.0	Suicide by hanging
12	Male	18	9.5	1.5	Heart rupture (car accident)
13	Male	32	16.5	3.6	Suicide by hanging
14	Male	28	17.5	0.0	Suicide by hanging

LC-MS/MS analysis

Concentrations of unconjugated steroids were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS), as previously described (Keski-Rahkonen, Huhtinen, Poutanen, & Auriola, 2011). Dry frozen brain samples (5–10 mg) were cryo-ground in 2-mL microcentrifuge tubes using a TissueLyser II (Qiagen Finland, Helsinki, Finland) with 5-mm stainless-steel beads in pre-cooled adapters, and shaken for 30 s at 30 Hz. Tissue samples were homogenized in 200 μL of deionized water. An aliquot of 150 μL was taken, and non-conjugated steroids were extracted from the aliquot by methyl tert-butyl ether (MTBE) and were then analyzed with LC-MS/MS (Keski-Rahkonen et al., 2011). We analyzed six brain regions: NAC, AINS, ACC, FC, HIPP, and AMY. The hypothalamus was not analyzed, because the region is relatively small and the tissue yield from this region was therefore low. At least two ion transitions were used for the measurement of each steroid (quantifier ion and qualifier ion). Samples and standards were spiked with deuterated steroids and the quantitation by LC-MS/MS was based on the use of these isotope-labeled internal standards. Use of deuterated steroids as internal standards also enabled compensation for variation in the matrix effect between the samples (Stokvis, Rosing, & Beijnen, 2005). The method used has been previously validated for body fluid and tissue samples (Huhtinen et al., 2014; Keski-Rahkonen et al., 2011). The main aim of the present research was to compare relative levels of steroids in the post-mortem brain samples between alcoholics and non-alcoholic controls. This aim was achieved with the use of internal standards to compensate the matrix effect from the samples. Because of the limited availability of the sample material, we were not able to conduct tissue-specific validations for the method.

Statistical analyses

The aim of the present research was to compare relative levels of steroids between alcoholics and non-alcoholic controls in the post-mortem brain samples. In order to estimate overall differences in steroid levels, the measured concentrations of individual steroids were standardized by the mean and standard deviation of the control group, and the mean of these standardized scores (from individual brain regions) was calculated for each subject. The 95% confidence intervals (95% CI) were obtained with bias-corrected bootstrapping. Statistical significance was evaluated by a permutation-type analysis of variance (Monte-Carlo p values), followed by multiplicity adjustment with Holm's method for comparison of three groups, and by a Student's t test for comparing two groups. Measured values, without normalization, were used to evaluate statistical significance in the individual brain regions. Spearman's method was used to calculate correlations between measured steroid concentrations and AGE, BAC, and PMI, as well as previous reports of receptor binding values and endocannabinoid levels (Kärkkäinen et al., 2013; Kupila et al., 2015; Laukkanen et al., 2013, 2015; Lehtonen et al., 2010). Cohen's methods were used to calculate effect sizes (d for comparison of two groups and f for comparison of three groups). The α level was set at 0.05, and STATA (release 13.1, College Station, TX) was used for statistical analyses.

Results

Representative peaks for DHEA, PREGN, and T were obtained from brain samples spiked with deuterated steroids as internal standards (Fig. 1). Peaks from the endogenous steroids had the same retention time and similar peak shape when compared to the deuterated internal standards.

Statistically significant differences were observed between the study groups in DHEA and PREGN levels, but not in T levels (Fig. 2). Significantly increased DHEA and PREGN levels were seen in alcoholics when compared to non-alcoholic controls (Monte-Carlo p values 0.0056 and 0.019, respectively). DHEA, PREGN, and T concentrations from individual brain regions are shown in Table 2. DHEA levels were significantly increased in all measured brain regions in alcoholics when compared to controls (t test p values between 0.026 and 0.002). PREGN levels were significantly increased in alcoholics when compared to controls in all measured brain regions except in the amygdala (significant t test p values between 0.018 and 0.005). The significantly increased DHEA levels were 71–161% higher and the significantly increased PREGN levels were 71–164% higher in alcoholics when compared to controls. There were no significant correlations between AGE, PMI, and BAC and the measured steroid levels. Other steroids (e.g., progesterone and androstenedione) were below the quantitation limit of the assay for most of the controls' samples, and were therefore excluded from further analysis.

