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Neurochemical Effects of Long-Term Ingestion of Ethanol by Sinclair (S-1) Swine

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HARRIS, R A, D FENNER, D FELLER, G SIECKMAN, S LLOYD, M MITCHELL, J D DEXTER, M E TUMBLESON AND D B BYLUND *Neurochemical effects of long-term ingestion of ethanol by Sinclair (S-1) swine* PHARMACOL BIOCHEM BEHAV 18(3) 363–367, 1983 —Sinclair (S-1) miniature swine were given access to a mixture of ethanol and beer for three years Control swine were fed an isocaloric diet with corn starch substituted for ethanol Both groups had free access to tap water The alcohol group consumed about 4 g ethanol/kg/day (about 50% of their caloric intake) resulting in plasma ethanol concentrations of about 100 mg/dl Brain membranes were prepared for analysis of neurotransmitter receptor binding, membrane lipid composition and physical properties Receptor studies demonstrated an increase in the binding of ^{3}H-GABA to cortical and cerebellar membranes from the alcoholic pigs as compared to control Binding of ^{3}H-ligands to muscarine cholinergic, β-adrenergic, α-adrenergic, dopamine and benzodiazepine receptors was not changed by chronic ethanol ingestion These results are similar to those obtained in studies of human alcoholics by other investigators The cholesterol content of myelin and synaptic plasma membranes was not altered by ethanol consumption The fluorescence polarization of diphenylhexatriene, a measure of membrane order, did not detect any differences in the membranes from control or alcohol-treated swine either before or after *in vitro* exposure to ethanol These results are different from those reported for rodents after short-term ethanol treatments and emphasize the importance of evaluating different models of experimental alcoholism

Alcoholism Neurotransmitter receptors GABA Membrane fluidity

THE neurochemical effects of chronic alcohol consumption have been the subject of many recent investigations Two of the main areas of interest are neurotransmitter receptor binding, and membrane composition and physical properties Administration of ethanol to mice or rats for one or two weeks increases muscarinic cholinergic receptors in brain [15,22], although this change is not seen after four days of treatment [10] Treatment with ethanol for four to nineteen days does not alter rodent brain α-adrenergic, benzodiazepine, γ-aminobutyric acid (GABA), β-adrenergic or dopamine receptor binding [7, 10, 12, 15, 21, 23] However, β-adrenergic receptor binding is decreased in rat brain and heart after consumption of ethanol for 60 days [1] and mouse brain benzodiazepine receptor binding is decreased after 7 months of alcohol ingestion [7] In addition, increased GABA receptor binding is observed in brains of human alcoholics [24] These results suggest that prolonged ethanol consumption may produce changes in neurotransmitter receptor binding which are not observed after short-term treatment

Some changes in rodent membrane physical properties and composition have been reported Consumption of ethanol increases synaptic plasma membrane (SPM) cholesterol [2], alters the acyl composition of SPM phospholipids [13], increases the rigidity of SPM [19] and decreases the perturbation of brain membranes produced by *in vitro* exposure to ethanol [3]

With the exception of the study of GABA binding in human alcoholics and benzodiazepine binding in mice, the studies discussed above used force consumption of ethanol for short periods of time as a model of chronic ethanol exposure The relevance of these paradigms to human alcoholism remains controversial An animal model which incorporates several aspects of human alcoholism is the Sinclair (S-1) miniature swine This species will voluntarily consume intoxicating quantities of ethanol over a long period of time [4,25] Neurochemical correlates of alcohol dependence have not, however, been reported for this paradigm The purpose of the present study was to investigate the effects of long-term consumption of alcohol on neurotransmitter receptor binding, and membrane composition and physical properties For these studies, we obtained brain tissue from two groups of swine one which had voluntarily consumed ethanol for 3 years (alcohol group) and one (control group) whose caloric, carbohydrate and protein-intake was matched to that of the alcohol group

