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European Journal of Pharmacology 281 (1995) 229–239

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Chronic alcoholization alters the expression of 5-HT_{1A} and 5-HT_{1B} receptor subtypes in rat brain

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Received 7 April 1995; accepted 13 April 1995

Abstract

The expression of central 5-HT_{1A} and 5-HT_{1B} receptors was studied in several brain areas of rats subjected to a 2-week period of chronic alcoholization, followed by 18 h withdrawal. Quantitative autoradiography indicated that the ethanol treatment provoked an increase ($\sim +30\%$) in the labeling by [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) and [^{3}H]*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide ([^{3}H]WAY-100635) of 5-HT_{1A} autoreceptors in the dorsal raphe nucleus, accompanied by a concomitant decrease in the labeling of postsynaptic 5-HT_{1A} receptors in the hippocampus ($\sim -20\%$), anterior ($\sim -30\%$) and posterior ($\sim -32\%$) cortices. These changes were associated with a tendency toward an increase and decrease in 5-HT_{1A} mRNA levels in the anterior raphe area and hippocampus, respectively, suggesting that the changes observed are due to modifications in 5-HT_{1A} receptor protein synthesis. The autoradiographic labeling of 5-HT_{1B} receptors by serotonin-*O*-carboxymethylglycyl[^{125}I]iodotyrosinamide ([^{125}I]GTI) was found to increase (+55%) in the globus pallidus of alcoholized rats. Interestingly, a significant increase (+57%) in 5-HT_{1B} receptor mRNA levels was observed in the striatum, which contains cell bodies of neurons projecting into the globus pallidus. These data suggest that altered sensitivity of chronically alcoholized rats to 5-HT_{1A} and 5-HT_{1B} receptor ligands may result from alcohol-induced changes in the transcription of the genes encoding these receptors.

Keywords: Alcoholization; 5-HT (5-hydroxytryptamine, serotonin); Receptor; mRNA; Binding; Autoradiography; (Rat)

1. Introduction

Numerous central neurotransmitter systems have been implicated in various ways in alcohol consumption-related phenomena (for reviews, see Samson and Harris, 1992; Nutt and Peters, 1994; Nevo and Hamon, 1995). Among the different systems studied, that mediated by serotonin (5-hydroxytryptamine; 5-HT) has come under particular scrutiny, subsequent to the first evidence of the implication of the indoleamine in alcohol-related consummatory behavior by Myers and Veale (1968). Since then, numerous studies have focused on the ability of a number of pharmacological agents capable of modifying serotonergic transmission to modulate alcohol intake. Thus, administration of substances which increase serotonergic neurotransmission, such as 5-HT precursors, uptake inhibitors or releasers, has generally been found to reduce ethanol consumption; conversely, pharmacological manipulations which reduce cerebral 5-HT neurotransmission, such as the lesion of serotonergic neurons with 5,7-dihydroxytryptamine, provoke an increase in alcohol intake (Sellers et al., 1992; McBride et al., 1993; Le Marquand et al., 1994; Nevo and Hamon, 1995).

In view of the mediation of the central actions of 5-HT through the activation of several receptor subtypes (Hoyer et al., 1994), subsequent studies attempted to establish which of these receptors may be

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SSDI 0014-2999(95)00238-3

involved in the molecular mechanisms by which alterations of serotonergic transmission can modulate alcohol consumption, and conversely, examined the effects of alcohol consumption on the central serotonergic system. Notably, a reduction in alcohol consumption has been observed following administration of 5-HT_{1A} receptor agonists to rats and monkeys (Collins and Myers, 1987; Kostowski and Dyr, 1992) and man (Bruno, 1989), or of 5-HT_{2A} receptor antagonists to alcohol-preferring rats (Panocka and Massi, 1992; Myers et al., 1993). In addition, antagonists of the 5-HT_3 receptors have been found not only to attenuate ethanol consumption in alcohol-preferring rats (Fadda et al., 1991; Meert, 1993) and in man (Toneatto et al., 1991), but also to prevent the behavioral consequences of ethanol withdrawal (Fozard, 1992; Kostowski et al., 1993). Further studies with non-selective ligands showed that the 5-HT agonist *m*-chlorophenylpiperazine (mCPP), which acts at 5-$HT_{1D\beta}$, 5-HT_{2A} and 5-HT_{2C} receptors, produces an ethanol-like behavioral response in abstinent alcoholics (George et al., 1990), while *m*-trifluoromethylphenylpiperazine (TFMPP), which acts at 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1A} and 5-HT_{2C} receptors, is able to substitute for the discriminative stimulus effects of ethanol (Grant and Colombo, 1993). A predominant role for the 5-$HT_{1D\beta}$ receptor (of which the 5-HT_{1B} receptor is the rodent homologue; see Hoyer et al., 1994) in these effects is likely, as investigations into the discriminative stimulus properties of TFMPP have minimized the implication of 5-HT_{1A} and 5-HT_{2C} receptors (Grant and Colombo, 1993).

Knowledge of whether certain characteristics of these various receptor types are modified upon acute or chronic alcoholization, or in genetically alcohol-preferring rat strains, would provide an insight into the molecular mechanisms by which the different receptor ligands mentioned above may act in order to produce their effects. To date, however, the results of such studies have been contradictory and far from conclusive (see Nevo and Hamon, 1995). For instance, binding studies using membranes from the brains of alcohol-preferring P rats have shown that the density of 5-HT_1 (Wong et al., 1988) or 5-HT_{1A} (Wong et al., 1990,1993) binding sites in the hippocampus and cerebral cortex was increased in these animals with respect to their non-preferring NP counterparts. By contrast, using another strain of alcohol-preferring rats, Korpi et al. (1992) found no difference in the labeling of 5-HT_1 receptors in various brain regions of the alcohol-preferring AA rats as compared to their non-preferring ANA counterparts. A few studies have also examined the effects of alcoholization on 5-HT receptors in unselected rats. For instance, Ulrichsen (1991) observed a decrease in the labeling of 5-HT_{1A} receptors in hippocampal membrane preparations upon both chronic alcoholization and withdrawal. Using a similar protocol, Pandey et al. (1994) noted an increased labeling of 5-HT_{1B} receptors in the globus pallidus, also in the alcoholization as well as the withdrawal phases.

