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Chronic Effect of Ethanol Ingestion on Flunitrazepam Binding to GABA Receptors of Rat Brain Synaptosoms

Effect of ethanol on GABA receptors

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Abstract:

Purpose*: Changes in the CNS functions associated with ethanol ingestion are suggested to be related to the involvement of several neurotransmitter receptor systems, including the GABA receptor complex. The aim of this study is to investigate the effects of chronic exposure of ethanol ingestion on gamma-aminobutyric acid (GABA) - benzodiazepine receptor complex in four rat brain regions.*

Materials and Methods: *Male Wistarrats were housed with food and drinking water/ethanol (85%+15% v/v). After 13 months of chronic ethanol consumption, the rats were killed by decapitation and the brain regions dissected and the synaptosome fractions prepared on Ficoll-sucrose gradient centrifugation procedure(4°C). The resulting pellet was resuspended in 1 ml of Tris-Hclbuffer (pH 7.4) and binding assay carried out in the presence of [^{3}H] Flunitrazepam,*

Results: *Chronic ethanol ingestion by the rat resulted in an increase of receptor affinity in the striatum and hippocampus, and a decrease in the receptor affinity in the cerebellum and frontal cortex. GABA receptor density, as assessed by GABA-benzodiazepine binding (B max), was decreased insignificantly in the all brain regions studied. The different responses of GABA receptor affinity of the brain regions to chronic ethanol ingestion may result from quantitative differences in the distribution of GABA-ergic neurons in the tested brain areas.*

Conclusions: *The increase in the affinity of the striatum and hippocampus and the decreased affinity of the cerebellum and frontal cortex suggested that up-regulation of these receptors may be Induced by chronic ethanol ingestion.*

1. Keywords: Brain regions; Chronic ethanol; GABA benzodiazepine receptors; GABA receptors

2. Introduction

Repeated exposure to ethanol causes a variety of

complex changes in the central nervous system (CNS), some of which are suggested as behavioral tolerance and physical dependence **[1-3]**. Clinical and experimental evidence have demonstrated that ethanol interferes with many molecular, neurochemical and cellular actions. It is suggested that ethanol interrupts the normal functioning of certain neurotransmitters, and that would have an effect on certain types of behavior **[4-6].**A number of evidences are in favor of involvement of ethanol on gamma amino butyric acid (GABA) and its specific receptors **[7-9].** However, many studies in the literature have described the effects of ethanol on the GABA receptors; sometimes with conflicting results **[10-12]**.Interpretation of these results is difficult in part due to the complexity and heterogeneity of the brain and in some cases treatment protocols, as well as the multiple actions of ethanol *in vivo*. Furthermore, the extensively distributed inhibitory GABA is also believed to play a fundamental role in mediating the effects of ethanol **[13, 14]**. Because, some brain areas are more affected than others, and even within regions, some cell populations and/or cell fractions are more vulnerable than others **[15]** and ethanol ingestion in man is considered as a long-term process [, this study was undertaken to examine the effects of long-term consumption of ethanol on the binding affinity of [^{3}H] flunitrazepam onto the synaptosomal membrane isolated from hippocampus, cerebellum, frontal cortex and striatum of rat brain. The results reveal marked differences in the binding of the brain areas of rats exposed to ethanol for 13 months.

3. Materials and Methods

3.1. Chemicals

[N-methyl-^{3}H] Flunitrazepam, 84 Ci/m. Mol, was purchased from Amersham. Clonazepam was obtained from Roche Ltd, Basel, Switzerland. All other reagents used were of ANALAR grade (or the highest grade available) unless stated otherwise and made up in double distilled water.