TABLE 1

CONDITIONS FOR BINDING OF RADIOACTIVE LIGANDS TO BRAIN MEMBRANES FROM MINIATURE SWINE

^{3}H-Ligand	Buffer	Assay Vol (ml)	Protein (mg/ml)	Assay Temp (°C)	Assay Time (min)	Conc Range Liquid (nM)	Blank (μM)
Prazosin	40 mM Tris pH 7 4	1	0 25	25	30	0 2 -6	(-)NE (100)
p-Aminoclonidine	40 mM Tris pH 7 4	1	0 12	25	30	0 1 -2	(-)NE (1)
Yohimbine	25 mM Glygly* pH 7 4	1	0 08–0 12	25	30	0 1 -1	(-)NE (10)
Spiroperidol	40 mM Tris pH 8	2	0 033	25	60	0 05–7	(+)Butaclamol (1)
Quinuclidinyl benzilate	40 mM Tris pH 8	1	0 03–0 05	25	30	0 2 -3	Atropine (1)
Dihydroalprenolol	40 mM Tris pH 8	1	0 25	25	30	0 1 -2	(-)Propranolol (0 3)
Flunitrazepam	40 mM Tris-citrate pH 7 2	0 5	0 25–0 5	4	15	0 3 -10	Diazepam (10)
γ-Aminobutyric Acid (GABA)	40 mM Tris-citrate pH 7 2	0 5	0 25–0 5	4	15	8 -64	GABA (100)

*Glycylglycine

METHOD

Animals

Male and female Sinclair (S-1) miniature swine (9 months of age at the beginning of the study) were housed individually The lifespan of these animals is about 12 years Initial body weights averaged 36 kg for the control group and 31 kg for the alcohol group Final body weights were 70 kg (control) and 51 kg (alcohol) The ethanol group was given free access to 10% w/v ethanol in beer Beer was delivered by Lixit spouts which reduced spillage to about 10% of the total consumption Spillage was collected in a pan under the spout and measured daily Both ethanol and control groups were allowed free access to tap water and were given 53 g of protein/day in addition to 500 g/day pig chow To compensate for the calories derived from ethanol and beer, the control group was given 450 g/day of corn starch Animals were maintained on their respective regimens for 35–37 months They consumed about 4 g of ethanol/kg/day which was equivalent to about 50% of their caloric intake Plasma ethanol concentrations were determined weekly (2–4 p m) and averaged about 140 mg/dl for males and about 100 mg/dl for females for the last 12 weeks of the study The animals consumed about 50% of their daily ethanol between 8 a m and 2 p m and blood ethanol levels were near maximal at the time of measurement These data will be presented in detail elsewhere (Tumbleson and Dexter, in preparation) Animals were not withdrawn from ethanol and the blood ethanol concentrations at the time of death (9–11 a m) was 50 to 100 mg% The animals showed no signs of withdrawal The swine were given 12 mg/kg ketamine HCl 0 088 mg/kg atropine sulfate and sufficient thiamytal sodium to produce light anesthesia They were killed by exsanguination and decapitated The brain was removed, placed on ice and dissected The brain regions were weighed and homogenized for membrane preparation as described below

Membrane Preparation

All steps were done at 0–5°C For receptor binding assays, tissue was homogenized for 30 sec in 50 mM Tris HCl (pH 7 4) with a Tissumizer (Tekmar Co , Cincinnati OH) and centrifuged at 48,000×g for 10 min The supernatant was discarded and the pellet was homogenized again This suspension was centrifuged as before and the pellet was again homogenized This suspension was centrifuged as before, and the pellet was stored at −70°C For binding studies, the membranes were suspended in the appropriate buffer (Table 1) and used without further treatment, except in the case of GABA receptor binding For GABA binding, membranes were homogenized with 0 01% Triton X-100 and incubated at 37° for 30 min [5] Membranes were pelleted and resuspended in 50 mM Tris citrate (pH 7 2) buffer for the binding assay

For analysis of membrane lipid composition and physical properties, brain tissue (cerebellum and cortex) was homogenized in 0 32 M sucrose with 10 mM HEPES, pH 7 4 Myelin and synaptic plasma membranes (SPM-2) were prepared by discontinuous gradient centrifugation as described previously [6,8]

Receptor Binding

The binding of tritiated ligands (New England Nuclear, Boston, MA) to brain membranes was measured by incubating the membrane with 5–7 concentrations of each ligand Each membrane was assayed in duplicate or triplicate Radioactivity bound to the membranes was determined after filtration (Whatman GF/B filters) and washing for all ligands except GABA, which was determined by centrifugation (30,000×g for 10 min) Nonspecific binding was determined by addition of an appropriate excess of a competing ligand The assay conditions used for each ligand are given in Table 1 The number of binding sites (B_{max}) and the affinity of binding