For several 5-HT receptors, the current availability of new tools and methods allows for more reliable investigations. In particular, [^{3}H]WAY-100635 ([^{3}H]*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide) has been shown to be a highly selective antagonist radioligand of 5-HT_{1A} receptors (Khawaja et al., 1994; Gozlan et al., 1995), and [^{125}I]GTI (serotonin-*O*-carboxymethylglycyl [^{125}I]iodotyrosinamide) allows the specific labeling of 5-HT_{1B} receptors in the rat brain (Boulenguez et al., 1992). In addition, the reverse transcriptase-polymerase chain reaction (PCR) technique can be used for the quantification of specific mRNAs encoding receptors in selected brain areas (Siebert and Larrick, 1992). These approaches were used in the present study to reinvestigate the effects of chronic alcohol consumption on the expression of central 5-HT_{1A} and 5-HT_{1B} receptors in the rat.

2. Materials and methods

2.1. Chemicals

[*O*-methyl-^{3}H]WAY-100635 (69 Ci/mmol) was synthesized by Amersham International (Buckinghamshire, UK) for Wyeth Labs (Taplow, UK) and generously provided by Dr. Colin T. Dourish (Wyeth Research UK). [^{3}H]8-OH-DPAT ([^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin, 139 Ci/mmol) was provided by the Service des Molécules Marquées of the CEA (Gif-sur-Yvette, France), and [^{125}I]GTI (2000 Ci/mmol) was a generous gift from Immunotech (Marseille, France).

CP 93,129 (3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo [3,2-*b*]pyrid-5-one) was kindly provided by Pfizer (Groton, CT, USA). Other compounds and reagents used were of the highest grade commercially available: Taq polymerase (Hi-Taq, Bioprobe Systems, Paris, France); reverse transcriptase from Moloney murine leukemia virus (Stratascript, Stratagene, La Jolla, CA, USA); RNAse inhibitor (Boehringer Mannheim, Germany); 5-HT-creatinine sulfate, corticosterone (Sigma, St. Louis, MO, USA); pargyline (Abbott, Chicago, IL, USA).

2.2. Alcoholization protocol

Experiments were performed on adult (~ 200 g body weight), male Sprague-Dawley rats (Centre d'Elevage R. Janvier, 53940 Le Genest-St. Isle, France; ICO: OFASD [IOPS], Iffa Credo, France). Animals were housed in hanging metal cages with a metal grid floor

(Sufraco, Bagneux, France) and maintained under standard laboratory conditions (22 ± 1°C, 60% relative humidity, 12 h/12 h light-dark cycle, food and water ad libitum) for one week prior to the beginning of the experiment. All rats were handled in agreement with the ethical rules of experimentation on laboratory animals (recommended by the guide for the care and use of laboratory animals (1980), DHEW Publication 80-23, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205, USA).

For a period of 14 days, the rats were given access, as their only source of nutrition, to a liquid diet (Bio-Serv Liquid Rat Diet, Frenchtown, NJ, USA) containing 9% (v/v) ethanol, or a control diet in which ethanol was substituted isocalorically with dextrose, in 100 ml calibrated drinking bottles (BioServ, Frenchtown, NJ, USA). Animals were assigned to the ethanol or control group according to a stratified procedure balancing body weight. Water was available ad libitum. In order to gradually introduce the rats to the ethanol diet, animals from the ethanol group received a liquid diet containing 5% ethanol on the first day, 6.7% ethanol on the second day, and 9% ethanol from the third day on. Liquid diet consumption was measured daily between 4:00 p.m. and 5:00 p.m., and the liquid diet was then replaced with a fresh solution. Animals from the control group received an amount of control liquid diet equivalent to the average volume of ethanol liquid diet consumed the previous day by animals from the ethanol group.

In the morning of the 14th treatment day, blood samples were collected from the tail vein and frozen at −30°C for determination of ethanol levels using gas phase chromatography (Lal et al., 1988; Kleven et al., 1995). In the afternoon of the same day, rats from the ethanol group received 5 g/kg ethanol prepared in liquid diet by oral gavage (40 ml/kg), while pair-fed controls received the same volume of control liquid diet. Tolerance to the ethanol treatment was assessed by measuring rectal temperature at several time points following the oral gavage, as compared to an additional control group also receiving the ethanol gavage, used for this purpose only. Animals of the ethanol-fed group and of the pair-fed control group then had access to 50 ml control diet until the next morning. The degree of severity of the withdrawal syndrome was assessed according to previously described procedures (Lal et al., 1988; Kleven et al., 1995) 18 h following the last exposure to ethanol. The rats were then killed by decapitation, trunk blood was collected for determination of serum corticosterone levels, and the brains were either dissected on ice (for preparation of mRNAs for reverse transcriptase-PCR or for preparation of membranes for radioligand binding assays) or frozen immediately at −30°C in isopentane cooled by dry ice and stored at −80°C (for autoradiography).

2.3. Radioligand binding assays

Preparation of membrane suspensions

Tissues were dissected on ice and homogenized in 40 vols. (v/w) of 50 mM Tris-HCl ice-cold buffer, pH 7.4, with a Polytron (type PT10 OD) tissue disrupter. The resulting homogenates were centrifuged at 40 000 × *g* for 20 min at 4°C, and the pellets were washed twice by resuspension in 100 vols. of ice-cold buffer, followed by centrifugation. The sedimented material was then resuspended in 40 vols. of Tris-HCl and incubated at 37°C for 10 min to remove endogenous 5-HT (Nelson et al., 1978). Membranes were centrifuged and washed three more times as above, and the final pellet was resuspended in 10 vols. of Tris-HCl buffer. The membranes were used immediately after preparation for the binding experiments, and any remaining membranes were frozen at −80°C for subsequent use if necessary.

