

Endogenous cannabinoids in amygdala and hippocampus in post-mortem brains of Cloninger type 1 and 2 alcoholics

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Abstract

Accumulating evidence continues to link certain aspects of the endogenous cannabinoid (EC) system with alcohol dependence, negative reinforcement learning and the modulation of stress response. Specific alterations in brain regions that are related to stress and negative reinforcement learning have been reported to exist in Cloninger type 1 and 2 alcoholics. To study possible differences in profiles of ECs between Cloninger type 1 (n=9), type 2 (n=8) alcoholics and non-alcoholic control subjects (n=10), we analyzed post-mortem amygdala and hippocampus brain samples for several ECs by quantitative liquid chromatography with triple quadrupole mass spectrometric detection. A significant difference was found between these three groups in terms of EC profiles in the amygdala ($p = 0.037$). In particular, this difference was prominent for variations in docosahexaenoylethanolamine levels, which were significantly higher in type 1 alcoholics ($p = 0.022$) when compared to controls. There was also a large negative correlation between anandamide concentration and mGlu1/5 receptor density in the hippocampus of Cloninger type 1 alcoholics ($R = -0.88$, $p = 0.002$), which was not seen in type 2 alcoholics or in controls. Although preliminary, and from relatively small diagnostic groups, these results suggest that the EC profile may be altered in the hippocampus and amygdala of type 1 alcoholics.

Keywords

Endocannabinoids; Anandamide; 2-Arachidonoylglycerol; Docosahexaenoylethanolamide; Late onset alcoholism

Introduction

Alcoholism has become the most harmful drug of abuse, especially when harm to the alcoholic individual and society are taken into account (Nutt et al., 2010). Moreover, alcohol consumption is one of the leading risk factors of death among the working age population (World Health Organization, 2011). Alcoholics are a heterogeneous group with a wide spectrum of emotional, social and medical problems. In Cloninger's typology, alcoholics are divided into two groups (Cloninger, 1995); type 1 alcoholics who are anxiety prone and characterized by adult onset alcoholism, and type 2 alcoholics who are characterized by impulsive, antisocial behavior and teenage onset alcoholism. We previously reported on decreased dopamine function in post-mortem brains of Cloninger type 1 alcoholics (Tupala et al., 2000; Tupala et al., 2001; Tupala and Tiihonen, 2004) and alterations in the serotonergic signaling in both type 1 and type 2 alcoholics (Leggio and Addolorato, 2008; Mantere et al., 2002; Storvik et al., 2006; Storvik et al., 2009).

The behavioral characteristics seen in type 1 and type 2 alcoholics suggest that both groups may have problems in modulating appropriate stress responses (Cloninger, 1995). Dysfunctional stress responses, including allostatic alterations in the function of the stress-mediating amygdala and stress-inhibiting hippocampus, have been hypothesized to be one of the key neural changes in the pathology of addiction, which proceeds from a negative reinforcement of learning processes (Funk et al., 2006; Koob and Le Moal, 2008; Vendruscolo et al., 2012). Alterations in the stress systems of Cloninger type 1 and type 2 alcoholics have been reported, for example, as altered metabotropic glutamate receptor 1/5 (mGluR1/5) densities in the hippocampus (Kupila et al., 2012), altered serotonin transporter (SERT) densities in the amygdala (Storvik et al., 2007) and altered ratios between SERT densities in the amygdala and hypothalamus (Storvik et al., 2008).

Endocannabinoids (ECs) have been associated with alcohol dependence (Pava and Woodward, 2012), with the regulation of stress response (Ruehle et al., 2012) and with the modulation of stress-enhanced negative reinforcement learning (Campolongo et al., 2009; Ramot and Akirav, 2012). ECs have both anxiogenic and anxiolytic properties, and this biphasic response has been suggested to results from changes in cannabinoid receptor type 1 (CB1) expression in both glutamatergic and GABAergic axon terminals (Millan, 2003). Furthermore, different brain regions seem to be responsible for the anxiogenic and anxiolytic properties of ECs (Rubino et al., 2008a). It has also been suggested that ECs can activate other receptors, such as the transient receptor potential vanilloid 1 (TRPV1), which may contribute to anxiogenic effects (Rubino et al., 2008b). Recently, we reported on significantly altered endocannabinoid levels in the Cloninger type 1 nucleus accumbens, cingulate cortex and frontal cortex (Lehtonen et al., 2010).

