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Original Anticonvulsant Urea Derivative Alters the Properties of Benzodiazepine Receptors "Central" and "Peripheral" Types in the Cerebral Cortex of "Heavy Drinkers" Rats

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Abstract

Objective: studies of anticonvulsants have a stimulating effect on neuronal receptors; in particular $GABA_A$/benzodiazepine receptor complex ($GABA_A$/BzDR) can be the basis for the development of new approaches to the treatment of alcohol withdrawal syndrome (AWS) and alcohol addiction. Benzodiazepine receptors (BzDRs) of the cerebral cortex of Wistar male rats with a different preference for alcohol and BzDRs in the brain "heavy drinkers" rats, treated with original anticonvulsant meta-chloro-benzhydryl urea (m-ch-BHU) were examined in these study.

Materials and methods: Wistar male rats (n=250) were used in an experimental model of alcoholism. Properties of the BzDRs of the "central" (synaptic) and "peripheral" (mitochondrial) type were examined in membrane fractions obtained from the cerebral cortex of rats under various experimental groups using radio receptor binding assays (RRA) selective ligands: [^{3}H]flunitrazepam and [^{3}H] Ro5-4864 to these receptors respectively.

Results: our study has shown that the binding affinity of [^{3}H] flunitrazepam and [^{3}H] Ro5-4864 with synaptosomal and mitochondrial membranes was decreased, but capacity of receptors was increased in the cerebral cortex of rats prefer alcohol. Administration of m-ch-BHU increased affinity of BzDRs in the cortex of "heavy drinkers" rats that can enhance the mediation of GABA in the brain of these animals.

Conclusion: Our data showed that m-ch-BHU has a stimulating effect on $GABA_A$/BzDRs in the brains of "heavy drinkers" rats and may provide a new pharmacotherapeutic approach to the treatment of alcohol addiction.

Keywords: Alcohol; Anticonvulsant; Benzodiazepine receptor; Brain; GABA; Mitochondria; Synapse

Abbreviations:

AWS: Alcohol Withdrawal Syndrome; BzD: Benzodiazepine; BzDR: Benzodiazepine Receptor; CNS: Central Nervous System; GABA: Gamma-Aminobutyric Acid; $GABA_A$ receptor: Receptor for Gamma-Aminobutyric Acid Type A; m-ch-BHU: Meta-Chloro-Benzhydryl Urea; Kd: Dissociation Constant; Bmax: Density of binding sites; LD50: Lethal Dose; RRA: Radio Receptor Assay

Introduction

The problem of the treatment of alcohol addiction is very difficult due to the occurrence of relapses and the complexity of understanding the mechanisms of their formation. Elucidation of these mechanisms is important for the prevention of alcohol and the development of alcohol dependence. This problem arises from the fact that there are both direct pharmacological effects of ethanol, as well as long-term compensatory changes that occur in response to those pharmacological effects.

The currently accepted position is that the adverse effects of ethanol are also linked with interactions with specific proteins, ion channels, and receptors leading to changes in their functions [1,2]. According to modern concepts in the pathogenesis of alcohol dependence GABA receptors type A ($GABA_AR$) play central role in both the short- and long-term effects of ethanol in the brain [3-5].

$GABA_AR$ belong to a family of trans - membrane ligand-gated ion channels. These receptors are responsible for rapid neuronal transmission in the mammalian CNS. $GABA_AR$ primarily occur in the pre- and post- synaptic membranes, although there is evidence that certain subtypes may occur extra-synaptically [6]. The $GABA_AR$ are pentameric receptors having 5 subunits including various isoforms of subunits: 6α, 4β, 3γ, 2ρ, δ, ε, θ and π [7]. Activation of $GABA_AR$ is followed by selective inward current of CL- through the central pore, which leads to hyperpolarization of the neuronal membrane and reduces the neuronal excitability. They have a rich pharmacology, and this is dependent upon the particular subunits that are present within the receptor pentamer [7]. An important point in the functioning of the $GABA_A$ receptor complex is that this oligomeric protein complex contains various allosteric binding sites modulating the activity of the receptor [8]. These allosteric binding sites are the targets for a variety of agents, including benzodiazepines and ethanol. Benzodiazepines,

binding with specific sites benzodiazepine receptors (BzDR) on $GABA_A$ receptors alter its conformation and affinity [9-11] and play an important role on neuropharmacology of inhibitory processes in CNS modulatory fast and tonic inhibition [6,12].

