AMPA receptors in post-mortem brains of Cloninger type 1 and 2 alcoholics: A whole-hemisphere autoradiography study

Olli Kärkkäinen a, b; Virpi Laukkanen a, b; Jukka Kupila a, b; Merja Häkkinen c; Erkki Tupala a, b, †; Hannu Kautiainen d, e; Jari Tiihonen b, f; Markus Storvik a, *

a Pharmacology and Toxicology, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland

b Department of Forensic Psychiatry, Niuvanniemi Hospital, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland

c Department of Biosciences, University of Eastern Finland, P.O. Box 1627, Kuopio, Finland

d Unit of Family Practice, Central Finland Central Hospital, Jyväskylä, Finland

e Unit of Primary Health Care, Kuopio University Hospital, Finland

f Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden.

† Deceased

* Corresponding author: Markus Storvik, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland; E-mail: markus.storvik@uef.fi

ABSTRACT

Dysfunction of the brain glutamate system has been associated with alcoholism. Ionotropic glutamatergic alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) play an important role in both neurotransmission and post-synaptic plasticity. Alterations in AMPAR densities may also play a role in the neurobiological changes associated with alcoholism. In the present study, [^{3}H] AMPA binding density was evaluated in the nucleus accumbens (NAc), frontal cortex, anterior cingulate cortex (ACC), dentate gyrus and hippocampus of Cloninger type 1 (n=9) and 2 (n=8) alcoholics, and compared with non-alcoholic control subjects (n=10) by post-mortem whole-hemisphere autoradiography. The [^{3}H] AMPA binding density was significantly higher in the ACC of early onset type 2 alcoholics when compared with controls (p=0.011). There was also a significant negative correlation between [^{3}H] AMPA binding and previously published results of dopamine transporter (DAT) density in the ACC in these same brain samples (R=-0.95, p=0.001). Although preliminary, and from a relatively small diagnostic group, the present results help to further explain the pathology of alcohol dependence and impulsive behaviour in type 2 alcoholics.

Key words: Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, Anterior cingulate cortex, Alcohol dependence, Addiction, Impulsive behavior, Early onset alcoholism

1. Introduction

The habitual consumption of alcohol is one of the leading risk factors of death among the working age population (World Health Organization, 2011), and alcohol misuse is now considered to be the most harmful drug of abuse when harm to both the individual and society are taken into account (Nutt et al., 2010). Alcoholics are a heterogeneous group with a wide spectrum of emotional, social and medical problems. In Cloninger's typology of alcoholism, alcoholics are divided in to two groups; type 1 and 2 alcoholics (Cloninger, 1988,Cloninger, 1995). The main characteristics for these two groups of alcoholics are the age of the onset (<25 years in type 2 and >25 years in type 1) and behavioral traits such as impulsive and antisocial behavior in type 2 alcoholics, contrasted with a susceptibility to anxiety in type 1 alcoholics. Different receptor binding profiles have been reported between type 1 and 2 alcoholics; e.g., dopaminergic and serotonergic receptors and transporters (Tupala and Tiihonen, 2004,Leggio and Addolorato, 2008).

Cloninger's typology of alcoholism has a superficial resemblance to the two phase model of addiction proposed by Koob and Le Moal, where phase 1 substance abuse is impulsive and driven by positive reinforcement learning, which is similar to the euphoria seeking behavior seen with type 2 alcoholics, while phase 2 substance abuse is characterized by compulsive behaviors and influenced by negative reinforcement learning, which is similar to the anxiety-prone type 1 alcoholics (Cloninger, 1995,Koob and Le Moal, 2008). Positive and negative reinforcement learning are at the heart of pathologic addictions and, on a neural network level, synaptic plasticity with the addition and removal of glutamatergic alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) in the post-synaptic dendrite, which is thought to be a key mechanism for learning (Koob and Le Moal, 2008,Barry and Ziff, 2002,Bredt and Nicoll, 2003,Collingridge and Singer, 1990,Kessels and Malinow, 2009,Malinow and Malenka, 2002,Scannevin and Huganir, 2000). Evidence from both animal and human studies associates the function of the fast-acting ionotropic AMPARs to the pathology of alcoholism and other forms of addiction (Di Ciano and Everitt, 2001,Johnson et al., 2003,Johnson et al., 2007). In clinical studies, topiramate, an antagonist of AMPA and kainate receptors and agonist for GABA-A receptor, has been shown to be effective in the treatment of alcoholism (Johnson et al., 2003,Johnson et al., 2007). Conversely, AMPAR potentiators have also been studied in the pharmacologic treatment of addiction. For example, upregulation of AMPAR in the nucleus accumbens (NAc) seems to increase the extinction of cocaine-seeking behavior in rodents