To our knowledge, EC profiles in the amygdala and hippocampus of Cloninger type 1 and 2 alcoholics have not been previously reported. The main aim of the present study was to examine endocannabinoid profiles in the amygdala and hippocampus by measuring 2-arachidonoyl glycerol (2-AG), arachidonylethanolamine (anandamide, AEA), dihomono- γ -linolenylethanolamine (LEA), docosatetraenylethanolamine (DHEA), palmitoyl ethanolamine (PEA) and oleoyl ethanolamine (OEA) levels in post-mortem brain samples of type 1 and 2 alcoholics and a control group. Secondary aim of the present study was to determine possible correlations between EC levels and previously published mGluR1/5 densities from these same individuals (Kupila et al., 2012), because activation of mGluR5 has been associated with the release of ECs (Chavez et al., 2010).

Materials and methods

Study subjects, sampling and diagnostics

The selection and collection of post-mortem human brains, psychological diagnostics and sample preservation methods have already been described in detail (Mantere et al., 2002; Tupala et al., 2001). 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 Kuopio, Finland. The Ethics Committee of the University of Oulu and the National Institute of Medico-legal Affairs, Helsinki, Finland, approved the study. The brains were removed, cleaned of the dura, and divided at the midsagittal plane. The left hemisphere was placed with the midsagittal plane on a glass plate before freezing at $-75\text{ }^{\circ}\text{C}$. None of the hemispheres exhibited damage or neuroanatomical abnormalities. Brain samples were cryosectioned into 100- μm horizontal (cantomeatal) slices (Tupala et al., 2001). These sections were allowed to air dry before they were stored with dehydrating agents at $-25\text{ }^{\circ}\text{C}$ until use.

Medical records on the cause of death, previous diseases and medical treatments were collected. Alcoholism was coded according to DSM-IV criteria (APA, 1994), and sub-classified as type 1 or 2, according to Cloninger's typology of alcoholism (1995). Two physicians reviewed medical records and anamnestic data, which included extant criminal records. The two main criteria for defining type 2 alcoholics were early onset of alcohol abuse (i.e., before the age of 25) and a record of severe antisocial behavior. The Cohen's kappa coefficient of diagnostic agreement subjects was 0.9; i.e., one of the type 2 alcoholics was diagnosed as a type 1 alcoholic by the second physician. Otherwise, diagnoses were unanimous. Subjects with psychotic disorders or any neurological diseases, or those taking medication that could affect the CNS (such as neuroleptics or antidepressants), or the use of

substances that directly affect the dopaminergic system (e.g., psychostimulants or opioids) were excluded.

The study groups consisted of 17 alcoholics that were further sub-classified as type 1 alcoholics (N=9, seven males, two females; mean age 52.7 years; post-mortem interval (PMI) 11.9 ± 4.5 h; mean \pm SD), and type 2 alcoholics (N=8, all males; mean age 34.6 years; PMI 14.1 ± 3.4 h; mean \pm SD). The control group consisted of 10 non-alcoholic subjects (eight males, two females; mean age 53.5 years; PMI 14.8 ± 9.2 h; mean \pm SD) who were free of psychiatric diagnosis. All subjects died of sudden causes, and individual data are shown in Table 1. Alcoholism was determined by frequent admissions to emergency stations and other medical appointments due to alcohol-related problems. Eight of the nine type 1 alcoholics had ethanol in their blood at their time of death, and one had an abstinence period of 10 h while incarcerated. 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 alcoholics had traces of diazepam in their blood samples. Six type 2 alcoholics had ethanol in their blood at the time of death, and three had traces of benzodiazepines. One type 2 alcoholic had an abstinence period of five days and another abstained for three to seven days. Mean blood alcohol concentration (BAC) was $2.0 \pm 1.7\%$ and $1.9 \pm 1.4\%$ for the type 1 and 2 alcoholics at the time of death, respectively, and these two groups did not differ in this regard [$F(0.174)$, $p = 0.90$, independent samples t-test](Tupala et al., 2008). 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.