There were negative Spearman correlations between previous reports of [^{3}H]naloxone binding (Laukkanen et al., 2015) and PREGN levels in the AINS, ACC, NAC, and FC (Fig. 3). AMY was excluded from the analysis, because [^{3}H]naloxone binding was not measured from this brain region. In comparison, negative correlations between DHEA and previous reports of [^{3}H]naloxone binding to MOR were observed only in the ACC and AINS (R values −0.49 and −0.48; p values 0.014 and 0.018, respectively) but not in the other brain regions (Supplementary Fig. 1). There were no significant correlations between previous reports of [^{3}H]naloxone binding and T levels measured in the present study (data not shown).

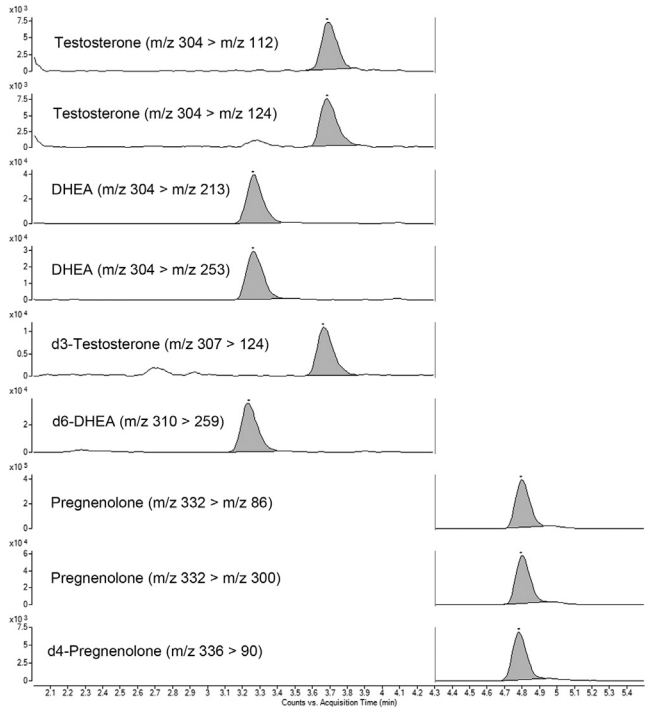

Fig. 1. Representative MRM chromatograms of testosterone, dehydroepiandrosterone (DHEA), and pregnenolone in a brain tissue sample. At least two product ions from precursor ions were used for each steroid. Quantifier ions and qualifier ions were 213 and 253 for DHEA, 124 and 112 for testosterone, and 86 and 300 for pregnenolone, respectively. Deuterated steroids (d3-testosterone, d6-DHEA, and d4-pregnenolone) were added to the brain samples to serve as internal standards in the quantitation. Retention time and the peak shape were similar between endogenous steroids from the brain samples and the internal standards.

