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EFFECT OF PRENATAL ALCOHOL EXPOSURE ON ^{3}H-DIAZEPAM BINDING TO CEREBRAL CORTICAL SYNAPTIC MEMBRANES AT VARIOUS STAGES OF POSTNATAL DEVELOPMENT

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KEY WORDS: prenatal period; rat cerebral cortex; ethanol; benzodiazepine receptors

According to clinical observations, alcoholism in the parents leads to the appearance of cerebral developmental disturbances in children, including oligophrenia and delayed mental development [16]. Experiments on animals have shown that intrauterine alcoholization induces a combination of disturbances in postnatal development. The animals concerned are retarded in growth and weight [12], development of various parts of their brain is delayed [11], and metabolic changes arise [2], and all these disturbances are accompanied by impairment of formation and preservation of conditioned reflexes [4].

One possible cause of the effects of ethanol may be its interaction with the benzodiazepine system of the brain. In the first place ethanol, like benzodiazepines, has an anxiolytic, sedative, and hypnotic action [8]; the development of behavioral cross-tolerance between ethanol and benzodiazepines has been noted in animals [5]. Second, it has been shown that changes at the benzodiazepine receptor level in the brain of pregnant rats lead to specific changes in the analogous receptor system and behavior of the offspring [7, 15].

It was accordingly decided to study the effect of prenatal exposure of rats to ethanol on ^{3}H-diazepam binding with synaptic membranes of the brain in the offspring.

EXPERIMENTAL METHOD

Pregnant albino rats from the 5th to the 20th days of pregnancy were given 2.5-3 ml of 40% ethanol daily by gastric tube. Instead of alcohol, control animals received the equivalent volume of water. When the offspring of both groups (males) reached the age of 14 days (or 2 months) they were decapitated between 10 a.m. and 12 noon, the cerebral cortex was removed and homogenized in a Potter's homogenizer with Teflon pestle (25-30 transmissions) in 20 volumes of isolation medium, consisting of 0.32 M sucrose, 0.05 M Tris-HCl (pH 7.4), and 1 mM EDTA. The homogenate was centrifuged for 10 min at 1000g, the residue was discarded, and the supernatant was centrifuged for 20 min at 20,000g. The residue was suspended in 20 ml of 0.05 M Tris-HCl (pH 7.4) and frozen overnight at −8°C. Next day the material was sedimented by centrifugation at 20,000g for 20 min and the residue was again washed with 0.05 M Tris-HCl (pH 7.4) under the same conditions. The coarse fraction of synaptic membranes thus obtained was resuspended in 18 ml of 0.05 M Tris-HCl (pH 7.4).

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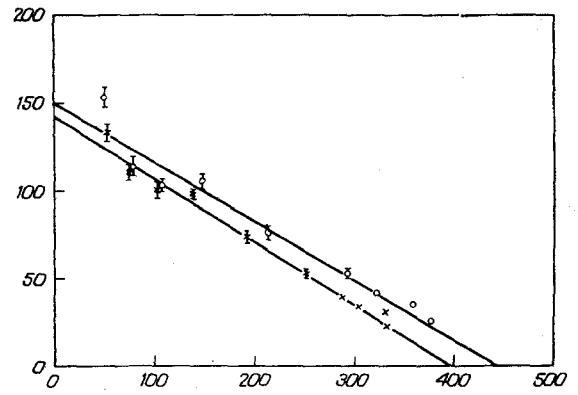

Fig. 1

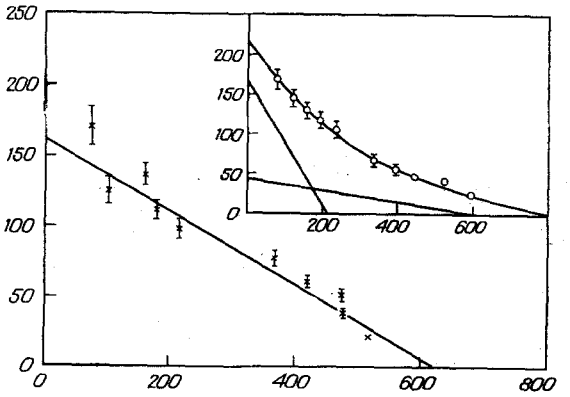

Fig. 2

Fig. 1. Scatchard plot for binding of ^{3}H-diazepam with brain synaptic membranes of rats aged 14 days whose mothers were given alcohol during pregnancy. Abscissa, specific binding (in fmoles/mg protein); ordinate, ratio of specific binding/unbound label (in fmoles/mg protein·nM). Circles — control, crosses — experiment.