(Sutton et al., 2003), and the AMPAR potentiator 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide has been shown to improve the preservation of extinction learning after cocaine self-administration (LaLumiere et al., 2010).

A limited amount of research has been published on the putative link between alcoholism and AMPARs. Chronic ethanol exposure seems to increase the AMPAR subunit protein expression in cortical cell cultures and AMPAR binding in rat cortical membranes (Chandler et al., 1999,Haugbol et al., 2005). Conversely, a recent study reported no alterations in AMPAR subunit expression in the prefrontal cortex of mice after chronic alcohol exposure (Kroener et al., 2012). In the hippocampus, AMPAR subunit mRNA levels were up-regulated during chronic alcohol exposure in rats (Bruckner et al., 1997). Previously, one human post-mortem autoradiography study was published on AMPARs in the frontal cortex (FC), where no significant difference was seen in AMPA binding between alcoholics and a non-alcoholic control group (Freund and Anderson, 1996). However, in that study only the superior frontal cortex AMPAR densities were measured, and the alcoholics were not divided into subgroups.

The purpose of the present study was to examine AMPAR densities from human post-mortem brain regions in Cloninger type 1 and 2 alcoholics and non-alcoholic controls by whole-hemisphere autoradiography. In this study we examined five different brain regions related to addiction and behavior control, namely the anterior cingulate cortex (ACC), the dentate gyrus (DG), the FC, the hippocampus (Hipp) and the NAc. Secondarily, we aimed to examine correlates of AMPAR densities to previously published results of dopamine transporter (DAT) (Tupala et al., 2000,Tupala et al., 2001,Tupala et al., 2006), serotonin transporter (SERT) (Mantere et al., 2002,Storvik et al., 2006a,Storvik et al., 2006b), dopamine D1 (Tupala and Tiihonen, 2005,Tupala and Tiihonen, 2008), dopamine D2 (Tupala et al., 2004), metabotropic glutamate 1/5 (mGluR1/5)(Kupila et al., 2012), serotonin 5-HT_{1A} (Storvik et al., 2009) and 5-HT_{1B} (Storvik et al., 2012) receptor densities from these same study subjects.

2. Methods

The methodology of this study has been described earlier (Mantere et al., 2002,Storvik et al., 2009,Tupala et al., 2001). In brief, post-mortem brain left hemispheres (17 alcoholics and 8 controls) were obtained during clinical autopsy from the Department of Forensic Medicine, University of Oulu, Finland, and two of the non-alcoholic control brains were obtained from the Department of Forensic Medicine, University of Eastern Finland, Kuopio, Finland. The recovery procedure was essentially the same in both locations. This portion of the study was approved by the Ethics Committees of the University of Oulu and the National Institute of Medico-legal Affairs, Helsinki, Finland. A post-mortem analysis for drugs, which included alcohol, and the normal necropsy protocol were performed. None of the hemispheres exhibited damage or gross neuroanatomical abnormalities, as judged from a series of Nissl stained sections. Medical records on the cause of death, previous diseases, and medical treatments of the controls and alcoholics were also collected.

2.1. Diagnostics

Diagnoses were made by two physicians independently of each other. Medical record data were available for all 27 subjects. Mental disorders were coded according to DSM-IV criteria (APA, 1994), and alcoholic subjects were sub-classified as type 1 or 2, according to criteria established by Cloninger which closely resemble the Babor and Early/Late onset typologies (Cloninger, 1988,Cloninger, 1995,Leggio et al., 2009). The kappa coefficient of diagnostic agreement for the subjects was 0.9; i.e., one type 2 alcoholic was diagnosed as a type 1 alcoholic by the other physician. Otherwise, diagnoses were unanimous. The most important criteria for defining the two groups of alcoholic subjects were early onset (before the age of 25) of alcohol abuse and documented severe antisocial behavior among the type 2 alcoholic subjects. Subjects having psychotic disorders or any neurological diseases (such as epilepsy), or those taking medication that could affect the CNS (such as neuroleptics or antidepressants, including the SSRIs) were excluded. A history of tobacco smoking and years of abuse, based only on medical records, were considered unreliable and were not included in the final criteria.