TABLE 2

RECEPTOR DENSITY (B_{Max}) AND AFFINITY (K_D) IN MINIATURE SWINE BRAIN AFTER CHRONIC CONSUMPTION OF ALCOHOL

Receptor	Ligand	Brain Area	K_D (nM) Control	K_D (nM) Alcohol	B_{Max} (pmol/mg Protein) Control	B_{Max} (pmol/mg Protein) Alcohol
Benzodiazepine	Flunitrazepam	Cortex	5 4 ± 0 9	5 0 ± 0 3	2 0 ± 0 2	2 4 ± 0 1
		Cerebellum	5 5 ± 0 3	5 6 ± 0 4	1 0 ± 0 1	1 0 ± 0 1
Muscarinic	QNB	Caudate	0 09 ± 0 01	0 11 ± 0 02	1 3 ± 0 2	1 5 ± 0 1
Dopamine	Spiroperidol	Caudate	0 03 ± 0 02	0 03 ± 0 03	0 58 ± 0 06	0 63 ± 0 02
β-Adrenergic	DHA	Cerebellum	0 28 ± 0 02	0 32 ± 0 02	0 13 ± 0 01	0 15 ± 0 01
α-Adrenergic	Yohimbine	Cortex	0 27 ± 0 02	0 26 ± 0 02	0 17 ± 0 02	0 16 ± 0 02
	PAC	Cortex	1 2 ± 0 1	1 1 ± 0 1	0 08 ± 0 01	0 08 ± 0 01
	Prazosin	Cortex	0 13 ± 0 02	0 10 ± 0 01	0 22 ± 0 01	0 23 ± 0 02

Values represent mean ± SEM N=8–9 There are no significant differences between alcohol and control

(K_D) were determined by a Rosenthal [18] analysis of the binding curves

Membrane Composition and Physical Properties

Lipids were extracted from SPM and myelin membranes of cortex and cerebellum as described previously [6] The cholesterol content was determined by gas chromatography [6] The "fluidity" of the SPM and their sensitivity to *in vitro* exposure to ethanol was studied using diphenylhexatriene (DPH) as a fluorescent probe of the membrane core [8] Fluorescence polarization of DPH was measured as described previously [8] at 37°C using an HH-1 spectrofluorimeter (BHL Associates, Burlingame, CA)

Other Assays

Protein was determined by the method of Lowry [14] using BSA as the standard

RESULTS

Receptor Binding

Chronic consumption of ethanol increased the binding of ^{3}H-GABA to brain membranes In the cortex and cerebellum of female swine, the increased binding was due to a decrease in K_D with no change in B_{max} (Fig 1) In male swine, the K_D was decreased in cerebellum while B_{max} was increased in cortex For the control group, there was a clear sex difference in ^{3}H-GABA binding, with females displaying twice as many binding sites as males for both cortex and cerebellum The binding affinity did not differ between males and females (Fig 1) With the concentrations of ^{3}H-GABA used in the present study, we detected only 1 population of binding sites

In contrast to the changes in ^{3}H-GABA binding, chronic alcohol consumption did not alter the binding of ligands for benzodiazepine, muscarinic cholinergic, dopamine, β-adrenergic or α-adrenergic receptors (Table 2) The data in Table 2 are the average of values obtained from both male and female swine When analyzed separately, there was no significant difference between males and females

Cholesterol Content of Brain Membranes

The cholesterol content of SPM and myelin was determined (Table 3) Chronic alcohol ingestion did not alter the cholesterol content of SPM or myelin from either cortex or cerebellum In addition, chronic alcohol consumption did not change the acyl composition of SPM or myelin (not shown)

