Numerous studies have provided evidence for the role of serotonin (5-hydroxytryptamine, 5-HT) in alcohol dependence, as this neurotransmitter may be actively involved in anxiety, depressive mood, impulsive, aggressive and suicidal behaviours, brain reward systems, and consummatory binges, which are often included in the co-morbid clinical picture of alcoholism (Roy et al., 1987; Meltzer, 1990; Engel et al., 1992; Koob et al., 1994). Furthermore, the effect of the specific serotonin reuptake inhibitors (SSRIs) in alcohol-dependent patients has brought strong arguments favouring the role of these neurotransmitters in alcoholism, while opening new therapeutic perspectives (Arranz and San, 1997).

Accordingly, recent research in the field of alcohol dependence has focused on the study of biological markers linked to the serotonergic system. So far, reduced platelet (Rolf et al., 1978; Bailly et al., 1990, 1993) and plasma (Banki, 1978; Simonsson et al., 1992) 5-HT concentrations, higher 5-HT affinity (lower $K_m$) for its platelet transporter, together with increased 5-HT platelet uptake ($V_{max}$) (Baccino et al., 1987; Boismare et al., 1987; Neiman et al., 1987, 1988; Lhuintre et al., 1993; Daoust et al., 1991; Ernouf et al., 1993) have been noted in alcohol-dependent patients. Furthermore, reduced 5-hydroxyindol-3-ylacetic acid (5-HIAA) concentrations have also been observed in plasma, urine (Helander et al., 1993), and cerebrospinal fluid (Ballenger et al., 1979; Banki, 1981; Banki et al., 1984; Virkkunen et al., 1994) from different subgroups of alcoholic patients, i.e. those with suicide ideation, depressive symptoms, and impulsive behaviour, while 5-HIAA concentrations may be increased during alcohol withdrawal (Ballenger et al., 1979).

The 5-HT uptake mechanism or 5-HT transporter is an important part of the 5-HT system which can be measured by the number of binding sites for antidepressant drugs located on the 5-HT transporter in the membranes of serotonergic neurones or platelets. So far, studies using tritiated imipramine have shown reduced (Suranyi-Cadotte et al., 1989), unchanged (Gentsch et al., 1985), or increased (Patkar et al., 1995) platelet binding sites ($B_{max}$) in alcoholic patients. Other reports using [3H]paroxetine have noted increased (Mellerup...
et al., 1993) or unchanged (Daoust et al., 1991) numbers of 5-HT uptake sites in these patients.

With regard to postsynaptic binding sites, it has been suggested that acute alcohol consumption might alter postsynaptic 5-HT$_{2A}$ receptor function, as evidenced by diminished inositol monophosphate production secondary to 5-HT$_{2A}$ receptor activation (Simonsson and Alling, 1988), though no change in 5-HT$_{2A}$ receptor number has been noted so far (Pandey et al., 1992).

The present study is the first that simultaneously addresses serotonergic function through measurement of the 5-HT uptake sites and the 5-HT$_{2A}$ receptor binding parameters in platelets from alcohol-dependent patients. Although the platelet constitutes a peripheral site that does not share the same micro-environment as central 5-HT neurones, platelet 5-HT transporter sites have been reported to be structurally identical to those existing in human brain neurones (Lesch et al., 1993). Both tritiated paroxetine and imipramine were chosen to measure 5-HT presynaptic uptake sites, because, although $[^{3}H]$paroxetine appears to be more selective than other radioligands (Arranz and Marcusson, 1994), $[^{3}H]$imipramine has been shown to be a more sensitive radioligand to detect variations in the platelet 5-HT reuptake sites from some psychiatric patients (Rosel et al., 1997a, 1999).

SUBJECTS AND METHODS

Subjects

Alcohol dependent patients. Twenty-four male subjects (age 42.5 ± 1.4 years; mean ± SEM) fulfilling DSM-IV criteria for alcohol dependence as determined by the Structured Clinical Interview for DSM-IV (American Psychiatric Association, 1994) and admitted for inpatient detoxification participated in the study. All patients met criteria for type II alcoholism (Cloninger, 1987). Medical history, physical examination, electrocardiogram, blood analysis (including platelet count), serum biochemical analysis (including hepatic tests), and urinalysis (including drug screening tests) were performed for each patient.

All patients included in the study stayed in the detoxification unit for at least 14 days. Blood samples were collected during acute alcohol intoxication (day 0), as the patients were not asked to avoid alcohol consumption prior to admission; during withdrawal (day 1), as whenever possible the patients were maintained with placebo during the first 24 h; and after 2 weeks of abstinence (day 14). Detoxification was performed with decreasing doses of oral diazepam, as this has not been reported to affect serotonergic measurements (Lingjaerde, 1978). Written informed consent was obtained from all patients after explaining the study procedures fully.