Measurement of ECs by liquid chromatography/mass spectrometry

Post-mortem brain EC levels were measured from amygdala and hippocampus by a liquid chromatography/tandem mass spectrometric (LC/MS/MS) method (Lehtonen et al., 2011). Briefly, frozen post-mortem brain tissues (1–3 mg) were removed from the glass slide with a scalpel and transferred to a pre-weighed Eppendorf tube, then weighed again to determine the precise amount of sample. Methanol (500 μ l) and the deuterated internal standards AEA-d8 (50 μ l, 50 nM) and 2-AG-d8 (50 μ l, 460 nM) were added to the sample and homogenized with ultrasonification. Lipids were extracted by adding chloroform and water to yield a final methanol/chloroform/water ratio of 1:2:1 (v/v/v), then centrifuged at $1500 \times g$ for 10 min at 10 °C. The upper aqueous layer was discarded and the lower organic layer was transferred to a screw capped glass test tube. The liquid extraction was repeated, the chloroform phases were combined and then evaporated to dryness under nitrogen at 20 °C. The residue was reconstituted in 70 μ l of an ice-cold acetonitrile water solution (5:2, v/v). The sample was then transferred to a sample vial for the separation of ECs by liquid chromatography (Agilent 1200 Series Rapid Resolution LC System, Agilent Technologies, Waldbronn, Germany), and measured by electrospray ionization (ESI) triple quadrupole mass spectrometric detection (Agilent 6410 Triple Quadrupole LC/MS, Agilent Technologies, Palo Alto, CA, USA). For each analysis, 10 μ l of sample solution was injected onto a reversed phase HPLC column (Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 \times 50 mm, 1.8 μ m) (Agilent Technologies, Palo Alto, CA, USA) using an isocratic mobile phase that consisted of 67% of 0.1% formic acid in acetonitrile and 33% of 0.1% formic acid in water. Ionization was performed with positive ESI and detection was by multiple reaction monitoring (MRM). This analytical method was selective, accurate and precise for concentrations within a calibration range of 0.4–40 nM for N-acylethanolamines (NAEs: i.e., AEA, DHEA, LEA, PEA, and OEA) and 40–5000 nM for 2-AG.

Statistical analyses

The measured concentrations of individual endocannabinoids were standardized to same mean and distribution within variable and across the study groups by standard deviation. The results are expressed as standardized values to enable comparison of EC profiles for six different ECs between the groups. Statistical significance of the differences in the EC concentrations between the three groups were evaluated by permutation type multivariate test of variance (MANCOVA). The PMI was used as a covariate because it has an effect on brain EC concentrations (Palkovits et al., 2008) and because it was observed that within the present data there was moderate correlation between EC concentrations and PMI (Pearson correlation). The 95% confidence intervals for the most important outcomes were obtained by bias-corrected bootstrapping (5,000 replications).

In order to study the possible correlations of measured EC levels to the regulatory mGlu1/5 receptor system, Spearman correlations were calculated to determine direct correlations of EC levels to previously published mGlu1/5 receptor densities (Kupila et al., 2012). The differences in Spearman correlations between the subject groups were tested by Fisher's (z)-transformation of correlations to evaluate the overall differences, followed by a Tukey-type test to compare individual groups. Correlations greater than 0.5 were considered to be large (Cohen, 1988). STATA (release 11.2, College Station TX) was used for all statistical analyses.

Results

The LC/MS/MS method was able to quantify the four most common ECs (i.e., 2-AG, AEA, LEA, and DHEA) and two other cannabimimetic compounds (PEA and OEA) in the amygdala and hippocampus of all subjects. The quantitative amounts of measured compounds were specific for both the brain regions and the subject groups. 2-AG was the most abundant EC found in both studied brain regions of all groups. The results of measured endocannabinoid profiles are presented in Figure 1 and in Supplementary table 1.

There was a significant difference in the endocannabinoid profiles between the three groups in the amygdala ($p = 0.037$, Figure 1). Further, this difference in the amygdala endocannabinoid profiles was especially prominent for DHEA between type 1 alcoholics and the control group ($p = 0.022$), with the type 1 alcoholics showing significantly higher DHEA levels. There were no significant differences in hippocampal endocannabinoid profiles between the three groups ($p = 0.21$, Figure 1).

There was a large negative correlation between AEA levels in hippocampus and previously published mGluR1/5 receptor concentrations (Kupila et al., 2012) in the hippocampal CA1 region ($R=-0.88$; Figure 2) in Cloninger type 1 alcoholics. This correlation was not seen in the control group or in type 2 alcoholics, and the difference between the three study groups was significant ($\chi^2=9.403$, $p=0.009$). Because of the limitations in the sampling method, we were not able to measure EC levels from only the CA1 region of the hippocampus.

Discussion

In this study, EC levels were measured in the amygdala and hippocampus of Cloninger type 1 and 2 alcoholic post-mortem brains, and compared with analogous samples from a group of non-alcoholic controls. A statistically significant ($p=0.037$) difference in EC profiles between the groups was found in the amygdala (Figure 1). In the hippocampus, there were no significant differences in the EC profiles between the groups. The main difference in the amygdala EC profiles was localized to DHEA levels, where type 1 alcoholics had increased DHEA levels when compared to the control group. The Cohen's effect size for this finding was large (-0.98), which increases the validity of this result, despite the p-value being only 0.022.

