Increased Metabotropic Glutamate 2/3 Receptor Binding in the Perigenual Anterior Cingulate Cortex of Cloninger Type 2 Alcoholics: A Whole-Hemisphere Autoradiography Study

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ABSTRACT — Aims: Metabotropic glutamate receptors 2 and 3 (mGluR2/3) contribute to control the level of glutamate in the synapse. In rodents, mGluR2/3 agonists attenuate the reinstatement of alcohol-seeking behavior. Linking possible alterations of the mGluR2/3 system to the etiology and type of alcoholism could provide valuable information for the development of novel mGluR2/3 function modulating therapies in addiction treatment. To date, mGluR2/3 binding density has not been studied in human alcoholics. We aimed to investigate the possible differences in mGluR2/3 binding between Cloninger type 1 anxiety-prone and type 2 impulsive alcoholics and controls. Methods: We performed a post-mortem whole-hemisphere autoradiography to study the mGluR2/3 binding density of 9 type 1 alcoholics, 8 type 2 alcoholics and 10 controls. [3H]LY341495, a potent group II metabotropic glutamate receptor antagonist, was used as the radio-ligand with t-glutamate as a displacer. Results: [3H]LY341495 binding density was statistically significantly increased (P = 0.046) in the perigenual anterior cingulate cortex (pACC) of type 2 alcoholics when compared with controls. In other brain areas, no significant difference between the groups was found. Conclusion: This preliminary study suggests that impulsive type 2 alcoholics might have alterations in the mGluR2/3 function in the pACC, a brain area presumed to be involved in the control of drug-seeking behaviors and self-control.

INTRODUCTION

Alcoholics are a heterogeneous group of people that present a wide spectrum of problems, which negatively impact both the alcoholic and society. In our study, alcoholics have been divided into two subgroups according to Cloninger’s typology (Cloninger et al., 1988); i.e. type 1 and type 2. The onset age of the alcoholism is typically <25 years for Cloninger type 2 alcoholics and over 25 years for type 1 alcoholics. Cloninger type 1 alcoholics tend to be anxiety-prone, cautious and low in novelty-seeking, whereas type 2 alcoholics score low in harm-avoidance and high in novelty-seeking. Type 2 alcoholics are often socially hostile, and they present problems with self-regulation, which leads to impulsivity, a characteristic trait of this alcoholic subgroup. Type 2 alcoholics often have a criminal record (Tiihonen and Hakola, 1994). Type 2 alcoholism has a high hereditary component. By comparing these alcoholic subtypes and controls by using whole-hemisphere autoradiography, our research group has previously reported several type-specific differences in neurotransmitter systems that have been linked with alcoholism and other forms of addiction; i.e. in the dopaminergic, serotonergic and glutamatergic systems (Tupala et al., 2001; Storvik et al., 2009; Kärkkäinen et al., 2013). Furthermore, we have found differences between alcoholics and controls in GABAergic, serotonergic and dopaminergic systems (Tupala et al., 2000; Mantere et al., 2002; Laukkanen et al., 2013).

Glutamate is the main excitatory neurotransmitter of the brain, and glutamatergic neurotransmission plays a key role in addiction and relapse (Kalivas and Volkow, 2005; Moussawi and Kalivas, 2010; Hovelsø et al., 2012). Metabotropic glutamate receptors (mGluRs) modulate the rate of excitation set by the ionotropic glutamatergic system (Olive, 2009; Hovelsø et al., 2012), mGluRs are also present in synapses that release neurotransmitters other than glutamate (Hovelsø et al., 2012). The group II metabotropic glutamate receptor mGluR2 is located presynaptically, outside of the active synaptic zone, where it regulates neurotransmitter release from the presynaptic axon terminal (Schoepp, 2001). The other group II receptor, mGluR3, is found both pre- and post-synaptically, and on glia, thus negatively regulating neurotransmitter release (Tamaru et al., 2001). mGluR2 and mGluR3 often form dimers (Xi et al., 2002) and recent studies suggest that mGluRs can form intra- and intergroup heteromeric receptors (Doumazane et al., 2011). The mGluR2/3s are highly expressed in hippocampus and moderately so in brain areas essential in addiction; i.e. cerebral cortex, nucleus accumbens, dorsal striatum and amygdala (Olive, 2009). mGluR2/3s regulate homosynaptically the release of glutamate and heterosynaptically the release of GABA (γ-amino-butyric acid) in hippocampal interneurons and the cerebellum, and the release of dopamine in the nucleus accumbens, striatum and dentate gyrus (Schoepp, 2001; Karasawa et al., 2006; van Berckel et al., 2006; Xi et al., 2010; Gangarossa and Valjent, 2012).