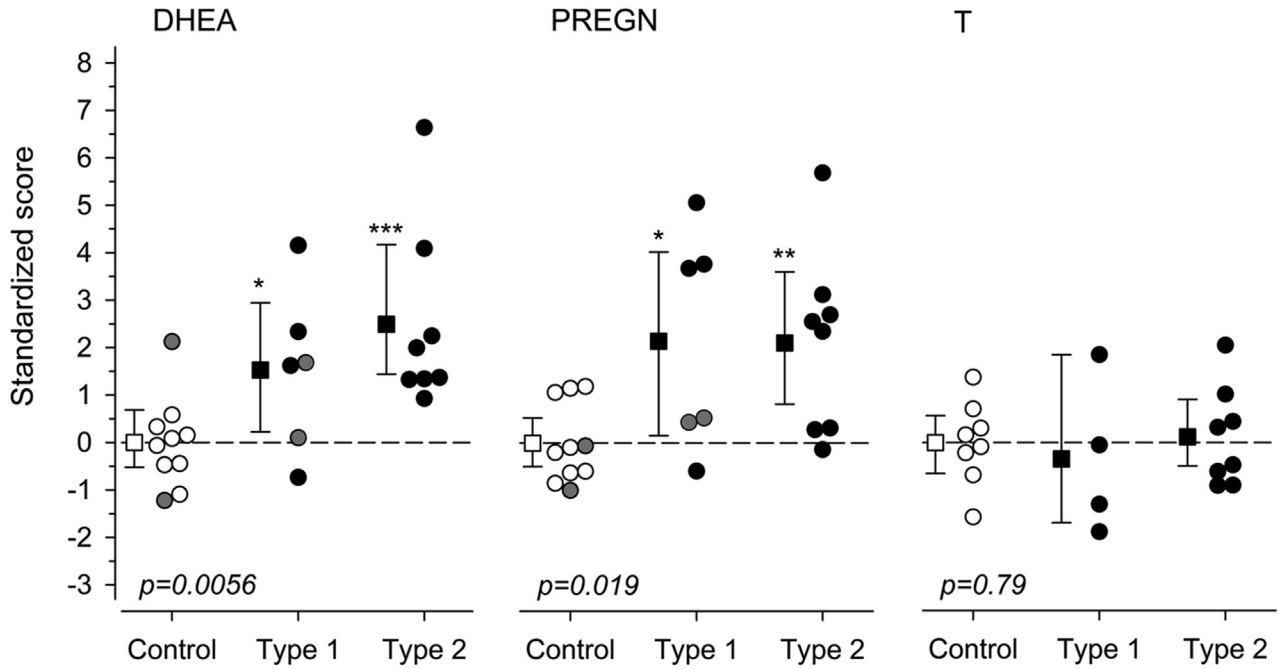

Fig. 2. Comparison of dehydroepiandrosterone, pregnenolone, and testosterone level averages for all measured brain regions between alcoholics and non-alcoholic controls. DHEA, PREGN, and T levels were measured in six different brain regions of post-mortem brain samples. The measured concentrations of individual steroids in different brain regions were standardized by the mean and standard deviation of the control group to compare overall differences in steroid levels. The means of these standardized scores were calculated for each subject (circles) and for study groups (boxes with whiskers represent group means with bootstrap bias-corrected 95% confidence intervals). Alcoholics were divided into two subgroups according to Cloninger's typology (Cloninger, 1995). Gray circles are female subjects, white circles are male controls and black circles are male alcoholics. Statistical significance was evaluated by a permutation-type analysis of variance (Monte-Carlo *p* values shown in the figure), followed by multiplicity adjustment with Holm's method. DHEA, dehydroepiandrosterone; PREGN, pregnenolone; T, testosterone; multiplicity adjusted *p* value when compared to controls: * = <0.05; ** = <0.01; *** = <0.001.

Furthermore, there were positive correlations between previous reports of [^{3}H]ifenprodil binding to NR2B (Kupila et al., 2015), and levels of PREGN and T (R values 0.73 and 0.59; p values < 0.001 and 0.007, respectively) in the FC. There was a negative correlation between previous reports of values for [^{3}H]flunitrazepam binding to GABA-A receptors (Laukkanen et al., 2013) and DHEA levels ($R = -0.52$; p value $= 0.013$) in the FC (Supplementary Fig. 2). Correlations between the steroid levels and previous reports of NR2B, GABA-A receptor binding values, and endocannabinoid levels (Kärkkäinen et al., 2013; Kupila et al., 2015; Laukkanen et al., 2013, 2015; Lehtonen et al., 2010) were not significant in other brain regions measured (data not shown).

Discussion

In the present study steroid levels were measured from the post-mortem brain samples of alcoholics and non-alcoholic controls. Increased levels of DHEA and PREGN were observed in the post-mortem brain samples of alcoholics when compared to non-alcoholic controls (Fig. 2). DHEA levels were significantly increased in all measured brain regions and PREGN levels were significantly increased in alcoholics when compared to controls in all measured brain regions except the amygdala (Table 2). These results are in agreement with previous results of increased peripheral DHEA and PREGN levels associated with alcohol consumption (Pierucci-Lagha et al., 2006; Välimäki et al., 1984; Wemm et al., 2013).