Alcohol abuse induces neuroadaptive alterations of BzDR that modulate $GABA_AR$, and GABA mediation in brain regions [2,13,14] associated with reward function in the brain [15] that serves alcohol addictions [16]. Studying the effects of drugs that have modulatory effects on neuronal receptors, in particular the $GABA_A$/BzDR can be the basis for understanding the formation of alcohol motivation and addiction and to develop new approaches to the treatment of this disease.

The purpose of this study was to examine the properties of BzDR "central" (synaptic) and "peripheral" (mitochondrial) types in the cerebral cortex of rats with different preference to alcohol and the effect of original anticonvulsant meta-chloro-benzhydryl urea (m-ch-BHU) on the properties of the BzDR in the cerebral cortex of "heavy drinkers" rats. There are numerous animal models that have been employed to study the effects of alcohol. Methodological basis of our study was an experimental model of alcoholism, which takes into account the stage of development and genetic predisposition.

Materials and Method

All animals before the study were placed in a separate room to the adaptation period (14 days). During this period, the animals were monitored manifestation of variations in health status according to standard operating procedures (SOPs) laboratory "Reception animal quarantine adaptation". Animals were distributed randomly into groups, using as a criterion of body weight so that the weight of the individual animals did not differ by more than 20% of the average weight of animals. Experiments conducted on 250 male rats Wistar line, weighing 150-180 g. Each animal was assigned a unique number, in accordance with which the animal put labels coloring dyes (eosin methylene blue) on the surface of the tail developed schemes. The label cells of a certain color indicate the group number of the number of the animal, tag, code research supervisor. Basic rules of maintenance and care consistent with the standards given in the manual Committee for the Update of the Guide for care and use of laboratory animals (ILAR publication, 1996, National Academy Press; eighth edition, Copyright 2010 by the National Academy of Sciences) and agreed with the Commission in the establishment of bioethical. All procedures for the routine care of animals were performed in accordance with SOP laboratory.

Animals were tested for preference (severity of alcohol motivation) in a free choice between 15% ethanol and water for 14 days (two bottle oral test), the amount of fluid from each measuring bottle was recorded every day. Animals, preferring ethanol (consumption of 15% ethanol solution was more than 50% in a day), were divided into two groups: rats from group No. 1 for 10 months subject to forced alcoholism (15% ethanol solution as the sole source of fluid) rats "heavy drinkers"; rats from group No. 2 had no access to ethanol entire experimental period rats "non-heavy drinkers". Animals in which the level of 15% ethanol solution consumption was less than 10% in a day of the total amount of fluid formed the group included "non-preferring" ethanol rats (group No. 3) and held without access to the entire period of ethanol.