In animal models, the mGluR2/3 receptors seem to play a critical role in the reinstatement of alcohol seeking. Preclinical studies in rodents have demonstrated that mGluR2/3 agonists attenuate both alcohol intake and the reinstatement of alcohol-seeking behavior (Bäckström and Hyytiala, 2005) and prevent relapse into alcohol use that derives from both stress and alcohol-related cues (Zhao et al., 2006; Kufahl et al., 2011). Several studies suggest that group II mGluR ligands may have beneficial effects in the treatment of alcoholism, in particular, and for addictions in general (Olive, 2009; Moussawi and Kalivas, 2010; Holmes et al., 2013). Clinical phase testing of some mGluR group II ligands are ongoing as potential...
treatments for schizophrenia, depression and anxiety (Patil et al., 2007; Dunayevich et al., 2008).

In the present study, we aimed to see if there are significant differences in the mGluR2/3 receptor binding densities between Cloninger alcoholic subtypes, or between the alcohols and the controls, in brain areas that are essential for self-regulation and addiction. There are very few human autoradiography studies of group II mGluR binding density, aside from the study by Frank et al. (2011) in schizophrenia patients and Samadi et al. (2009) in patients with Parkinson’s disease. To our knowledge, there are no human autoradiography studies of mGluR2/3 density of alcohols.

MATERIALS AND METHODS

Post-mortem brain left hemispheres of the study subjects (9 type 1 alcoholics, 8 type 2 alcoholics and 10 controls) were obtained during forensic autopsies in the Department of Forensic Medicine, University of Oulu, Finland, and the Department of Forensic Medicine, University of Eastern Finland, Kuopio, Finland. The recovery procedure was essentially the same in both locations. This part of the study was approved by the Ethics Committees of the University of Oulu (reference number 12/29/1997) and the National Institute of Medico-legal Affairs in Helsinki, Finland (reference number 5/07/1998). A post-mortem analysis for drugs was performed according to the normal necropsic protocol, including a test for alcohol. None of the hemispheres exhibited damage or gross neuroanatomical abnormalities. Medical records concerning the cause of death, previous diseases and medical treatments for both controls and alcoholics were also collected.

Diagnoses were made by two psychiatrists independently of each other. Medical records’ data were available for all 27 subjects. Mental disorders were coded according to DSM-IV criteria (APA, 1994); and alcohols were sub-classified as type 1 and 2, according to the criteria established by Cloninger (Cloninger et al., 1988; Cloninger, 1995). 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 alcohols were early onset of alcohol abuse (before the age of 25) and documented severe antisocial behavior for the type 2 alcoholics. Subjects having psychotic disorders or any neurological diseases, or those taking medication that could affect the CNS (such as anti-psychotics or antidepressants) were excluded. A history of tobacco smoking was considered unreliable and was not included in the final evaluation.

