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The Influence of Alcoholism and Cirrhosis on Benzodiazepine Receptor Function

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FLUCK, E., C. FERNANDES, S. E. FILE, H. V. CURRAN AND J. MARSHALL. *The influence of alcoholism and cirrhosis on benzodiazepine receptor function.* PHARMACOL BIOCHEM BEHAV **59**(4) 949–954, 1998.—In a previous study we reported that the affinity of the platelet benzodiazepine receptor was greater in alcoholic cirrhotic patients compared with normal controls and that there were detectable ligands for the neuronal benzodiazepine receptor in plasma from both alcoholic and nonalcoholic cirrhotic patients. The aim of the present study was to assess the separate contributions of alcoholism and cirrhosis to the presence of ligands in plasma for the neuronal and peripheral benzodiazepine receptors and to changes in peripheral benzodiazepine receptor binding in platelets. These parameters were measured in 10 alcoholic cirrhotics, 9 nonalcoholic cirrhotics, 7 alcoholics with a normal liver function, and 15 nonalcoholic subjects and normal liver function. Both groups of alcoholics had been abstinent for several months and the nonalcoholic groups had abstained for 24 h before the study. The concentration of ligands for the peripheral benzodiazepine receptor were significantly higher in both cirrhotic groups compared with the other two groups, suggesting that cirrhosis was responsible for this accumulation. Furthermore, the cirrhotic patients with detectable concentrations of these ligands had significantly poorer episodic memory than those without ligands. However, the presence of ligands for the peripheral benzodiazepine receptor did not correlate with the change in receptor affinity, which was increased in the alcoholic cirrhotic group compared with all other groups. Neither cirrhosis nor alcoholism altered the peripheral benzodiazepine receptor number. The cirrhotic patients with detectable ligands for the neuronal benzodiazepine receptor showed psychomotor slowing and executive dysfunction. The results suggest that the ligands for the peripheral benzodiazepine receptor may contribute to some of the cognitive deficits seen in hepatic encephalopathy, but are not responsible for the receptor affinity change seen in the alcoholic cirrhotics. This affinity change is not solely due to the effects of alcohol and could possibly serve as a marker for those at risk for developing alcoholic cirrhosis. © 1998 Elsevier Science Inc.

Cirrhosis Alcohol Peripheral benzodiazepine receptor Endogenous ligands Cognition

PHARMACOLOGICALLY active concentrations of benzodiazepine receptor ligands have been found in serum and CSF from patients with hepatic encephalopathy (31). Evidence that these ligands play a role in the cognitive impairments of these patients comes from the effects of the benzodiazepine receptor antagonist, flumazenil. There are clinical reports that flumazenil improves neurological signs (2,15) in patients with hepatic encephalopathy (HE), and in a double-blind placebo controlled study, flumazenil reversed the episodic memory impairments of alcoholic cirrhotic patients (18). In a recent study on alcoholic and nonalcoholic cirrhotics we found greater cognitive impairments in the patients with detectable plasma concentrations of endogenous ligands for the neuronal benzodiazepine receptor. We also measured benzodiazepine receptor binding in platelets and found that the alcoholic cirrhotics had a higher receptor affinity than the controls and that the change in receptor affinity correlated with the extent of psychomotor slowing (19). The peripheral benzodiazepine receptors (PBR) on platelets are the same as those found in the brain on glial cells, but these so-called PBR are different in structure from the neuronal receptors and have different pharmacological characteristics (23). It is therefore possible that the change in PBR affinity was the result of an accumulation of ligands for this receptor, and that in HE, ligands for

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both the neuronal and PBR accumulate. The purpose of the present experiment was to measure the concentrations of ligands for the neuronal and PBR and PBR binding in abstinent alcoholic and nonalcoholic cirrhotics, in abstinent alcoholics without cirrhosis, and in normal controls. So far the evidence suggests a particularly strong link between cognitive impairment and a changed benzodiazepine receptor function in alcoholic cirrhotics, but the separate contributions of alcoholism and cirrhosis have not really been assessed. Because our measures were taken during a period of abstinence from alcohol, we were assessing long-term effects of chronic drinking and not the immediate effects of the presence of alcohol.