At the end of the 10 month long experimental period all experimental rats were re-tested in a free choice between 15% ethanol and water for 14 days (two bottle oral tests) and they were separated by the following main groups: "heavy drinkers" rats (1^{st}), "non-heavy drinkers" rats (2^{nd}) and "non-preferring" alcohol rats (3^{rd}). The group was further highlighted No. 4 - "heavy drinkers" rats (for 10 months subject to forced alcoholism) and treated with anticonvulsant meta - chloro - benzhydryl urea (m-ch-BHU) for 14 days in a dose of 100 mg/kg intragastrically in a 1% starch suspension using a probe of 1 ml suspension per 100 gram of animal body weight. The rats in the 3^{rd} group (control group) "non-preferring" alcohol were administered 1% starch mucilage 1 ml via intragastric probe. Selection dose of 100 mg/kg corresponds to 1/20 LD50 m-ch-BHU, which is considered the closest approximation to the therapeutic dose range for its anticonvulsive effect [12]. Choosing the route of administration (oral) caused a major route of administration of anticonvulsants in the clinic - through the mouth, as well as high hydrophobic m-ch-BHU preventing getting drug dosage forms for parenteral administration. For conducting radio receptor assay (RRA) of BzDRs properties in the brain cortex of rats in the different groups at the end of the experimental period, the rats were decapitated under light ether anaesthesia, the brain is removed; the cerebral cortex was separated, frozen and stored in liquid nitrogen thermoses. Separation of samples of rat brain tissue to membrane fraction (synaptosomal and mitochondrial) was carried out by preparative ultracentrifugation. The obtained membrane fractions was frozen and stored at t= -80°C. The study of BzDR binding properties in synaptosomal and mitochondrial membranes was conducted by RRA with selective ligands.

[^{3}H] Flunitrazepam binding procedure with specific binding sites on the synaptosomal membrane derived from rat brain cortex

Properties of BzDRs "central" type from rat brain cortex examined by RRA binding of [^{3}H] flunitrazepam (85 Ci/mmol, "Amersham") with a synaptosomal membranes fraction of the brain tissue during 60 min. at t=0°C. Concentrations of [^{3}H] flunitrazepam were 0.2-15 nM in incubation volume. The concentration of the membranes was 0.2 mg protein/ml in 0.25 ml samples of the incubation. Nonspecific binding was performed with flunitrazepam cold in concentration 10 μM in incubation volume.

[^{3}H] Ro5-4864 binding procedure with specific binding sites on the mitochondrial membrane derived from rat brain cortex

Properties of BzDRs "peripheral" type from rat brain cortex was investigated by RRA binding of [^{3}H] Ro5-4864 (90 Ci/mmol, NEN, USA) (0.2-25.0 nM in incubation volume) to mitochondrial membranes of rat brain cortex for 120 min. at t=0°C. The concentration of the membranes was 0.6 mg protein/ml in 0.25 ml samples of the incubation. Nonspecific binding was performed with Ro5-4864 cold in concentration 10 μM in incubation volume.

Bound ligands was separated in all cases filtration through GF/B filters ("Whatman", UK) following vacuum filtration using a system "Harvester-Skatron" (USA) in 15 ml Tris - HCl (50 mM, pH=7.4 at t=0°C), the filters were placed in glass vials containing 10 ml of scintillator. Radioactive analysis of the amount of bound ligands was carried out in β-scintillation counter - "Rack-beta" (LKB, Sweden). Nonspecific binding (<10%) was similar in control and test samples. The dissociation constant (Kd) and the maximum number of specific binding sites (Bmax.) was determined by analysis of saturation curves

in Scatchard coordinates. Kd expressed in nM, Bmax in fmol/mg protein. Linear Scatchard blots were analysed in all cases which confirm the presence of only a specific population of binding sites. Distribution of signs did not differ significantly from normal, so the statistical data used parametric method of variation statistics (t-test) using the program Statistika 10.0, the differences were considered significant ($p<0.05$).

Anticonvulsant m-ch-BHU is designed and synthesized in the Department of Biotechnology and Organic Chemistry, National Research Tomsk Polytechnic University. Experimental work was carried out in the Laboratory of Neuroimmunology and Neurobiology Mental Health Research Institute (Tomsk) and Laboratory of Clinical Biochemistry Research Center for Mental Health Sciences (Moscow). All the studies were approved by the Ethics Committee of the Mental Health Research Institute.