2.2. Study subjects

All 27 subjects were Finns. The study groups consisted of type 1 alcoholics (N=9, seven men and two women; age: mean= 52.7 years, SD= 12.4; post-mortem delay (PMI): mean= 11.9 hours, SD= 4.5);

type 2 alcoholics (N=8, all men; age: mean= 34.6 years, SD= 12.2; PMI: mean= 14.1 hours, SD= 3.4); and non-alcoholic controls (N=10, eight men and two women; age: mean= 53.5 years, SD= 10.7; PMI: mean= 14.8 hours, SD= 9.2) (Table 1). All of the controls were free of a psychiatric diagnosis. Intervals between death and autopsy were not significantly different between the groups (P= 0.62 – 0.98, Scheffe's test for multiple comparisons, two-tailed). Six of the eight type 2 alcoholics had a criminal record or a history of violent offences (physical or sexual). Alcoholism in both type 1 and type 2 groups was severe, as judged by frequent admissions to emergency stations and doctors' appointments due to alcohol-related problems. Eight of the nine type 1 alcoholics had alcohol in their blood at the time of death, and one alcoholic had an abstinence period of 10 hours. One of the control subjects had a small amount of alcohol in his blood at the time of death (0.04%). Two of the type 1 alcoholics had traces of diazepam in their blood samples. Six of the eight type 2 alcoholics had alcohol in their blood at the time of death, and three had traces of benzodiazepines. One type 2 alcoholic tested positive for cannabinoids by radioimmuno assay, which was not confirmed by a more definitive test and could have been a false positive for ibuprofen. All subjects died of sudden causes.

Cryosectioning and autoradiography were performed at the Department of Pharmacology and Toxicology, University of Eastern Finland, Kuopio, Finland (as previously described in (Mantere et al., 2002,Tupala et al., 2001)). Individual variations in brain size were also considered when selecting representative sections. Each cryosection was coded for a subsequent blind analysis of the data.

2.3. AMPA binding assay

The method used for [^{3}H] AMPA binding was modified from our previous experiments with other radioligands for these same samples (Storvik et al., 2012,Tupala et al., 2001). Cryosections were pre-incubated for 30 minutes in the 50mM tris-citrate buffer, pH 7.4, containing 1M citric acid (at 4° C). To reach equilibrium, the sections were incubated for 45 minutes at 4°C in a tris-citrate buffer solution containing 26nM of [^{3}H] AMPA (Perkin Elmer Life and Analytical Sciences NET 833, Lot 3589093, specific activity = 40.0 Ci/mmol, GE Healthcare UK Ltd. Amersham, UK.) and 100 mM potassium thiocyanate.

Non-specific binding was determined by incubating adjacent sections using 10µM 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) as a displacer. Washing was made in a cold tris-citrate buffer for 3 x 2 min, followed by a brief dip into ice-cold distilled water. After washing, sections were dried under a gentle stream of warm air for 10 minutes and left for 2 hours at room temperature before

exposure to phosphor imager plates (BAS IP-TR 2040, Fuji Photo Film, Co., LTD, Japan) for 14 days prior to scanning (Storm 860 PhosphorImager scanner, Amersham). The autoradiograms were analyzed by using phosphor imager analysis (ImageQuaNT TL v. 2003, Amersham), and the resulting photo-stimulated luminescence values from the binding data were mathematically transformed by an exponential calibration equation into tissue properties (fmol/mg) with ^{3}H-calibrating scales (cat. no. RPA 507, Amersham). All analyses were made blind to the clinical classification of the samples. An adjacent section from the respective level was stained with cresyl violet (Nissl staining) to serve as an anatomical correlate to the autoradiography.