Several effects of alcohol have been attributed to an action at the $GABA_A$–benzodiazepine receptor complex (30,35), and numerous studies have reported changes in this receptor following chronic ethanol treatment in both animals and humans (13,14,22,29). The presence of endogenous ligands for the neuronal benzodiazepine receptor in patients with HE has been well documented (31,34), and it is thought that these ligands accumulate because metabolism is impaired due to liver damage. So far, there have been no reports of the presence of such ligands in alcoholics with normal liver function.

Although it is not clear whether alcohol interacts directly with PBR, in vitro studies have shown it alters PBR affinity in astrocytes (17) and furthermore, changes in PBR density have been reported following chronic ethanol exposure (5,20,40–42). Diazepam binding inhibitor (DBI) is an endogenous ligand for the PBR (9), and chronic alcohol consumption increases DBI mRNA in the cortex of mice (21). Also, alcohol-preferring rats have higher levels of DBI, which elevate even further after ethanol consumption (1). However, no difference in DBI levels were found in human alcoholics (38). Ligands for the PBR have also been reported to be elevated in both patients with liver cirrhosis and HE (25), and although it is not entirely clear what the functional role of these ligands are, administration of chlorodiazepam, the prototype PBR agonist to animals with experimental liver cirrhosis, results in astrocyte swelling (33), which indicates that such ligands may be promote pathogenesis of the disease.

METHOD

Subjects

All subjects gave written informed consent and the study had approval from the local ethics committees.

Cirrhotic patients. Nineteen inpatients (10 alcoholic and 9 nonalcoholic) from the Liver Unit, King's College Hospital, with grade-I hepatic encephalopathy participated in the study. The patients had not used benzodiazepines within 1 month of the study. The nonalcoholic cirrhotic group had primary biliary cirrhosis ($n = 2$), primary sclerosing cholangitis ($n = 5$), hepatitis B ($n = 1$), or hepatitis C ($n = 1$). ICD-10 criteria were used to confirm a psychiatric diagnosis of alcohol abuse or dependence in the alcoholic cirrhotic group, as well as to exclude this in the other group. The alcoholics had been drinking in excess of 80 g/day for at least 5 years, but had been abstinent for a mean of 4 months.

Noncirrhotic alcoholics. Seven alcoholic inpatients at the National Alcohol Unit, Maudsley Hospital, who had normal liver function also participated. They had been drinking in excess of 80 g/day for at least 4 years, but had been abstinent and benzodiazepine free for at least 4 months.

Normal controls. A group ($n = 15$) of noncirrhotic, nonalcoholic volunteers with normal liver function was matched to the patients for age and sex. They had not used benzodiazepines in the previous 6 months and had not consumed alcohol for 3 days before the blood sample was taken.

Blood Sampling

Venous blood samples (40–50 ml) were collected into heparinized plastic tubes and spun at $180 \times g$ for 15 min and then at $190 \times g$ for 5 min, at 23°C. Platelet-rich plasma (supernatant) was removed and spun at $1500 \times g$ for 15 min. The plasma was removed and stored at −20°C. The remaining pellet, containing the platelets, was also frozen at −20°C and the platelet binding measured within 6 weeks of storage. Ligands for the central benzodiazepine receptor were measured within 2 months of storage and ligands for the PBR were measured 10 months after storage. The effects of time of storage on the concentration of peripheral ligands was assessed in the samples from the control and noncirrhotic alcoholic group.

Benzodiazepine Binding in Platelets

The number and affinity of PBR was determined in the platelet-enriched pellet, after one freeze-thaw and three washes to remove any circulating endogenous ligands. The receptor binding parameters were determined by Scatchard analysis, using the method described by Kapczinski et al. (19), using eight concentrations of [^{3}H]-PK-11195 (0.3–20 nM) to achieve full saturation. Nonspecific binding was determined in the presence of 1 μM Ro-54864. The binding was quantified per mg of platelet protein, using the method of Lowry et al. (26). The data from the saturation studies were individually converted for Scatchard analysis and checked for one- or two-site fits (Enzfitter Elsevier-Biosoft). The data best fitted a single site and were fitted by linear regression analysis. The receptor affinity and number values were then calculated from the reciprocal of the slope and the intercept on the abscissa, respectively.

