A. STASIAK, W. A. FOGEL

HIGH VOLUNTARY ALCOHOL CONSUMPTION, IN EXPERIMENTAL LIVER CIRRHOSIS, IS HARDLY RESPONSIVE TO OPIOID ANTAGONIST TREATMENT

Department of Hormone Biochemistry, Medical University of Lodz, Lodz, Poland

**Background:** Rats with liver cirrhosis, evoked by chronic administration of thioacetamide (TAA), consumed voluntarily more alcohol than their healthy counterparts. Seeking the mechanisms underlying this phenomenon, the opioid system was screened for involvement and alterations. **In vivo**, the influence of chronically administered Naloxone and Naltrexone, non-specific opioid receptor antagonists, on alcohol intake was examined in free choice tests between 10% alcohol and tap water and **ex vivo** receptor binding studies were performed on cerebral membrane preparations. **Methods:** TAA rats, selected for the study, had confirmed liver insufficiency: their plasma bilirubin concentrations were about 3 times higher, the prothrombin time was 50% longer and they consumed voluntarily 3 times more alcohol than the control animals. The drugs were given s.c. for five days, at the beginning of the dark phase of a 24h cycle, in a daily dose of 10 mg per kg body mass. Throughout the treatment, the rats were kept individually in metabolic cages with a free access to water, alcohol solution and food. Feed and fluid consumption, as well as the urine outputs, were recorded on the 2h, 4h, 6h and 24h after the drug administration. The mu opioid ligand – [3H]- (D-Ala², -N-MePhe⁴, Glyol⁵) Enkephalin was used to obtain binding characteristics of the control and TAA rat brain membranes. **Results:** The drugs, if modified drinking behaviours, they did it transiently; alcohol, water and thus the total fluid intake by the cirrhotic and control rats was significantly less after 2h – 6h from either naloxone or naltrexone administration. Both drugs decreased general fluid consumption as such rather than the consumption of alcohol only, as observed from the recordings related to TAA rats. The binding data: $K_d$ of 2.62 ± 0.98 nM and $B_{max}$ of 43.71 ± 6.12 fmol/mg protein for cirrhotic rats, versus $K_d$ of 4.63 ± 1.98 nM and $B_{max}$ 95.61 ± 18.33 fmol/mg protein for the control ones, suggest that while the affinity of radioligand to cerebral mu receptors was similar for the two groups, there was a lower density of those receptors in the cirrhotic rats. **Conclusions:** The results indicate some disturbances in the opioid system in cirrhotic rats. However, the low response to opioid therapy suggests that the opioid system may have only be partly involved in the development of the observed increased alcohol drinking in the rats with liver cirrhosis.
INTRODUCTION

It is well known that ethyl alcohol is one of the causative factors of liver disease (1 - 3); about 10 to 15 percent of alcoholics develop cirrhosis (1). On the other hand, liver insufficiency itself leads to an increased alcohol preference in rats (4 - 11). This latter phenomenon was shown on two different models of experimental liver damage, the organ atrophy and liver cirrhosis (5, 7). Based on that, it was suggested that the participation of pathophysiological factors, associated with liver diseases in alcoholism, should also be considered.

Up to now, the pathophysiology of abnormal alcohol consumption by rats with liver dysfunction has remained, however, unexplained. The similarities between ethyl alcohol and opioid effects have been demonstrated in numerous animal studies. Alcohol consumption is thought to enhance the release of endogenous opioids, acting at opioid receptors in the central nervous system (12, 13). In rats, exhibiting high alcohol consumption (14, 15), and also in human alcoholics (16), some disturbances in the density of $\mu$ and $\delta$ opioid receptors have been found. An evidence has been provided that alcohol preference, evoked by various experimental procedures (9, 17 - 23, 24 - for review), could be attenuated by the administration of nonspecific (naloxone, naltrexone) or specific (beta-funaltrexamine, naloxonazine, CTOP, nalmefene, naltrindole) opioid receptor antagonists. Opioid antagonists also suppressed alcohol consumption in rodents with genetically determined alcohol preference (C57/BL6 mice; P, AA and Fawn-Hooded rats) (22, 25 - 27). Naltrexone treatment reduced alcohol consumption in human alcoholics (12, 24, 28 - 31); the compound has been approved by the U.S. Food and Drug Administration for the treatment of alcohol dependence. Interestingly enough, various liver diseases have been shown to be associated with opioid system alterations (8, 32 - 35).