Control group. Within 1 week of the first blood collection from each patient, 40 ml of blood were also collected from a supposedly healthy age- and sex-matched individual (age 42.6 ± 1.3 years; mean ± SEM; n = 24). With this procedure, it was our purpose to compare each patient with a corresponding control, while avoiding the possible influence of sex, age, seasonal, and diurnal variations (Arranz et al., 1993; Rosel et al., 1996a). Volunteers were asked about their alcohol consumption in the past 3 weeks, and were excluded whenever a consumption of >21 U was reported. Subjects with a physical disorder or those taking serotonergic drugs were also excluded from the study.

Platelet membrane preparation

Between 09:00 and 10:00, 40 ml of whole blood were drawn by antecubital venipuncture in tubes containing ethylenediaminetetraacetic acid (EDTA). Platelet-rich plasma was obtained by centrifuging blood tubes at 200 g for 30 min at room temperature and was then centrifuged (10 000 g, 10 min, 4°C) so as to obtain the platelet pellet. Each pellet was then washed twice (10 000 g, 10 min, 4°C) in 8 ml of washing buffer (50 mM Tris–HCl, 150 mM NaCl, 20 mM EDTA, pH 7.4) and lysed twice in 8 ml of lysis buffer (5 mM Tris–HCl, 5 mM EDTA, pH 7.4). The final pellet was suspended in 2 ml of incubation buffer (50 mM Tris–HCl, 3 mM KCl, 120 mM NaCl, pH 7.4), and kept at –80°C until analysis.

Binding assays

Binding assays were performed following the methods previously described (Arranz and Marcusson, 1994; Arranz et al., 1994; Rosel et al., 1996b, 1997b). In summary, for the $[^{3}H]$paroxetine binding assay, platelet membrane homogenates were incubated (60 min, 25°C) with six increasing concentrations of $[^{3}H]$paroxetine (0.015 to 0.5 nM) in a total volume of 1600 µl. Non-specific binding was determined in the presence of 100 µM 5-HT. The $[^{3}H]$imipramine binding assay was performed...
by incubating the samples (90 min, 0°C) with different concentrations of this radioligand (0.30 to 10 nM) in a total volume of 0.5 ml. Non-specific binding was also determined with 10 μM 5-HT.

The 5-HT$_{2A}$ binding parameters were determined by incubating the samples (3 h, 25°C) with [³H]ketanserine (0.3 to 10 nM). Non-specific binding was assessed with 10 μM mianserin.

After incubation, experiments were stopped rapidly by the addition of 6 ml (2 ml for the [³H]ketanserine binding) of ice-cold Tris–HCl incubation buffer to each tube. Separation of the bound and free ligand was achieved by a quick filtration of the diluted homogenates through Whatman GF/C filters using a 24-channel cell harvester (Brandel, Gaithersburg, MD, USA). Filters were then washed with two 6-ml rinses of the same buffer, and the radioactivity trapped in each filter was counted by liquid scintillation spectrometry at 45–55% efficiency.

All experiments were performed in duplicate tubes. All three samples from each patient and the matched control were run in the same series so as to avoid between-run differences. Binding accounted in all cases for less than 10% of the total [³H]ligand added. Membrane protein concentrations were determined by a modification of the Lowry procedure, as described by Markwell et al. (1978).

**Analysis of binding data**

The apparent dissociation constant ($K_d$) and the maximum number of binding sites ($B_{max}$) for [³H]paroxetine and [³H]ketanserine binding were estimated using a computerized least squares linear regression Scatchard procedure (Scatchard, 1949), where bound [³H]ligand/free [³H]ligand was plotted as a function of bound [³H]ligand. Curve fittings were performed on ‘specific’ binding, i.e. binding displaced in the presence of 100 μM 5-HT or 10 μM mianserin. Binding data were subsequently analysed with the non-linear curve fitting program LIGAND in order to assess the existence of one or two binding sites. Values for $B_{max}$ and $K_d$ obtained through both linear and non-linear analysis were in all cases in good agreement.

**RESULTS**

Consumption patterns of the alcoholic patients studied are summarized in Table 1. In summary, our patients showed an approximately 20-year history of regular alcohol consumption, with a maximum abstinence of only 6.8 months. It is important to note that the mean abstinence interval before the first blood collection was only 4 h, suggesting that the serotonergic parameters obtained on day 0 reflect the acute intoxication state.