2.4. Statistical analyses

In order to have same distribution for each brain area, the variables were normalized to same mean and distribution within region and across the study groups by standard deviation and the results are expressed as z-scores. The normality of the variables was tested by using the Shapiro-Wilk test. Statistical significance between groups was evaluated by a permutation analysis of co-variance (ANCOVA), as the sample size was insufficient for a standard statistical analysis. Hochberg's post-hoc test was used to correct levels of significance for multiple testing when appropriate.

Possible correlations between [^{3}H] AMPA binding and previously published results of several key macromolecule densities (Tupala et al., 2000,Tupala et al., 2001,Mantere et al., 2002,Storvik et al., 2006a,Storvik et al., 2006b,Tupala and Tiihonen, 2005,Kupila et al., 2012,Storvik et al., 2009,Storvik et al., 2012) were calculated by the Spearman method, as binding values for different radioligands could not be assumed to be directly comparable. The differences in Spearman correlations between the 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 where considered to be large (Cohen, 1988). We used STATA (release 11.2, College Station TX) for all statistical analyses.

3. Results

[^{3}H] AMPA binding was observed in all the measured brain regions (Figure 1, Supplementary Table 1). Increased [^{3}H] AMPA binding was observed in the ACC of type 2 alcoholics when compared to controls (p=0.011; Figure 2). There were no significant differences between the study groups for [^{3}H] AMPA binding in the other measured brain regions. All variables were normally distributed.

No significant correlations between [^{3}H] AMPA binding in any of the studied brain regions and age at the time of death, blood alcohol concentration at the time of death or post-mortem interval were observed in this study. However, there were a trends towards a negative correlation between age at the time of death and [^{3}H] AMPA binding in the FC of type 2 alcoholics (R=-0.67, p=0.071) and between age at the time of death, and [^{3}H] AMPA binding in the ACC of controls (R=-0.62, p=0.056).

When comparing measured [^{3}H] AMPA binding to previously published densities of key macromolecules in these same individuals, a large negative correlation between [^{3}H] AMPA binding and DAT density (Tupala et al., 2006) was observed in the ACC of type 2 alcoholics (R=-0.952). This correlation was not observed in type 1 alcoholics or control subjects, and the difference between these three groups was highly significant (χ^2=13.84, p=0.001) (Figure 3). A large positive correlation between [^{3}H] AMPA binding in the NAc and previously published SERT density in the ACC (Mantere et al., 2002) was observed in the control group (R=0.76) and type 1 alcoholics (R=0.73), whereas, type 2 alcoholics showed a large negative correlation (R=-0.69) (Figure 4). This difference between groups was highly significant (χ^2=11.89, p=0.003). Furthermore, there was a significant difference in correlations between [^{3}H] AMPA binding and previously published D1 receptor density (Tupala and Tiihonen, 2008) in the ACC between the studied groups (χ^2=7.12, p=0.028); i.e., there was a large positive correlation in the control group (R=0.90), which was absent in both of the alcoholic groups (type 1, R=0.02; type 2, R=0.19). There was also a significant difference in correlation between the groups for [^{3}H] AMPA binding and previously published D1 receptor density (Tupala and Tiihonen, 2008) in the FC (χ^2=6.77, p=0.034); a large positive correlation in Cloninger type 2 alcoholics (R=0.71), a large negative correlation in type 1 alcoholics (R-0.58) and a small positive correlation in controls (R=0.20). There were no significant differences between the groups in correlations between [^{3}H] AMPA binding and previously published dopamine D2, mGluR1/5, 5-HT_{1A} or 5-HT_{1B} receptor densities (data not shown).

4. Discussion

In this study, postmortem whole-hemisphere autoradiography was used to study AMPAR densities in Cloninger type 1 and 2 alcoholics and controls. [^{3}H] AMPA binding was significantly increased in type 2 alcoholics in the ACC when compared to controls (Figure 2). The Cohen's effect size for this finding was large (1.319), thus increasing the validity of this result. There were no statistically significant differences between the groups in any of the other measured brain regions.

