Dopamine D₂ Receptors and Transporters in Type 1 and 2 Alcoholics Measured With Human Whole Hemisphere Autoradiography

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Abstract: Increasing evidence implies the involvement of the dopamine (DA) system in the pathogenesis of alcoholism. We measured striatal DA D₂ receptors in Cloninger type 1 and 2 alcoholics by using [¹²⁵I]epidepride in human postmortem whole hemispheric autoradiography (WHA), which provides high-resolution images corresponding to positron emission tomographic (PET) studies. We also evaluated the correlation between transporter and receptor DA binding site densities and putative correlation of [¹²⁵I]epidepride binding between the dorsal striatum and nucleus accumbens. In the type 1 alcoholics, the DA D₂ receptor density was 21.4–32.6% lower in all dorsal striatal structures (caudate, putamen, globus pallidus) when compared with the controls. Type 2 alcoholics had 19.6–21.4% lower binding in other dorsal striatal structures, except medial globus pallidus, where they were not significantly different from controls. The density of DA D₂ receptors and DAT had a significant positive correlation only in the putamen of type 1 alcoholics. The binding of [¹²⁵I]epidepride showed also consistent and statistically significant positive correlation between nucleus accumbens and all dorsal striatal areas in type 1 alcoholics but not in the controls. In the type 2 alcoholics, the correlation was weaker than that observed in the type 1 alcoholics, and no correlation was observed between nucleus accumbens and globus pallidus. Our results show that these two subgroups of alcoholics have stark differences in their DA D₂ receptor binding characteristics. Type 2 alcoholics may have selective deficiency in the dorsal striatum, whereas in limbic structures they may not differ significantly from controls. Moreover, WHA provides a useful tool for

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detailed mapping of neuronal receptors in healthy as well as diseased brain, and can also be used in radioligand development for PET. *Hum. Brain Mapp.* 20:91–102, 2003. © 2003 Wiley-Liss, Inc.

Key words: alcohol-induced disorders; ethanol-induced nervous system disorders; dopamine; alcoholism; autoradiography; human brain; receptors; transporters

INTRODUCTION

Increasing evidence implies the involvement of the dopaminergic transmission in the pathogenesis of alcoholism. Animal studies have shown that acute ethanol administration in rats induces dopamine (DA) release in the nucleus accumbens (NAC) (di Chiara and Imperato, 1985; Yoshimoto et al. 1992). Low endogenous DA content in the terminals of the mesolimbic and mesostriatal DA neurons predisposes mice to high alcohol preference and consumption, and reversing this effect by augmenting synaptic DA concentrations leads to a marked decrease in this behavior (George et al., 1995). DA D₂ receptor antagonist spiperone increases ethanol intake at modest doses (Dyr et al. 1993), whereas complete blockade of DA D₂ receptors by high doses of pimozide and haloperidol (Pfeffer and Samson, 1988) or gene knockout (Phillips et al. 1998) produces a decrease in alcohol consumption.

In late-onset type 1 alcoholism, the emphatic, compassionate, and socially dependent character traits have been suggested to be associated with an underlying DAergic deficit, as opposed to the serotonergic deficit observed in teenage onset type 2 alcoholism, which has been linked to vengeful and socially hostile character leading to antisocial behavior (Cloninger, 1995). Positron emission tomographic (PET) and single photon emission tomographic (SPET) studies have indicated alterations in DA D₂ receptor binding characteristics in the striatum of unclassified (type 1 vs. type 2) alcoholics (Hietala et al., 1994; Volkow et al., 1996a) and endocrinological measurements have shown decreased DAergic activity among unclassified alcoholics (Balldin et al., 1992, 1993; Dettling et al., 1995; Schmidt et al., 1996; Wiesbeck et al., 1995). Human genetic studies have suggested an association between the DA D₂ receptor gene (DRD2) allele A1 and alcoholism (Blum et al., 1990; Noble et al., 1991; Noble, 2000) and with receptor binding characteristics in alcoholism (Noble et al., 1991), although contradictory results have been presented also (Edenberg et al., 1998; Gejman et al., 1994; Gelernter et al., 1993).

[¹²⁵I]Epidepride ((S)-N-((1-ethyl-2-pyrrolidinyl)methyl)-5-iodo-2,3-dimethoxybenzamide) has a high affinity for DA D₂ receptors, with low nonspecific binding, and has been widely used to label DA D₂ receptors in the brain (Hall et al., 1996; Murray et al., 1994). It has affinity also to DA D₃ receptors, but with the concentration used in the present study [¹²⁵I]epidepride labels predominantly DA D₂ receptors as demonstrated by competition studies (Hall et al., 1996). Human postmortem whole hemisphere autoradiography (WHA) provides high-resolution images from different