The subjects of the reported study included rats, exhibiting sustained exaggerated alcohol preference, evoked by liver cirrhosis. A possible contribution of the opioid system alterations was examined. The non-specific opioid receptor antagonists (naloxone, naltrexone) therapy and receptor binding research studies were performed.

MATERIALS AND METHODS

Animals

Male Wistar rats (weighing 180-250 g at the beginning of the experiments) were selected for the study, showing with preference for alcohol, as determined by an initial choice test. The experimental procedures were undertaken, according to EU directives and local ethical regulations. The rats were housed in standard cages with liquid and food available ad libitum, under artificial 12-h light-dark cycle.
Induction of cirrhosis

The liver cirrhosis was induced by chronic exposure to 0.03% thioacetamide (TAA), given instead of tap water (36). Additionally, TAA was administered three times a week by gastric tube in a gradually increased dose from 2.5–6 mg/kg body weight (5, 7).

Cirrhosis, induced by TAA, was claimed to be morphologically well defined and uniform, reflecting major features of the human disease (36 - 38). It corresponded first to micronodular and later to macronodular cirrhosis (39) and was associated with progressive liver fibrosis (40, 41).

Voluntary alcohol intake was evaluated in a series of choice tests. Free choice tests were performed in metabolic cages (Gazzada, Italy), equipped with two bottles: one containing a 10% ethyl alcohol solution and the other tap water. The position of the bottles was exchanged daily. The volumes of consumed fluids were recorded every day on the last hour of the light phase of the 24-h cycle and expressed in ml of the consumed fluid per kg of body weight. Typically, the test lasted 3 days (72 h) and its results were expressed as means with SEM calculated for 24h. The rats, subjected to the 5-day treatment with opioid antagonists, were kept in metabolic cages during the entire course of therapy and records were obtained every two hours during 6 and 24-h intervals after the drug administration. The final results are given as means with SEM, computed from 5-day recordings.

Evaluation of liver function

The liver function was systematically monitored by measurement of plasma bilirubin concentrations and of the prothrombin time (standard procedures, commercially available kits).

Assessment of liver fibrosis

The post mortem liver index was calculated and hepatic concentration of hydroxyproline was measured. In cirrhotic rats, the liver index (liver weight/body weight x 100) increased due to liver weight gain (5, 7, 36). Hydroxyproline is an iminoacid, present virtually only in collagen, and its concentration reflects collagen infiltration of liver parenchyma (the degree of liver fibrosis) (5, 7, 41). Hepatic hydroxyproline concentration was measured spectrophotometrically, using Woessner’s method (42).

Opioid Antagonists Treatment

The treatments were performed on rats, rendered cirrhotic (18 weeks on TAA), and expressing high voluntary preference for alcohol. As reference, control rats were used, matched for age and not treated with hepatotoxin. Naloxone and naltrexone (naloxone hydrochloride, naltrexone hydrochloride; RBI, Natick, MA, USA) were dissolved in 0.9% NaCl solution just before use. The drugs were given subcutaneously, 10 mg/kg/day for five consecutive days, at the beginning of the dark phase of 24-h cycle. Throughout the therapy, the rats were kept individually in metabolic cages with a free access to water, alcohol solution and food. The fluid and food consumption, as well as urine outputs, were recorded after 2, 4, 6 and 24 h from the drug administration.

Receptor binding assay

The binding characteristics of the mu opioid ligand – [3H]-(D-Ala², -N-MePhe⁴, Gly⁵)Enkephalin ([3H]DAGO, Amersham Pharmacia Biotech, Buckinghamshire, England) were studied with rat cerebral membranes, prepared from brain of the TAA and the control rats. The saturation binding experiments were performed, using a modification of the previously reported techniques.
Membrane preparation

The brain (without the cerebellum) was homogenised in 30 volumes of 50 mM Tris HCl buffer, pH 7.4 at 4°C. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 50,000 x g. The resulting pellet was resuspended in Tris HCl buffer and incubated at 37°C for 40 min to get free from the endogenous ligands. The suspension was then recentrifuged under the same conditions (43, 44). The final brain membrane pellet was frozen and stored at -70°C until use.

