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

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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 [3H]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.

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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 et al., 2012). Intra-tissue levels of steroid hormones (e.g., dehydroepiandrosterone [DHEA] and pregnenolone [PREGN]) have been 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...
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 (Iamamura & Prasad, 1998; Maninger, Wolkowitz, Reus, Epel, & Mellon, 2009; Pérez-Neri, Montes, & Rios, 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; Revzani & 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 affect the CNS (such as neuroleptics or antidepressants), and subse-

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, Hakkinen, Tupalpa, & Tiitinen, 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 Medical Affairs, Helsinki, Finland (Dnro 3020/322/96 and 3141/32/200/98). The brains were removed, cleaned of the dura, and divided at the mid sagittal 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 [1 mass/mass % is equivalent to approximately 106 mg of alcohol in 1 ml 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, Bonfanti, & 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

<table>
<thead>
<tr>
<th>Group and subject</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PMI (h)</th>
<th>BAC (%)</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-alcoholic controls</td>
<td>Male</td>
<td>55</td>
<td>5.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>1</td>
<td>Male</td>
<td>45</td>
<td>9.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>77</td>
<td>7.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>57</td>
<td>11.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>50</td>
<td>18.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>60</td>
<td>12.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>49</td>
<td>33.0</td>
<td>0.4</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>53</td>
<td>29.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>53</td>
<td>11.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>36</td>
<td>11.0</td>
<td>0.0</td>
<td>Dissection of aorta</td>
</tr>
</tbody>
</table>

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 steroidal 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, PMI, and BAC, 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 method was 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 [3H]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 [3H]naloxone binding was not measured from this brain region. In comparison, negative correlations between DHEA and previous reports of [3H]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 [3H]naloxone binding and T levels measured in the present study (data not shown).
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 $[^3]H$unitrazepam 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. 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: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

![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 in 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: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.](image-url)
DHEA, dehydroepiandosterone; PREGN, pregnenolone; T, testosterone; NAC, nucleus accumbens; AINS, anterior insula; HIPP, hippocampus; ACC, anterior cingulate cortex; 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 harmful-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, & 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 fluids.

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 [1H] 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
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 [3H]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.

Acknowledgments

The authors declare that they have no conflicts of interest in this study.

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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.

References