brain levels, corresponding to in vivo studies (i.e., PET and SPET) and enables a more detailed study of various structures (Hall et al., 1996, 1998, 1999; Tupala et al., 2000, 2001a,b). Here we studied [¹²⁵I]epidepride binding in caudate, putamen, and globus pallidus separately in type 1 and 2 alcoholics and controls and also the correlations between DA transporter (DAT) and receptor binding sites by comparing the present results to our previous studies (Tupala et al., 2000, 2001a,b) on these same subjects. [¹²⁵I]N-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropine ([¹²⁵I]PE2I) is a high-affinity radioligand that has proven to be an extremely selective DAT ligand with low affinity for other monoamine transporters (30- and >60-fold selectivity over serotonin and noradrenaline transporters, respectively) (Emond et al., 1997). This makes [¹²⁵I]PE2I superior to old cocaine derivatives like β-CIT, frequently used in previous studies and [¹²⁵I]PE2I has been used successfully in both in vitro and in vivo imaging of DAT (Hall et al., 1999; Kuikka et al., 1998; Tupala et al., 2000, 2001a,b).

In the present study, we tested several hypotheses. First, we studied whether the DA D₂ receptor densities in the dorsal striatum (caudate, putamen, globus pallidus) differ between the alcoholic subgroups and controls in a similar manner as in our earlier study on mesolimbic structures (Tupala et al., 2001b). Second, we compared in the same subjects the densities of DAT, which can be used as a marker of neuronal density in these regions (Boja et al., 1994) to densities of mostly postsynaptic DA D₂ receptors to test whether there is a positive correlation between these binding sites in all groups. Third, as we have previously shown striatal DAT binding heterogeneity to be lower in type 1 alcoholics (Tupala et al., 2001a), whereas type 2 alcoholics may have higher heterogeneity when compared with controls (Kuikka et al., 1998) and animal studies have indicated that individual differences in the number of DA-releasing neurons within DA cell group (e.g., DA neurons in A9 or A10) are maintained across all DA cell groups in the brain (Depue et al., 1994), we evaluated also the correlations of [¹²⁵I]epidepride binding between the dorsal striatum and mesolimbic NAC to test whether individual densities of these receptors are maintained in different brain areas in these three groups.

MATERIALS AND METHODS

Brain Sampling and Diagnostics

Human brains used were obtained for clinical necropsy at the Department of Forensic Medicine, University of Oulu, Finland, with the permission from the National Institute of

TABLE I. Demographic data and descriptions of the brains used*

Case no.	Sex	Age (yr)	Post mortem delay (hours)	Cause of death
Controls				
2	M	55	5.5	Acute myocardial infarction
5	M	45	9.5	Acute myocardial infarction
11	M	77	7.5	Acute myocardial infarction
14	F	57	11.0	Acute myocardial infarction
17	M	50	18.5	Acute myocardial infarction
19	F	60	12.0	Acute myocardial infarction
25	M	49	33.0	Acute myocardial infarction
26	M	53	29.0	Acute myocardial infarction
29	M	53	11.0	Acute myocardial infarction
24	M	36	11.0	Dissection of aorta
Type-1 alcoholics				
1	M	39	12.5	Pneumonia
4	M	48	4.5	Acute pancreatitis
7	M	45	12.0	Suicide by hanging
8	M	42	14.8	Acute myocardial infarction
12	M	76	10.5	Acute myocardial infarction
15	F	56	19.0	Ethanol intoxication
23	M	48	6.5	Pneumonia
28	M	69	16.0	Ethanol intoxication
31	F	51	11.0	Right subdural hemorrhage
Type-2 alcoholics				
3	M	49	12.0	Fibrotic degeneration of myocardium
6	M	37	9.5	Gunshot wound
13	M	47	15.5	Knife wound
16	M	20	14.5	Knife wound
20	M	46	18.0	Suicide by hanging
21	M	18	9.5	Heart rupture (car accident)
27	M	32	16.5	Suicide by hanging
30	M	28	17.5	Suicide by hanging

* Case no. 31 was included only in the [125 I]epidepride study due to methodological limitations.

Medicolegal Affairs, Helsinki, Finland. A postmortem analysis for drugs, including alcohol, and the normal necropsy protocol were also performed. All psychiatric diagnoses were based on both hospital and outpatient records, which were collected with the permission from the Ethics Committee of the University of Oulu, Finland. Two physicians, independently of each other made diagnoses. Mental disorders were coded according to DSM-III-R diagnostic criteria and alcoholics were classified as type 1 or 2 according to Cloninger (1995). Alcoholism among these subjects was severe judging by the frequent admissions to emergency stations and doctors appointments due to alcohol-related problems and the diagnosis of alcoholism itself was not a difficult task even without interviews. Kappa coefficient of diagnostic agreement was 0.9; i.e., the second diagnostician diagnosed one type 2 alcoholic included in the study as a type 1 alcoholic. Otherwise diagnoses were unanimous. Subjects having psychotic disorders or any diseases (such as epilepsy), or taking medication that affect the central nervous

system (such as neuroleptics or antidepressants), were excluded.