DHEA is an ethanolamide derivative of the *omega*-3 fatty acid docosahexaenoic acid (DHA), and in the context of addiction, low plasma levels of DHA have been associated with increased relapse vulnerability (Buydens-Branchey et al., 2009). DHEA is referred as an endocannabinoid, even though it has only a weak affinity to cannabinoid receptors (Felder et al., 1993). Therefore, it is possible that DHEA has still unknown primary target molecule. Current knowledge on the role of DHEA in the central nervous system, especially in the amygdala, is limited. DHEA has been found to promote synaptic development and glutamatergic synaptic activity in hippocampal neurons (Kim et al., 2011a; Kim et al., 2011b). DHEA oxidative metabolism products have also been associated with anti-inflammatory and neuroprotective properties (Yang et al., 2011). Increased levels of DHEA in the amygdala could be associated with anxiety-prone behavior seen in type 1 alcoholics, but before future research clarifies the role of DHEA in the amygdala no valid conclusion can be made.

In the present study, a large negative correlation between AEA levels in the hippocampus and previously published mGluR1/5 densities in the CA1 hippocampal region (Kupila et al., 2012) of

Cloninger type 1 alcoholics was observed (Figure 2). Superficially this Spearman correlation seems to be biased by two observations with high mGluR1/5 densities, but these observations are not outliers (detection performed) and therefore there is no justification of removing them (Figure 2). Furthermore, even if these two observations are removed there still is a large negative correlation between AEA levels in the hippocampus and previously published mGluR1/5 densities in the CA1 hippocampal region (Kupila et al., 2012) in type 1 alcoholics ($R=-0.79$). Activation of mGluR5 and further release of AEA are necessary for TRPV1 mediated synaptic plasticity (Chavez et al., 2010). Therefore, the altered correlation between AEA levels and the previously published mGluR1/5 densities (Kupila et al., 2012) in type 1 alcoholics could be associated with TRPV1 mediated synaptic plasticity.

Consumption of alcohol has been associated with alterations in 2-AG and AEA levels in both amygdala and hippocampus (Gonzalez et al., 2004; Malinen et al., 2009; Rubio et al., 2007). However, in the present study the differences in the EC profiles were not localized to these two endocannabinoids and altered correlation between EC levels and previously published mGluR1/5 densities was only seen in the hippocampal AEA levels. In a previous human post-mortem brain study, differences were only found in AEA levels of cortical regions and the NAc (Lehtonen et al., 2010). Together, these findings suggest that in human alcoholism the changes in 2-AG may not be as important as suggested from animal models (Gonzalez et al., 2004; Lehtonen et al., 2010; Malinen et al., 2009; Rubio et al., 2007).

In the present study, we included PMI as a covariant to minimize its influence on the results, because it has an effect to the EC levels in post-mortem brain samples and that this effect is EC specific (Palkovits et al., 2008). There were moderate correlations between PMI and EC levels in the present data (Supplementary Figures 1 and 2) further justifying the use of PMI as a covariant. Limitation of

the present study, like all post-mortem studies, is that the EC levels from post-mortem samples might not fully represent the levels prior to death. Therefore, future research is needed to verify the present results in vivo and to examine EC binding sites, e.g. CB1 and TRPV1, in alcoholics.

Although the results from the present study are preliminary, and from diagnostic groups with a relatively small number of subjects, these findings reveal interesting alterations in the post-mortem brain profiles of ECs for Cloninger type 1 and 2 alcoholics. Our results show increased levels of DHEA in the amygdala and a large negative correlation between AEA and previously published mGluR1/5 densities (Kupila et al., 2012) in the hippocampus of type 1 alcoholics. Together, with previous findings of altered function of the amygdala and hippocampus (Kupila et al., 2012; Storvik et al., 2007; Storvik et al., 2008), these results suggest that altered function of the stress system might enforce the typical behavioural patterns seen in the anxiety-prone type 1 alcoholics. Further study is needed to clarify the role of stress systems and ECs in the pathology of alcoholism.