Results

In this study it was found that affinity of synaptosomal BzDR for [^{3}H] flunitrazepam (1/Kd) significantly decreased in the 1st and 2nd rat's groups compared with the 3rd comparison group. This suggests the presence of initially reduced affinity of synaptosomal BzDR "central" type in the cerebral cortex of rats preferring ethanol compared to animals, to reject it. Significant differences in Bmax in all examined groups of animals were not found. However, one can note an increase in receptor density in brain cortex of rat's 1st and 2nd groups as compared with the 3rd group (Table 1 and Figure 1).

Group of rats	Binding parameters of [^{3}H] flunitrazepam with synaptosomal membranes (M ± m)		Binding parameters of [^{3}H] Ro5-4864 with mitochondrial membranes (M ± SE)	
	Kd^1 (nM)	Bmax^1 (fmole/mg prot)	Kd^2 (nM)	Bmax^2 (fmole/mg prot)
1st (n=13)	2.43 ± 0,38*	3065 ± 550	9.46 ± 1,17**	1064 ± 178**
2nd (n=11)	2.12 ± 0,28*	3024 ± 615	6.22 ± 0,85**	1027 ± 171**
3rd (n=12)	1.41 ± 0,19	2882 ± 453	4.71 ± 0,56	665 ± 76
4th (n=13)	2.10 ± 0,25*	2739 ± 568	5.86 ± 0,75*	854 ± 162
Notes: Bmax^1 - density of binding sites of [3Hflunitrazepam with synaptosomal membranes; Kd^1 - constant of dissociation of the ligand-receptor complex [^{3}H]flunitrazepam with synaptosomal membranes; Bmax^2 - density of binding sites [^{3}H]Ro5-4864 with mitochondrial membranes; Kd^2 - constant of dissociation of the ligand-receptor complex [^{3}H]Ro5-4864 with mitochondrial membranes; n - the number of cases studied; * - Statistically significant difference indicators binding [^{3}H]flunitrazepam and ** - [^{3}H]Ro5-4864 in the experimental groups compared with control group ($p<0.05$).				

Table 1: Properties of [^{3}H]flunitrazepam and [^{3}H] Ro5-4864 binding to the synaptosomal and mitochondrial membranes from cerebral cortex of rats in different groups.

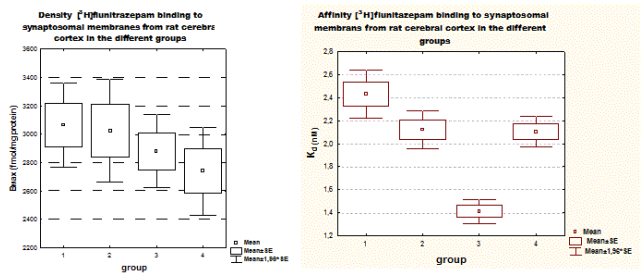

Figure 1: Statistical analysis of [^{3}H] flunitrazepam binding parameters {Kd (nM) – constant of dissociation ligand-receptor complex] and [Bmax (fmol/mg of protein) – density of binding sites] with synaptosomal membranes of rat cerebral cortex in the different experimental groups.

We found an increase in the number of binding sites of [^{3}H] flunitrazepam in the rat brain in the 1st and 2nd groups that can wear compensatory a deficit in GABAergic function in connection with a reduction in the affinity of the "central" type BzDR. At comparing the parameters of binding of [^{3}H] flunitrazepam to BzDR of synaptosomal membranes of rat brain cortex (1st and 2nd groups) it should be noted that they are close to each other by values (Table 1 and Figure 1). Thus, the properties of "central" type BzDR in brain rats in the 1st and 2nd groups that were in different conditions during experimental period differ from the properties of the BzDRs of rats revealed no alcoholic motivation. This can be explained by differences primordial affinity receptors in the brain cortex of rats that prefer alcohol ("heavy drinkers" and "non-heavy drinkers") compared to animals reject it (rats "non-preferring" alcohol).