Study Subjects

All 27 cases were Caucasians (Table I). The study groups consisted of 9 type-1 alcoholics in the [125 I]epidepride binding study (7 males, 2 females; mean age 53.5 ± 10.6 years; postmortem delay 11.9 ± 4.5 hr; mean $\pm$ SD), 8 type 1 alcoholics in the [125 I]PE2I binding study (7 males, 1 female; mean age 52.9 ± 13.2 years; postmortem delay 12.0 ± 4.8 hr; mean $\pm$ SD), and 8 type 2 alcoholics (males; mean age 34.6 ± 12.2 years; postmortem delay 14.1 ± 3.4 hr; mean $\pm$ SD) and 10 controls in both the [125 I]epidepride and [125 I]PE2I study (8 males, 2 females; mean age 53.5 ± 10.6 years; postmortem delay 14.8 ± 9.2 hr; mean $\pm$ SD) free of psychiatric diagnosis. Postmortem delays between the groups were not significantly different ($P = 0.62$ – 0.98 , Scheffé's test for multiple comparisons, two tailed). From one female type 1 alcoholic, only DA D_2 -receptors were measured, due to

methodological limitations. Eight of the nine type 1 alcoholics had alcohol in their blood at their time of death, and one (included in both ligand binding studies) had an abstinence period of 10 hr. One of the controls had a small amount of alcohol in his blood at the time of death (0.036%). Two of the type 1 alcoholics had traces of diazepam in their blood samples. Six type 2 alcoholics had alcohol in their blood at the time of death. One had an abstinence period of 3–7 days and one of 5 days. Three had traces of benzodiazepines and one was positive for cannabinoids. Mean alcohol concentration in the blood was $0.20 \pm 0.17\%$ (mean $\pm$ SD) in the type 1 alcoholic group and $0.19 \pm 0.14\%$ in the type 2 alcoholic group and the two groups did not differ in this respect [$F(0.174)$, $P = 0.90$, independent samples t-test]. Evaluation of the duration of heavy alcohol use or smoking based only on medical records was considered unreliable and was not included in the analysis.

Cryosectioning

For cryosectioning, the frozen (-75°C) left hemisphere was put in a specially designed rubber box with a specimen holder (Hall et al., 1998). A semi-liquid gel (approximate concentration 5 g/l, $+5^{\circ}\text{C}$) of carboxymethylcellulose (CMC) was added and the hemisphere was inserted with the midsagittal plane of the hemisphere facing up, carefully checking the horizontal level of the midsagittal surface. The positioning of the hemisphere to obtain parallel sections to the bicommissural line of Talairach was achieved by adjusting the hemisphere parallel to a ruler over the upper margin of the anterior and lower margin of the posterior commissura and the surface of the cryostat specimen holder (the cutting plane). When the hemisphere-CMC block was totally frozen, the rubber box was removed. The serial sectioning of the block was performed with a heavy-duty cryomicrotome (LKB 2250, LKB, Stockholm, Sweden) into 100- μm horizontal (canto-meatal) sections. The sections were allowed to air dry for 4 hours at room temperature and 24 hours in an excicator before they were stored with dehydrating agents at -25°C until use. Study level was chosen according the size and shape of the corpus striatum structures (caudate, putamen, globus pallidus) and the width of the internal capsule.

Autoradiography

[^{125}I]Epididride and [^{125}I]PE2I were obtained from MAP Medical Technologies Oy, Helsinki, Finland. The radiolabeling was performed using stannylated precursors with [^{125}I]NaI according to standard methods (Hall et al., 1996, 1999). β -CIT was obtained from the Department of Chemistry, Kuopio University, Finland. (+)-Butaclamol was obtained from RBI, Natick, MA.

After preincubation (2×10 min) the first series of sections (8 controls, 7 type 1 alcoholics, 6 type 2 alcoholics) were incubated for 1 hr to reach equilibrium in a solution (volume 2.5 l) containing 24 pM of [^{125}I]epididride (concentration was chosen to be close to K_d) in Tris-HCl buffer, pH 7.4, containing 0.1% (weight/volume) ascorbic acid, 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , and 1 mM MgCl_2 (Hall, 1994;

