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Selective inhibition of [^{3}H]lysergic acid diethylamide binding to mouse brain membranes by ethanol

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Recent reports have indicated that ethanol inhibits the binding of a number of ligands to brain membranes in vitro (Ross et al 1977; Ciofalo 1978, 1980; Fairhurst & Liston 1979; Hruska & Silbergeld 1980; Shanley et al 1980). During screening of drugs dissolved in ethanol, I observed that 5-hydroxytryptamine (5-HT) receptors in mouse brain membranes labelled with [^{3}H]lysergic acid diethylamide (LSD) and [^{3}H]5-HT were also sensitive to this alcohol. Further work revealed that 5-HT receptors labelled with [^{3}H]LSD were about three times more sensitive to ethanol than those labelled with [^{3}H]5-HT. Moreover, ethanol lowered the B_{max} for [^{3}H]-LSD binding while increasing the affinity (K_D) for the ligand, suggesting uncompetitive inhibition. I observed no change in the Hill coefficient for [^{3}H]LSD binding in the presence of ethanol. [^{3}H]LSD binding to dopamine receptors in mouse brain membranes was also inhibited by ethanol. A preliminary report of this data has appeared (Hirsch 1980).

Materials and Methods

Male HAM/ICR CD-1 mice (Charles River Breeding Laboratories, Portage, MI). 20–25 g, were decapitated and the whole brains were removed, washed free of blood and homogenized in 50 volumes of 50 mM Tris-HCl buffer (pH 7·4, 30 °C) with a Brinkmann Polytron PCU-2-110 (Brinkmann Instruments, Westbury, NY) at a setting of 6·5 (full scale of 10) for 20 s. Membranes were recovered by centrifugation at 36000 × *g* for 10 min in a refrigerated Sorvall RC2-B centrifuge and re-homogenized as described above. Following another centrifugation, membranes were resuspended with the Polytron in 40 volumes of 50mM Tris-HCl buffer (pH 7·4, 30 °C) containing 0·1% (w/v) ascorbic acid and 10 μM pargyline, incubated at 30 °C for 20 min and chilled. A fresh preparation of membranes was made for each experiment.

For the binding assays, triplicate glass incubation tubes contained 100 μl of membrane suspension (150–200 μg protein), 20 μl of either [^{3}H]LSD or [^{3}H]5-HT, various volumes of absolute ethanol (21·7 M), and the remaining volume as 50 mM Tris-HCl buffer (pH 7·4). The final volume was 200 μl. Unless otherwise indicated, the final concentrations of [^{3}H]LSD and [^{3}H]5-HT were 4·6 and 10·0 nM respectively. With each ligand, non-specific binding was determined in the presence of 10 μM unlabelled 5-HT. In these experiments, [^{3}H]LSD binding was effected in the presence of 10 μM dopamine and 1 μM pimozide to prevent association of the ligand with dopamine receptors (Hirsch 1980). When [^{3}H]LSD was used to label dopamine receptors, assays were performed in the presence of 100 μM unlabelled 5-HT and non-specific binding was determined in the presence of 100 μM dopamine.

All binding assays were incubated at 30 °C for 20 min. After incubation, the tubes were chilled briefly in an ice bath, the contents were diluted with 2 ml of ice-cold 50 mM Tris-HCl buffer (pH 7·4, 4 °C) and rapidly filtered under vacuum through Whatman GF/C glass fiber filters. Each filter was washed twice with 2 ml of buffer, dried at 50 °C for 30 min and counted in 10 ml of PCS (Amersham Corporation) in a Mark III Liquid Scintillation System (Searle Analytic, Inc., Elk Grove Village, IL) with an efficiency of 38%.

[*N*-methyl-^{3}H]Lysergic acid diethylamide (32·3 Ci mmol^{-1}) and 5-[1,2-^{3}H(N)]hydroxytryptamine creatinine sulphate (29·8 Ci mmol^{-1}) were obtained from New England Nuclear (Boston, MA) and diluted for use in 50 mM Tris-HCl buffer (pH 7·4, 30 °C) containing 0·1% (w/v) ascorbic acid. Unlabelled 5-HT and dopamine

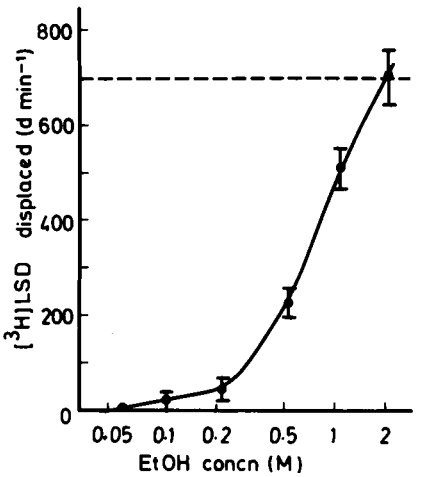

FIG. 1. Effect of ethanol (EtOH) on [^{3}H]LSD binding to mouse brain membranes. [^{3}H]LSD was present at 4·6 nM. Dopamine (10 μM) and pimozide (1 μM) were also added to prevent association of the ligand with dopamine receptors. (-----) represents displacement obtained in the presence of 10 μM unlabelled 5-HT. Results are means ± s.e.m. from 3 experiments.