All 27 subjects were Finns. For details of the study subjects, see Table 1. The study groups consisted of 9 type 1 alcoholics (age: mean = 52.7 years, SD = 12.4; post-mortem interval: mean = 11.9 h, SD = 4.5); 8 type 2 alcoholics (age: mean = 34.6 years, SD = 12.2; post-mortem interval: mean = 14.1 h, SD = 3.4); and 10 non-alcoholic controls (age: mean = 53.5 years, SD = 10.7; post-mortem interval: mean = 14.8 h, SD = 9.2). None of the controls had a psychiatric diagnosis. There was no statistically significant difference in the time between death and autopsy between the three groups \( P = 0.62\text{–}0.98 \), Scheffe’s test for multiple comparisons, two-tailed). Six type 2 alcoholic subjects 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. Two of the type 1 alcohols and three of the type 2 alcohols had traces of diazepam or its metabolites in their blood samples. A history of tobacco smoking was considered unreliable and was not included in the final evaluation. All subjects died unexpectedly and therefore, a forensic autopsy was performed.

Cryosectioning and autoradiography were performed at the Department of Pharmacology and Toxicology, University of Eastern Finland, Kuopio, Finland (for detailed methods, see Tupala et al., 2000, 2001). Individual variations in brain size were considered when selecting representative sections. In all, six brain areas were selected to for this study; i.e. the frontal cortex, nucleus accumbens, perigenual anterior cingulate cortex (pACC), hippocampus, dentate gyrus and amygdala. \( ^{[3]H} \)LY341495, a potent group II metabotropic glutamate receptor antagonist binding mainly to mGluR2 and mGluR3 with a minor binding to mGluR8 (Johnson et al., 1999), was used as a binding ligand, according to the procedures described in the literature (Kingston et al., 1998; Wright et al., 2001; Samadi et al., 2009), subsequent to our previous experiments with other ligands for these brain samples (for example, see Tupala et al., 2000, 2001; Storvik et al., 2009). Cryosections were pre-incubated for 1 × 30 min in ice-cold 10 mM potassium phosphate buffer containing Tris-ultrapure with 100 mM potassium bromide, pH 7.6. To reach equilibrium, sections were incubated for 90 min in a solution with 5 nM of \( ^{[3]H} \)LY341495, specific

<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></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 Male</td>
<td>55</td>
<td>5.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>2 Male</td>
<td>45</td>
<td>9.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>3 Male</td>
<td>77</td>
<td>7.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>4 Female</td>
<td>57</td>
<td>11.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>5 Male</td>
<td>50</td>
<td>18.5</td>
<td>0.0</td>
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<td></td>
</tr>
<tr>
<td>6 Female</td>
<td>60</td>
<td>12.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
<td></td>
</tr>
<tr>
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<td>49</td>
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<td>0.4</td>
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<td></td>
</tr>
<tr>
<td>8 Male</td>
<td>53</td>
<td>29.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
<td></td>
</tr>
<tr>
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<td>53</td>
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<td>0.0</td>
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<tr>
<td>10 Male</td>
<td>36</td>
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<td>0.0</td>
<td>Dissection of aorta</td>
<td></td>
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<td>Type 1 alcoholics</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1 Male</td>
<td>39</td>
<td>12.5</td>
<td>0.0</td>
<td>Pneumonia</td>
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<td>0.1</td>
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<td>1.5</td>
<td>Suicide by hanging</td>
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<tr>
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<td>42</td>
<td>14.8</td>
<td>0.8</td>
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<tr>
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<td>76</td>
<td>10.5</td>
<td>3.2</td>
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<tr>
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<td>Ethanol intoxication</td>
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<tr>
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<tr>
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<td>4.7</td>
<td>Ethanol intoxication</td>
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<tr>
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<td>0.0</td>
<td>Right subdural hemorrhage</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>49</td>
<td>12.0</td>
<td>1.7</td>
<td>Fibrotic degeneration of myocardium</td>
<td></td>
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<tr>
<td>2 Male</td>
<td>37</td>
<td>9.5</td>
<td>3.0</td>
<td>Gunshot wound</td>
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<tr>
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<td>15.5</td>
<td>3.0</td>
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<td></td>
</tr>
<tr>
<td>4 Male</td>
<td>20</td>
<td>14.5</td>
<td>1.3</td>
<td>Knife wound</td>
<td></td>
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<tr>
<td>5 Male</td>
<td>46</td>
<td>18.0</td>
<td>0.0</td>
<td>Suicide by hanging</td>
<td></td>
</tr>
<tr>
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<td>18</td>
<td>9.5</td>
<td>1.5</td>
<td>Heart rupture (car accident)</td>
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</tr>
<tr>
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<td>32</td>
<td>16.5</td>
<td>3.6</td>
<td>Suicide by hanging</td>
<td></td>
</tr>
<tr>
<td>8 Male</td>
<td>28</td>
<td>17.5</td>
<td>0.0</td>
<td>Suicide by hanging</td>
<td></td>
</tr>
</tbody>
</table>