Hall et al., 1996; Murray et al., 1994). Concentrations were ascertained by γ -scintillation counter (1271 RiaGamma, LKB-Wallac Ltd., Turku, Finland). (+)-Butaclamol (15 μM) was used as a specific displacer to determine nonspecific binding. Washing was performed in cold buffer for 3×10 min, followed by a brief cold wash by dipping the sections into distilled water. The sections were dried for 10 min with a gentle stream of warm air and then for 3 hr at room temperature before exposure to radiation-sensitive film (^3H -Hyperfilm; Amersham Pharmacia Biotech Limited, Buckinghamshire, UK) for 5 days before development (developer, Kodak D19; fixation, Kodak Fixer 3000; Kodak). The autoradiograms were analyzed by computerized densitometry, using a Mikrotek Scanmaker E6 (Microtec International, Taiwan, R.O.C.) connected to an Osborne PC Pentium II (Oy Microlog Ltd., Espoo, Finland). Software packages included Photoshop v. 5.0.2 (Adobe, Mountain View, CA) and Scion Image for Windows, v. beta 3b (Frederick, MD). The resulting pixel values of binding data were mathematically transformed by an exponential calibration equation into relative radioactivity values by the use of [^{125}I]calibrating scales (cat. no. RPA 523L; Amersham), and background was subtracted. All analyses were made blind to the clinical classification of the subjects. An adjacent section from the respective level was stained with cresyl violet (Nissl staining) to serve as an anatomical correlate to the autoradiography.

Autoradiography with [^{125}I]PE2I, using β -CIT as a displacer for the first series of sections (7 controls, 7 type 1 alcoholics, and 6 type 2 alcoholics), was performed essentially as described above and has been described in our previous reports (Hall et al., 1999; Tupala et al., 2001a,b). The second autoradiographic series (2 subjects from each group with [^{125}I]epididride; 3 controls, 1 type 1 alcoholic, and 2 type 2 alcoholics with [^{125}I]PE2I) was made according to the same protocol, but only in an incubation volume of 750 ml.

Statistical Analyses

Univariate analysis of co-variance (ANCOVA) (Anderson et al., 1980) with the age as a covariate followed by Bonferroni's test for multiple comparisons was used to compare the mean values of [^{125}I]epididride and [^{125}I]PE2I binding in each group, because type 2 alcoholics were substantially younger than the two other groups, and a negative correlation between brain DAT and DA D₂ receptor binding with age has been well documented (Rinne et al., 1993; Volkow et al., 1996b). The two experiment series (the first and second) were also used as a covariate (see Figs. 3 and 4) due to the fact that different batches of films and ligands had to be used in the different series. We have not previously used blood alcohol levels as a covariate because alcoholic subgroups do not differ in this respect as mentioned above. No correlation has been found between the ligand binding and blood alcohol level in limbic structures or in dorsal striatal DAT (Tupala et al., 2001a,b). However, there was a tendency towards positive correlation between the blood alcohol level and the binding values of [^{125}I]epididride to DA D₂ receptors in the dorsal striatal structures (caudate, putamen, globus palli-

pus). The correlations were 0.45 to 0.70 ($P = 0.054$ – 0.27) in the type 2 alcoholics and 0.43 to 0.63 ($P = 0.07$ – 0.25) in the type 1 alcoholics, who also showed statistically significant correlation in the lateral globus pallidus ($r = 0.68$, $P = 0.045$). Therefore, the blood alcohol level was added as a covariate to compare the mean values of [125 I]epidepride and [125 I]PE2I binding in each group.

Because Shapiro-Wilk's test of normality (Shapiro et al., 1968) showed a normal distribution of binding values within the groups (data not shown), the correlations between binding values and age were explored with Pearson's correlation coefficient, two-tailed. A P value smaller than 0.05 was considered significant. The kappa coefficient of agreement was calculated with regard to the matching of diagnoses between two diagnosticians (Cohen, 1960), where a value over 0.75 is considered excellent (Landis and Koch, 1977). The differences between correlations were tested by using Fisher's (z) transformation of correlations to test the overall difference followed by Tukey-type test to compare individual groups (Zar, 1984).

RESULTS

[125 I]Epidepride showed very dense labeling in the caudate and putamen, indicating high densities of DA D_2 -receptors (Fig. 1). Labeling in the lateral and medial globus pallidus was weaker (approximately 31 and 5% of the putamen radioactivity, respectively). [125 I]Epidepride did not bind or accumulate in the white matter. Addition of the D_2/D_3 dopamine receptor antagonist (+)-butaclamol (15 μ M) totally (>95%) inhibited specific binding in the striatal area (data not shown). [125 I]PE2I bound with high intensity in the caudate and putamen indicating a high DAT density in these structures (Fig. 2) and the results of that experiment (excluding dorsal striatal DAT in type 2 alcoholics) have been described in our previous reports (Tupala et al., 2001a,b). The labeling of DAT with [125 I]PE2I in the caudate and putamen was totally (>95%) inhibited by the addition of the DA reuptake inhibitor β -CIT (2 μ M)(data not shown). [125 I]PE2I accumulated in the white matter, possibly due to its relatively high lipophilicity (Hall et al., 1999), and addition of β -CIT did not affect the accumulation. It accounted for approximately 27% of the specific binding in the putamen and there were no differences between the groups (data not shown). By analyzing the original data without the covariates mentioned above, all results regarding type 1 alcoholics and both DAT and DA D_2 receptors in all brain areas are about the same as mentioned below. However, type 2 alcoholics do not significantly differ from the controls regarding DAT in the caudate and the putamen and DA D_2 receptors in the globus pallidus.