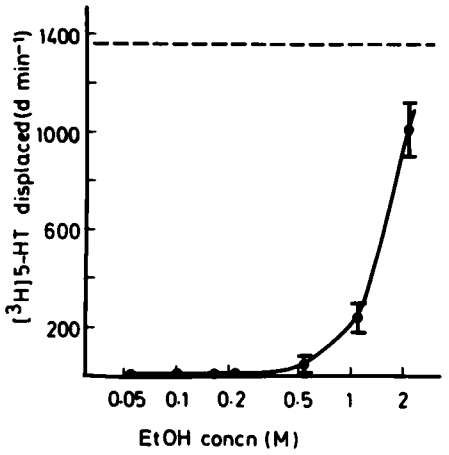

FIG. 2. Effect of ethanol (EtOH) on [³H]5-HT binding to mouse brain membranes. [³H]5HT was present at 10·0 nM. (- - - - -) represents displacement obtained in the presence of 10 μM unlabelled 5-HT. Results are means ± s.e.m. for 3 experiments.

were purchased from Calbiochem. The following gifts are acknowledged: pargyline (Abbott) and pimozide (McNeil).

Results

Inhibition of [³H]LSD *and* [³H]5-HT *binding by ethanol.* Ethanol inhibited [³H]LSD binding to 5-HT receptors in a dose-responsive fashion with 100% inhibition observed at 2·17 M (Fig. 1). Log-logit analysis of the ethanol dose-response curves revealed an IC50 of 0·63 M.

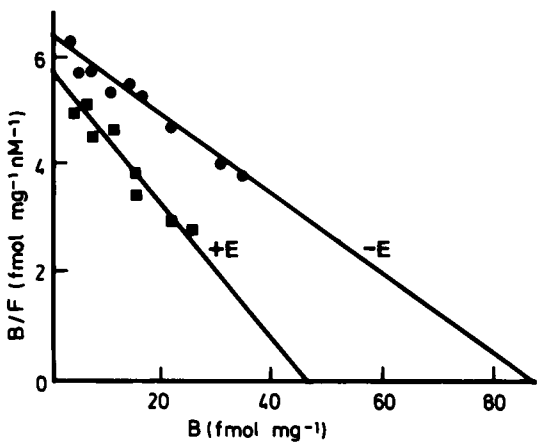

FIG. 3. Scatchard plots of saturable specific [³H]LSD binding to mouse brain membranes in the absence (−E) and presence (+E) of 0·54 M ethanol. [³H]LSD binding was determined over the range of 0·77–10·8 nM and non-specific binding was measured in the presence of 10 μM unlabelled 5-HT. Dopamine and pimozide were present as described in the legend to FIG. 1. $K_D(-E)$ = 13·8 nM; $B_{max}(-E)$ = 86 fmol mg^{-1} protein. $K_D(+E)$ = 7·8 nM; $B_{max}(+E)$ = 46 fmol mg^{-1} protein. Results are means from 3 experiments.

In contrast, [³H]5-HT binding to 5-HT receptors was more resistant to the alcohol with only 73% inhibition occurring at 2·17 M (Fig. 2). An estimated IC50 for ethanol in this case was 1·8 M.

When [³H]LSD was used to label dopamine receptors in the membranes (5-HT receptors blocked by 100 μM unlabelled 5-HT), ethanol had an IC50 of 0·96 M (not shown).

Type of inhibition by ethanol. Saturable specific binding of [³H]LSD (0·77–10·8 nM) to 5-HT receptors was determined in the presence and absence of 0·54 M ethanol. Scatchard plots of the saturation isotherms obtained revealed that this concentration of ethanol lowered the B_{max} for binding by 47%, while enhancing the affinity (K_D) of the remaining receptors by a similar amount (43%) (Fig. 3). This suggests an uncompetitive type of inhibition. Double reciprocal plots of the data led to the same conclusion (not shown).

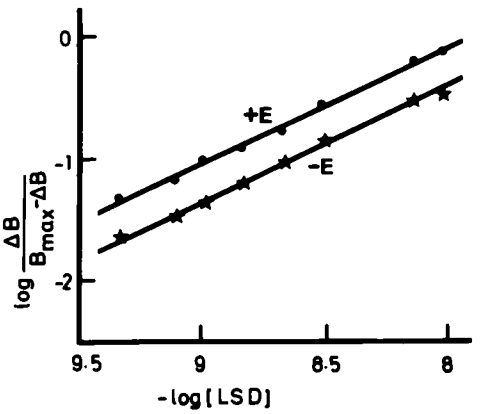

FIG. 4. Hill plots of saturable specific [³H]LSD binding to mouse brain membranes in the absence (−E) and presence (+E) of 0·54 M ethanol. [³H]LSD binding was determined as described in the legend to FIG. 3. $B_{max}(-E)$ = 86 fmol mg^{-1} protein. $B_{max}(+E)$ = 46 fmol mg^{-1} protein. Results are means from 3 experiments.