5-HT$_{1A}$ receptors

[^{3}H]8-OH-DPAT and [^{3}H]WAY-100635 binding assays were performed as described by Hall et al. (1985) and Gozlan et al. (1995), respectively. Briefly, 50 μl aliquots (corresponding to ~ 0.25 mg protein) of cortical or hippocampal membrane suspensions were incubated at 25°C for 60 min in 0.5 ml (final volume) of 50 mM Tris-HCl buffer, pH 7.4, containing 4.0 nM [^{3}H]8-OH-DPAT or 2.0 nM [^{3}H]WAY-100635 (concentrations producing the saturation of high affinity [^{3}H]8-OH-DPAT or [^{3}H]WAY-100635 specific binding sites; Hall et al., 1985; Gozlan et al., 1995). In some experiments, assays were performed with eight different concentrations of [^{3}H]8-OH-DPAT (0.25–5.0 nM) or [^{3}H]-WAY-100635 (0.03–1.4 nM). In all cases, non-specific binding was defined in the presence of a saturating concentration (10 μM) of 5-HT. The incubation was terminated by the addition of 3.5 ml of ice-cold Tris-HCl buffer and rapid vacuum filtration using a Brandel Cell Harvester. After two additional washings with 3.5 ml of ice-cold Tris-HCl buffer, the glass fiber filters (GF/B) were removed, dried and immersed in 4.5 ml of Aquasol scintillation liquid (New England Nuclear, Boston, MA, USA) for radioactivity counting. For [^{3}H]WAY-100635 binding assays, the glass fiber filters were presoaked in 0.5% polyethylenimine for 30 min in order to reduce the level of non-specific binding (Gozlan et al., 1995). All determinations were performed in triplicate.

Experiments with only one (saturating) concentration of each radioligand allowed the estimation of respective B_{max} values according to the equation: Estimated $B_{max} = B$ (in fmol/mg protein) × (K_d + [ligand])/[ligand], where B is the specific binding measured at the saturating concentration ([ligand]) of each radioligand ([^{3}H]8-OH-DPAT, [^{3}H]WAY-100635). The

GraphPad and InPlot 4 programs were used for the calculation of K_d and B_{max} values from saturation curves (Gozlan et al., 1995).

5-HT_{1B} receptors

Aliquots (20 μl, corresponding to ~0.10 mg protein) of nigral and striatal membrane suspensions were incubated at 15°C for 60 min in 0.2 ml (final volume) of 50 mM Tris-HCl buffer, pH 7.4, containing 4 mM $CaCl_2$, 10 μM pargyline, 0.1% ascorbic acid, and 25 pM [^{125}I]GTI. Non-specific binding was defined in the presence of a saturating concentration (10 μM) of the selective 5-HT_{1B} receptor agonist CP 93,129 (Macor et al., 1990). The incubation was terminated by the addition of 3.5 ml of ice-cold Tris-HCl buffer and rapid vacuum filtration through polyethylenimine-pretreated filters (as above) using a Brandel Cell Harvester. All determinations were performed in triplicate.

2.4. Measurement of proteins

Proteins were determined according to the method of Lowry et al. (1951) using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

2.5. Quantitative autoradiography

Preparation of brain sections

Coronal brain sections (20 μm) were cut at −15°C in a cryostat, thaw-mounted onto gelatin-coated glass slides, dried at 4°C for 30 min on silica gel, and stored at −20°C for less than 2 weeks before being used for radiolabeling.

5-HT_{1A} receptors

Sections were preincubated for 30 min at 20°C in 170 mM Tris-HCl, pH 7.6, and then incubated for 60 min at 20°C in the same (fresh) buffer, containing 1.2 nM [^{3}H]8-OH-DPAT or 1.0 nM [^{3}H]WAY-100635. Non-specific binding was determined on adjacent sections processed in the same way, except that a saturating concentration (10 μM) of 5-HT was added to the incubation medium (Vergé et al., 1986; Gozlan et al., 1995). After incubation, the labeled sections were washed twice for 5 min in ice-cold Tris-HCl buffer, quickly dipped in cold (4°C) distilled water, dried in a stream of cold air, and apposed to [^{3}H]Hyperfilm (Amersham) in an X-ray cassette for one month.

5-HT_{1B} receptors

Sections were preincubated for 30 min at 20°C in Krebs' buffer (15 mM Tris-HCl, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 7.6), and then incubated for 60 min at 20°C in the same (fresh) buffer, supplemented with 0.1% bovine serum albumin and 36 pM [^{125}I]GTI. Non-specific binding was determined on adjacent sections processed in the same way, except that a saturating concentration (10 μM) of CP 93,129 was added to the incubation medium. After incubation, the labeled sections were washed twice for 1 min in cold (4°C) distilled water, dried in a stream of cold air, and apposed to [^{3}H]Hyperfilm (Amersham) in an X-ray cassette for 10 days.

Film development and quantification

Autoradiographic films were developed in Microdol X (Kodak) for 10 min at 20°C, and fixed in Agefix (Agfa) for 10 min. Quantification of the labeling by the various ligands was performed with a Biocom densitometer. Optical density was converted into fmol radioligand bound per mg tissue using appropriate ^{3}H and ^{125}I standards (Amersham).