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Reference List

- Buydens-Branchey, L., Branchey, M., and Hibbeln, J.R. (2009). Low plasma levels of docosahexaenoic acid are associated with an increased relapse vulnerability in substance abusers *Am. J. Addict.* *18*, 73-80.
- Campolongo, P., Roozendaal, B., Trezza, V., Hauer, D., Schelling, G., McGaugh, J.L., and Cuomo, V. (2009). Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory *Proc. Natl. Acad. Sci. U. S. A.* *106*, 4888-4893.
- Chavez, A.E., Chiu, C.Q., and Castillo, P.E. (2010). TRPV1 activation by endogenous anandamide triggers postsynaptic long-term depression in dentate gyrus. *Nat. Neurosci.* *13*, 1511-1518.
- Cloninger, C.R. (1995). The psychobiological regulation of social cooperation. *Nat. Med.* *1*, 623-625.
- Cohen, J. (1988). *Statistical power analysis for the behavioral sciences* (Hillsdale, N.J.: L. Erlbaum Associates).
- Felder, C.C., Briley, E.M., Axelrod, J., Simpson, J.T., Mackie, K., and Devane, W.A. (1993). Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. U. S. A.* *90*, 7656-7660.
- Funk, C.K., O'Dell, L.E., Crawford, E.F., and Koob, G.F. (2006). Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. *J. Neurosci.* *26*, 11324-11332.
- Gonzalez, S., Valenti, M., de Miguel, R., Fezza, F., Fernandez-Ruiz, J., Di Marzo, V., and Ramos, J.A. (2004). Changes in endocannabinoid contents in reward-related brain regions of alcohol-exposed rats, and their possible relevance to alcohol relapse. *Br. J. Pharmacol.* *143*, 455-464.
- Kim, H.Y., Moon, H.S., Cao, D., Lee, J., Kevala, K., Jun, S.B., Lovinger, D.M., Akbar, M., and Huang, B.X. (2011a). N-Docosahexaenylethanolamide promotes development of hippocampal neurons. *Biochem. J.* *435*, 327-336.
- Kim, H.Y., Spector, A.A., and Xiong, Z.M. (2011b). A synaptogenic amide N-docosahexaenylethanolamide promotes hippocampal development. *Prostaglandins Other Lipid Mediat.* *96*, 114-120.
- Koob, G.F., and Le Moal, M. (2008). Addiction and the brain antireward system. *Annu. Rev. Psychol.* *59*, 29-53.
- Kupila, J., Karkkainen, O., Laukkanen, V., Tupala, E., Tiihonen, J., and Storvik, M. (2012). mGluR1/5 receptor densities in the brains of alcoholic subjects: A whole-hemisphere autoradiography study. *Psychiatry Res* (Epub ahead of print).
- Leggio, L., and Addolorato, G. (2008). Serotonin transporter (SERT) brain density and neurobiological Cloninger subtypes model: a lesson by human autoradiography studies. *Alcohol Alcohol.* *43*, 148-150.

- Lehtonen, M., Storvik, M., Malinen, H., Hyytia, P., Lakso, M., Auriola, S., Wong, G., and Callaway, J.C. (2011). Determination of endocannabinoids in nematodes and human brain tissue by liquid chromatography electrospray ionization tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.* *879*, 677-694.
- Lehtonen, M., Storvik, M., Tupala, E., Hyytia, P., Tiihonen, J., and Callaway, J.C. (2010). Endogenous cannabinoids in post-mortem brains of Cloninger type 1 and 2 alcoholics. *Eur. Neuropsychopharmacol.* *20*, 245-252.
- Malinen, H., Lehtonen, M., and Hyytia, P. (2009). Modulation of brain endocannabinoid levels by voluntary alcohol consumption in alcohol-preferring AA rats. *Alcohol. Clin. Exp. Res.* *33*, 1711-1720.
- Mantere, T., Tupala, E., Hall, H., Sarkioja, T., Rasanen, P., Bergstrom, K., Callaway, J., and Tiihonen, J. (2002). Serotonin transporter distribution and density in the cerebral cortex of alcoholic and nonalcoholic comparison subjects: a whole-hemisphere autoradiography study. *Am. J. Psychiatry* *159*, 599-606.
- Millan, M.J. (2003). The neurobiology and control of anxious states. *Prog. Neurobiol.* *70*, 83-244.
- Nutt, D.J., King, L.A., Phillips, L.D., and Drugs, on behalf of the Independent Scientific Committee on. (2010). Drug harms in the UK: a multicriteria decision analysis. *The Lancet* *376*, 1558-1565.
- Palkovits, M., Harvey-White, J., Liu, J., Kovacs, Z.S., Bobest, M., Lovas, G., Bago, A.G., and Kunos, G. (2008). Regional distribution and effects of postmortal delay on endocannabinoid content of the human brain. *Neuroscience* *152*, 1032-1039.
- Pava, M.J., and Woodward, J.J. (2012). A review of the interactions between alcohol and the endocannabinoid system: Implications for alcohol dependence and future directions for research. *Alcohol* *46*, 185-204.
- Ramot, A., and Akirav, I. (2012). Cannabinoid receptors activation and glucocorticoid receptors deactivation in the amygdala prevent the stress-induced enhancement of a negative learning experience. *Neurobiol. Learn. Mem.* *97*, 393-401.
- Rubino, T., Guidali, C., Vigano, D., Realini, N., Valenti, M., Massi, P., and Parolaro, D. (2008a). CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour. *Neuropharmacology* *54*, 151-160.
- Rubino, T., Realini, N., Castiglioni, C., Guidali, C., Vigano, D., Marras, E., Petrosino, S., Perletti, G., Maccarrone, M., Di Marzo, V., and Parolaro, D. (2008b). Role in anxiety behavior of the endocannabinoid system in the prefrontal cortex. *Cereb. Cortex* *18*, 1292-1301.
- Rubio, M., McHugh, D., Fernandez-Ruiz, J., Bradshaw, H., and Walker, J.M. (2007). Short-term exposure to alcohol in rats affects brain levels of anandamide, other N-acyl ethanolamines and 2-arachidonoyl-glycerol. *Neurosci. Lett.* *421*, 270-274.
- Ruehle, S., Rey, A.A., Remmers, F., and Lutz, B. (2012). The endocannabinoid system in anxiety, fear memory and habituation. *J. Psychopharmacol.* *26*, 23-39.

