CORRELATION BETWEEN THE SERT BINDING DENSITIES IN HYPOTHALAMUS AND AMYGDALA IN CLONINGER TYPE 1 AND 2 ALCOHOLICS

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Abstract — Serotonin plays a role in the regulation of emotional states in amygdala which in turn affect the function of hypothalamus. The physiological effects of emotions are mediated to autonomic nervous system by the hypothalamus, also innervated by the serotonergic Raphe nuclei. Aims: We evaluated the putative alterations of the serotonin transporter (SERT) density in the paraventricular nucleus (PVN) of alcoholics of Cloninger type 1 and 2 (early onset, anti-social) alcoholics and controls. Methods: The study was performed by human whole-hemisphere autoradiography with [3H]citalopram. Results: Substantially sparser SERT density (∼26%) with a moderate effect size (0.53) was observed in the hypothalamus of alcoholic subjects in relation to non-alcoholic comparison subjects, although the result failed to reach statistical significance. In type 2 alcoholics, there was a trend towards decreased SERT binding with large effect size (0.86), and no correlation between the SERT binding and the age at the time of death. There was a strong positive correlation between the SERT binding in amygdala and in PVN in type 2 alcoholics (P = 0.001), and negative correlation in type 1 alcoholics (P = 0.05), and no correlation in the control subjects. The difference between the groups was significant (χ² = 16.75, P = 0.0002). Conclusions: Taken together, these preliminary results support the hypothesis that the serotonergic regulation in the hypothalamus and amygdala are defected especially in type 2 alcoholics.

INTRODUCTION

The neurochemical model of alcoholism by Cloninger proposes that type 2 alcoholics have serotonergic deficits with no defect in the dopaminergic system, whereas type 1 alcoholics have defective dopaminergic system (Cloninger, 1995; Tupala and Tiihonen, 2004). The Cloninger type 1 alcoholism (∼80% of alcoholics) is characterized by the development of dependence in adulthood on the anxiety-reducing effects of alcohol, and is not associated with anti-social behaviour (Cloninger, 1995). In contrast, type 2 alcoholism (∼20% of alcoholics) is more strongly heritable and is characterized by adolescent or early onset of alcoholism (Cloninger, 1995). A large proportion of severe violent offences such as homicides are attributable to type 2 alcoholism (Tiihonen et al., 1993; Eronen et al., 1996).

The Cloninger type 2 alcoholics are almost exclusively men, and their behaviour can be viewed as extreme examples of innate male sensation-seeking behaviour, which is suggested to be an evolutionary conservated trait related to limbic system reactivity, as described by Joseph (2000). In human brain, the responses for aggression and fear stimuli are mediated by a network that is centred in the amygdala and other limbic regions. Projections from those areas to hypothalamic and brainstem sites explain many of the observed signs of emotional responses. Serotonin system is suggested to inhibit this network from the level of the amygdala through its projections to the hypothalamus and the brainstem (Weidenfeld et al., 2005). We have previously reported decreased serotonin transporter (SERT) density in the perigenual anterior cingulate cortex (pACC) (Mantere et al., 2002), in the dorsal striatum (Storvik et al., 2006), and in the dorsal level of the amygdala in alcoholics (Storvik et al., 2007). Many of the effects of the amygdala on the emotional states are mediated through the hypothalamus and the hypothalamic-pituitary-adrenocortical (HPA) axis. Alcoholics are reported to have alterations in the hypothalamic neurons (Sivukhina et al., 2006), and the function of the HPA axis may alter during anti-addictive drug treatments (Kiefer et al., 2006).