In the autoradiography study done by Freund and Anderson (1996) there was no significant difference between alcoholics and controls for AMPAR densities in the superior FC. However, results from the present study indicate that the increased [^{3}H] AMPA binding was specifically located in the ACC, and was only seen in Cloninger type 2 alcoholics. This further highlights the previously stated view that alcoholics need to be divided into subgroups for research and treatment (Tupala and Tiihonen, 2004,Leggio and Addolorato, 2008). Previous studies have reported several alterations between alcoholics and non-alcoholic controls for both serotonin and endocannabinoid signaling in the ACC (Mantere et al., 2002,Storvik et al., 2009,Lehtonen et al., 2010). However, to our knowledge, the present study is the first to report on specific changes in the ACC that are only associated with type 2 alcoholics. Increased [^{3}H] AMPA binding in the ACC has also been observed in subjects with major depressive disorder (MDD) (Gibbons et al., 2012), however, in that study most of the MDD subjects died from suicide, which suggests that increased [^{3}H] AMPA binding in the ACC might be more associated with the risk of suicide and impulsive behavior than MDD.

Increased [^{3}H] AMPA binding could be explained by increased synaptic activity, as increased synaptic activity leads to long-term potentiation (LTP), which can be observed as increased AMPAR density in the postsynaptic dendrite (Barry and Ziff, 2002). In the cortical regions this synaptic strengthening by LTP is considered to be long lasting, or even permanent (Kessels and Malinow, 2009,Gold et al., 1996,Petralia and Wenthold, 1992). In the context of addiction, the ACC has been lately associated with reward-related learning and the process of optimal-decision making (Kennerley et al., 2006,Kennerley and Wallis, 2009). Increased activity in the ACC has been associated with impulsive behavior, where the value of reward greater is than the effort needed to obtain it, and with a preference for smaller rewards that can be obtained more easily (Kennerley et al., 2006,Kennerley

and Wallis, 2009,Croxson et al., 2009,Prevost et al., 2010). Increased [^{3}H] AMPA binding in the ACC could therefore be associated with the impulsive behavior seen in type 2 alcoholics and to the development of alcohol dependence by positive reinforcement learning at early age (Cloninger, 1995,Koob and Le Moal, 2008).

We also observed large negative correlations between [^{3}H] AMPA binding and previously published DAT density (Tupala et al., 2006) in the ACC, and between [^{3}H] AMPA binding in the NAc and previously published SERT density in the ACC (Mantere et al., 2002) of type 2 alcoholics (Figures 3 and 4). Moreover, in the control group we observed a large positive correlation between [^{3}H] AMPA binding and previously published D1 receptor densities in the ACC (Tupala and Tiihonen, 2008), which was absent in both alcoholic groups. These findings suggest an alteration in the interactions between the ACC and the NAc in alcoholics, especially in type 2 alcoholics, and this alteration might contribute to a dysfunction in optimal-decision making and reward-related learning processes (Kennerley et al., 2006,Kennerley and Wallis, 2009). Dysfunction of these processes could contribute to the development of addiction, especially in the positive reinforcement state (Koob and Le Moal, 2008).

Since type 2 alcoholics usually die young because of their antisocial and impulsive behavior (Repo-Tiihonen et al., 2001) using age at the time of death as a covariant is not a statistically valid procedure as it is an integral part of the studied phenomenon (Cloninger, 1988,Cloninger, 1995,Miller and Chapman, 2001). Furthermore, there was no significant correlation between age at the time of death and [^{3}H] AMPA binding which suggest that age at the time of death does not have direct influence to [^{3}H] AMPA binding in the present study. From medical records it is impossible to determine years of abuse reliably and therefore it was not used as a covariant in the present study. It should also be noted, that, as in other autoradiography studies, the observed binding densities might not necessarily fully reflect to changes in the receptor densities and these results should be confirmed by other methods, e.g. in vivo imaging studies.

Although preliminary, and from a relatively small diagnostic group, the present results show that Cloninger type 2 alcoholics seem to have higher [^{3}H] AMPA binding and an altered correlation

between observed [^{3}H] AMPA binding and previously published DAT densities in the ACC when compared to non-alcoholic control subjects. These results support the idea that type 2 alcoholics have some altered function in the ACC, possibly reflecting to a dysfunction in optimal-decision making and reward-related learning processes with these individuals. Such a dysfunction in the ACC could be one of the neural changes leading to the addiction and impulsive behavior already seen with type 2 alcoholics at an early age.