The mean binding of [125 I]epidepride to DA D_2 -receptors in the caudate was 24.1% lower in the type 1 alcoholic group (437 ± 74 Bq/mg; mean $\pm$ SD) and 21.4% lower in the type 2 alcoholic group (453 ± 73 Bq/mg) when compared with the control group (576 ± 99 Bq/mg), and these differences were statistically significant ($P < 0.001$ and $P = 0.001$, respectively) (Fig. 3). The binding values in the putamen were

Figure 1.

Color-coded autoradiograms of human postmortem brain sections showing the binding of [125 I]epidepride (DA D_2 receptors) in type 1 alcoholic (A), type 2 alcoholic (B), and control (C) brains. Adjacent section of each brain was stained with cresyl violet (Nissl staining) to serve as an anatomical correlate (D). Nc: caudate; Put: putamen; Gp: globus pallidus.

532 ± 111 Bq/mg in the type 1 alcoholics (23.5% lower; $P = 0.001$) and 546 ± 80 Bq/mg (21.4% lower; $P = 0.002$) in the type 2 alcoholics vs. 695 ± 127 Bq/mg in the controls (Fig. 3). In the lateral globus pallidus, type 1 alcoholics had 32.6% lower (151 ± 61 Bq/mg; $P < 0.001$) and type 2 alcoholics 19.6% lower (181 ± 28 Bq/mg; $P = 0.018$) binding when compared with the controls (225 ± 36 Bq/mg) (Fig. 3). In the medial globus pallidus, only type 1 alcoholics had statistically significantly lower binding (23.6 ± 7.6 Bq/mg vs. 33.8 ± 8.3 Bq/mg, 30.2% lower in alcoholics; $P = 0.007$) and type 2 alcoholics were not significantly different from healthy controls.

The mean binding of [125 I]PE2I to DAT in the caudate was 35% lower in the type 1 alcoholic group when compared with the control group and this difference was statistically significant ($P = 0.001$; the univariate analysis of co-variance (ANCOVA) with the experiment series (the first or second) and blood alcohol level as a covariate), and we have reported these results regarding type 1 alcoholics elsewhere (Tupala et al., 2001a). In further analyses, [125 I]PE2I-binding

Figure 2.

Color-coded autoradiograms of human postmortem brain sections showing the binding of [125 I]PE2I (DAT). See Fig. 1 legend for details.

(DAT) in the putamen was reduced by 28% in the type 1 alcoholic group when compared with the control group ($P = 0.001$). In the type 2 alcoholics, the binding of [125 I]PE2I to DAT was also significantly lower in the caudate, but not in the putamen (619 ± 90 Bq/mg; 5.9% difference), although also in the putamen the finding was almost significant ($P = 0.060$) (Fig. 4). When comparing the values of type 2 alcoholics in the caudate (503 ± 81 Bq/mg) to controls (584 ± 94 Bq/mg) (14% difference) the P value was 0.054.

The correlation between the pre-synaptic DAT and mostly post-synaptic DA D_2 receptor sites was significant in the putamen of the type 1 alcoholic group ($r = 0.78$, $P = 0.02$), but not in the caudate ($r = 0.43$, $P = 0.29$). In the two other groups, the DAT and DA D_2 receptor correlations were: $r = -0.01$, $P = 0.98$ in the caudate and $r = 0.23$, $P = 0.59$ in the putamen of the type 2 alcoholic group and $r = 0.53$, $P = 0.12$ in the caudate and $r = 0.25$, $P = 0.48$ in the putamen of the control group. Otherwise in the type 1 alcoholic group there was consistent significant positive correlation in all other measures. Correlations between NAC and dorsal striatal structures were: caudate $r = 0.76$, $P = 0.018$, putamen $r = 0.82$, $P = 0.007$, lateral globus pallidus $r = 0.79$, $P = 0.11$ and medial globus pallidus $r = 0.81$, $P = 0.008$). In the type

2 alcoholic group the correlation of [125 I]epidepride binding between the NAC and dorsal striatal structures was weaker, and no correlation was observed regarding globus pallidus: caudate $r = 0.69$, $P = 0.057$, putamen $r = 0.73$, $P = 0.039$, lateral globus pallidus $r = 0.40$, $P = 0.33$, medial globus pallidus $r = 0.13$, $P = 0.77$. In the control group, there was no significant correlation between any measured structures ($r = -0.019$ – 0.52 , $P = 0.12$ – 0.96). However, Fischer's transformation of correlations and Tukey type test did not show significance between the groups in any of the measures mentioned above ($\chi^2 = 0.59$ – 3.85 , $P = 0.15$ – 0.74).