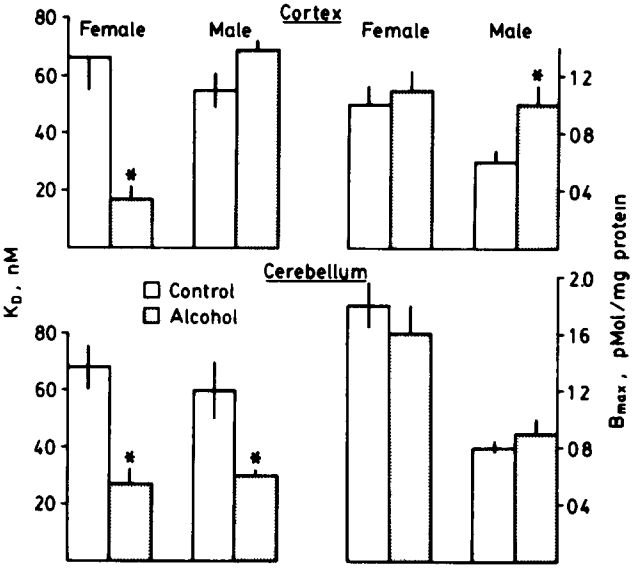

FIG 1 Effects of chronic alcohol treatment in the binding of ^{3}H-GABA to brain membranes from S-1 miniature swine The left half represents binding affinity (K_d, nM), the right half represents the number of binding sites (F_{max}, pmol/mg protein) Values in the upper panels were obtained from cortical membranes, values in the lower panels from cerebellar membranes Open bars represent control animals, shaded bars represent chronic alcohol Values are mean±SEM, n = 8–9 *indicates significantly different from control, $p<0$ 02

Membrane Physical Properties

The fluorescence probe molecule DPH was used to measure the order of the hydrophobic core of SPM Fluorescence

TABLE 3

EFFECTS OF CHRONIC ALCOHOL CONSUMPTION ON THE CHOLESTEROL CONTENT OF BRAIN MEMBRANES FROM MINIATURE SWINE

Membrane	Brain Region	μ Mol Cholesterol/mg Protein Control	Alcohol
SPM	Cortex	0 52 ± 0 04	0 52 ± 0 03
	Cerebellum	0 49 ± 0 03	0 51 ± 0 05
Myelin	Cortex	0 69 ± 0 04	0 59 ± 0 04
	Cerebellum	0 73 ± 0 05	0 66 ± 0 05

Values are mean ± SEM, n=8–9

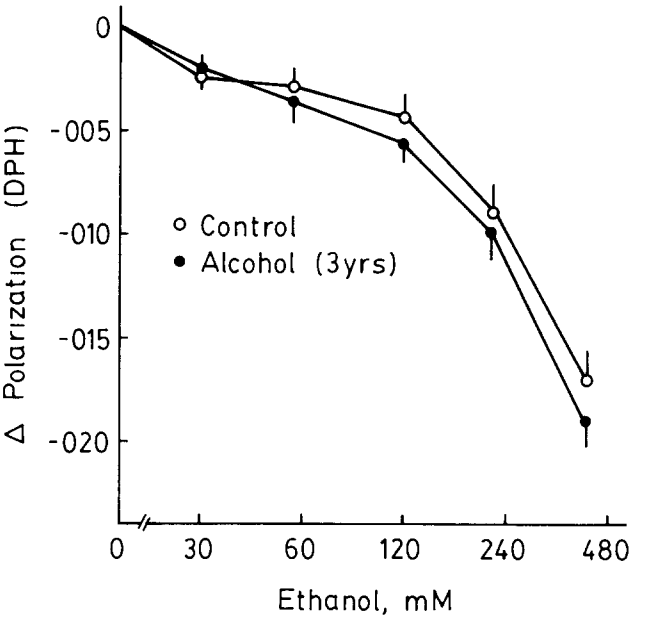

FIG 2 Effects of *in vitro* addition of ethanol on the fluorescence polarization of DPH in synaptic plasma membranes from S-1 miniature swine The abscissa represents the concentration (mM) of ethanol added, the ordinate represents the change in fluorescence polarization produced by each addition Open circles represent membranes from control swine filled circles represent membranes from chronic alcohol swine Values represent mean±SEM n = 8–9

polarization values indicated that chronic alcohol exposure did not affect the rigidity of the membranes (control, 0 256±0 005, alcohol, 0 259±0 004) *In vitro* exposure to ethanol decreased fluorescence polarization, indicating a disordering of the membrane (Fig 2) The perturbation produced *in vitro* by ethanol was the same for membranes from both control and alcohol swine The data in Fig 2 represent an average of values from male and female swine and from cortex and cerebellum Separate analysis of each sex and brain region (by analysis of variance for repeated measures) indicated no significant effect of chronic alcohol exposure