2.6. mRNA measurement (quantitative reverse transcriptase-PCR)

The method used to measure mRNAs is based on the competitive PCR technique (Siebert and Larrick, 1992), in which mRNAs are reverse-transcribed and amplified in the presence of internal standards consisting of the same target RNAs, synthesized with deletions of about 100 bases to allow electrophoretic separation. These synthetic RNAs were obtained by sense transcription of 5-HT receptor cDNAs, in which the deletions were introduced by construction using PCR with composite primers. The deletions were of 92 and 115 bases for 5-HT_{1A} (Albert et al., 1990) and 5-HT_{1B} (Voigt et al., 1991) receptor mRNAs, respectively. Transcription with T7 or SP6 RNA polymerase from recombinant pGEM-4Z plasmids was performed using the Ampliscribe kit from Epicentre Technologies (Madison, WI, USA).

Total RNAs from various brain tissues were prepared according to Chomczynski and Sacchi (1987), electrophoresed on 1% agarose gel, ethidium bromide-stained, and quantified, using a scale of total RNAs prepared by cesium chloride gradient (Chirgwin et al., 1979), and measured from the optical density at 260 nm. About 0.5–1.0 μg of total tissue RNA were converted to cDNA with 5–10 units of reverse transcriptase from Moloney murine leukemia virus for 1–2 h at 42°C in 5 μl of 50 mM Tris-HCl, pH 8.3, in the presence of 5–10 units of RNAse inhibitor, 1.0 mM deoxynucleotides, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, corresponding deleted RNAs (0.01–10 pg), and 800 nM complementary oligonucleotides. The sequences of these oligonucleotides were 5′-ATGGATCCCCCAGAGTCTTCACCGTCTTC (nucleotides 1165-1144) and 5′-ATGAATTC-TGTGAAAGACCCAACTTGGTCCC (nucleotides 1708–1685) for 5-HT_{1A} and 5-HT_{1B} receptors, respectively (Albert et al., 1990; Voigt et al., 1991). Amplifi-

cation was then performed in 25 μl of 10 mM Tris-HCl, pH 8.3, containing 1 unit of Taq polymerase, 2.5 and 5 mM $MgCl_2$ for 5-HT_{1A} and 5-HT_{1B} receptors, respectively, 50 mM KCl, 0.1% Triton X-100, 0.2 mM deoxynucleotides, and 160 nM oligonucleotides: 5'-ATGAATTCCTCTACGGGCGCATCTTCAGA (nucleotides 761–782, 5-HT_{1A}) and 5'-AAGAATTCTGAAACAGACACCCAACAAGACC (nucleotides 1193–1216, 5-HT_{1B}). Cycle amplifications were performed at 94°C, 56°C and 72°C (1 min each, 30 cycles). PCR products (410 and 515 base pairs in size for 5-HT_{1A} and 5-HT_{1B} receptors, respectively) were electrophoresed on a 2% agarose gel, ethidium bromide-stained, and quantified with a GDS 5000 gel analyzer (UVP, Cambridge, UK). mRNA levels are expressed as attomol RNA standard per μg of total tissue RNA according to the method of Siebert and Larrick (1992), and corrected according to the respective sizes of tissue and standard RNA.

2.7. Corticosterone assay

Rats which had undergone the chronic alcoholization procedure, along with pair-fed controls and control rats having received food and water ad libitum, were decapitated and trunk blood was collected into non-heparinized tubes. Serum corticosterone content was assayed by an adaptation of the method of Solem and Brinck-Johnsen (1964). Briefly, 0.25 ml serum was diluted to 0.5 ml with distilled water (corticosterone standards were prepared in absolute ethanol and diluted to 0.5 ml in distilled water) and extracted with 7.5 ml dichloromethane. A back extraction was performed with 3.5 ml of ethanolic sulfuric acid (7 vols. H_2SO_4 : 3 vols. ethanol), and the acid extracts were allowed to stand for 90 min at room temperature before measuring the fluorescence (excitation wavelength, 470 nm; emission wavelength, 530 nm).

2.8. Statistical analyses

Data were analyzed by one-way analysis of variance and, in case of significance ($P < 0.05$), the F test for significant treatment effects was followed by a two-tailed Student's t-test to compare the ethanol-fed groups with their controls (Snedecor and Cochran, 1967). In the case of comparisons between more than two groups, one-way analysis of variance was followed by the Student-Newman-Keuls test for multiple comparisons.

3. Results

3.1. Alcohol consumption, tolerance and withdrawal

The average daily alcohol consumption of the rats from the ethanol-fed group during the last week of the alcoholization period was 14.1 ± 0.17 g/kg body weight, producing a mean blood alcohol concentration on the last treatment day of 2.55 ± 0.13 mg/ml. Alcoholized rats showed a significant mean weight loss of approximately 32 g in comparison to pair-fed controls (controls: 248 ± 9 g; alcoholized rats: 216 ± 8 g, means ± S.E.M., $n = 8$ in each group, $P < 0.05$). Measurements of core body temperature of animals from the ethanol group as compared to control animals, over a 180 min period following oral gavage of 5 g/kg ethanol to both groups, showed that this acute dose of ethanol induced a marked hypothermia in control rats (maximal ampli-