Biochemical and haematological parameters obtained before and after detoxification (Table 2) showed a significantly diminished platelet count during acute intoxication (day 0) in comparison to the values obtained after detoxification (day 14) ($P = 0.02; t = −2.42; Student’s paired t-test$). Significantly increased γ-glutamyltranspeptidase concentrations ($P = 0.02; t = 2.43; Student’s paired t-test$) were also observed. No significant differences were obtained with other biochemical markers of alcohol abuse or liver damage, such as mean cell volume, glutamic oxaloacetic transaminase or glutamic pyruvic transaminase.

Alcohol abuse (day 0) was found to be associated with an increased number and a lower binding
In this study, no alteration in the platelet imipramine binding sites and affinity from our alcoholic patients was observed. So far, studies using [3H]imipramine as the ligand to label the platelet 5-HT uptake sites in alcoholic patients have produced no clear picture. Suranyi-Cadotte et al. (1989) found that [3H]imipramine binding sites in platelets from 10 alcoholic patients (1229 fmol/mg), 10 abstinent former alcoholics (1031 fmol/mg) and 10 genetically high risk subjects (1103 fmol/mg) were lower than those found in controls (2150 fmol/mg). In a study by Gentsch et al. (1985), unchanged imipramine binding sites were noted in nine alcohol-dependent patients diagnosed according to the ICD-9 criteria, as compared to 11 control patients (1220 vs 1590 fmol/mg respectively), whereas other authors have reported increased platelet [3H]imipramine binding in 29 male alcohol-dependent patients and 29 male abstainers in comparison with 20 healthy volunteers (Patkar et al., 1995).

The use of [3H]imipramine in radioligand experiments presents several drawbacks. In the first place, the drug used to measure non-specific binding can considerably alter the binding parameters (Rosel et al., 1995). In this respect, the existence of a low affinity component of the [3H]imipramine binding displaced with desipramine which is not related to the 5-HT uptake site has been widely reported (Marcusson et al., 1985). In the three studies reported so far on [3H]imipramine binding, desipramine (Gentsch et al., 1985) and fluoxetine (Patkar et al., 1995) were the compounds of

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Parameter</th>
<th>Control group</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Imipramine</td>
<td>$B_{\text{max}}$</td>
<td>1225 ± 62</td>
<td>1147 ± 53</td>
<td>1212 ± 76</td>
<td>1133 ± 69</td>
</tr>
<tr>
<td></td>
<td>$K_d$</td>
<td>0.95 ± 0.06</td>
<td>0.98 ± 0.08</td>
<td>0.93 ± 0.07</td>
<td>0.99 ± 0.06</td>
</tr>
<tr>
<td>[3H]Paroxetine</td>
<td>$B_{\text{max}}$</td>
<td>1294 ± 57a</td>
<td>1551 ± 95</td>
<td>1525 ± 79</td>
<td>1326 ± 61b</td>
</tr>
<tr>
<td></td>
<td>$K_d$</td>
<td>0.048 ± 0.003c</td>
<td>0.061 ± 0.003</td>
<td>0.055 ± 0.003</td>
<td>0.056 ± 0.004</td>
</tr>
<tr>
<td>[3H]Ketanserin</td>
<td>$B_{\text{max}}$</td>
<td>143 ± 17d</td>
<td>136 ± 14</td>
<td>157 ± 22</td>
<td>98 ± 12c</td>
</tr>
<tr>
<td></td>
<td>$K_d$</td>
<td>1.42 ± 0.05</td>
<td>1.48 ± 0.09</td>
<td>1.49 ± 0.08</td>
<td>1.11 ± 0.08f</td>
</tr>
</tbody>
</table>

Data are means ± SEM, with $B_{\text{max}}$ values expressed in fmol/mg protein and $K_d$ values expressed in nmol/l.

aSignificant differences between the control group and day 0 ($P = 0.03$) and day 1 ($P = 0.02$) respectively (Student’s t-test).
bSignificant differences between day 14 and values obtained on days 0 and 1 ($P = 0.04$; paired ANOVA).
cSignificant differences between the control group and the values obtained on day 0 ($P = 0.003$; Student’s t-test).
dSignificant differences between the control group and the values obtained on day 14 ($P = 0.03$; Student’s t-test).
eSignificant differences between the values obtained on day 14 and those from day 0 and day 1 ($P = 0.008$; ANOVA).
fSignificant differences between control and day 14 values ($P = 0.004$; Student’s t-test) and between patient’s values ($P = 0.001$; ANOVA).
choice to measure non-specific binding. No information was given in the study by Suranyi-Cadotte et al. (1989). Furthermore, drug residues remaining in the platelet membrane preparations can influence imipramine binding kinetics considerably by means of competitive as well as non-competitive inhibition, resulting in higher $K_d$ and lower $B_{\text{max}}$ values respectively (Mellerup and Plenge, 1990).