Fig. 2. Scatchard plots for ^{3}H-diazepam binding with brain synaptic membranes of rats aged 2 months whose mothers were given alcohol during pregnancy. Inset: analysis of experimental points into two linear components (see text). Legend as to Fig. 1.

The reaction of binding of ^{3}H-diazepam with benzodiazepine receptors was started by addition of 0.4 ml of a suspension of membranes to 0.1 ml of an aqueous solution of ^{3}H-diazepam (90 Ci/mmole, Amersham International, England) and it was stopped after 30 min by filtration of the sample through a GF/B filter, and washing three times with 2 ml of cold 0.05 M Tris-HCl (pH 7.4): 10 points were chosen within the concentration range 0.3-30 nM, three parallel determinations were made at each point, the filtration time for one sample was 5 sec, and incubation was carried out at a temperature of 1-2°C. The dried filters were placed in flasks containing 8 ml of ZhS-8 liquid scintillator (Reakhim, USSR) and radioactivity was counted on a "RackBeta" liquid scintillation counter (LKB, Sweden). To account for nonspecific binding, dependence of binding of ^{3}H-diazepam with the synaptic membrane fraction was studied in the presence of $4 \cdot 10^{-7}$ M unlabeled 7-bromodesmethyldiazepam, the binding inhibition constant of which is 3.4 nM [1]. For a concentration of 0.3 nM ^{3}H-diazepam, nonspecific binding did not exceed 8%, whereas for a concentration of 30 nM it did not exceed 30% of total binding. In each experiment the slope of the graph of nonspecific binding was verified at several points, and correction also was introduced for the protein concentration. Specific binding was then calculated as the difference between total and nonspecific binding, determined from the slope of the corresponding graph (this method of determination of nonspecificity under conditions of standardized isolation and binding procedures gives a smaller error than the traditional method). The protein concentration, calculated by the method in [10], varied from 0.2 to 0.4 mg per sample.

Each experimental and control group consisted of five or six animals (one or two rats were taken from each litter).

The binding data were averaged and displayed on a Scatchard plot. The number of accessible binding sites (B_{max}) and affinity (K_d) were calculated. The significance of differences was estimated by Student's test. For graphic interpretation of the data the coefficient of correlation was calculated with linear, exponential, and natural logarithmic functions by the method of least squares. Analysis of the curve corresponding to the group of control animals aged 2 months was carried out by the method in [3].

EXPERIMENTAL RESULTS

To compare the results for binding between different groups of animals, the method of graphic interpretation of the data on a Scatchard plot had to be assessed. Coefficients of correlation with linear and nonlinear functions were therefore calculated for all the graphs. On the basis of the values of these coefficients, data relating to groups of 2-week-old and experimental 2-month-old rats were approximated by linear regression (Figs. 1 and 2). Mean-

while the Scatchard plot for the group of control 2-month-old animals was biphasic in character (r for linear regression was 0.959, and for exponential and natural logarithmic functions it was 0.985 and 0.988, respectively).

Prenatal alcoholization led to some decrease (11.2%; $p < 0.05$) in the number of accessible binding sites without any significant change in its affinity in animals aged 14 days (control: B_{max} = 442.2 ± 18.7 fmoles/mg protein, K_d = 2.96 ± 0.10 nM; experiment: B_{max} = 392.5 ± 15.0 fmoles/mg protein, K_d = 2.80 ± 0.10 nM). With age, binding of ^{3}H-diazepam increased, in agreement with data in the literature [14]. The level of binding in rats aged 2 months belonging to both subgroups was 1.5 times higher than in rats aged 2 weeks, if a monomolecular mechanism of ligand-receptor binding (the straight line on the Scatchard plot) is accepted. In this case a tendency remains for the number of accessible binding sites to fall in the group of experimental animals (control: B_{max} = 626.5 ± 45.3 fmoles/mg protein, K_d = 3.68 ± 0.13 nM; experiment: B_{max} = 608.3 ± 34.9 fmoles/mg protein, K_d = 3.73 ± 0.22 nM).

However, as was pointed out above, the Scatchard plot for the group of control 2-month-old rats is biphasic in character, and it evidently corresponds to a more complex mechanism of binding. When concave Scatchard plots are interpreted, a model of two independent binding sites is most frequently used. On the one hand, this choice is determined by the fact that under conditions of invariable experimental binding parameters (pH, temperature, protein concentration) it is impossible to express preference for any particular model, and on the other hand, the model indicated enables the maximal level of binding and the quantitative ratio between high- and low-affinity components to be estimated. Using the method in [3], the curve for the corresponding group of 2-month-old control animals was resolved into two linear components: high-affinity (B_{max} = 209.6 ± 12.1 fmoles/mg protein, K_d = 1.27 ± 0.7 nM; 26.2%) and low-affinity (B_{max} 590.4 ± 34.0 fmoles/mg protein, K_d = 13.3 ± 0.78 nm; 73.8%) with a total binding level of 800.0 ± 46.1 fmoles/mg protein (see Fig. 2, inset).