Administration of m-ch-BHU within 14 days (100 mg/kg/day) to rats "heavy drinkers" (4th group) caused an increase in the binding affinity (1/Kd) of [^{3}H] flunitrazepam - reduces the Kd values, but does not reach the values of Kd in the group of rats, who rejected ethanol. Kd values in rats from 4th group were comparable to those of the rats in 2nd group ("non-heavy drinkers"). Therefore, m-ch-BHU increases the affinity synaptosomal BzDR agonist ([^{3}H] flunitrazepam), thereby improving neuromediation GABA in the cerebral cortex of rats. Introduction of m-ch-BHU to rats "heavy-drinkers" caused a decrease in Bmax that was comparable with those in the 3rd group (Table 1 and Figure 1).

Perhaps improved neuromediation GABA by increasing the affinity of the BzDR leads to decreased expression of the receptors, which was

a compensatory response. Administration of m-ch-BHU caused a sharp decline in ethanol consumption in rats with free access to 15% ethanol, starting with the 2nd and 3rd day of its application, saving this level of consumption during the 14 days of observation [12]. So, m-ch-BHU alters the properties of the synaptosomal "central" type BzDR, closely connected with $GABA_AR$ in brain cortex of rats, preferring alcohol and long under his influence, increasing the affinity of the receptor agonist and several reducing their density. Investigation of the properties of mitochondrial "peripheral" type BzDR that not associated with $GABA_A$ receptors was studied using selective ligand for these receptors [3H] Ro5-4864 (Table 1 and Figure 2). Analysis of the data showed a statistically significant increase in the binding sites of [^{3}H] Ro5-4864 (Bmax) in the mitochondrial membrane fraction of the cerebral cortex rats from1st and 2nd groups as compared with the 3rd group on 54.8-59.4%, respectively. Increase of mitochondrial density of "peripheral" type BzDR in rat brain, preferring alcohol, due to the simultaneous reduction receptor affinity (1/Kd).

We found an increase in Kd values in the 1st and 2nd groups compared with the 3rd, indicating a decrease in the affinity of the BzDR, and the severity of these changes in "non-heavy drinkers" and "heavy drinkers" rats was different. Thus, we have found that as all rats prefer alcohol (1st and 2nd groups) had elevated values of Kd compared to rats not prefer alcohol that showed significantly lower affinity of mitochondrial BzDR in the cerebral cortex of rats prefer alcohol (Table 1 and Figure 2).

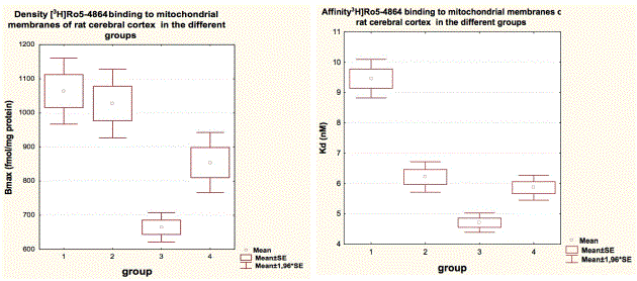

Figure 2: Statistical analysis of [^{3}H] Ro5-4864 binding parameters {Kd (nM) – constant of dissociation ligand-receptor complex] and [Bmax (fmol/mg of protein) – density of binding sites] with mitochondrial membranes of rat cerebral cortex in the different experimental groups.

Comparative study of the properties of mitochondrial "peripheral" type BzDR in rats prefer ethanol from different groups (1st and 2nd group) showed that the affinity of the receptors (1/Kd) in brain cortex of rats "heavy drinkers" was significantly lower than affinity of BzDR in rats "non heavy drinkers". Prolonged exposure to alcohol causes a significant decrease in receptor affinity (≈ 2 times) (Table 1 and Figure 2). Density of mitochondrial "peripheral" type BzDR (Bmax) in rats "non-heavy drinkers" was comparable to that of rats "heavy drinkers", and was significantly much higher than in rats of group that includes rats "non-preferring" alcohol. Density level of mitochondrial "peripheral" type BzDR is the most sensitive index of damage to the nervous tissue, especially glial cells.