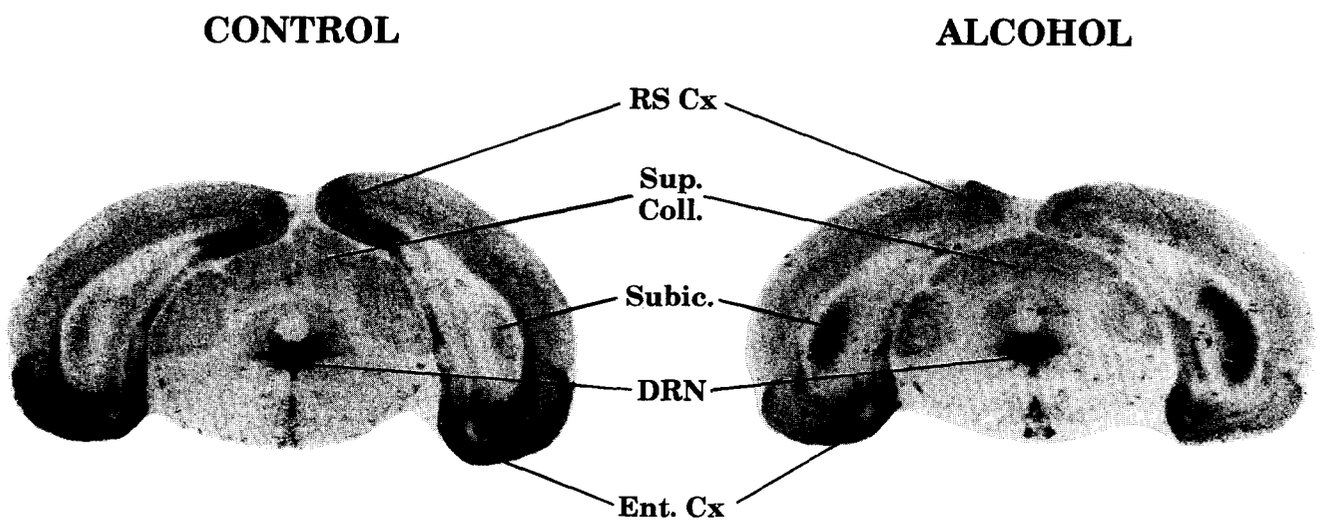

Fig. 1. Autoradiograms of brain sections from a control (left) and an alcoholized (right) rat taken at a level containing both the dorsal raphe nucleus (DRN) and the entorhinal cortex (Ent. Cx), showing the labeling of 5-HT_{1A} binding sites by [^{3}H]WAY-100635. Abbreviations: RS Cx: retrosplenial cortex; Sup. Coll.: superior colliculus; Subic.: subiculum.

Table 1
Chronic ethanol-induced modifications in 5-HT_{1A} receptor binding in various rat brain regions as assessed by quantitative autoradiography

Brain region	Radioligand	Control group (fmol/mg tissue)	Ethanol group (fmol/mg tissue)	% Change
Dorsal raphe	[^{3}H]8-OH-DPAT	117.9 ± 3.1	151.5 ± 5.1 [a]	+28.5
	[^{3}H]WAY-100635	181.6 ± 4.1	243.7 ± 5.1 [a]	+34.2
Frontal cortex (layer IV)	[^{3}H]8-OH-DPAT	43.9 ± 0.8	32.2 ± 0.5 [a]	−26.6
	[^{3}H]WAY-100635	80.5 ± 1.9	50.4 ± 0.9 [a]	−37.4
Entorhinal cortex	[^{3}H]8-OH-DPAT	183.3 ± 4.2	113.8 ± 2.6 [a]	−37.9
	[^{3}H]WAY-100635	254.1 ± 4.8	177.9 ± 4.1 [a]	−30.0
Dentate gyrus (hippocampus)	[^{3}H]8-OH-DPAT	170.7 ± 3.3	140.3 ± 1.4 [a]	−17.8
	[^{3}H]WAY-100635	285.6 ± 4.1	206.8 ± 4.0 [a]	−27.6

Results (in fmol ^{3}H-ligand bound per mg tissue) are the means ± S.E.M. of 5–8 animals per group. The percent change due to chronic alcoholization is indicated in the right-hand column. [a] Ethanol group significantly different from pair-fed controls ($P < 0.05$).

tude: −2.1 ± 0.3°C, mean ± S.E.M., $n = 5$) but not in alcoholized animals (−0.3 ± 0.2°C, mean ± S.E.M., $n = 5$), indicating the development of tolerance to ethanol in the latter group (see Lal et al., 1988; Kleven et al., 1995). Finally, in agreement with previous observations in rats subjected to the same alcoholization protocol (Lal et al., 1988), behavioural alterations typical of mild ethanol withdrawal such as rigidity, caudal tremor and general tremor were noted in alcoholized rats deprived of alcohol since 18 h. At this time, alcohol was no longer detectable in the blood (Kleven et al., 1995).

3.2. Serum corticosterone levels

Eighteen hours after ethanol withdrawal, the serum corticosterone levels of the ethanol-fed group were significantly higher (25.1 ± 3.3 μg/100 ml, mean ± S.E.M., $n = 5$; $P < 0.05$, Student-Newman-Keuls test) than those of the ad libitum-fed control group (12.3 ± 0.9 μg/100 ml, mean ± S.E.M., $n = 5$) and of pair-fed controls (16.8 ± 2.7 μg/100 ml, mean ± S.E.M., $n = 5$). In contrast, the slight difference between the latter two groups was not significant.

3.3. Receptor binding (autoradiographic and membrane studies)

The chronic alcoholization protocol produced significant changes in the labeling of 5-HT_{1A} and 5-HT_{1B} receptors in several brain structures, as assessed by quantitative autoradiography. Notably, increases in the labeling of 5-HT_{1A} binding sites by both [^{3}H]8-OH-DPAT and [^{3}H]WAY-100635 (Fig. 1) were seen in the dorsal raphe nucleus (+28.5% and +34.2%, respectively) of alcoholized rats with respect to pair-fed controls (Table 1). Conversely, significant decreases in [^{3}H]8-OH-DPAT and [^{3}H]WAY-100635 labeling of 5-HT_{1A} receptors were observed in layer IV of the frontal cortex (−26.6% and −37.4%, respectively), in the entorhinal cortex (−37.9% and −30.0%, respectively), and in the dentate gyrus of the hippocampus (−17.8% and −27.6%, respectively) (Table 1). Fig. 1 depicts typical autoradiograms from brain sections taken from a control and an alcoholized rat at the level of the dorsal raphe nucleus and entorhinal cortex, where a concomitant increase in labeling of the raphe and decrease in labeling of the cortex is apparent.

Whereas the [^{125}I]GTI labeling of 5-HT_{1B} receptors was increased in the globus pallidus (+55.0%) of rats from the ethanol group, no changes were observed in the substantia nigra or subiculum as compared to control animals (Table 2).