DISCUSSION

Perhaps the most difficult problem in this line of studies is the low availability of suitable brains, which is not helped by our strict inclusion criteria, especially the demand of relatively short postmortem time. In the present study, the mean age and age-range could not be matched between the type 2 alcoholics and the two other groups, which may complicate the interpretation of the results. However, this is not merely a consequence of the lack of availability of postmortem material. By definition, the onset of type 2 alcoholism is typically before and in type 1 alcoholism after 25 years of age (Cloninger, 1995) and the life-expectancy of type 2 alcoholics is markedly shorter than in the normal population due to the fact that antisocial personality is associated with a higher death rate at young age (Repo-Tiitonen et al., 2001). This may make it difficult to arrange even an age-matched clinical PET study in the issue, and in the present study the difference of the mean ages between type 2 alcoholics and the two other groups was controlled by using co-variance analysis with age as a co-variate (Anderson et al., 1980). However, considering the relatively small sample size in the present study, one has to bear in mind that co-variance analysis may not be able to correct for the age-difference, so there might be a type 1 error here. Additional studies with larger sample sizes are needed to establish these differences between the alcoholic subtypes. Another possible confounding factor could be the duration of alcohol misuse as type 2 alcoholics in the present study were substantially younger than type 1 alcoholics or controls, and the quantity of heavy use was another factor difficult to reliably determine from the medical records only. Needless to say, the type 2 alcoholics may have had shorter exposure than the rest of the index subjects, but the trend for the possible age of onset was as also by definition much younger in the type 2 alcoholics, which diminishes the difference of exposure times to ethanol. Furthermore, there is some evidence that the number of years of alcohol abuse does not correlate between DAT or DA D_2 binding values (Hietala et al., 1994; Volkow et al., 1996a).

Our results are in line with previous in vivo (PET) studies with [11 C]raclopride on striatal DA D_2 receptors of unselected alcoholic material (Hietala et al., 1994; Volkow et al., 1996a) and with one autoradiographic study on alcoholics classified as severe (Noble et al., 1991). However, it is not clear whether those changes observed in DA D_2 receptor

Figure 3.

Scatter plots showing the binding of $[^{125}\text{I}]$ epidepride to DA D_2 receptors in caudate (A), putamen (B), lateral globus pallidus (C), and medial globus pallidus (D) of alcoholics and controls. *m*: the mean value; full circles represent cases from the first and open circles cases from the second experiment series.

availability (also called binding potential or B_{max}/K_d ratio) reflect differences in receptors or in the level of intrinsic DA, because $[^{11}\text{C}]$ raclopride is sensitive to intrinsic DA and it has actually been used to measure the levels of intrinsic DA in the human brain (Di Chiara 2002; Hietala et al., 1994; Seeman et al., 1996; Volkow et al., 1997).

However, the present study with a very high affinity ligand ($K_d \sim 24$ pM) and a preincubation procedure to remove the intrinsic DA from the cryosections supports the assumptions of the aforementioned groups that their results reflect changes in DA D_2 receptors and not in the levels of intrinsic DA (Hall et al., 1996). We noted a statistically

significant 24 and 21% difference in the caudate of type 1 and type 2 alcoholics, respectively, which is of the same magnitude as observed in the earlier PET studies (20–22%). Interestingly, our present and former findings (Tupala et al., 2000, 2001a,b) suggest that type 2 alcoholics may have a selective decrease in DA D_2 receptors in the dorsal striatal structures and DAT in the caudate, but in the limbic structures NAC and amygdala they do not differ significantly from controls. One previous SPET study did not find differences in striatal DAT between type 2 alcoholics who had been abstinent for at least 6 months and controls (Tiihonen et al., 1995), so the reduction observed in the present study

Figure 4. Scatter plots showing the binding of [125 I]PE2I to DAT in caudate (A) and putamen (B) of alcoholics and controls (See Fig. 3 legend for details).

could reflect rapid DAT regulation associated with the acute effects of ethanol on type 2 alcoholics as they were mostly intoxicated at the time of death. The magnitude of the DAT decrease was 14%, while type 1 alcoholics had 35% lower DAT density in the caudate, which in turn could reflect also, in part, acute effects of ethanol and additional “trait” effect in type 1 alcoholics, i.e., to have lower DAT even without the acute effect of ethanol. In order to determine whether the lower DAT represents fewer DA terminals or regulation of DAT, other measures of the DA system markers have to be evaluated. However, there is some preliminary evidence from a PET study on seven unclassified alcoholics and controls of lower striatal vesicular monoamine transporter 2 (VMAT2), which is supposed to be a more permanent marker of DA nerve terminal integrity than DAT (Gilman et al., 1998). Therefore, the next rational step for our group to investigate the present issue further is to perform an autoradiography on VMAT2 in these subjects.