The increased DHEA and PREGN levels in post-mortem alcoholic brain samples could be due to release from the periphery, alterations in the steroid metabolism, or increased *de novo* steroidogenesis (Adinoff et al., 1990; Pierucci-Lagha et al., 2006; Sánchez et al., 2014; Sanna et al., 2004; Välimäki et al., 1984; Wemm et al., 2013). There is some evidence that the CYP11A1 and CYP17A1 enzymes, which are needed for synthesis of PREGN and DHEA, may not be active in the adult human brain (Steckelbroeck et al., 2010). Although there is evidence to suggest that DHEA synthesis can be independent of CYP11A1 activity in the mouse brain (Liu, Pocivavsek, & Papadopoulos, 2009), it still seems likely that the presently observed high PREGN and DHEA values in brain samples of alcoholics could be due to increased peripheral release and altered steroid metabolism. The role of altered steroid metabolism is supported by the present observation that T levels were not increased, although T is synthesized from DHEA by 3- and 17-β-hydroxysteroid dehydrogenase (3β-HSD and 17β-HSD, respectively). Perhaps chronic alcohol consumption causes a depletion of the metabolic co-factors nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH), which are required for function of 3β-HSD and 17β-HSD similar to that of alcohol dehydrogenase (Crabb, Matsumoto, Chang, & You, 2004; Helms et al., 2012; Krause & Karavolas, 1980).

High levels of unconjugated DHEA have been associated with many factors that could affect ethanol consumption, including antagonism of GABA-A receptors and activation of NMDA receptors (Imamura & Prasad, 1998; Maninger et al., 2009), in addition to decreased MAO activity and dopamine turnover in the NAC of rats (Pérez-Neri, Méndez-Sánchez, Montes, & Ríos, 2008; Pérez-Neri et al., 2009). Furthermore, administration of DHEA reduces the amount of ethanol consumed by rats (Gurkovskaya, Leonard, Lewis, & Winsauer, 2009). Similarly, high levels of unconjugated PREGN levels have also been implicated in the inhibition of both GABA-A receptor activity (Majewska, 1992; Majewska, Demirgören, & London, 1990) and the endocannabinoid system (Vallée et al., 2014), and decreased acute response to alcohol (Besheer et al., 2010; Rezvani & Levin, 2014). Correlations between steroid levels and previously reported levels of [^{3}H]ifenprodil binding to both NR2B (Kupila et al., 2015) and [^{3}H]flunitrazepam binding to GABA-A

Table 2
Measured dehydroepiandosterone, pregnenolone, and testosterone levels (pmol/g tissue) in different brain areas.