Estimation of Endogenous Ligands for the Neuronal Benzodiazepine Receptor

Endogenous ligands were extracted from the plasma samples using chloroform and determined as described by Lund (27) using [^{3}H]-Ro-154513 (2 nM), which labels 100% of the sites (i.e., both diazepam-sensitive and diazepam-insensitive sites) on the neuronal benzodiazepine receptor; 100 μM (−)bicuculline was added to block any action of GABA that remained after extraction. Nonspecific binding was determined in the presence of 100 μM flumazenil. A standard curve was constructed using known concentrations of flumazenil and the concentration of benzodiazepine receptor ligands estimated from this curve.

Estimation of Endogenous Ligands for the PBR

Endogenous ligands were extracted from plasma samples using chloroform and determined as described by Lund (27) using [^{3}H]-PK-11195 (5 nM), which labels the PBR. Nonspecific binding was determined in the presence of 10 μM Ro-54684 (chlorodiazepam). A standard curve was constructed using known concentrations of Ro-54684 and the concentration of the ligands for the benzodiazepine receptor was estimated from this curve.

Chemicals

[^{3}H]-Ro-154513 (20.8 Ci/mmol) and [^{3}H]-PK-11195 (83.5Ci/mmol) were purchased from DuPont, NEN (Steven-

age, UK). Ro-54864 was purchased from RBI (Semat, St. Albans, Herts, UK), (−)bicuculline was purchased from Sigma (Poole, Dorset, UK), and diazepam and flumazenil were a gift from Roche Products Ltd (Welwyn Garden City, UK). Chloroform was purchased from BDH (Dagenham, Essex, UK). Tris HCl and Tris Base buffers were used for all the binding assays and were obtained from Sigma (Poole, UK).

Statistics

The concentrations of endogenous ligands were compared using Mann–Whitney *U*-tests, and the changes over time were compared with Wilcoxon *T*-tests because these data were not normally distributed. The affinity ($1/K_D$) and number for the PBR binding were analysed with two-way analyses of variance with alcoholism and cirrhosis as the two factors; comparisons between individual groups were then made using Duncan's tests.

RESULTS

Endogenous Ligands for the Neuronal Benzodiazepine Receptor

It can be seen from Table 1 that the highest concentration of ligands for the neuronal receptor was in the alcoholic cirrhotic group and this was significantly higher than in the normal controls [$Z = 2.7$, $p < 0.01$] and just missed significance when compared with the alcoholics with normal liver function [$Z = 1.8$, $p = 0.06$]; neither of the groups with normal liver function had any detectable ligands. The difference between the two cirrhotic groups did not reach significance ($Z = 0.7$), and the nonalcoholic cirrhotic group also had significantly higher concentrations of ligands than the normal controls [$Z = 2.0$, $p < 0.05$].

Endogenous Ligands for the PBR

The alcoholic cirrhotic patients had higher levels of these ligands for the PBR compared with the normal control group [$Z = 3.1$, $p < 0.01$] and the alcoholics with normal liver function [$Z = 3.8$, $p < 0.01$]. At the 10-month comparison point, there were no detectable ligands in the groups with normal liver function. The nonalcoholic cirrhotic patients also had higher concentrations of ligands compared with the normal control group [$Z = 3.8$, $p < 0.01$], but they did not differ from the alcoholic cirrhotic patients [$Z = 0.1$] (see Table 1).

These analyses were performed on samples stored for 10 months. The effect of storage is shown in Table 2, and it can be seen that there was a steady decline from 2–6 months ($Z = 2.3$, $p < 0.05$) and from 6–10 months ($Z = 2.4$, $p < 0.05$). The rate of decline was the same in samples from the control group and the alcoholics with normal liver function and at no point did these two groups differ (in all cases $Z \leqslant 1.1$)

Platelet Benzodiazepine Receptor Binding

It was not possible to measure binding in five nonalcoholic and five alcoholic cirrhotic patients, and these patients also had protein levels below detection in our assay. There was a trend for the other cirrhotic patients to have a lower protein concentration compared with those with normal liver function, $F(1, 27) = 3.3$, $p = 0.08$. There were no differences in receptor number between any of the groups. There was an overall difference in affinity in the cirrhotic patients compared with subjects with normal liver function, $F(1, 27) = 4.6$, $p < 0.05$, but no overall difference between the alcoholics with normal liver function and our normal control group, $F(1, 27) = 0.7$. There was a nearly significant cirrhosis × alcoholism interaction, $F(1, 27) = 3.9$, $p = 0.08$, because cirrhosis significantly ($p < 0.05$) increased affinity for the PBR in alcoholics, but was without effect in those drinking normally (see Table 1).