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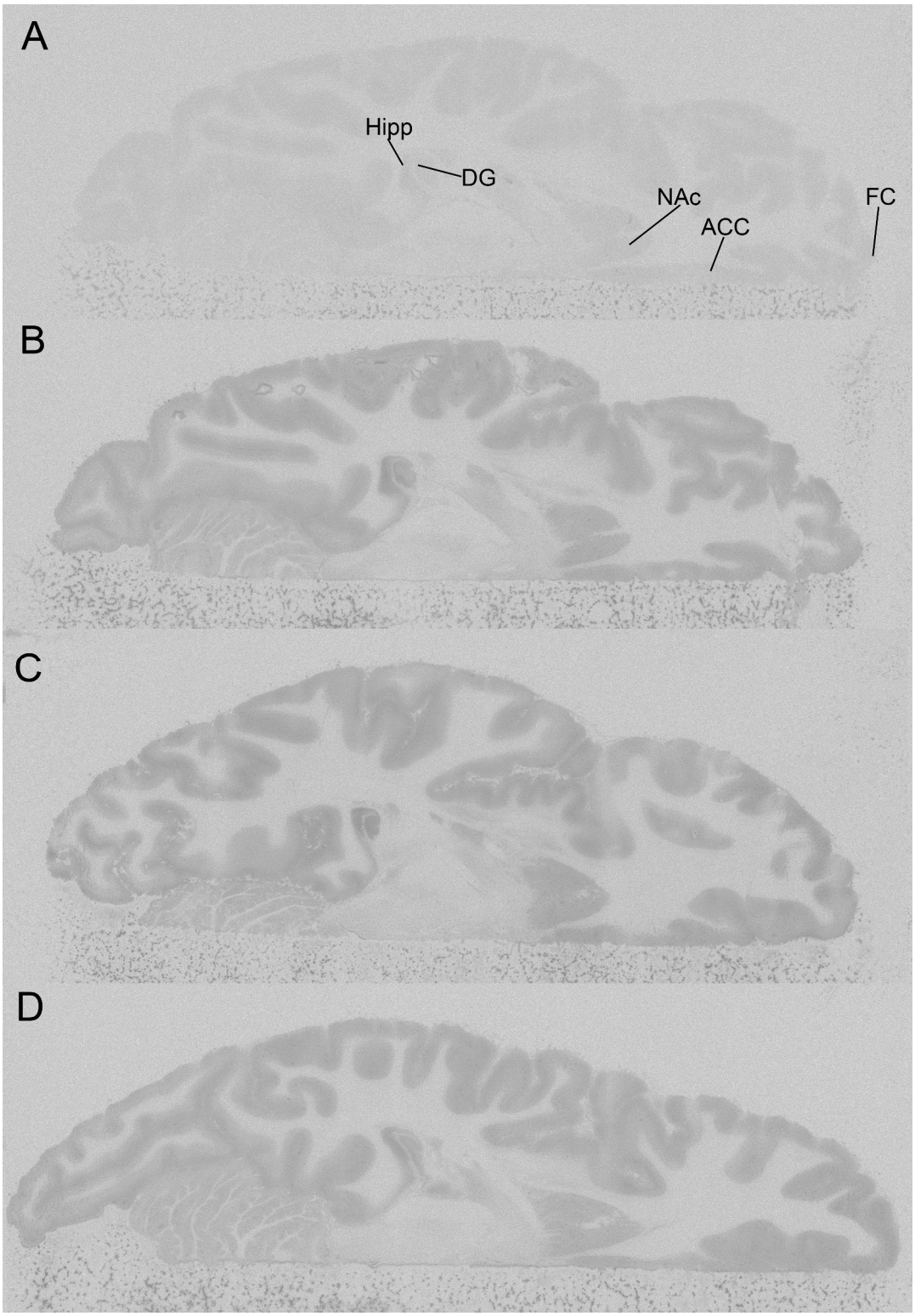

Figure 1. [^{3}H] AMPA binding in post mortem whole-hemisphere autoradiography. Representative images of non-specific [^{3}H] AMPA binding in controls (A) and total [^{3}H] AMPA binding in controls (B), type 1 (C) and type 2 alcoholics (D). ACC, anterior cingulate cortex; DG, dentate gyrus; FC, frontal cortex; Hipp, hippocampus; NAc, nucleus accumbens.