Steroid	Area	Subjects	Controls			All alcoholics			*t* test		Type 1 alcoholics			Type 2 alcoholics			ANOVA	
			Mean	SD	*n*	Mean	SD	*n*	*d*	*p*	Mean	SD	*n*	Mean	SD	*n*	*f*	*p*
DHEA	NAC	All	115.45	61.79	10	248.44	100.47	14	1.64	0.002	232.00	89.51	6	260.78	106.31	8	0.77	0.007
		Males	110.02	38.68	8	249.74	105.88	12	1.93	0.003	227.66	101.48	4	260.78	106.31	8	0.82	0.013
	AINS	All	109.32	59.28	10	260.85	116.35	14	1.73	0.002	214.34	115.56	6	295.72	104.10	8	0.88	0.002
		Males	103.22	36.74	8	280.57	113.92	12	2.35	<0.001	250.28	126.03	4	295.72	104.10	8	0.99	0.003
	HIPP	All	93.57	49.52	10	187.59	87.58	14	1.37	0.008	147.63	50.62	6	217.55	96.98	8	0.77	0.008
		Males	86.06	24.65	8	195.63	89.51	12	1.92	0.005	151.79	48.38	4	217.55	96.98	8	0.88	0.008
	ACC	All	111.68	49.82	10	291.20	197.82	14	1.45	0.013	238.79	170.38	6	330.51	207.62	8	0.63	0.030
		Males	105.89	37.99	8	320.31	199.16	12	1.81	0.011	299.92	179.34	4	330.51	207.62	8	0.68	0.040
	AMY	All	92.47	42.28	10	157.83	74.87	14	1.12	0.026	151.15	81.66	6	162.85	68.91	8	0.51	0.085
		Males	88.91	27.49	8	157.11	73.11	12	1.36	0.028	145.65	79.63	4	162.85	68.91	8	0.58	0.087
	FC	All	117.22	79.25	9	228.07	100.50	13	1.23	0.014	194.47	92.66	6	253.27	98.76	8	0.65	0.029
		Males	101.32	36.47	7	241.38	102.39	11	2.02	0.004	217.61	105.34	4	253.27	98.76	8	0.82	0.015
PREGN	NAC	All	296.85	132.99	10	579.77	274.17	14	1.39	0.008	589.51	294.79	6	572.47	257.37	8	0.62	0.034
		Males	304.09	137.99	8	609.12	285.71	12	1.44	0.016	682.40	323.04	4	572.47	257.37	8	0.66	0.046
	AINS	All	317.22	131.37	10	812.83	526.37	14	1.51	0.011	762.30	452.62	6	850.73	572.59	8	0.61	0.039
		Males	340.23	133.65	8	876.45	541.55	12	1.59	0.018	927.91	469.20	4	850.73	572.59	8	0.62	0.064
	HIPP	All	306.12	99.28	10	522.38	192.01	14	1.48	0.005	478.55	209.55	6	555.26	170.49	8	0.70	0.015
		Males	323.56	103.43	8	553.79	188.82	12	1.58	0.008	550.84	220.93	4	555.26	170.49	8	0.70	0.033
	ACC	All	304.66	158.75	10	805.80	527.37	14	1.46	0.011	842.51	638.42	6	778.28	423.39	8	0.60	0.040
		Males	317.61	175.02	8	892.00	521.16	12	1.65	0.011	1119.46	615.40	4	778.28	423.39	8	0.77	0.019
	AMY	All	286.47	157.62	10	356.10	163.70	14	0.43	0.329	407.29	186.46	6	317.7	131.82	8	0.31	0.391
		Males	314.56	164.27	8	353.80	171.79	12	0.23	0.635	426.00	214.39	4	317.70	131.82	8	0.27	0.555
	FC	All	325.71	165.58	9	620.04	286.81	13	1.30	0.014	674.43	331.73	6	579.24	239.86	8	0.61	0.043
		Males	341.47	174.90	7	651.07	297.62	11	1.31	0.030	794.74	345.98	4	579.24	239.86	8	0.69	0.045
T	NAC	Males	17.37	6.22	8	15.93	4.92	12	−0.26	0.590	15.69	6.08	4	16.05	4.22	8	0.13	0.864
	AINS	Males	13.54	3.60	8	13.23	4.41	12	−0.08	0.874	12.08	5.30	4	13.80	3.76	8	0.16	0.806
	HIPP	Males	12.43	3.04	8	12.52	3.41	12	0.03	0.959	11.20	2.63	4	13.17	3.56	8	0.23	0.654
	ACC	Males	14.36	2.63	8	16.53	7.25	12	0.44	0.452	14.30	6.58	4	17.64	7.32	8	0.28	0.521
	AMY	Males	13.90	2.72	8	12.93	4.91	12	−0.25	0.635	11.55	5.93	4	13.63	4.14	8	0.22	0.674
	FC	Males	14.51	4.09	7	12.79	3.89	11	−0.43	0.402	13.47	5.52	4	12.45	2.68	8	0.23	0.661

DHEA, dehydroepiandosterone; PREGN, pregnenolone; T, testosterone; NAC, nucleus accumbens; AINS, anterior insula; HIPP, hippocampus; ACC, anterior cingulate cortex; AMY, amygdala; FC, frontal cortex; SD, standard deviation; *n*, number of samples above the quantitation limit of the assay; *d*, Cohen's *d* effect size (comparison between all alcoholics and controls); *f*, Cohen's *f* effect size (comparison between controls, type 1 and type 2 alcoholics); *p*, *p* value from *t* test (comparison between all alcoholics and controls) or ANOVA (comparison between controls, type 1 and type 2 alcoholics).