Binding experiments

The pellet was resuspended in the initial volume of ice-cold Tris HCl buffer. The saturation binding assay mixture consisted of 1.86 to 11 nM concentrations of 

\[ ^{3}H \]DAGO, 400 µl of the brain membrane suspension (ca 1 mg of protein per tube) and buffer to a final volume of 500 µl. Nonspecific 

\[ ^{3}H \]DAGO binding was determined in presence of 1 µM naloxone (44). The incubation was at 25°C for 60 min in duplicate and in triplicate to determine the non-specific and total binding of radioligand, respectively. Following incubation, the samples were filtered through Whatman glass filters (GF/B) and washed with two 5 ml volumes of ice cold Tris HCl buffer. To decrease the nonspecific radioligand binding, the filters were soaked before use in 0.05% polyethyleneimine solution (45). The radioactivity was counted in 4.5 ml scintillation fluid prepared, according to Simantov et al. (46). Protein contents in the samples were estimated by Lowry’s method (47).

The equilibrium binding constants (\(K_d\), \(B_{\text{max}}\)) for 

\[ ^{3}H \]DAGO binding to mu receptors were determined with the GraphPad Prism program, using non-linear and linear regression techniques. The presented data were obtained in three separate experiments. In each experiment, the membranes used for binding characteristics, were prepared from three brains of TAA and control rats, respectively.

Statistical Analysis

Results are expressed as means ± SEM. Statistical significance was assessed with the one-way analysis of variance, followed by the Student-Newman-Keuls test. The efficacy of pharmacological therapy was determined by the Student Paired-t test. \(P\) value of 0.05 or less was considered significant.

RESULTS

TAA treatment and voluntary alcohol consumption

Chronic treatment with TAA leads to development of progressive liver insufficiency, see our earlier studies (5, 7). The TAA rats expressed significantly higher plasma bilirubin concentration (3 folds) and longer prothrombin time (by 50%), as compared with healthy animals (Table 1). The severity of organ damage is further illustrated in Table 1 by the high hepatic hydroxyproline concentration and the liver-to-body weight ratio.

Free Choice Tests revealed that the cirrhotic rats drank voluntarily more alcohol than the control ones did (Table 2, 3; Fig. 1A, 2A: Before therapy). They also showed a significantly higher total fluid intake (Table 2, 3; Fig. 1C, 2C). The contribution of ethyl alcohol to total fluid intake was within the range 72-77% for TAA while only 10-52% for the control rats.
The opioid antagonists therapy

Neither naloxone nor naltrexone altered 24 h alcohol intake by TAA rats (Fig. 1A, 2A). However, more detailed recordings showed that alcohol, water and thus
the total fluid intake by TAA and control rats was significantly smaller after 2 - 6 hours from naloxone or naltrexone administration. The suppression was the highest at the 2nd hr, being evidently more distinctive for naloxone. The extent of intake suppression was similar for alcohol and water and, for example, at the 2 h suppression it achieved almost 90%, being, 90.39 ± 4.61% and 76 ± 13.44 %,
respectively. Six hours after naloxone the corresponding values were 41.45 ± 5.64% and 59.72 ± 7.59%, respectively. In TAA rats, the suppression of fluid consumption after naloxone lasted for some hours (more than 6), whereas in the control rats, significantly decreased water intake and, thereby, the total fluid intake was present during the whole 24 h period (Paired t-test, p = 0.035 and p = 0.02 for water and total fluid intake during 24 h). During the naltrexone treatment TAA rats drank, as before, significantly more alcohol and total fluid compared with the control group (p<0.05, Keuls-Newman test). ETOH – 10% ethanol

Fig. 2. Consumption of fluids by cirrhotic (TAA) and control rats in free choice tests; the effect of in vivo opioid receptor antagonist. The left chart columns (Before therapy) are means with SEM of data from 3 days prior to naltrexone administration. The right chart columns (Naltrexone) are means with SEM of records taken during 5 days treatment. Naltrexone was given s.c. at the beginning of dark phase of 24 h cycle. Fluid consumption was monitored at indicated times points following the drug administration. Alcohol, water and total fluid intake by TAA and control rats were significantly smaller after 2-6 h from naloxone administration with p<0.05 at least. Paired t-test (Naltrexone vs. Before therapy), p = 0.02 for water and total fluid intake during 24 h.