Receptor binding assays performed on brain membranes revealed significant decreases in the estimated B_{max} of 5-HT_{1A} binding sites labeled by [^{3}H]WAY-100635 in the hippocampus and posterior cortex of rats from the ethanol-fed group as compared to controls (−15.0% and −18.0%, respectively, $P < 0.05$, Fig. 2). In contrast, no difference was found in the estimated B_{max} of 5-HT_{1A} binding sites labeled by [^{3}H]8-OH-DPAT in these structures between the ethanol-fed group and pair-fed control rats (Fig. 2). In addition, neither the specific binding of [^{3}H]WAY-100635 nor that of [^{3}H]8-OH-DPAT were altered in the anterior cortex of alcoholized rats as compared to controls (Fig. 2). Saturation studies with increasing concentrations of [^{3}H]WAY-100635 indicated that only the B_{max} of 5-

Table 2
Chronic ethanol-induced modifications in 5-HT_{1B} receptor binding in various rat brain regions as assessed by quantitative autoradiography

Brain region	Control group (fmol/mg tissue)	Ethanol group (fmol/mg tissue)	% Change
Globus pallidus	4.1 ± 0.1	6.4 ± 0.2 [a]	+55.0
Substantia nigra	9.3 ± 0.5	9.2 ± 0.3	−1.3
Subiculum	10.5 ± 0.5	10.4 ± 0.3	−1.3

Results (in fmol [^{125}I]GTI bound per mg tissue) are the means ± S.E.M. of 5–8 animals per group. The percent change due to chronic alcoholization is indicated in the right-hand column. [a] Ethanol group significantly different from pair-fed controls ($P < 0.05$).

HT_{1A} binding sites was significantly reduced in hippocampal membranes from alcoholized rats (controls: 348.9 ± 19.6 fmol/mg protein; alcoholized rats: 288.6 ± 18.3 fmol/mg protein, means ± S.E.M., $n = 4$, $P < 0.05$) as no change in the K_d value was found between the two groups (controls: 0.12 ± 0.03 nM; alcoholized rats: 0.13 ± 0.02 nM, means ± S.E.M., $n = 4$). Parallel saturation studies with [^{3}H]8-OH-DPAT confirmed that the alcoholization procedure did not alter the characteristics of the corresponding specific binding sites in the same hippocampal membrane preparations as those used in [^{3}H]WAY-100635 binding assays (B_{max} of [^{3}H]8-OH-DPAT specific binding sites = controls: 228.1 ± 17.2 fmol/mg protein; alcoholized rats: 233.2 ± 20.7 fmol/mg protein; K_d = controls: 0.78 ± 0.11 nM; alcoholized rats: 0.69 ± 0.12 nM, means ± S.E.M., $n = 4$).

Membrane binding assays with [^{125}I]GTI for the specific labeling of 5-HT_{1B} receptors showed no significant change in the striatum (controls: 23.8 ± 0.8 fmol/mg protein; alcoholized rats: 23.9 ± 1.1 fmol/mg protein, means ± S.E.M., $n = 5$) as well as in the area of the substantia nigra (controls: 21.3 ± 1.0 fmol/mg protein; alcoholized rats: 21.4 ± 0.7 fmol/mg protein, means ± S.E.M., $n = 5$) after the chronic alcoholization procedure.

3.4. Messenger RNA levels

Quantitative reverse transcriptase-PCR revealed a significant increase (+57.3%, $P < 0.01$) in 5-HT_{1B} receptor mRNA levels in the striatum of alcoholized rats with respect to their pair-fed controls (Table 3). Similarly, an increase in 5-HT_{1A} receptor mRNA levels was found in the anterior raphe area of ethanol-fed rats, whereas, in contrast, a decrease in the levels of this mRNA was noted in the hippocampus of these animals (Table 3). However, these changes did not reach the critical level of statistical significance ($P < 0.05$) because of the large variations in absolute values in both the ethanol-fed group and pair-fed controls.

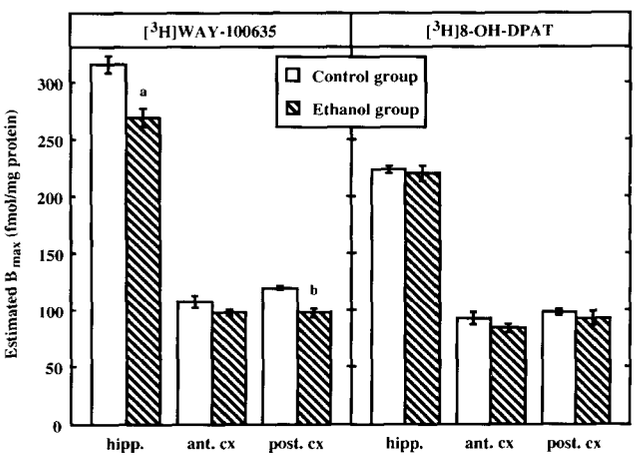

Fig. 2. Chronic ethanol-induced modifications in the estimated B_{max} of 5-HT_{1A} binding sites in various rat brain regions as assessed by membrane binding assays. B_{max} values are expressed in fmol of each [^{3}H]radioligand specifically bound per mg membrane protein. Each bar is the mean ± S.E.M. of five independent determinations. [a,b] Ethanol group significantly different from pair-fed controls ([a] $P < 0.01$, [b] $P < 0.001$). Abbreviations: hipp.: hippocampus; ant. cx: anterior cortex; post. cx: posterior cortex.