receptors (Laukkanen et al., 2013) in the FC (Supplementary Fig. 2) could be associated with these interactions between steroids and both NMDA and GABA-A receptors (Imamura & Prasad, 1998; Majewska, 1992; Majewska et al., 1990; Maninger et al., 2009). Overall, DHEA and PREGN can counteract some of the neuropharmacological effects of alcohol that include GABA-A and NMDA receptor function, monoamine transmitter activity, and effects mediated by the endocannabinoid system (Allan & Harris, 1986; Lovinger, White, & Weight, 1989; Lovinger & Zhou, 1994; Pava & Woodward, 2012; Suzdak, Glowa, et al., 1986; Suzdak, Schwartz, Skolnick, & Paul, 1986). Thus, high DHEA and PREGN levels in the post-mortem brain samples of alcoholics, observed in the present study, could be associated with an acquired tolerance toward alcohol-induced positive effects, which could contribute adversely to the pathology of alcoholism (Koob, 2013; Leggio & Addolorato, 2008; Tupala & Tiihonen, 2004).

In the present study, some alcoholics had relatively normal PREGN levels while the others had significantly increased brain tissue levels of PREGN when compared to non-alcoholic controls (Fig. 2). Furthermore, these differences in PREGN levels do not follow the Cloninger's typology of alcoholism. Both female alcoholics in the present study had relatively normal PREGN levels when compared to controls, but there were no clear associations between background information (e.g., cause of death, AGE, BAC, or PMI) and the difference in PREGN levels between the alcoholics. However, there were significant negative Spearman correlations between brain tissue levels of PREGN and previous reports of [^{3}H] naloxone binding in the AINS, ACC, NAC, and FC (Fig. 3). Although correlation does not imply causation, these results suggest a possible role for the opioid system in regulation of PREGN levels. This agrees with reports that pharmacological challenge with the MOR antagonist naloxone increases plasma levels of PREGN in non-human primates (Porcu et al., 2006) and that chronic morphine administration decreases PREGN levels, while a naloxone challenge increases PREGN levels in the rat brain tissue (Yan & Hou, 2004). Future research projects should study the possibility that PREGN levels are associated with binding and possibly efficacy of the MOR antagonist naltrexone and nalmefene in the treatment of alcoholism (Nutt, 2014).

In the present study, we did not detect any differences in the brain tissue levels of T between non-alcoholic controls and alcoholics or between antisocial Cloninger type 2 alcoholics and harm-avoiding type 1 alcoholics (Fig. 2 and Table 2). This contrasts with previous studies showing altered peripheral and cerebrospinal fluid T levels in association with alcoholism and antisocial behavior (Eriksson, Kaprio, Pulkkinen, & Rose, 2005; La Grange, Jones, Erb, & Reyes, 1995; Lenz et al., 2012; Virkkunen et al., 1994; Yildirim & Derksen, 2012). However, peripheral T levels do not differ between alcoholics and controls at the onset of withdrawal, and increased T levels are observed only after a period of abstinence (Walter et al., 2007). Most of the alcoholics in the present study were intoxicated at the time of death (Table 1). High intoxicating doses of alcohol have been associated with decreased peripheral T levels (Mendelson et al., 1977), but conversely with increased T levels in the rat brain (Alomary et al., 2003). Therefore, phase of alcohol intoxication and altered metabolism of T in the brain are possible explanations for the differences between present results and previous results from body fluids.

Because of the small number of subjects in these diagnostic groups, the present results should be considered preliminary. Intra-tissue concentrations of DHEA and PREGN were quantifiable in almost all brain samples by LC-MS/MS. T levels were quantifiable in