- Storvik, M., Hakkinen, M., Tupala, E., and Tiihonen, J. (2009). 5-HT(1A) receptors in the frontal cortical brain areas in Cloninger type 1 and 2 alcoholics measured by whole-hemisphere autoradiography. *Alcohol Alcohol.* *44*, 2-7.
- Storvik, M., Haukijarvi, T., Tupala, E., and Tiihonen, J. (2008). Correlation between the SERT binding densities in hypothalamus and amygdala in Cloninger type 1 and 2 alcoholics. *Alcohol Alcohol.* *43*, 25-30.
- Storvik, M., Tiihonen, J., Haukijarvi, T., and Tupala, E. (2007). Amygdala serotonin transporters in alcoholics measured by whole hemisphere autoradiography. *Synapse* *61*, 629-636.
- Storvik, M., Tiihonen, J., Haukijarvi, T., and Tupala, E. (2006). Nucleus accumbens serotonin transporters in alcoholics measured by whole-hemisphere autoradiography. *Alcohol* *40*, 177-184.
- Tupala, E., Hakkinen, M., Storvik, M., and Tiihonen, J. (2008). Striatal dopaminergic terminals in type 1 and type 2 alcoholics measured with [3H]dihydrotetrabenazine and human whole hemisphere autoradiography. *Psychiatry Res.* *163*, 70-75.
- Tupala, E., Hall, H., Bergstrom, K., Sarkioja, T., Rasanen, P., Mantere, T., Callaway, J., Hiltunen, J., and Tiihonen, J. (2001). Dopamine D(2)/D(3)-receptor and transporter densities in nucleus accumbens and amygdala of type 1 and 2 alcoholics. *Mol. Psychiatry* *6*, 261-267.
- Tupala, E., Hall, H., Sarkioja, T., Rasanen, P., and Tiihonen, J. (2000). Dopamine-transporter density in nucleus accumbens of type-1 alcoholics. *Lancet* *355*, 380.
- Tupala, E., Kuikka, J.T., Hall, H., Bergstrom, K., Sarkioja, T., Rasanen, P., Mantere, T., Hiltunen, J., Vepsalainen, J., and Tiihonen, J. (2001). Measurement of the striatal dopamine transporter density and heterogeneity in type 1 alcoholics using human whole hemisphere autoradiography. *Neuroimage* *14*, 87-94.
- Tupala, E., and Tiihonen, J. (2004). Dopamine and alcoholism: neurobiological basis of ethanol abuse. *Prog. Neuropsychopharmacol. Biol. Psychiatry* *28*, 1221-1247.
- Vendruscolo, L.F., Barbier, E., Schlosburg, J.E., Misra, K.K., Whitfield, T.W., Jr, Logrip, M.L., Rivier, C., Repunte-Canonigo, V., Zorrilla, E.P., Sanna, P.P., Heilig, M., and Koob, G.F. (2012). Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. *J. Neurosci.* *32*, 7563-7571.
- World Health Organization. (2011). Global status report on alcohol and health.(Switzerland: WHO Press).
- Yang, R., Fredman, G., Krishnamoorthy, S., Agrawal, N., Irimia, D., Piomelli, D., and Serhan, C.N. (2011). Decoding functional metabolomics with docosahexaenoyl ethanolamide (DHEA) identifies novel bioactive signals. *J. Biol. Chem.* *286*, 31532-31541.