3.2. Dissection of rat brain

Male adult Wistar rats from a colony bred in our laboratory were used. Animals were maintained with respect to the animal welfare regulation in animal house with food and ethanol 15% volume ad libitum under a 12 hour light-dark cycle (light on 07.00, local time). After 13 months of chronic ethanol consumption, the rats were killed by decapitation between 8 to 9 and the brain regions were dissected over an ice plate, by the method of Glowinski and Iversen **[16]**. The brain was carefully removed and placed on a petri-dish over ice. The cerebellum was separated from the forebrain. The forebrain was the divided into three parts by two transverse sections. The frontal cortex was separated from the first transection. The striatum including caudate nucleus, putamen nucleus and globuspallidus was dissected with the external wall of the lateral ventricles. The hippocampus was dissected out from the midbrain. The dissected regions were immediately placed in ice-cold isolation medium (0.32M-sucrose /l mM-potassium EDTA/10 mM-Tris HCI, pH7.4).

3.3. Preparation of Synaptosomes

The synaptosomes were prepared from the dissected brain regions essentially as described by Booth and Clark **[17]**. Briefly the dissected brain regions from 6 rats were dropped into ice-cold isolation medium (0.32M-sucrose /l mM-potassium EDTA/10 mM-Tris HCI, pH7.4). The tissue was chopped into small pieces with scissors. The blood and other debris were washed off. This was homogenized in a glass homogenizer using a glass pestle with 0.1 mm clearance. The homogenate was diluted to 60ml with isolation medium. This was centrifuged at 1200g for 3 min at 4°C. The supernatant was then centrifuged at 16000g for 10min. The crude mitochondrial/synaptosomal pellet was resuspended in 30m1 of the Ficoll / sucrose medium [12% (w/w) Ficoll, 0.32M-sucrose, 50M potassium EDTA, pH7.4] and homogenized. The suspension was transferred into a centrifuge tube. Above this 5 ml of 7.5% Ficoll/sucrose medium [7.5% (w/w) Ficoll, 0.32M-sucrose, 50 pM-potassium EDTA, pH 7.4] was carefully layered. Finally, on top of this 5ml of isolation medium was layered. The tubes were centrifuged at 70000g for 40min at 4°C. Synaptosomes were gently sucked off from the second interface. The sucked off layer was resuspended in 10 ml. cooled Tris-Hcl (50 mM) buffer pH 7.4 and centrifuged at 30000g for 10 min at 4°C. The resulting pellet was washed 3 times with the Tris-HCl buffer and resuspended in 1 ml of the buffer. The determination of synaptosomal protein concentration was performed by the method of Lowry et al. **[16]**, after lysing the synaptosomal membrane in a 2% (w/v) Na-deoxycholate solution.

3.4. Binding Assay

[^{3}H] Flunitrazepam binding was carried out by incubating 0.5 ml a1iquots of the purified synaptosomal preparation (200 µg protein) with [^{3}H] flunitrazepam in a range of concentrations of 0.50 µM to 10.00 µMin a final volume of 0.7 ml. The non-

specific binding was determined in the presence of c1onazepam (about 5%of total bound). The assay tubes were incubated for 30 min in a crushed ice bath. The incubation was terminated by the addition of 5ml ice-cold 50 mMTris- HCI buffer (pH 7.4) and filtered under a constant vacuum through Whatman GF/B fiberglass discs. Incubation tubes and filters were washed three times with 5 ml of ice-cold buffer and treated with 6 ml ACS scintillation cocktail. The bound radioactivity was counted by a liquid scintillation counter. Specific binding was calculated by subtracting the non-specific binding from the total binding was expressed as f mol/mg protein.

3.5. Data Analysis

The receptor affinity (KD) and maximum binding capacity (B max) were estimated by the Eadie-Hofstee analysis. The standard deviation of the background in such estimation and the standard errors of B max and Kd were calculated according to Zivin and Waud **[17]**. The statistical significance between Kd values was established by testing the common variant hypothesis, then the covariance analysis.