Table 3
Chronic ethanol-induced alterations in 5-HT_{1A} and 5-HT_{1B} receptor mRNA levels in various rat brain regions as measured by quantitative reverse transcriptase-PCR

Brain region	Serotonin receptor	Control group	Ethanol group	% Change
Anterior raphe area	5-HT_{1A}	0.71 ± 0.14	1.05 ± 0.21	+47.9
Hippocampus	5-HT_{1A}	0.93 ± 0.07	0.67 ± 0.14	−28.0
Striatum	5-HT_{1B}	8.9 ± 0.9	14.0 ± 0.8 [a]	+57.3

Results (in attomol brain receptor mRNA per μg total RNA) are the means ± S.E.M. of five animals per group. The percent change due to chronic alcoholization is indicated in the right-hand column. [a] Ethanol group significantly different from pair-fed controls ($P < 0.01$).

4. Discussion

The results of this study show that a 2-week period of chronic alcoholization followed by 18 h withdrawal is able to induce changes in the expression of 5-HT_{1A} and 5-HT_{1B} receptors in the rat brain. Autoradiographic experiments revealed that the labeling of somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus was significantly higher in alcohol-treated rats than in their pair-fed controls. In contrast, the labeling of postsynaptic 5-HT_{1A} receptors in certain terminal projection areas of the raphe serotonergic neurons (frontal cortex, entorhinal cortex, dentate gyrus) was decreased in alcoholized rats as compared to controls. These modifications could be due to an enhanced or reduced expression, respectively, of the 5-HT_{1A} receptor gene in these areas, as the levels of the mRNA encoding this receptor tended to increase in the anterior raphe area and to diminish in the hippocampus.

Interestingly, the same alcoholization protocol as that used in the present study was found to affect several of the well-characterized 5-HT_{1A} receptor-mediated symptoms of the '5-HT behavioral syndrome' (Tricklebank et al., 1985). Notably, withdrawal of the liquid diet following the 2-week period of chronic alcoholization provoked a reduction of 8-OH-DPAT-induced flat body posture in alcoholized rats with respect to controls, whereas, on the contrary, the ethanol treat-

ment appeared to enhance the effects of 8-OH-DPAT on lower lip retraction (Kleven et al., 1995). These results fit well with those found in the present study, if one considers the mechanisms of action by which these elements of the 5-HT syndrome are evoked. Indeed, knowing that 5-HT agonists provoke flat body posture by acting on 5-HT_{1A} receptors located *postsynaptically* (Tricklebank et al., 1985), it goes to reason that the down-regulation of postsynaptic 5-HT_{1A} receptors observed in the present study would lead behaviorally to a reduction in the effects of 8-OH-DPAT acting upon these receptors. Similarly, as lower lip retraction is believed to be provoked by activation of *somatodendritic* 5-HT_{1A} autoreceptors (Berendsen et al., 1989), the apparent up-regulation of these receptors noted here would logically account for a sensitization to 8-OH-DPAT for inducing this element of the 5-HT syndrome in alcoholized rats, as observed by Kleven et al. (1995).

Previous studies have shown that corticosterone, through its action at both mineralocorticoid and glucocorticoid receptors, can affect the expression of postsynaptic 5-HT_{1A} receptors in rat forebrain (Mendelson and McEwen, 1992), by decreasing the transcription of the 5-HT_{1A} receptor gene (Chalmers et al., 1993; Meijer and De Kloet, 1994; Tejani-Butt and Labow, 1994). Therefore, the down-regulation of hippocampal and cortical 5-HT_{1A} receptors in alcohol-fed rats might well result from an inhibitory influence of this corticosteroid at the genomic level, as chronic alcoholization (and withdrawal) is known to activate the hypothalamic-pituitary-adrenal axis (Ylikahri et al., 1980; Noth and Walter, 1984; Keith and Crabbe, 1992). Indeed, a significant increase in the serum levels of corticosterone was also noted in rats subjected to the alcoholization protocol in the present study.

In addition to reducing the synthesis of postsynaptic 5-HT_{1A} receptors (Chalmers et al., 1993; Meijer and De Kloet, 1994; Tejani-Butt and Labow, 1994), corticosterone has recently been shown to affect the functional status of somatodendritic 5-HT_{1A} receptors. Thus, a reduced efficacy of 8-OH-DPAT to inhibit the firing of serotonergic neurons through the stimulation of these autoreceptors has been found by Lanfumey et al. (1993) in brain stem slices pretreated with the corticosteroid. Such a functional desensitization of inhibitory 5-HT_{1A} autoreceptors might also contribute to the alcohol-induced changes observed in the present study, since the enhanced secretion of corticosterone in alcoholized rats could thus lead to an increased release of 5-HT at the terminal level, and thence to a possible adaptive down-regulation of postsynaptic 5-HT_{1A} receptors, as that observed in the hippocampus and cerebral cortex. Within the raphe area, serotonergic neurons might attempt to compensate for such a corticosterone-induced alteration in the functional status of somatodendritic 5-HT_{1A} autoreceptors by an increased synthesis of these receptors. In line with this hypothesis, a decrease in the density of somatodendritic 5-HT_{1A} autoreceptors was, in contrast, reported in the dorsal raphe nucleus following corticosterone removal by adrenalectomy in rats (Tejani-Butt and Labow, 1994). In any case, further investigations in adrenalectomized rats and/or in rats treated with metyrapone to suppress corticosterone synthesis (Jenkins et al., 1985) have to be performed in order to investigate the possible involvement of corticosterone in the changes in somatodendritic and postsynaptic 5-HT_{1A} receptors after chronic alcoholization.

The membrane binding data also support a down-regulation of certain populations of postsynaptic 5-HT_{1A} receptors, as the estimated B_{max} of specific 5-HT_{1A} binding sites labeled by the highly selective 5-HT_{1A} antagonist [^{3}H]WAY-100635 (Khawaja et al., 1994; Gozlan et al., 1995) was significantly lower in the hippocampus and posterior cortex of ethanol-treated rats than in their pair-fed controls (with no change in the anterior cortex). However, no decrease was observed in the binding of the agonist radioligand [^{3}H]8-OH-DPAT in any of these structures, in contrast to the autoradiographic data. Discrepancies between the results of binding studies using membranes and those of autoradiographic experiments probably reflect the fact that membranes were prepared from rather large areas, whereas quantitative autoradiography was limited to discrete zones (i.e. dentate gyrus in the hippocampus, layer IV in the frontal cortex) in these areas. Accordingly, alcoholization-induced changes in 5-HT_{1A} receptor expression might be restricted to some neuronal types within the hippocampus and cerebral cortex.