The role of serotonin in the function of the hypothalamus in alcoholics is however not fully understood. The paraventricular nucleus (PVN) of the hypothalamus is located at the anterior medial hypothalamus, and it receives controlling serotonergic input from the raphe nuclei. Serotonergic activity in the PVN modulates the effect that the amygdala activation has on the HPA axis (Feldman et al., 2000; Weidenfeld et al., 2002). In addition, the amygdala has a facilitatory effect on the function of the dorsal raphe serotonergic neurons which project to the PVN (Weidenfeld et al., 2002), and the serotonin system may be a mechanism by which the amygdala may modulate the function of the HPA axis (Weidenfeld et al., 2002). The relationship is two-directional as the corticotropin releasing hormone is secreted by the PVN of the hypothalamus in response to stress and the release is modulated by the serotonergic transmission (Jorgensen et al., 2002), but the glucocorticoids may regulate the expression of SERT, as the promoter of the SERT gene (SLC6A4) contains a glucocorticoid response element (Glatz et al., 2003).

The aim of the present study was to evaluate the SERT binding densities in the PVN in alcoholics. To further evaluate the serotonergic system in the brain areas that are important for the regulation of the impulsive aggression, the SERT binding between the PVN and the limbic areas were compared in the alcoholic groups and controls to determine if serotonergic alterations in these functionally coupled brain areas are correlated with each other in a similar or different way in type 1 and type 2 alcoholics.

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MATERIALS AND METHODS

The brain sampling, diagnostics, study subjects, and cryosectioning have been described in detail previously (Storvik et al., 2006). The subjects were the same as in our previous human post-mortem auto-radiography articles (Mantere et al., 2002; Tupala and Tiihonen, 2004; Storvik et al., 2006, 2007). Human brain left hemispheres used 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 Medicolegal Affairs, Helsinki, Finland approved the study. Medical records on the cause of death and previous diseases and medical treatments of controls and alcoholics were collected. Alcoholism was coded according to DSM-IV criteria (APA, 1994), and sub-classified as type 1 or 2, according to Cloninger (1995). The kappa coefficient of diagnostic agreement subjects was 0.9; i.e. one type 2 alcoholic was diagnosed as type 1 alcoholic by the second physician. Otherwise, diagnoses were unanimous.

All 27 subjects were Caucasians. The study groups consisted of 9 type 1 alcoholic subjects (seven men and two women; age: mean = 52.7 years, SD = 12.4; post-mortem delay: mean = 11.9 h, SD = 4.5); eight type 2 alcoholic subjects (all men; age: mean = 53.5 years, SD = 10.7; post-mortem delay: mean = 14.8 h, SD = 9.2) who 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). Nine controls died because of myocardial infarction and one of aorta rupture. The causes of death in the type 1 alcoholic group were pneumonia (2), lethal ethanol intoxication (2), myocardial infarction (2), suicide by hanging (1), pancreatitis (1), and subdural haematoma (1). The causes of death in the type 2 alcoholic group were suicide by hanging (3), knife wound (2), gunshot wound (1), rupture of heart due to car accident (1), and cardiac death (1). Alcoholism among these subjects was severe, judging by the frequent admissions to emergency stations and doctors’ appointments due to alcohol-related problems. Evaluation of the duration of alcohol use was based on medical records and was considered unreliable and not included in the analysis. Eight of the nine type 1 alcoholic subjects had alcohol in their blood at the time of death, and one alcoholic subject had an abstinence period of 10 h. One of the non-alcoholic comparison subjects had a small amount of alcohol in his blood at the time of death (0.04%). Two of the type 1 alcoholic subjects had traces of diazepam in their blood samples. Six type 2 alcoholic subjects had alcohol in their blood at the time of death, three had traces of benzodiazepines, and one was tested positive for cannabinoids.

Subjects having psychotic disorders or any neurological diseases (such as epilepsy) or taking medication that could affect the central nervous system (CNS) [such as neuroleptics or anti-depressants including Selective serotonin reuptake inhibitors (SSRIs)] or using substances with direct effect on the monoaminergic systems (such as psychostimulants or opioids) were excluded. A history of tobacco smoking, based only on medical records, was not included in the final criteria.