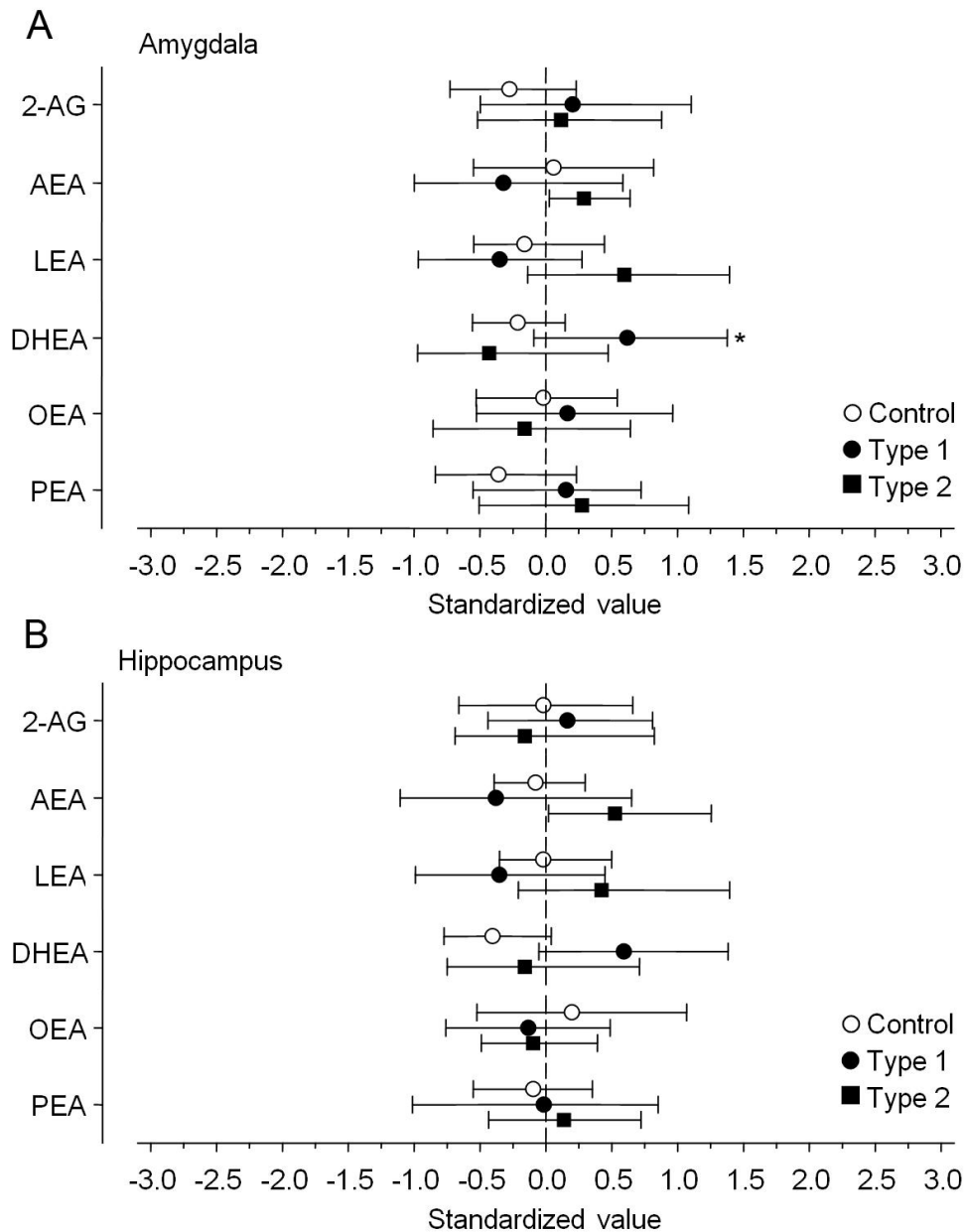


Figure 1. Amygdala and hippocampus endocannabinoid profiles of the nonalcoholic control group, with Cloninger type 1 and type 2 alcoholics.

Amygdala (A) and Hippocampus (B) endocannabinoid profiles of the control group, type 1 and type 2 alcoholics presented as standardised values with 95% confidence intervals. In the amygdala there was a significant difference in the EC profiles between the groups ($p=0.037$). The main difference between the EC profiles was localised to DHEA concentrations between type 1 alcoholics and the controls ($p=0.022$). In hippocampus there were no statistically significant differences between the groups ($p=0.21$). EC, endocannabinoid; 2-AG, 2-arachidonoyl glycerol; AEA, arachidonylethanolamine ; LEA, dihomogamma-linolenylethanolamine; DHEA, docosahexaenylethanolamine; OEA, oleoyl ethanolamine; PEA, palmitoyl ethanolamine; *, $p<0.05$.