With regard to the differences between the data obtained with [^{3}H]WAY-100635 or [^{3}H]8-OH-DPAT as specific radioligands of 5-HT_{1A} receptors, one must consider the respective binding characteristics of these two molecules. Indeed, [^{3}H]8-OH-DPAT, which is an agonist at 5-HT_{1A} receptors, is as such only able to bind to 5-HT_{1A} receptors as long as they are physically coupled to G-proteins (Emerit et al., 1990). In contrast, the antagonist [^{3}H]WAY-100635 binds with the same high affinity to both the G-protein-coupled 5-HT_{1A} receptors and the free 5-HT_{1A} receptor binding subunits (Gozlan et al., 1995). This explains why higher B_{max} values were found with [^{3}H]WAY-100635 than with [^{3}H]8-OH-DPAT as 5-HT_{1A} receptor radioligand (Gozlan et al., 1995; see also Fig. 2). Thus, the fact that no decrease in binding was observed in the membranes labeled with [^{3}H]8-OH-DPAT, whereas a lower degree of labeling of 5-HT_{1A} receptors by [^{3}H]WAY-100635 was found in certain brain regions of rats from the ethanol group, suggests that the ethanol treatment modified the equilibrium between G-protein-coupled

and free 5-HT_{1A} receptors. Indeed, as the ratio of [^{3}H]8-OH-DPAT binding to that of [^{3}H]WAY-100635 is an index of the proportion of 5-HT_{1A} receptor binding subunits coupled to G-proteins in a given membrane preparation (Gozlan et al., 1995), the significantly ($P < 0.05$) higher values of this ratio in the hippocampus and posterior cortex of ethanol-fed rats (0.81 ± 0.03 and 0.93 ± 0.03, respectively; see Fig. 2) than of pair-fed controls (0.71 ± 0.02 and 0.80 ± 0.02, respectively) support the idea that the relative coupling of 5-HT_{1A} receptors to G-proteins increased in chronically alcoholized animals. As the G-protein-coupled receptors are the functional receptors (Kobilka, 1992), such an increased coupling might compensate for the decreased synthesis of 5-HT_{1A} receptor binding subunits in these two regions. However, this mechanism might not compensate for the inhibitory influence of alcoholization on the synthesis of 5-HT_{1A} receptors in all forebrain areas since not only the specific binding of [^{3}H]WAY-100635 but also that of [^{3}H]8-OH-DPAT was found to be significantly reduced within the layer IV of the frontal cortex, for instance, in alcoholized rats. Further investigations aimed at directly assessing the coupling of 5-HT_{1A} receptor binding subunits with G-proteins have to be performed to possibly substantiate the inference, only based on binding data, of changes in this coupling after chronic alcoholization and/or withdrawal.

Another noteworthy finding of this study concerns the 5-HT_{1B} receptor. Thus, autoradiographic analyses revealed an increased labeling of this receptor in the globus pallidus. Interestingly, this increase corresponded to a concomitant, significant elevation of 5-HT_{1B} receptor mRNA levels in the striatum; this latter finding is not unexpected, given the predominant localization of 5-HT_{1B} receptors in the globus pallidus on the terminals of neurons whose cell bodies are situated in the caudate putamen (Boschert et al., 1994). Peculiarly, however, no significant increase in 5-HT_{1B} receptor labeling was noted in the substantia nigra, where 5-HT_{1B} receptors are also predominantly located on the terminals of neurons originating in the striatum (Hamon et al., 1990; Boschert et al., 1994). This would suggest that striato-pallidal and striato-nigral neurons are differently affected by the chronic alcoholization and withdrawal procedure used in the present study. Furthermore, as 5-HT_{1B} receptors are involved in local controls of neurotransmitter (γ-aminobutyric acid, substance P, dynorphin, enkephalins, etc.) release from the terminals of these neurons (Boschert et al., 1994), the present data also suggest that differential alterations in these controls might occur in the globus pallidus versus the substantia nigra (and subiculum, see Table 2).

In conclusion, marked alterations in the expression of 5-HT_{1A} and 5-HT_{1B} receptors were found in various brain areas of chronically alcoholized rats after an 18 h withdrawal. Because of the important role of some of these receptors, especially the somatodendritic 5-HT_{1A} autoreceptors and the presynaptic 5-HT_{1B} receptors, in the control of the synthesis and release of 5-HT and other neurotransmitters (see Hoyer et al., 1994), one can wonder whether these alterations can account for at least some of the various effects of alcoholization on neurotransmitter metabolism and function in the central nervous system (Samson and Harris, 1992; Nutt and Peters, 1994; Nevo and Hamon, 1995).

Acknowledgements

This research was supported by grants from IREB (contract No. 94/05), INSERM, DRET (contract No. 93/047/BC), MESR (contract No. 92.C.0412) and the European Community (Biotech No. BIO2-CT93-0179). We are grateful to the Service des Molécules Marquées of the CEA (Gif-sur-Yvette, France), Dr. J. Chauveau (Immunotech, Marseille, France) and Dr. Colin T. Dourish (Wyeth Research, Taplow, UK) for their generous gifts of [^{3}H]8-OH-DPAT, [^{125}I]GTI and [^{3}H]-WAY-100635, respectively. Pharmaceutical companies (Abbott, Pfizer) are gratefully acknowledged for generous gifts of drugs. L.L. was supported by IVIC, CONICIT and the Fondation pour la Recherche Médicale.

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