Cryosectioning and auto-radiography

Cryosectioning and auto-radiography were performed at the Department of Pharmacology and Toxicology, Kuopio University, Kuopio, Finland. (For detailed methods, see Tupala et al., 2003). Individual variations in brain size were considered when selecting sections parallel to each other. Each cryosection was coded for a blind analysis of the data. The incubation concentration for [3H]citalopram (specific activity = 82 Ci/mmol, NEN Life Science Products Inc., Boston, MA, USA) in phosphate buffer solution was 1.2 nM. The buffer solution contained 137 mM of sodium chloride, 2.7 mM of potassium chloride, 1.8 mM of potassium phosphate, and 10.1 mM of hydrosodium phosphate pH 7.4. Cryosections were pre-incubated for 15 min in the phosphate buffer at room temperature. Each section was surrounded with plasticine before the addition of the incubation solution (14 ml). The incubation lasted 90 min at room temperature, followed by washings (3 × 10 min) in ice-cold phosphate buffer solution which was followed by a brief dip in ice-cold distilled water. Non-specific binding was determined by incubating adjacent sections with 10 μM of fluoxetine (Research Biomicals International, Natick, MA, USA) with 0.1% ascorbic acid. After washing, sections were dried under a gentle stream of cool air for 10 min and left for 5 days at room temperature. Sections were exposed to radiation-sensitive film ([3H]-Hyperfilm, discontinued, Amersham, Buckinghamshire, UK) for 4 weeks in x-ray cassettes together with tritium calibration standards (Amersham Microscales). The auto-radiograms were analysed by means of computerized densitometry. The resulting pixel values of the SERT binding in PVN (located at the anterior part of the hypothalamus at its uppermost level) were mathematically transformed by an exponential calibration equation into tissue properties (pmol/g) by the use of calibration standards (RPA 507, Amersham). An adjacent section from the respective specimen’s level was stained with cresyl violet (Nissl staining) to serve as an anatomical correlate to the auto-radiography.

Statistical analysis

SPSS for Windows, version 13 was used for the analyses. Because the literature has suggested that striatal monoamine transporter densities may decline with age (Mantere et al., 2002; Hesse et al., 2003; Tupala et al., 2003), and a significant age correlation was observed in the caudate body (Storvik et al., 2006), univariate analysis of covariance (ANCOVA) with age as a covariate was used for comparing the [3H]citalopram binding to SERT between each group. The effect size was calculated as the difference between the mean SERT binding in the alcoholics and controls divided by the pooled standard deviation of the respective groups. A value of 0.5 was considered as a medium, and a value of 0.8 as a large effect. Correlations of the SERT binding values between the brain areas were studied with the two-tailed Pearson’s correlation
et al. in PVN and our previously published data from the amygdala serotonergic system in an alcoholic brain, the SERT binding in type 2 alcoholics. In order to further study the putative alterations in the serotonergic alterations in alcoholics, Comparison of SERT binding in PVN and brain areas with serotonergic alterations in alcoholics

In order to further study the putative alterations in the serotonergic system in an alcoholic brain, the SERT binding in PVN and our previously published data from the amygdala (Storvik et al., 2007), dorsal striatum (Storvik et al., 2006), and from the prefrontal cortical regions (Mantere et al., 2002) were compared using correlation analysis. The correlations of selected regions with differences between the correlations are presented in detail in Fig. 2.

There was a significant correlation between the PVN and both dorsal ($R = 0.931, P = 0.001$) and ventral ($R = 0.898, P = 0.002$) regions of amygdala in type 2 alcoholics (Fig. 2). On the contrary, in type 1 alcoholics there was significant negative correlation between the dorsal amygdala and the PVN. In control subjects, no correlation between these areas was observed. The area-to-area correlations between the SERT binding in the PVN and the dorsal or ventral levels of amygdala were significantly different ($\chi^2 = 9.18, P = 0.01$, and $\chi^2 = 16.75, P = 0.00023$, respectively). In both comparisons, the difference between the correlations between type 1 and 2 alcoholics ($q = 3.810–5.722$), and between type 2 alcoholics and controls ($q = 3.768–3.732$) were significant in Tukey-type test.