4. Results

Specific binding of increasing concentrations of [^{3}H] Flunitrazepam to the synaptosoms prepared from the brain regions was saturable. Scatchard plots of specific [^{3}H]-Flunitrazepam binding were linear. **(Table 1)** shows the results of Scatchard analysis, using the samples from alcoholic rats after a 13 month exposure to ethanol and corresponding controls. The relative Kd values for the four different brain regions of the control were as follows; hippocampus> frontal cortex >striatum> cerebellum. The relative binding capacities (B max) of the four brain regions were different and were as follows; frontal cortex > cerebellum> hippocampus> striatum. KD values were decreased significantly in the striatum and hippocampus, but increased in the cerebellum and frontal cortex of the ethanol-treated rats. However, there seemed to be no significant change in the B max values, though there are indications of lower values in all the regions as compared with non-alcoholic controls.

Brain Regions	Control Rats		Ethanol-Treated Rats	
	KD	B max	KD	B max
Frontal cortex	8.9 ± 1.6	356 ± 46	10.5 ± 1.6^	323 ±35
Striatum	5.7 ±0.8	183 ± 15	1.6 ± 0.1*	164 ± 5
Hippocampus	9.1 ± 2.4	216 ± 40	5.6 ± 0.9*	183 ± 17
Cerebellum	4.9 ± 0.5	332 ± 17	6.4 ± 0.2*	313 ± 34

Table 1: Effect of chronic ethanol ingestion on specific binding of [3H] flunitrazepam in different regions of the rat brain.

[3H] Flunitrazepam binding to the purified synaptosomal preparations was performed in the Tris-HCL buffer pH 7.4. KD values are given as mM. And B max values are given as fmol/mg protein. Values (means ± SD of four determinations) are the results of Scatchard analysis of saturation isotherms using [3H]-flunitrazepam concentrations of 0.5-10 μM.*significantly different from the control value ($P<0.05$).

5. Discussion

The systems studied in this paper will approximate closely to the optimal conditions at which ethanol induces changes on the synaptic terminals in different brain regions. The relative increase in specific binding affinity of [^{3}H]-flunitrazepam (decreased KD) in the striatum and hippocampus, and the decrease in the specific binding affinity (increased KD) in the cerebellum and frontal cortex after chronic ethanol ingestion, may be explained by the specific changes in the concentrations of GABA, as well as the number and the type of GABA receptors in the different brain regions. GABA concentrations have been shown to be higher in the striatum and hippocampus as compared with the frontal cortex and cerebellum **[18]**. The increase in the receptor affinity in the striatum and hippocampus may be explained by the up-regulation phenomenon of the GABA receptors in the straitum and hippocampus. However, the decrease of affinity of the receptors in the cerebellum and frontal cortex following exposure to chronic ethanol is inconsistent with this view. Therefore, the number and type of the GABA receptors in the synaptosomal membrane of the cerebellum and frontal cortex might be lower and different.

Flunitrazepam is a well-known benzodiazepine and has a high affinity for the benzodiazepine receptor that is associated with $GABA_A$ receptors **[19- 21]**. The results indicated that the main sites of action of chronic ethanol consumption are $GABA_A$ receptors located in the stratum and hippocampus. The increased affinity observed in the striatum and hippocampus would suggest that chronic ethanol consumption may enhance GABA agonists' binding affinity and then facilitate GABA transmission in these areas. However, the decrease of affinity of the receptors in the cerebellum and frontal cortex following exposure to chronic ethanol is inconsistent with this view. The selective increase in benzodiazepine receptor affinity in the brain regions induced by chronic treatment with ethanol has important implications with respect to the inhibitory nature of GABA transmission. It has been demonstrated that the GABA-ergic neurons seem to be mediated by at least two pharmacologically distinct benzodiazepine receptor subtypes; a type I receptor enriched in the cerebellum, and a type II receptor enriched in hippocampus and some other brain regions **[22- 26]**. It is therefore possible that chronic exposure to ethanol leads to different alternations of GABA-benzodiazepine receptor affinity in different brain regions **[i.e. 27, 28]**.

It is concluded that chronic ethanol treatment modulates GABA-benzodiazepine binding, so that the altered efficacy of the receptor complex in different brain regions leads to altered behavior of GABA-benzodiazepine receptor complexes in the brain.

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