There are several animal receptor studies on alcoholism reporting altered DA D_2 -like receptor and/or transporter densities (B_{max}), while affinities (K_d) have remained unchanged (e.g., Korpi et al., 1987; McBride et al., 1993; Stefanini et al., 1992), and also human PET studies have not reported significant changes in K_d (Hietala et al., 1994; Volkow et al., 1996a). Therefore, we set the ligand concentration to be close to the K_d value, in order to optimize binding dynamics. By increasing the concentration to, e.g., two or three times higher than the K_d value would have increased nonspecific binding substantially, which could have disturbed the detection of possible differences between the groups, and considering the aforementioned reports, we still had a reasonable basis to assume that possible differences in binding would reflect the difference in B_{max} and not in the K_d .

Most benzamides including [125 I]epidepride have affinity also for the DA D_3 receptor (Halldin et al., 1995). Previous data have implied that as much as 34% of the sites labeled by [125 I]epidepride in the NAC are DA D_3 receptors, as opposed to the caudate, where only a very small percentage of the DA receptors are D_3 -like (Murray et al., 1994). However, based on our earlier inhibition studies with the DA D_2 receptor antagonist domperidone and the DA D_3 receptor agonist 7-OH-DPAT we may assume that [125 I]epidepride binding with the present study concentration labels mainly DA D_2 receptors also in the NAC, because only high concentrations (10 μ M) of 7-OH-DPAT were able to block the binding of [125 I]epidepride whereas lower concentrations had only a minor effect. The predominantly DA D_2 receptor antagonist domperidone inhibited binding also in extrastriatal regions at concentrations that also inhibited binding in striatal regions. In addition, in our previous study [125 I]epidepride binding with this concentration was lower among type 1 alcoholics and not significantly different among type 2 alcoholics in NAC and also in the amygdala, where the density of DA D_3 receptors is negligible (Tupala et al., 2001b).

The lower density of DA D_2 receptors in alcoholics resembles the situation where animals are administered intermediate doses of DA antagonists, which results in an increase of ethanol intake. The interpretation proposed for this phenomenon was that an intermediate dose of these antagonists may cause a partial blockade of DA D_2 receptors, the effect of which would logically be a reduction in the reinforcing efficacy of ethanol (Levy et al., 1991; Oyr et al., 1993). This would lead the rats to increase their already high ethanol intake in an attempt to overcome the reduced reinforcing efficacy of ethanol. On the other hand, the complete blockade of DA D_2 receptors produced by higher doses (or by

gene knockout) would completely eliminate the reinforcing effects of ethanol, thus producing a decrease in alcohol consumption (Pfeiffer and Samson, 1988; Phillips et al., 1998). Extrapolating this to our results, we could speculate that alcoholics due to their lower density of DA D₂ receptors are vulnerable to excessive drinking in order to restore their subnormal level of DA function by the DA-releasing effect of ethanol.

It is unclear, of course, whether this lower DA D₂ receptor density is a genetic trait marker or consequence of long heavy ethanol abuse. Human genetic studies have indicated that the DA D₂ receptor gene allele A1 is associated with alcoholism (Blum et al., 1990). However, there have now been numerous studies specifically addressing the issue of an association between alleles at DRD2 and alcoholism and they have been divided into those supporting the association (Blum et al., 1990; Noble et al., 1991; Noble, 2000) and those maintaining the contrary view (Edenberg et al., 1998; Gejman et al., 1994; Gelernter et al., 1993) leaving the issue still controversial. By using [³H]7-OH-DPAT as a radioligand in autoradiography, Staley and Mash (1996) have shown an increase in DA D₃ receptors in NAC of six overdose victims of cocaine, which like ethanol has NAC as a neuroanatomical substrate for reinforcement, and exerts its effects on DA D₃ receptors indirectly by increasing the concentration of intrasynaptic DA (Koob and Bloom, 1988; Self and Nestler, 1995). These authors suggested that cocaine use might lead to an adaptive increase in DA D₃ receptors in response to elevated synaptic DA levels. DA D₂ receptors seem to have an opposite mode of regulation compared to DA D₃ receptors: denervation leads to DA D₂ receptor upregulation as a consequence of a lack of DAergic stimulation, whereas denervation evokes downregulation of DA D₃ receptors (Sokoloff and Schwarz, 1995). Furthermore, DA D₃ receptor is able to play an autoreceptor role in transfected cells. Therefore, our results may indicate downregulation of mostly post-synaptic DA D₂ receptors in response to elevated synaptic DA levels. However, in previous PET studies (Hietala et al., 1994; Volkow et al., 1996a), DA D₂ receptor availability in the striatum had no substantial correlation with the period of abstinence, suggesting their independence of withdrawal. Thus, our findings may reflect genetic or developmental variability related to addiction vulnerability. Further studies are needed to establish the extent of this observed decrease in DA binding site densities in alcoholics, and to study whether it relates to variabilities in neurodevelopment or to chronic ethanol exposure.