The SERT levels correlated significantly between the PVN and at the lower level of putamen and caudate in type 2 alcoholics (correlations presented in Fig. 2). The difference in the correlations between the SERT in the PVN and in the head of caudate in type 2 alcoholics and non-alcoholics was significant ($q = 3.419$). There was also a significant negative correlation observed between the SERT in the PVN and in the body of the caudate ($R = -0.711, P = 0.032$) in type 1 alcoholics, but the differences between the three subject groups were not significant.

There was significant positive correlation between the SERT binding in the PVN and upper level of pACC in type 2 alcoholics. In addition, there was a significant negative correlation between the PVN and the superior frontal gyrus in

RESULTS

Binding values in all groups and brain areas were normally distributed (data not shown). In alcoholics, the SERT binding was $-16.3\%$ lower in the PVN when compared to the controls (Table 1). In the secondary analysis, the SERT binding was observed to be $-7.7\%$ lower in type 1 alcoholics when compared to controls (effect size 0.25), and in the type 2 alcoholics $-25.9\%$ lower than in the controls with large effect size of 0.88 (Fig. 1). Despite the moderate to large effect sizes, the differences did not reach significance. The SERT binding declined significantly with age ($R = -0.777, P = 0.016$) in type 1 alcoholics (age range 39–76 years), by 7.5 fmol/mg per decade, but increased with age ($R = 0.655, P = 0.042$) in control subjects (age range 36–77 years). In a Fisher’s (z)-transformation followed by Tukey-type test, the differences between the age correlations between the subject groups were statistically significant ($\chi^2 = 10.53, P = 0.005$). There was no correlation ($R = -0.12, P = 0.77$) between the age and SERT density in type 2 alcoholics.

Comparison of SERT binding in PVN and brain areas with serotonergic alterations in alcoholics

In order to further study the putative alterations in the serotonergic system in an alcoholic brain, the SERT binding in PVN and brain areas was observed. The area-to-area correlations between the PVN and the dorsal or ventral levels of amygdala and the PVN. In control subjects, no correlation between these areas was observed. The area-to-area correlations between the SERT binding in the PVN and the dorsal or ventral levels of amygdala were significantly different ($\chi^2 = 9.18, P = 0.01$, and $\chi^2 = 16.75, P = 0.00023$, respectively). In both comparisons, the difference between the correlations between type 1 and 2 alcoholics ($q = 3.810–5.722$), and between type 2 alcoholics and controls ($q = 3.768–3.732$) were significant in Tukey-type test.

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Fig. 2. (A) The correlation of the SERT binding in the PVN at the age at the time of death. Pearson correlation coefficient, two-tailed. B, C, D: The correlation of the SERT binding in the PVN and in the dorsal (B) and ventral (C) amygdala, and (D) in caudate in post-mortem brains of type 1 and 2 alcoholics, and control subjects. Pearson correlation coefficient, two-tailed.
type 1 alcoholics. In either case, the differences between the three subject groups were not significant (data not shown).

**DISCUSSION**

In type 2 alcoholics, the trend towards decreased SERT density together with the lack of decline with age suggest that the SERT may already be decreased in the PVN in early adulthood. Since type 2 alcoholics begin to use alcohol very early on, the finding is interesting. The finding supports the Cloninger model for the neurobiological typology of alcoholics (Cloninger, 1995). The trend towards decrease of SERT density in the PVN is in line with those reported from other brain areas (Mantere et al., 2002; Storvik et al., 2006) in the same subjects, although there is a caveat because of the selection of single set of subjects from the population. The large effect size suggests however, that it is likely that with larger subject groups, the observed decrease would have reached significance. The small effect size in type 1 alcoholics suggests on the other hand that the difference would not turn out to be significant in a study with a larger subject group in that subject group. The sizes of the subject groups were based on the original power calculations which were performed based on our previous data on dopaminergic receptors, but unfortunately the deviation turned out to be higher for SERT density. The ages, although not significantly different between the groups, could not be matched due the strict inclusion criteria. In addition, the life expectancy of type 2 alcoholics is shorter than in the normal population due to the anti-social life-style of these patients (Repo-Tiihonen et al., 2001). We controlled the effect of differences between the mean ages by using covariance analysis with age as covariates. Curiously, the SERT binding increased with age in the control subjects, which is opposite to the findings from most other brain areas where age-related declines of monoamine transporters are often reported (Kakिंchi et al., 2001; Hesse et al., 2003; Strother et al., 2005), even in the same subjects (Storvik et al., 2006).