Age (years), age at time of death; PMI, post-mortem interval; BAC, blood alcohol concentration.
activity 3400 mCi/mmol. Non-specific binding was determined by incubating adjacent sections with 1 mM L-glutamate as a displacer. Washing was made with ice-cold buffer, 3 × 2 min, followed by a brief dip into ice-cold distilled water. Sections were dried under a gentle stream of warm air for 10 min, and left for 5 days at room temperature before exposure to phosphor imager plates (BAS IP-TR 2040, Fuji Photo Film, Japan) for 14 days, and then scanned (Storm 860 PhosphorImager scanner, Amersham). The autoradiograms were analyzed by a phosphor imager analysis (Image J, National Institutes of Health, USA), and the resulting luminescence values for the binding data were transformed into tissue properties (pmol/mg) by the use of [3H]-calibrating scales (cat. no. RPA 507, Amersham). All binding assays and analyses were made blind to the clinical classification of the samples.

Correlation coefficients for [3H]LY341495 binding values between the brain areas, and also for the binding values and age, post-mortem interval and blood alcohol content (BAC), were determined by a two-tailed Pearson’s correlation coefficient. In anatomically, and functionally connected areas, trends in binding density were congruent and the binding in brain areas was not independent. In order to have same distribution for each brain area, the variables were normalized to the same mean and distribution within the region and across the study groups by standard deviation. The results are expressed as z-scores. The values of standardized data on these brain areas were analyzed by permutation-type ANOVA (analysis of variance) with the age as a covariate and using the appropriate contrast. P-values of <0.05 were considered to be statistically significant in all tests.

The effect size was calculated as the difference between the mean binding in type 1, type 2 and controls divided by the pooled standard deviation of the respective group (Cohen, 1988). An effect size of 0.5 was considered to be medium and a value of 0.8 was considered to be a large effect. We used STATA (release 11.2, College Station, TX, USA) for statistical analyses.

RESULTS

The [3H]LY341495 binding in whole hemisphere autoradiography is presented in Fig. 1. The study groups, i.e. controls, type 1 alcoholics and type 2 alcoholics, displayed a statistically significant difference (P = 0.042) in [3H]LY341495 binding density in the area of nucleus accumbens, frontal cortex and pACC. This difference was further localized to the pACC, where [3H]LY341495 binding density in type 2 alcoholics was statistically significantly increased (P = 0.046) when compared with controls (Fig. 2A and B). This finding had a large effect size of 1.09 in type 2 alcoholics. There was no significant correlation between [3H]LY341495 binding and age or post-mortem interval in the pACC for any of the groups. However, there was a significant correlation between [3H]LY341495 binding and BAC at the time of death in the pACC in type 2 alcoholics only (R = 0.86, P = 0.006).

No statistically significant differences in [3H]LY341495 binding densities between the three study groups were found in the hippocampus and the dentate gyrus (P = 0.58) (Fig. 3A and B). In amygdala, differences between groups did not approach significance in permutation-type ANOVA (P = 0.97, age adjusted).