Alcohol, water and total fluid intake by TAA and control rats were significantly smaller after 2-6 h from naloxone administration with p<0.05 at least. Paired t-test (Naltrexone vs. Before therapy), p = 0.02 for water and total fluid intake during 24 h. During the naltrexone treatment TAA rats drank, as before, significantly more alcohol and total fluid compared with the control group (p<0.05, Keuls-Newman test). ETOH – 10% ethanol
0.017, respectively) (Fig. 1B and 1C; Table 2). A similar effect was observed in healthy rats, treated with naltrexone (p = 0.02). Naltrexone also reduced 24 hr water intake (no statistical significance was attained) and, thereby, the total fluid consumption by TAA rats (p = 0.02). (Fig. 2B and 2C; Table 3). In none of the groups, were daily food consumption and urine outputs changed during the treatment (Table 2, 3).

**Receptor binding study**

Fig. 3 presents the saturation curves and Scatchard analyses, obtained for \[^{3}\text{H}]\text{DAGO,}\ using whole brain membrane preparations from TAA and control rats. For both groups, a single saturable binding site was observed. The $K_d$ of 2.62 ± 0.98 nM and $B_{\text{max}}$ of 43.71 ± 6.12 fmol/mg protein for TAA rats and $K_d$ of 4.63

![Receptor binding study graph](image-url)

*Fig. 3. Non-linear regression curves (A) and the corresponding Rosenthal-Scatchard plots (B) of \[^{3}\text{H}]\text{DAGO binding to brain membranes of TAA (closed symbols) and control rats (open symbols). Data are the means and SEM of three separate experiments. Nonspecific \[^{3}\text{H}]\text{DAGO binding was determined in a presence of 1 µM naloxone.})*
± 1.98 nM and $B_{\text{max}}$ 95.61 ± 18.33 fmol/mg protein for the control rats indicate that there was lower density of $\mu$ receptors (lower $B_{\text{max}}$ value) in the brain of cirrhotic (TAA) rats, while the affinity of radioligand to these receptors was similar to that, obtained for the control rats (similar $K_d$ value).

**DISCUSSION**

The results of the present study confirmed our previous observations on high alcohol preference, exhibited by cirrhotic rats (5, 7). Indeed, evoked by TAA liver damage, as evidenced by the measurement of relevant liver function parameters and by *post mortem* macroscopic and biochemical examinations (*Table 1*), was associated with a strongly pronounced exaggerated voluntary alcohol consumption (*Fig. 1, 2*). In an experimental model of liver atrophy, resulting from portocaval anastomosis (PCA), similarly, shunted rats drank much more alcohol than their control counterparts (4 - 11). In earlier studies, we have demonstrated that the improvement (7) or restoration of hepatic function (5) has a positive effect on such aberrant drinking behaviour. Its nature seems complicated and remains to be clarified. First, it is not certain whether the same pathomechanisms underlie various experimental models of increased alcohol preference. Alteration of brain serotoninergic activity (deficit) has been indicated from studies on P (alcohol-preferring) rats (48 - 50). However, up to now, the brain serotonergic activity alteration has been ruled out for alcoholic rats with liver insufficiency (5). Both the cirrhotic and PCA rats demonstrated high voluntary alcohol preference and no signs of serotonergic deficit, *i.e.* while TAA rats exhibited a similar brain level of 5-HT and 5-HIAA, the shunted rats had the same 5HT and more than doubled cerebral 5-HIAA level, as compared with the control rats (5).