Correlations With Cognitive Performance

It was not possible in this present study to measure the cognitive performance in all our groups. However, 16 of our 19 cirrhotic patients had participated in our previous study (19) and therefore it was possible to relate their performance to the concentrations of ligands for the PBR. The cirrhotic patients were therefore divided into two groups on the basis of the presence ($n = 10$) or absence ($n = 6$) of these ligands. Total recall (immediate plus delayed) in those without detectable ligands was significantly higher than those with ligands for the PBR, $F(1, 14) = 13.1$, $p < 0.005$ (see Fig. 1) I. There were no differences between the groups in their simple reaction times, in their performance in a digit-cancellation task or in the trails test, which measures executive function ($F < 1.0$ in all cases; see Table 3).

TABLE 1

MEAN (RANGE) CONCENTRATIONS OF LIGANDS FOR THE NEURONAL BENZODIAZEPINE RECEPTOR (FLUMAZENIL EQUIVALENTS, nM) AND THE PERIPHERAL BENZODIAZEPINE RECEPTOR (CHLORODIAZEPAM EQUIVALENTS, nM) AND MEAN (±SEM) K_D (1/AFFINITY) AND B_{max} (NUMBER) FOR THE PLATELET BENZODIAZEPINE RECEPTOR IN ABSTINENT ALCOHOLICS AND NONALCOHOLICS WITH CIRRHOSIS, ABSTINENT ALCOHOLICS WITH NORMAL LIVER FUNCTION, AND NORMAL CONTROLS

	Alcoholic Cirrhotics	Non-Alcoholic Cirrhotics	Alcoholic Normal Liver	Normal Controls
Neuronal ligands (2 months storage)	12.3† (0–70.6)	4.6* (0–39.1)	0	0
Peripheral ligands (10 months storage)	56§ (0–277)	16.7§ (0–80)	0	0
Platelet binding (2 months storage)				
K_D (nM)	3.4 ± 0.4‡	6.2 ± 1.5	7.4 ± 0.9	6.3 ± 0.6
B_{max} (fmol/mg)	6474 ± 314	9100 ± 1196	7216 ± 1830	7278 ± 826

*$p < 0.05$, †$p < 0.01$ compared with the normal controls.
‡$p < 0.05$, §$p < 0.01$ compared with the normal controls and the alcoholics with normal liver.

TABLE 2

MEAN (RANGE) CONCENTRATIONS (nM) OF LIGANDS FOR THE PBR IN SAMPLES FROM ALCOHOLICS WITH NORMAL LIVER FUNCTION AND CONTROLS STORED FOR 2, 6, OR 10 MONTHS

	Alcoholics, Normal Liver $n = 7$	Normal, Controls $n = 15$
2 months	148 (66–215)	107 (46–161)
6 months	54 (0–106)	41 (0–191)
10 months	0	0

We have previously reported (19) that when the cirrhotic patients were divided into groups with and without detectable concentrations of ligands for the neuronal receptor, the groups differed significantly ($p < 0.05$) in the digit-cancellation task, and there was a trend ($p < 0.07$) for greater impairment in the trails task in the group with ligands. There were no differences between the groups in their reaction times or recall of the words (see Table 2).

The change in affinity of the benzodiazepine receptor was found to correlate significantly for both reaction time and digit cancellation (Spearman's $\rho = 0.51$ in both cases, $p < 0.05$); however, there was no correlation between the concentrations of ligands for the peripheral receptor and the change in receptor affinity ($\rho = 0.06$).