DISCUSSION

The main finding of our study is a statistically significant increase in binding density for [3H]LY341495 in the pACC of Cloninger type 2 alcoholics when compared with controls. The main limitation of this study is the small number of study subjects. Cloninger type 2 alcoholics are a minor subgroup of alcoholics. According to our previous studies, the glutamatergic neurochemistry of this impulsive and often violent subgroup differs from type 1, the majority of alcoholics, in several aspects (Kärkkäinen et al., 2013; Kupila et al., 2013). Our finding of elevated [3H]LY341495 binding density in Cloninger type 2 alcoholics requires further studies. To the authors’ knowledge, group II glutamatergic receptor binding density in human alcoholics has not been studied before. Recently, Griffin and his co-workers (2014) demonstrated a positive correlation between elevated extracellular glutamate and escalation of drinking behavior in mice, which was attenuated by mGluR2/3-agonist LY379268. In rodent studies, exposure to ethanol has been reported to decrease, rather than increase, mGluR2/3 receptors. It has been suggested that the altered mGluR2/3-mediated function induced by ethanol exposure might be related to the length of the exposure and number of withdrawals (Kufahl et al., 2011; Meinhardt et al., 2013).

A study by Zhou and co-workers (2013) suggests a strong role of mGluR2 function in alcohol use. In their study, alcohol-prefering rats carried a variant of Grm2 stop codon with decreased levels of Grm2 transcripts, which impaired mGluR2-mediated inhibition of the glutamatergic system. In humans, the mGluR2-mediated neurotransmission of the ACC has been suggested to have a potent impact on relapse vulnerability (Meinhardt et al., 2013). In their recent study, Meinhardt et al. (2013) demonstrated that the mGlu2 receptor is downregulated in post-dependent rats’ infralimbic cortex, an area that has been suggested to be anatomically and functionally related to the human ACC (Uylings et al., 2003; Meinhardt et al., 2013). The downregulation of mGlu2 of rat’s infralimbic-accumbal projection neurons led to an
excessive drug-seeking, which could be restored by targeted mGluR2 gene therapy. Meinhardt et al. (2013) also found a decrease in mGluR2 transcript levels in the ACC of human alcoholics compared with controls. The study of Meinhardt and co-workers suggests that chronic alcohol causes dysregulation in the human ACC mGluR2 function and this dysregulation plays an essential role in relapse propensity and reinstatement of alcohol use. In the study of Meinhardt, alcoholics had not been divided into specific subgroups, which may explain the diverging results compared with our study, where [3H]LY341495 binding density in the pACC of type 2 alcoholics was increased. However, the ACC seems to play a central role in relapse.

Parallel to the role in relapse vulnerability, the dysfunction of the ACC is tightly connected to the main behavioral characteristics of Cloninger type 2 alcoholics, e.g. impulsivity and low deliberation. In the study by Nikiforuk et al. (2010), mGluR2 positive allosteric modulator LY487379 enhanced cognition and reduced impulsive behavior in rats. LY487379 also caused an increase in the levels of norepinephrine and serotonin in the rat medial prefrontal cortex. This finding of Nikiforuk and co-workers suggests that mGluR2-mediated neurotransmission has an impact on impulsivity, a main characteristic of type 2 alcoholics. Kennerley et al. (2006) state that the ACC is involved in decision making by sustaining rewarded actions and integrating the significance of the current errors and rewards with the history of actions. They found a damage in the ACC to interfere with this consideration process. In the light of the findings of Kennerley et al. (2006) and Nikiforuk et al. (2010) presented above, our finding of increased [3H]LY341495 binding density in the pACC of Cloninger type 2 alcoholics might be related to impulsivity, the characteristic trait of this alcoholic subgroup. In our recent study, Kärkkäinen et al. (2013) observed an increased AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor density in the pACC of type 2 alcoholics, which may further suggest altered glutamatergic activity in this area, and relate to the critical role of the ACC in reinforcement-guided behavior (Kennerley et al., 2006). The elevated mGluR2/3 density suggested by the present study could serve as a

![Fig. 2.](image-url) (A) Distribution of the standardized data in the brain areas. (B) Binding of [3H]LY341495 in the areas related to self-regulation. Nac, nucleus accumbens; pACC, perigenual anterior cingulate cortex; FC, frontal cortex. *P-value < 0.05 of [3H]LY341495 binding density difference between Cloninger type 2 alcoholics and controls.