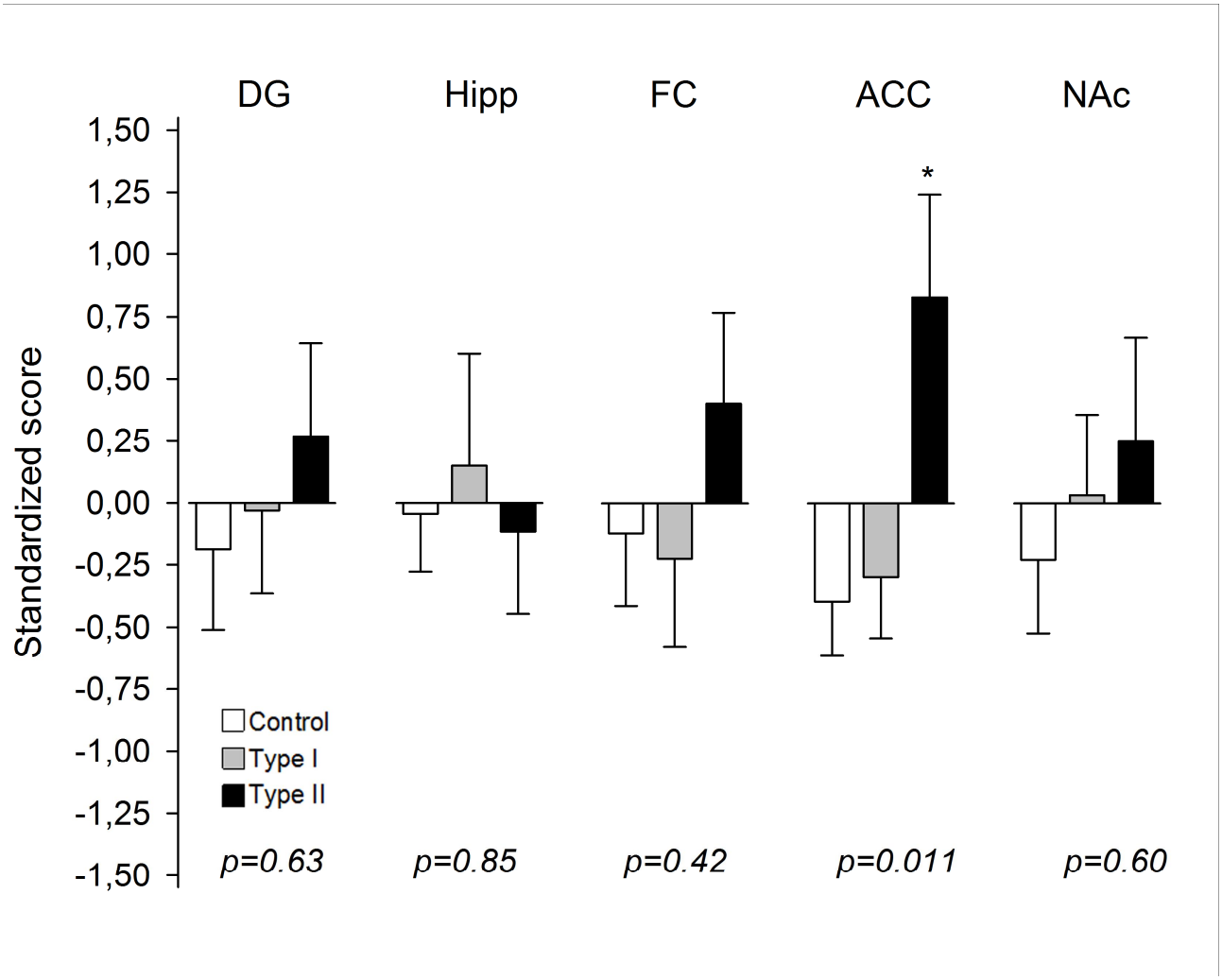

Figure 2. [^{3}H] AMPA binding in different brain regions for the non-alcoholic control group, and both type 1 and type 2 alcoholics, presented as standardized scores. There was a significant increase of [^{3}H] AMPA binding in the ACC of type 2 alcoholics when compared to controls (p=0.011). DG, dentate gyrus; Hipp, hippocampus; FC, frontal cortex; ACC, anterior cingulated cortex; NAc, nucleus accumbens; *, p<0.05 when compared to the control group.