All patients included in this study were type II alcoholics (Cloninger, 1987), with an early onset of the disorder, a family history of alcoholism, and impulsive behaviour. Biochemically, type II alcoholism has been associated with low levels of 5-HT metabolites in the cerebrospinal fluid, thus indicating diminished serotonergic function. The study by Patkar et al. (1995) including 11 type II alcoholics showed that the increased number of $[^3\text{H}]$imipramine binding sites only occurred in the type II subgroup, when compared to either controls or the type I subgroup.

A significantly increased number of $[^3\text{H}]$paroxetine binding sites was noted in our alcoholic patients in comparison to the control group, with the values returning to normal after 2 weeks of abstinence. Results published so far using this radioligand are in agreement with those reported in this study. Thus, increased $[^3\text{H}]$paroxetine binding sites (1400 vs 1280 fmol/mg) have been noted in 24 alcoholic patients who had been abstinent for at least 1 month before blood sampling, predominantly in those individuals with lower Newcastle scores (non-endogenous depression) (Mellerup et al., 1993). In the same patients, a lower binding affinity ($K_d = 0.06 \text{ nM}$) was found in comparison to the control group ($K_d = 0.04 \text{ nM}$), suggesting a connection between depressive symptoms of the non-endogenous type and high platelet paroxetine binding. In another study by Daooust et al. (1991), blood samples from alcoholic patients collected on days 1 and 5 of hospitalization and from control subjects showed no differences in either $B_{\text{max}}$ or $K_d$ values. However, a tendency to higher $B_{\text{max}}$ values was observed in alcoholic patients. In this latter study, mean $K_d$ values of 6 and 5 nM were obtained from the controls and the alcoholic patients respectively, these values being considerably higher than those previously reported by other authors. It is somewhat surprising that the highest $[^3\text{H}]$paroxetine concentration used in their binding assays was 3 nM (range 0.05–3 nM), a concentration unable to saturate the binding site given the $K_d$ values obtained. Furthermore, on the fifth day of hospitalization, only nine patients remained in the study.

Previous reports by our group (Rosel et al., 1997a, 1999) have indicated the existence of a diminished number of $[^3\text{H}]$imipramine but not of $[^3\text{H}]$paroxetine binding sites in melancholic patients. In this study, altered $[^3\text{H}]$paroxetine, but not $[^3\text{H}]$imipramine, binding sites were noted in alcoholic patients. Explanations for this difference between endogenously depressed and alcoholic patients may be that while treatment with tricyclic antidepressant drugs has been proved to be effective in severe melancholic hospitalized patients, SSRIs are successful in decreasing alcohol consumption in some alcohol-dependent patients (Naranjo et al., 1990, 1992).

Acute ethanol intoxication/withdrawal (day 0 and day 1) showed a similar number of 5-HT$_{2A}$ binding sites to the control group. However, a significantly diminished number of these receptors was observed after 2 weeks of alcohol withdrawal. It has been suggested that alcohol-dependent patients may have pre-existing low brain serotonin levels that are transiently raised by alcohol consumption, which in turn eventually lead to further depletion of brain serotonin (Ballenger et al., 1979). Increased imipramine or paroxetine binding may reflect a compensatory response to a central 5-HT deficiency (Langer et al., 1981), leading to higher 5-HT reuptake and to decreased synaptic 5-HT concentrations.

It would seem then that the compensatory increase in paroxetine binding and consequently the decrease in synaptic 5-HT, observed in our chronic ethanol-use patients (day 0 and day 1) are unable to produce changes at the postsynaptic receptor level, probably due to a reversible inhibition of the 5-HT$_{2A}$ receptor complex produced by chronic alcohol intake. In this respect, previous studies have reported that chronic ethanol treatment does not have any effect on the 5-HT$_{2A}$ receptor numbers, although it decreases 5-HT-stimulated phosphoinositide hydrolysis in the rat cortex (Pandey et al., 1992) and in platelets from alcoholic patients (Simonsson et al., 1992) according to the duration of the exposure. Abstinence for 2 weeks might both normalize the 5-HT transporter sites and lessen the inhibitory effect at the postsynaptic level, allowing the increase in synaptic 5-HT levels to down-regulate the 5-HT$_{2A}$ receptors.
In conclusion, though it may be tempting to conclude that chronic alcohol use modifies the 5-HT transporter density as evidenced by increased paroxetine binding, this finding seems to be a reversible phenomenon that normalizes after detoxification and withdrawal. Further studies are necessary to assess whether the changes noted in a peripheral model such as the human platelet are replicated successfully in human brain.

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