Thus whereas in 2-week-old rats exposed to prenatal alcoholization differences in ^{3}H-diazepam binding compared with the control were small, in animals aged 2 months they reached 24% ($p < 0.05$) and were not only quantitative, but also qualitative in character.

It is evident that a decrease in the number of benzodiazepine receptors ought to lead to general disinhibition. In fact, in rats exposed in utero to alcohol, motor activity and excitability were increased and the threshold of predisposition to convulsions lowered [13]. We also know from clinical observations that delay of mental development associated with antenatal exposure to alcohol is often accompanied by a hyperdynamic syndrome, and also by emotional impoverishment of behavior. This latter effect may be linked with qualitative readjustments at the level of the brain benzodiazepine receptors, for the ratio between the numbers of subtypes of receptors affects the emotional component of behavior [9].

Significant changes in ^{3}H-diazepam binding in prenatally alcoholized animals toward the age of 2 months evidently acquire parameters which persist during later development. Data on completion of development of benzodiazepine receptors toward the 21st day of postnatal development can serve as an argument in support of this suggestion. In the course of further development neither the over-all binding level nor the ratio between high- and low-affinity components changes [6].

Intrauterine exposure to alcohol thus leads to considerable modifications to the benzodiazepine receptors of the brain, to a disturbance of their normal functioning, and this may be the cause of some of the abnormalities accompanying delay of mental development.

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POSITIVE MODULATION OF DIAZEPAM ACTIVITY IN ALCOHOLIZED RATS BY CORTEXOLONE

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KEY WORDS: diazepam; cortexolone; ethanol

It was shown previously that glucocorticoids reduce, whereas antagonists of glucocorticoid receptors enhance the anxiolytic activity of diazepam [2]. Also we know that certain psychopathological disorders, including depressive states and chronic alcoholism, are accompanied by marked glucocorticoid hypersecretion with disturbance of regulatory feedback mechanisms [1, 4]. At the same time the effectiveness of tranquilizers of the benzodiazepine series is considerably weakened in rats with established physical dependence on ethanol and in the withdrawal state [1]. Doses of diazepam required to reduce the ethanol consumption in rats with physical dependence on it are correspondingly greatly increased by comparison with those for animals at the stage of formation of alcohol motivation [1].

To test the hypothesis of the antitranquilizing action of endogenous glucocorticoids in individuals with chronic alcoholism, experiments were carried out to study the effect of cortexolone — a glucocorticoid antagonist at the receptor level — on the effectiveness of diazepam, assessed on the basis of its tranquilizing activity and ability to reduce voluntary consumption of ethanol.

EXPERIMENTAL METHOD

Male rats weighing 450-500 g, consuming ethanol for 10 months, were used in the experiments. The anxiolytic activity of diazepam was studied by the method of motivated intraspecific aggression, which is a variant of the conflict situation, based on fighting between a pair of rats for the safe place on an electrode floor through which painful electric shocks are applied to the limbs [2]. Diazepam (Polfa) in a dose of 1 mg/kg and cortexolone (Calbiochem) in a dose of 20 mg/kg were injected intraperitoneally 30 and 45 min, respectively, before testing, in the form of a suspension with Tween-80. Animals of the control group received distilled water in equal volumes 30 min before the beginning of the experiment.

Before the experiments, animals consuming ethanol for not less than 10 months were placed in individual cages with free access to water and 15% ethanol solution, and for 14

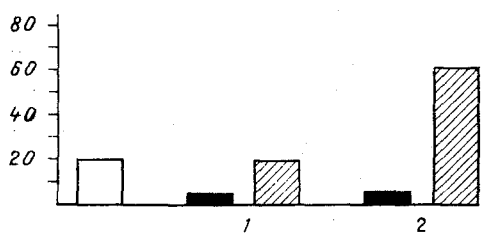

Fig. 1. Effect of acute (1) and chronic (2, for 5 days) administration of cortexolone on anxiolytic activity of diazepam in alcoholized rats. Abscissa: unshaded column — diazepam (1 mg/kg), black columns — cortexolone (20 mg/kg), shaded columns — diazepam (1 mg/kg) + cortexolone (20 mg/kg); ordinate, anxiolytic effect (in %).

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