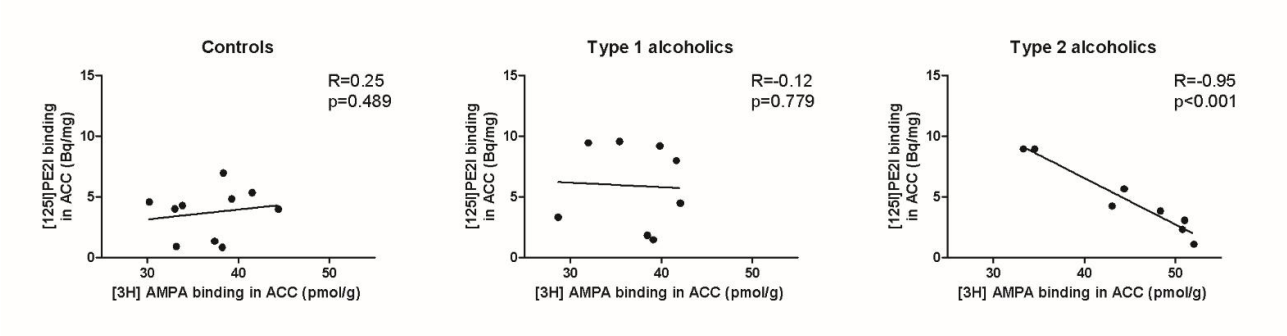

Figure 3. Type specific Spearman correlations between [^{3}H] AMPA binding and previously measured [^{125}I] PE2I binding to DAT in the ACC. ACC, anterior cingulate cortex; DAT, dopamine transporter.

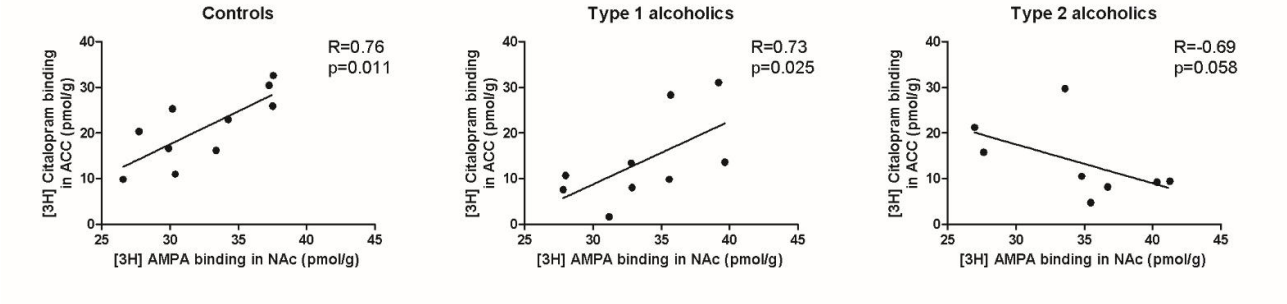

Figure 4. Type specific Spearman correlations between [^{3}H] AMPA binding in the NAc and previously measured [^{3}H] citalopram binding to SERT in the ACC. ACC, Anterior cingulate cortex; NAc, Nucleus accumbens; SERT, serotonin transporter.

Supplementary table 1: Specific [^{3}H] AMPA binding (fmol/mg) and Cohen's d effect sizes

	DG	Hipp	FC	NAc	ACC
Control	50.34 (9.42)	57.36 (9.58)	41.55 (7.32)	32.49 (4.10)	36.97 (4.34)
Type 1	51.82 (9.34)	59.86 (17.42)	40.72 (8.32)	33.65 (4.30)	37.59 (4.62)
Type 2	54.61 (9.75)	56.44 (12.10)	45.67 (8.16)	34.60 (5.21)	44.69 (7.36)*
Cohen's d:					
Control vs. type 1	0.158	0.186	0.105	0.277	0.137
Control vs. type 2	0.446	0.085	0.533	0.455	1.319#

The data is shown as means and standard deviations; *, $p<0.05$ when compared to controls; #, large effect size when compared to controls