DISCUSSION

We previously reported elevated levels of ligands for the neuronal benzodiazepine receptor in cirrhotic patients and that the presence of these ligands was linked to a psychomotor deficit and executive dysfunction (19). Although we cannot confirm the nature of these ligands, it is possible that they are agonists, as diazepam-like compounds in the plasma of cirrhotic patients with HE have been confirmed from HPLC analysis (34). Furthermore, the cognitive deficits as a result of HE were diazepam-like and have been reversed with the benzodiazepine receptor antagonist, flumazenil (18).

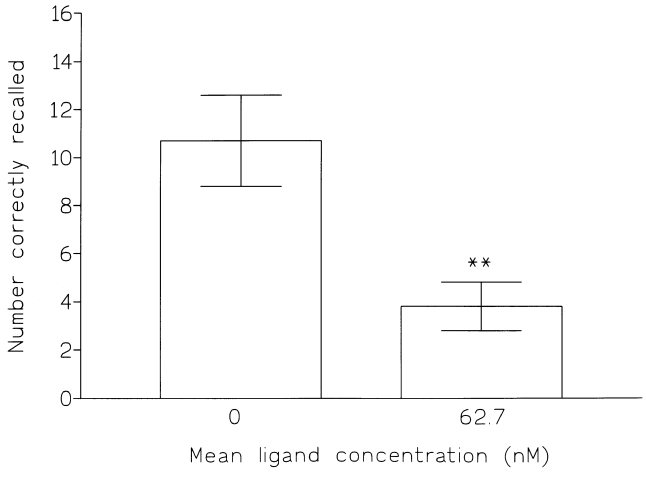

FIG. 1. Mean (±SEM) total (immediate plus delayed) number of words correctly recalled by cirrhotic patients with and without detectable concentrations of peripheral benzodiazepine receptor ligands.

TABLE 3

SIGNIFICANCE OF DIFFERENCES IN COGNITIVE PERFORMANCE OF CIRRHOTIC PATIENTS ACCORDING TO THE PRESENCE OR ABSENCE OF LIGANDS FOR THE NEURONAL AND PERIPHERAL BENZODIAZEPINE RECEPTOR AND SIGNIFICANT CORRELATIONS WITH PERIPHERAL BENZODIAZEPINE RECEPTOR AFFINITY

	Neuronal Ligands	Peripheral Ligands	Affinity
Word recall	NS	$p < 0.005$	NS
Digit cancellation	$p < 0.05$	NS	$p < 0.05$
Trails	$p < 0.07$	NS	NS
Reaction time	NS	NS	$p < 0.05$

NS = not significant.

After 2 months of storage we could not detect any measurable concentrations of neuronal benzodiazepine ligands. In samples from control subjects and from alcoholics with normal liver function there were high nanomolar concentrations of endogenous ligands for the PBR; however, these ligands are eventually unmeasurable following lengthy freezer storage. Endogenous ligands for the PBR have been reported in the plasma and urine of normal subjects (4), raising the possibility that these ligands play a significant role in normal subjects. Thus, although the levels of these ligands that we report in Table 1 are probably much lower than the levels that would have been present when the blood was taken, they are likely to reflect the relative levels between the groups, as we found that the rate at which the ligands for the PBR decrease is the same in both the groups. Although we report that ligands for the PBR occur in people with normal liver function, there is a possibility that in high concentrations some may be hepatotoxic. High doses of two agonists for the PBR, PK 8165, and PK 9084 caused liver damage in rats (12) and alpidem, which has a high affinity for the PBR (6), has been withdrawn from the market because of hepatotoxicity (3).

The present study shows that ligands for the PBR measured after 10 months storage are elevated only in the cirrhotic patients and are linked to the increased amnesia seen in these patients. No work investigating the direct effect of PBR ligands on memory in humans has been carried out, but they may indirectly alter cognition in patients with HE by attacking astrocyte function in two ways. Firstly, ligands for the PBR may be indirectly involved in the decreased glutamatergic neurotransmission that occurs as a result of HE. There is an increase in the number of PBRs and their ligand octadecaneuropeptide (ODN) in astrocytes of rats with experimental HE (7) and in the CSF of patients with HE (37), and it has been suggested that ODN is responsible for impaired astrocyte–neuronal metabolic trafficking, leading to decreased glutamate neurotransmission (8). Indeed, there is considerable evidence for reduced glutamate function having an amnesic effect [for examples, see (32,36)]. Secondly, ligands for the PBR stimulate steroid synthesis in the mitochondria of astrocytes, and therefore it may be that the PBR are indirectly modulating memory through neurosteroid modulation of neurotransmission. With the exception of porphyrins, all the ligands for the PBR (e.g., diazepam, zolpidem, and the peptides diazepam binding inhibitor (DBI), trikontatetraneuropeptide (TTN), and ODN) cross the blood–brain barrier and promote steroidogenesis through an action at PBRs (24).