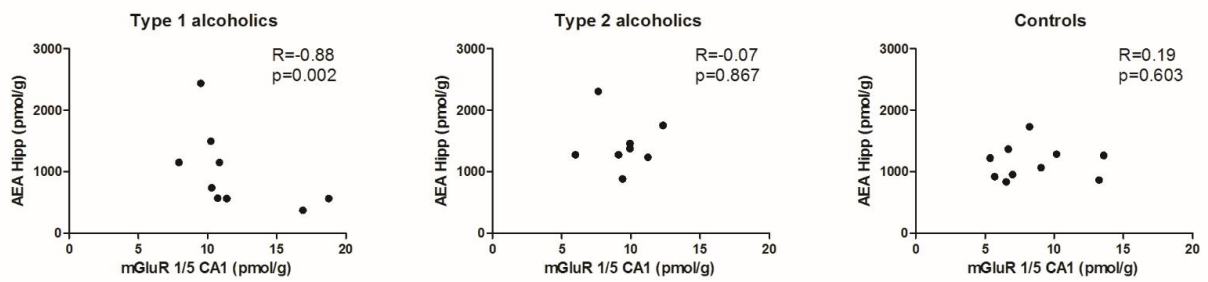
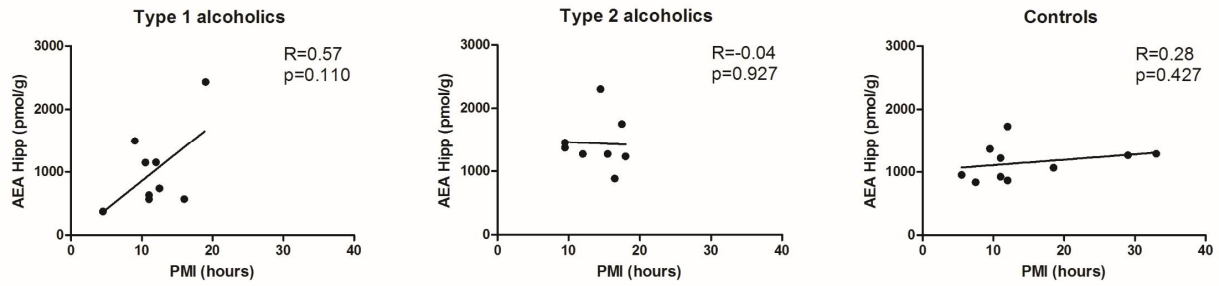


Figure 2. Spearman correlations between hippocampal AEA levels and previously published mGluR1/5 densities in the hippocampal CA1 region.

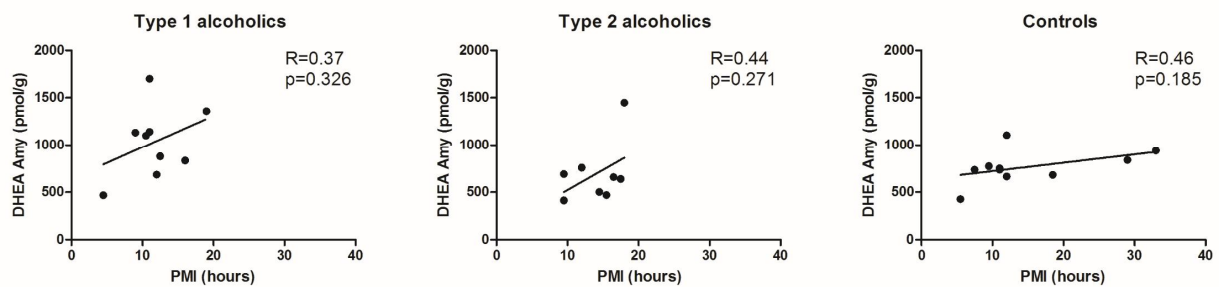
Type specific correlations can be seen between type 1, type 2 and controls in correlations between AEA levels and previously published mGluR1/5 densities (Kupila et al., 2012). AEA, arachidonylethanolamine (anandamide); Hipp, hippocampus; mGluR1/5, metabotropic glutamate receptor 1/5; CA1, CA1 regions of hippocampus.



Supplementary Figure 1. Pearson correlations between hippocampal AEA levels and post-mortem interval values.

Type specific correlations can be seen between type 1, type 2 and controls in correlations between AEA levels and post-mortem interval values.

AEA, arachidonylethanolamine (anandamide); Hipp, hippocampus; PMI, post-mortem interval.



Supplementary Figure 2. Pearson correlations between DHEA levels in amygdala and post-mortem interval values.

Type specific correlations can be seen between type 1, type 2 and controls in correlations between DHEA levels and post-mortem interval values.

Amy, amygdala; DHEA, docosatetraenoylethanolamine; PMI, post-mortem interval.