Vast literature concerns the relationship between alterations in the endogenous opioid system and increased alcohol preference (8, 12 - 16, 51) and also the efficacy of opioid antagonist therapy in various experimental conditions and clinical trials (12, 17 - 31). Alterations in the opioid system, caused by liver damage, have been evidenced in clinical investigations. Significant elevation in plasma levels of $\beta$-endorphin and enkephalins has been observed in patients, suffering from acute and chronic liver diseases (33 - 35). Accordingly, it has been suggested that alterations in the brain levels of opioid peptides might be involved in the pathomechanism of hepatic encephalopathy, associated with chronic liver disease (32, 52). The blockade of opioid receptors with naloxone normalised some of the abnormalities, characteristic for this disturbance (32, 53) and also improved liver function parameters (54). The lower density of $\mu$ opioid receptors in the whole brain as well as an increased density of $\delta$ opioid receptors in some regions of the brain, along with changes in the brain $\beta$-endorphin system, were observed in PCA rats, the animal model of hepatic encephalopathy in humans (8). It was reported that naloxone, administered to PCA rats, could bring down their abnormal alcohol consumption (9).
In the present work, in support of (8), the binding studies revealed a lower density of mu opioid receptors also in brain of TAA rats (Fig. 3). However, in contrast to (9), the therapy with the nonspecific opioid antagonists was not so successful. In our preliminary experiments, when we monitored consumed water and alcohol on daily basis, we obtained even more discouraging results, e.g., no modifying effect on drinking (data not shown). This apparent discrepancy of our and others’ data (8, 9) prompted us to perform more detailed observations with records, taken at few time points, following the drug administration. The overall 24 h consumption of alcohol was hardly affected by opioid antagonists, however, alcohol, water and thus the total level of fluid, consumed by TAA and the control rats, was significantly lower after 2 - 6 hrs from the blockers, naloxone seemingly producing bigger effects (Fig. 1, 2). At a given time interval, the extent of intake suppression was similar for the two fluids. This observation supports the hypothesis of nonselective drinking suppression, caused by naloxone (19), extending it on the other drugs of this type (naltrexone). On the other hand, it cannot be excluded that nonspecific opioid antagonists might be effective in the attenuation of alcohol consumption in rats with moderate alcohol preference. It should be noticed that PCA rats, used by Canadian authors (8, 9), drank voluntarily roughly half of the alcohol dose, ingested daily by the TAA rats, employed in this study. The investigations, performed on PCA rats, also expressing much higher alcohol preference, in which naltrexone (10 mg/kg b.w., 5 days) reduced the total fluid consumption, but had no effect on alcohol intake (6), further support this suggestion. Here, the amount of alcohol, consumed by the control rats over the 24-h period, was quite low (Table 2, 3; Fig. 2, 3). All those rats drank less than 1 g/kg b.w. per day. With such a small consumption level, it is difficult to evaluate suppressive effects of the drugs without more sophisticated instrumentation.

The modulatory effect of nonselective opioid antagonists on increased alcohol consumption is not that clear. In alcoholic patients, naltrexone was usually administered together with psychotherapy (30, 31). It is impossible to evaluate the relative contribution of any of the two components in the final effect of therapy. Moreover, the neurobiological mechanism, underlying the attenuation of ethanol intake by opioid antagonists, has still to be clarified. Some findings support their interactions with the brain dopaminergic reward system (55). It has also been suggested that ethanol acts at the level of opioid biosynthesis and increases the expression of pro-enkephalin mRNA in the rat mesocorticolimbic system (nucleus accumbens); this phenomenon may play the key role in ethanol reinforcement (51). Naltrexone has also been shown to modify the palatability of alcohol and basic tastes (56). The ability of naltrexone to increase the hypothalamo-pituitary-adrenocortical axis activity was also reported to be associated with its effects on reduced craving for alcohol (57).

At last, in the literature on related subjects, we can find conflicting results which do not support the efficacy of opioid receptor antagonists in lowering alcohol intake (58), while some data show even higher alcohol intake after
naltrexone treatment (59). The researchers found no differences between naltrexone and placebo group (58). The heterogenous response to naltrexone in clinical trials may be associated with polymorphism in the mu-opiate gene, OPRM1 (60, 61).

In the experiments, presented in this paper, naloxone and naltrexone produced unspecific and differential effects on alcohol consumption in cirrhotic rats. Although in the brains of TAA rats, lower density of mu opioid receptors was measured (Fig. 3), the low efficacy of opioid antagonist treatment indicates that alterations in the opioid system may only in part be involved in the excessive drinking behaviour of cirrhotic (TAA) rats. Thus, there must be another pathomechanism responsible. The damaged liver may, for example, facilitate the formation of different compounds and/or increase the half-life of metabolites, which could act at the brain level, promoting increased alcohol preference (52). Possible candidates are the amine-aldehyde adducts; the occurrence of these compounds has already been earlier associated with excessive alcohol intake (62, 63). In rats with liver insufficiency, higher synthesis of these compounds may be facilitated by disturbances in the metabolism of biogenic amines and aldehydes.

In conclusion, further investigations are needed to explain the biological mechanism(s), implicated in the development of excessive ethanol consumption by cirrhotic rats.

Acknowledgement: Supported by KBN 4P05A.141.14, UM 502-17-689 and 503-7085-1.

REFERENCES


42. Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961; 93: 440-447.


Received: October 9, 2007
Accepted: December 3, 2007

Author’s address: A. Stasiak, Ph. D., Department of Hormone Biochemistry, Medical University of Lodz, Żeligowskiego 7/9 Str., 90-752 Lodz, Poland. Tel.: +48 (42) 639 31 26, 639 31 25, Fax: +48 (42) 639 31 25; e-mail: an.stasiak@wp.pl