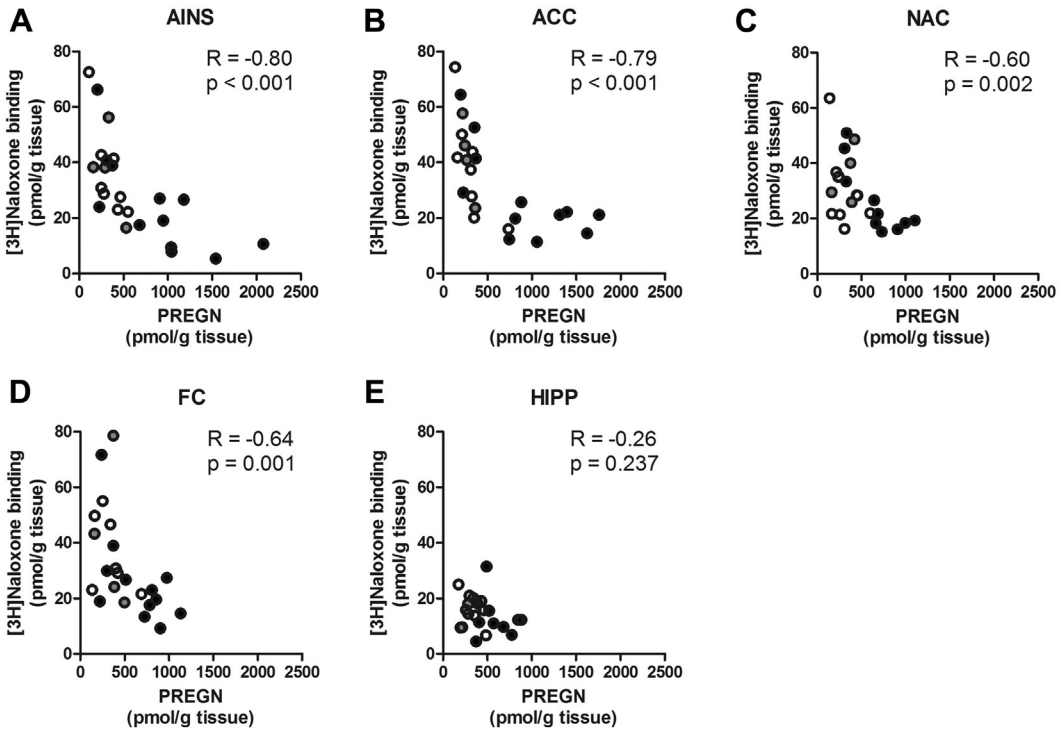

Fig. 3. Spearman correlations between pregnenolone levels and [^{3}H]naloxone binding in different brain regions measured from post-mortem brain samples of alcoholics and non-alcoholic controls. Measured brain tissue levels of pregnenolone compared to previous reports of [^{3}H]naloxone binding values (Laukkanen et al., 2015) measured from the same study subjects in anterior insula (A), anterior cingulate cortex (B), nucleus accumbens (C), frontal cortex (D), and hippocampus (E). Females are presented as gray circles, male alcoholics are black circles, and male non-alcoholic controls are white circles. Correlations were calculated using the Spearman method. AINS, anterior insula; ACC, anterior cingulate cortex; NAC, nucleus accumbens; FC, frontal cortex; HIPP, hippocampus; PREGN, pregnenolone.

all male subjects, but only in one female subject (a type 1 alcoholic) who was excluded from the statistical analyses for T results (Table 2). The quantitation limit of the assay for T concentration was 0.033 nM. Inclusion or exclusion of the female subjects did not have a large effect on the mean DHEA or PREGN levels (Table 2). Furthermore, dehydroepiandrosterone sulfate and pregnenolone sulfate, which have inhibitory effects on GABA-A receptor function (Finn, Ford, Wiren, Roselli, & Crabbe, 2004; Helms et al., 2012; Park-Chung, Malayev, Purdy, Gibbs, & Farb, 1999), were not measured in the present study because the method only enabled the measurement of unconjugated steroids (Keski-Rahkonen et al., 2011). Because of the limited availability of the sample material, we were not able to conduct tissue-specific validation of the method (Huhtinen et al., 2014; Keski-Rahkonen et al., 2011). In the present study we used internal standards to compensate for the potential differences in the matrix effect between the samples. For this reason, the present results could only be used to compare differences between the study groups, which was the main aim of the present study.

In conclusion, although preliminary, the present results show that DHEA levels seem to be increased in the brains of alcoholics when compared to non-alcoholic controls (Fig. 2). This difference could be associated with the chronic consumption of alcohol and the subsequent pathology of alcoholism, possibly contributing to allostatic changes in reward and stress responses (Koob, 2013). Furthermore, brain tissue levels of PREGN seem to be increased only in a subset of alcoholics when compared to controls (Fig. 2). Moreover, PREGN levels were negatively correlated with [^{3}H]naloxone binding (Fig. 3), suggesting an association between the opioid system and brain PREGN levels. More studies are needed to further clarify the likely relationship between alcoholism and alterations in steroid function, synthesis, and metabolism.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.alcohol.2016.03.002.

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