DISCUSSION

These results demonstrate an increase in GABA receptor binding in S-1 miniature swine brain following long-term consumption of ethanol This change was selective for GABA receptors as binding of ligands to adrenergic, cholinergic, dopamine and benzodiazepine receptors was not affected by alcohol consumption These results are very similar to those obtained by Tran *et al* [24] in studies of human alcoholics These investigators reported an increase in the number of GABA binding sites in cortical tissue from male alcoholics, while cholinergic β-adrenergic and benzodiazepine binding were not altered (A small decrease in β-adrenergic receptors was observed, but this was attributed to post-mortem changes) Likewise, we observed an alcohol-induced increase in the number of GABA binding sites in cortical tissue from male swine, the increased binding in cerebellar tissue from males and in cortical and cerebellar tissue from females was due to enhanced binding affinity Interpretation of these results is complex The GABA-receptor-ionophore contains several subunits, including benzodiazepine and barbiturate binding sites [16] An increase in GABA receptor binding might be expected to enhance GABA action *in vivo*, but it is important to note that it is necessary to remove endogenous inhibitors of GABA receptor binding prior to the binding assay If alcohol consumption affects the level of these inhibitors, then the binding observed *in vitro* may not reflect binding *in vivo* In the range of ligand concentrations used in this study and tested by Tran *et al* [24] there was evidence of only one population of binding sites, but it may be possible to detect other sites using a wider range of ligand concentrations [5] Regardless of the interpretation, it is important to note that the changes produced by long-term alcohol consumption in miniature swine are similar to those found in human alcoholics This is in contrast to studies with rats where 21 days of alcohol exposure did not alter GABA receptor binding [17 23] Likewise, the selective effect on GABA receptors is in contrast to the decrease in β-adrenergic receptor binding noted after 60 days of alcohol consumption by rats [1], the decrease in benzodiazepine binding reported in mice after 7 months of alcohol exposure [7], and the increase in muscarinic cholinergic receptor binding found in mice after 7 days of alcohol consumption [22]

In contrast to the studies of rodents and humans, animals in our study were anesthetized with a mixture of ketamine, atropine and thiamytal It is unlikely that these drugs altered the receptor binding because membranes were washed extensively prior to the *in vitro* studies Furthermore addition of these drugs directly to the assay solution did not alter the binding of ^{3}H-GABA under the conditions of the present study ([26] and unpublished results) Atropine can compete with QNB for muscarinic binding sites but in view of the high density of ^{3}H-QNB sites observed in the present study it is doubtful that administration of atropine reduced receptor binding

Changes in membrane lipid composition and physical properties have been suggested as mechanisms for tolerance and dependence [9] It is, however, unlikely that changes in membrane lipids were responsible for the increase in GABA receptor binding observed in the present study Analysis of the cholesterol content of synaptic and myelin membranes did not detect any effect of chronic alcohol consumption Likewise, chronic ingestion of alcohol failed to change the physical properties of synaptic membranes Fluorescence

polarization of DPH indicated that neither the intrinsic order of the membranes nor their sensitivity to the disordering effect of ethanol were altered by chronic ethanol exposure These results are also in contrast to the effects of short-term ethanol treatment in rodents, where changes in acyl saturation, cholesterol content and ethanol-induced membrane fluidization have been observed [2, 3, 11, 13, 19] Although several of the studies [11,13] of rodents have used a crude synaptosomal preparation, it is possible that our preparation of synaptic membranes was not sufficiently pure to allow detection of small changes in SPM Our membrane isolation procedure is well suited for rodent brain tissue [6], but the purity of the membranes obtained from swine brain has not been characterized In addition, there may be species differences in the responses of brain membranes to alcohol treatment, for example, the changes in lipid acyl composition measured by Sun and Sun [20] in guinea pig brain differ markedly from those reported by Littleton *et al* [13] for mouse brain Neurochemical responses to ethanol may also depend upon the length and level of exposure Studies with rodents have used techniques which achieve high (200–400 mg/dl) blood ethanol concentrations for a few days In contrast, the technique used in the present study maintained moderate blood ethanol (50–200 mg/dl) for three years Although there is evidence for tolerance and dependence in swine consuming these quantities of ethanol [4,25], it is possible that the degree of tolerance and dependence is not as great as that achieved during short-term treatment of rodents Perhaps the present techniques can only detect membrane changes which accompany a very high degree of tolerance and dependence Regardless of the reasons for the differences between the present results and those obtained in other studies, our findings emphasize the need for caution in extrapolating results from short-term studies in rodents to long-term consumption in other species, including human alcoholics

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