The fact that the binding of [¹²⁵I]epidepride binding correlated strongly between the NAC and dorsal striatum, and also between [¹²⁵I]PE2I binding (DAT) in the dorsal striatum of especially type 1 alcoholics but not controls is somewhat puzzling. Behavioral genetic studies on rodents have indicated that individual differences in the number of DA-releasing neurons, within the DA cell group (e.g., DA neurons in A9 or A10), are maintained across all DA cell groups in the brain (Depue et al., 1994). This is reflected in a strong correlation between the reactivity of hormonal and behav-

ioral indicators in different DA cell groups and this fact has been used to extrapolate results obtained with PET in the dorsal striatum to mesolimbic system in order to overcome the difficulty to differentiate the NAC from the rest of the striatum (Farde et al., 1997). Therefore, it is surprising that binding correlated between mesolimbic NAC and dorsal striatal structures in alcoholics, but not in controls. However, a similar observation in a SPET study has recently been made regarding striatal (striatum-to-cerebellum ratio) and extrastriatal (temporal pole-to-cerebellum ratio) DA D₂ receptors in the type 1 alcoholics (Repo et al., 1999). Perhaps this, as well as the correlation between the DAT and DA D₂ receptors only in the type 1 alcoholics, could be explained by the overall lower binding and the lower heterogeneity of DA binding (Kuikka et al., 1998; Tupala et al., 2001a) in the type 1 alcoholics as compared with the controls, which show also larger variability of DA binding site densities between different brain structures and between different binding sites. This could suggest that these two variables are connected, i.e., lower mean binding and correlation of binding between different brain structures and binding sites. [¹²⁵I]Epidepride binding correlated also among type 2 alcoholics between the NAC and dorsal striatal structures, but not in all of them and the correlation was weaker when compared with the type 1 alcoholics. Perhaps this could be explained by the fact that they did not have a significantly lower binding in NAC compared to controls (Tupala et al., 2001b).

A decrease in DA D₂ receptors should, in effect, result in an attenuation of the DA signal. Endocrinological measurements, in response to DA agonists, have been performed in unclassified alcoholics as a means of assessing brain tuberoinfundibular DA function. The growth hormone response to the DA agonist apomorphine has been shown to be decreased in alcoholics even after an abstinent period of two months (Balldin et al., 1992, 1993), and alcoholics showing a persistent, blunted response seem to be more vulnerable to relapse (Dettling et al., 1995; Schmidt et al., 1996), which supports an association between low levels of DA neurotransmission and addiction potential. Furthermore, this reduced DA D₂ receptor function in alcohol-dependent men may be not only a state marker of residual heavy drinking, but also a genetically determined trait marker (Wiesbeck et al., 1995), because alcoholics having a first-degree, alcohol-dependent relative seem to have more blunted response to apomorphine, when compared to alcoholics not having a first-degree alcohol-dependent relative. Considering the aforementioned studies showing that the number of DA-releasing neurons, within the DA cell group, are maintained across all DA cell groups in the brain (Depue et al., 1994) and the fact that receptor densities correlated strongly especially among type 1 alcoholics between NAC and dorsal striatal structures, we could speculate that this hypofunction of tuberoinfundibular DA system may also be true concerning mesolimbic and mesostriatal DA systems of the type 1 alcoholics.

CONCLUSION

Our results support Cloninger's neurogenetic model of two different alcoholic subtypes. Type 1 alcoholics seem to have more general and severe DA D₂ receptor and DAT deficit, whereas type 2 alcoholics have statistically significant differences compared to controls only in the dorsal striatum and not in the mesolimbic structures. This might provide some explanation of why there has been some, but not sufficient success in treating alcoholics with DAergic agents (Borg, 1983; Shaw et al., 1987) and why there has been controversy in studies on the association between DRD2 genotypes and alcoholism. The majority of alcoholics (80%) represent type 1 (Cloninger et al., 1981; Cloninger 1995) and obviously in most of these studies the index subjects were predominantly type 1 alcoholics. However, in at least one of the genetic studies not finding the link, a major portion of the study subjects were violent, suggesting type 2 alcoholism (Gejman et al., 1994). Because it seems that these two subgroups show different DA binding patterns, the heterogeneity of study subjects might be one reason for the previous controversial findings concerning the association between alcoholism and DAergic abnormalities or treatment outcomes.

Furthermore, although also other neurotransmitter systems play an active role in alcoholism, only some neuronal receptors and transporters have been extensively studied using brain imaging techniques. Of great importance for our understanding of psychiatric and neurological diseases is to characterize the distribution of receptors and transporters in normal and affected brains as well as to characterize the detailed regional pharmacology. Due to its high resolution, WHA provides a useful tool to explore brain regions that are not easily defined in vivo, thus being able to provide detailed maps of neuronal receptor distributions. Because direct comparisons of in vitro autoradiograms and in vivo PET results can be performed by using positron emitting ¹¹C- and ¹⁸F-labeled compounds (Hallidin et al., 1993), WHA can also be used in the early phase of radioligand development for PET.

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