The $GABA_A$–benzodiazepine receptor complex has specific steroid binding sites, which allows both positive and neg-

ative modulation of GABA transmission (28) and it is widely documented that increasing GABA transmission increases sedation and causes amnesia, although it still remains controversial as to whether the amnesia is secondary to the sedation or a separate process. The patients with measurable concentrations of ligands for the peripheral benzodiazepine receptor had impaired memory, without differences in the speed of performing. However, the possibility that the PBR plays a role in some of the sedative effects detected in the cirrhotic patients was indicated by the correlation between receptor affinity and motor and psychomotor slowing. In summary, the global cognitive impairment detected in our HE patients, comprising both psychomotor and memory deficits, could be accounted by the combined effects of neuronal ligands and steroids acting at the $GABA_A$–benzodiazepine receptor complex. Thus, the increased concentration of PBR ligands might indirectly, through steroidogenesis, influence the neuronal receptor.

We found that alcoholics with normal liver function did not differ in PBR binding compared with matched controls, and the only difference in binding was found in the alcoholic cirrhotic patients who had a higher affinity compared with all the other groups. Therefore, the alteration in affinity was not primarily due to the effect of alcohol. The change in affinity was not due the presence of endogenous ligands for the PBR as, firstly, we found there to be no correlation between affinity and the presence of these ligands and, secondly, the presence of these ligands was significantly higher in both groups of cirrhotic patients. An alteration in affinity due to an environmental factor, such as drugs or disease, or following exposure to a receptor agonist is uncommon, and it is more usual to find a decrease in receptor number. Indeed, a decrease in receptor number in lymphocytes of patients with HE has been reported (11); thus, it is possible that the changes in HE will vary in different tissues and/or cell types. It is not known why cirrhosis develops in only 10–15% of alcoholics, although alcohol consumption greater than 125 g/day, nutrition, and viral infections from hepatitis B and C appear to be factors (39). There is also some evidence for a genetic predisposition to developing alcoholic cirrhosis, as patients with alcoholic cirrhosis have an increased frequency of the ADH3*1 gene that encodes the γgr;1 ADH (aldehyde dehydrogenase) enzyme, thus causing an increase in aldehydes, which is thought to mediate some of the hepatotoxicity (10). Alcoholic cirrhotics also have increased incidence of polymorphisms of the promoter of the P4502E1 gene (16), which may alter the rate of metabolism of alcohol. This raises the intriguing possibility that the affinity difference in the patients with cirrhosis due to alcoholism did not change as a result of the alcohol and cirrhosis but is indicative of susceptibility to alcoholic cirrhosis. Interestingly, one of our seven noncirrhotic alcoholic subjects had an affinity for the PBR within the range of our alcoholic cirrhotic patients. This would be within the expected proportion of 10–15% of alcoholics who go on to develop cirrhosis. Because endogenous ligands for the PBR are present in normal controls, it is possible that a more sensitive PBR in the liver could play some role in promoting the development of cirrhosis. One possible mechanism would be an enhancement of the astrocyte swelling due to ammonia accumulation in liver failure, which is mediated by agonists at the PBR (33). A second possibility is an enhancement of the porphyrin-mediated decrease in metabolism, which would result in greater hepatotoxicity.

In conclusion, the alcoholic cirrhotics had a higher affinity for the PBR than the other groups, and we suggest that this may be one of many risk factors for developing alcoholic liver damage. However, as there has been little work published specifically investigating the role of PBR in patients with alcoholic cirrhosis and our group numbers are small, a longitudinal study in alcoholics is needed. Only then will it be possible to determine whether a changed affinity for the PBR is a useful marker for those at risk for developing alcoholic cirrhosis.

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