Lower binding of SERT in alcoholics has been in most of the recent studies (Heinz et al., 1998; Mantere et al., 2002; Szabo et al., 2004; Storvik et al., 2006). However, in a recent technically valid PET study with a moderately large set of subjects, in essence, no alteration was observed between the alcoholics and the controls (Brown et al., 2007). In that report, it was not mentioned if the subjects would have been Cloninger type 1 or 2 alcoholics, as the monoamine systems could be largely different between the two groups, as reviewed by Tupala and Tiihonen (2004). There was also no correlation observed between the aggression and the SERT levels, but since the alcoholics were not classified further (Brown et al., 2007), it is difficult to estimate whether the alterations were masked by the heterogeneity of the alcoholics. The abstinence period does not probably explain the conflicting observations, since the decrease has been reported from living subjects with years of abstinence (Szabo et al., 2004), and from deceased active drinkers (Mantere et al., 2002).

The finding for early decreased SERT in type 2 supports the hypothesis summarized by Barr et al. (2004) based on the macaque experiments, that the traits characteristic of types of alcoholism are related to early developed defects in serotonergic functioning. Serotonergic function is required during the refinement of brain connections (Gaspar et al., 2003) and in the development of normal emotional behaviour (Ansonge et al., 2004). The defect in the serotonergic system could then disrupt the development of the connections between the cortex and amygdala (Pezawas et al., 2005), which could further lead to altered function of the PVN, in addition to a lack of proper serotonergic regulation. The 5-HT1A receptor activation inhibits serotonin release and modulates the reactivity of amygdala (Fishet al., 2006). There is currently no data of alteration of the 5-HT1A receptor density in the hypothalamus in type 2 alcoholics, but the SERT and 5-HT1A may be affected together in type 2 alcoholics, causing the observed correlations.

The observed significant positive correlation between the SERT binding densities in the dorsal and ventral amygdala and in the PVN supports the hypothesis that serotonergic system is defective in both of these regions in type 2 alcoholics. On the contrary, in type 1 alcoholics the SERT seems to be decreased only in the amygdala (Storvik et al., 2007) and not in the PVN. This may further elucidate why type 2 alcoholics are especially prone to aggressive outbursts. In the previous report, the SERT binding was found to be decreased in the dorsal but not in the ventral amygdala in alcoholics (Storvik et al., 2007). Despite this, because majority of the output from the amygdala is derived from the ventral nuclei, the global defect in the serotonergic system is likely to cause a correlation between the SERT levels in the PVN and the ventral amygdala.

Type 1 alcoholics are reported to have decreased D2 receptor density in nucleus accumbens and amygdala, and decreased dopamine transporter density in several brain areas (Tupala and Tiihonen, 2004). This suggests that this group could benefit from medication to enhance dopaminergic activity. On the other hand, type 2 alcoholics do not have these defects in the dopaminergic system, but are reported to respond better to acamprosate and naltrexone treatments than type 1 alcoholics (Kiefer et al., 2005, 2007). The present findings suggest that enhancing the serotonergic control of the prefrontal cortex-amygdala-hypothalamus axis, which could lead to better control of behaviour, may be beneficial augmentive treatment for type 2 alcoholics.

As a conclusion, the results suggest that the correlations between the SERT binding and the age and between the SERT binding in the PVN and the amygdala differed between the alcoholic sub-types, and suggest that in type 2 alcoholics, the SERT decrease is present in early age, but in type 1 alcoholics the serotonergic alterations may be secondary and possibly reflect the alterations in the dopaminergic system. However, these results must be considered preliminary and should be re-examined in a larger sample before making any firm conclusions.

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