![Fig. 3.](image-url) (A) Distribution of the standardized data of hippocampus and gyrus dentatus. In these areas, the difference in normalized [3H]LY341495 binding density between the groups was not statistically significant. (B) Binding densities in hippocampus and gyrus dentatus. Hipp, hippocampus; GD, gyrus dentatus.
compensatory mechanism to the elevated AMPA receptor density and the impulsive trait of type 2 alcoholics.

Rodent studies suggest that there might be sensitization to a treatment modulating mGluR2/3 function in severe states of addiction, and that mGluR2/3 may serve as a target for treatment of addiction. In several studies, the mGluR2/3 agonist LY379268 has been shown to attenuate drug-seeking behavior in rats trained to self-administer for example cocaine or alcohol and also in rats exposed to chronic intermittent alcohol (Baptista et al., 2004; Bäckström and Hyytiä, 2005; Peters and Kalivas, 2006; Kufahl et al., 2011; Griffin et al., 2014). Ethanol-dependent rats respond stronger to LY379268 than non-dependent ones (Sidhpara et al., 2010; Griffin et al., 2014). The attenuating effect of this mGluR2/3 agonist on cue-induced reinstatement is more potent in rats with a history of extended access to methamphetamine when compared with the rats with more limited access (Kufahl et al., 2013). The same was shown for rats with long access to cocaine when compared with limited access rats (Hao et al., 2010). mGluR group II ligands are on clinical phase testing as a novel treatment for schizophrenia, anxiety and depression. The aforementioned studies and also the preliminary results of our study suggest this group of ligands could also serve as a future treatment for certain subgroups of alcoholics as well.

In our study, there was a statistically significant (R = 0.863, P = 0.006) positive correlation between BAC and [3H]LY341495 binding density in the pACC among type 2 alcoholics. In other brain areas or subgroups, no correlation between BAC and binding density was found. It seems implausible that BAC would have a specific acute effect on mGluR2/3 binding density in the pACC of type 2 alcoholics only, without any parallel positive effect on binding density in other brain areas of type 2 alcoholics, or among type 1 alcoholics, whose alcoholism also was severe in nature. In a small number of subjects, individual values may have a potent effect on correlation coefficient. Therefore, we expect this correlation to be a statistical artifact.

In the study by Frank and co-workers (2011) among schizophrenia patients, mGluR2/3 binding density correlated negatively with the age in Brodmann area 46. In our study, the mean age of the type 2 alcoholics (34.6 years) was lower than type 1 alcoholics (52.7 years) and controls (53.5 years). It is difficult to obtain post-mortem brain samples from older type 2 alcoholics, due to the high mortality in this population (Repo-Tiihonen et al., 2001). In our study, no correlation between [3H]LY341495 binding density and age in any of the groups in any of the six studied brain areas was found. Therefore, we expect our finding of elevated [3H]LY341495 binding density in the pACC of the type 2 alcoholics not to be due to their young age.

In conclusion, the present study suggests that there is an upregulation of the mGluR2/3 system in the pACC of Cloninger type 2 alcoholics, whose characteristic traits include impulsivity and antisocial behavior. The cingulate cortex is a brain area that is involved in self-regulation and inhibitory control (Volkow et al., 2004; Heatherton and Wagner, 2011). The alteration of the mGluR2/3 system of the pACC in type 2 alcoholics, reported herein, may be of importance for effectively treating problems related to impulse control in this population of alcoholics.

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Increased mGluR2/3 binding in the ACC of Cloninger type 2 alcoholics


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