Hill plots of the data yielded Hill coefficients of 0·95 (minus ethanol) and 0·91 (plus ethanol) indicating that [³H]LSD binding was noncooperative and that ethanol had no effect on this parameter (Fig. 4).

Discussion

The major finding of this study is that 5-HT receptors in mouse brain membranes labelled with [³H]LSD are about three times more sensitive to perturbation by ethanol in vitro than those labelled with [³H]5-HT. These results suggest that the accessibility of these binding sites to the alcohol is different. By extension, these data lend support to the notion that the two ligands label separate 5-HT receptors in the brain (Fillion et al 1978; Whitaker & Seeman 1978). Further biochemical work with more specific membrane active agents may reveal additional differences between 5-HT

binding sites, particularly on a brain regional basis, and may confirm the existence of separate 5-HT receptors.

As proposed by others (Hruska & Silbergeld 1980), the present data also show that caution should be exercised when drugs dissolved in ethanol are evaluated in receptor binding assays.

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(—)-*m*-Chlorophenyl-piperazine, a central 5-hydroxytryptamine agonist, is a metabolite of trazodone

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Trazodone an antidepressant introduced recently in clinical practice, was found to inhibit 5-hydroxytryptamine (5-HT) 5-HT uptake in the rat brain and blood platelets (Garattini et al 1976). In this study, a possible metabolite of trazodone, (−)-*m*-chlorophenylpiperazine (mCPP) also inhibited 5-HT uptake, suggesting that it might contribute to the effect of the parent compound. Subsequent studies with mCPP showed that it behaves like a central 5-HT-agonist (Samanin et al 1979; Rokosz-Pelc et al 1980) and this agrees with its effectiveness in displacing [^{3}H] 5-HT binding to brain membranes (Samanin et al 1980).

A recent article (Maj et al 1979) reports that trazodone at low doses has anti-5-HT properties, while at higher doses it acts as a central 5-HT agonist. This latter effect was attribute to the formation of mCPP in trazodone-treated animals (Maj et al 1980) although this compound was not measured directly. mCPP has been recently identified as the *N*-glucuronide in rat urine (Melzacka et al 1979) confirming this route of trazodone's metabolism.

The aim of the present study was to prove that substantial amounts of mCPP are formed in the brain after oral administration of trazodone. To obtain preliminary information on the significance of mCPP formation for the effects of trazodone, brain levels of mCPP in rats treated with trazodone were compared with those found after pharmacologically and biochemically effective doses of mCPP (Samanin et al 1979).

Male CD-COS rats (Charles River, Italy), ≈250 g, were treated orally with trazodone hydrochloride (12·5, 25 and 50 mg kg^{-1}) or mCPP hydrochloride (1 and 5 mg kg^{-1}) and killed at various times after. mCPP was extracted from plasma (2 ml) and brain homogenates (0·1 M HCl, 6: v/w) with 5 ml of benzene, after addition of 1 M NaOH and 4-amino-1-(6-chloro-2-pyridil)-piperidine as an internal marker. To 1 ml of the benzene phase, 50 μl of heptafluorobutyric anhydride (a 25% v/v solution methyl acetate) was added and the samples left to stand for 30 min at 60 °C. After the reaction the samples were washed with water (1 ml) and 5% aqueous ammonia solution (0·5 ml) and 1–2 μl of the benzene phase were injected into the gas chromatographic column.

Samples were analysed on a C. Erba Fractovap Mod. 2150 equipped with an electron capture detector, using a 2 m by 3 mm internal diam. column containing 3% OV 17 on Supelcoport (Supelco, Inc.). The conditions were as follows: column temp. 205 °C, injector and detector temperature 250 °C. Carrier gas was nitrogen at a flow rate of 35 ml mm^{-1}. Standard curves were determined for each experiment by adding known amounts of mCPP and internal standard to brain homogenates and plasma samples and determining the ratio of the mCPP to internal standard peak areas. Specificity of the analyses was confirmed by gas chromatography combined with mass spectrometry (g.l.c.-m.s.). The sensitivity of the method was 10 ng ml^{-1} plasma or 50 ng g^{-1} brain. The recovery from plasma and brain was 90 ± 5% and 83 ± 7% respectively.

Table 1 shows that the biotransformation of trazodone in rats yields measurable amounts of mCPP in both plasma and brain. After oral trazodone (12·5, 25 and 50 mg kg^{-1}) the metabolite reached peak plasma concentrations between 1 and 2 h, declining thereafter in a monoexponential manner with an apparent half-life

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