Administration of m-ch-BHU for 14 days at a dose of 100 mg/kg body weight to rats "heavy drinkers" exposed to long-term of 15% ethanol (4th group), caused decrease of Kd compared to the corresponding values of this parameter in rats "heavy drinkers" that not treated with m-ch-BHU (1st group). This indicates increased binding affinity (1/Kd) of [3H] Ro5-4864 with mitochondrial "peripheral" type BzDR in the cerebral cortex of rats "heavy drinkers" under the influence of m-ch-BHU. It was also revealed significant decrease in the density of the binding of [^{3}H] Ro5-4864 (Bmax) in the cerebral cortex of rats under the therapy with m-ch-BHU from 4th group compared to the 1st (Table 1 and Figure 2).

Parameters of the binding of [^{3}H] Ro5-4864 to BzDR (Kd and Bmax) in the cerebral cortex of rats "heavy drinkers" on a background of 14-day administration of m-ch-BHU "improved", but did not reach the values of the relevant parameters of the rats in the control group "non-preferring" alcohol (3rd group). Number of binding sites of [^{3}H] Ro 5-4864 (Bmax) in the 4th group was comparable to those in the 3rd group, whereas Kd values differed significantly (Table 1 and Figure 2). These findings suggest that m-ch-BHU increase the affinity of mitochondrial BzDRs "peripheral" type and reduced receptor density in rats in 1st group. Consequently, the 14-day administration m-ch-BHU (100 mg/kg/day) to rats "heavy drinkers" exposed to long-term (during 10 months) exposure of 15% ethanol, had effect of positive modulation of the binding of [^{3}H] Ro5-4864 with BzDR "peripheral" type in the cerebral cortex of these animals.

Increase the binding affinity of [^{3}H] flunitrazepam to BzDR causes conformational changes of the $GABA_A$ receptor complex and can stimulate $GABA_AR$ sensitivity to endogenous positive or reducing their sensitivity to negative $GABA_AR$ neuromodulators. Administration of m-ch-BHU during 14 days to "heavy drinkers" rats (4th group), increased the binding affinity of [^{3}H] flunitrazepam and [^{3}H] Ro5-4864 with BzDR in the rat cerebral cortex and decreased the number of receptors that had compensatory effect in conditions of GABAergic functional impairment. Anticonvulsant m-ch-BHU modulates the BzDR in the cerebral cortex of rats "heavy drinkers" that can increase the affinity of $GABA_AR$ to GABA and stimulate GABA - mediation in the cerebral cortex of these rats.

Discussion

One of the leading currently accepted hypotheses is the development of tolerance to ethanol by enhancing the action of GABA, which corresponds to the existing assumption that genetic tolerance to ethanol may be due to reduced sensitive to GABA and $GABA_AR$-modulators [17]. In the present study, data were obtained reinforces the notion of biological determinism of the properties of both types BzDR ("central" and "peripheral" types) in the cerebral cortex of rats with different preference to ethanol: rats "preferring alcohol" with and without alcohol consumption during long experimental period and rats "non-preferring" alcohol, reject it.

We observed a reduced affinity of BzDR in the cerebral cortex of rats "preferring ethanol"; besides affinity of BzDR in the cerebral cortex of rats preferring alcohol but not exposed to alcohol "non-heavy drinkers" was more comparable to the affinity of BzDR "heavy drinkers" rats than in "non-preferring" rats rejected alcohol. As BzDR "central type" is part of the $GABA_A$/BzDR complex it can modulate the function of $GABA_AR$ in the brain and can be associated with a reduction GABAergic inhibitory neurotransmission in the brain of animals that can stimulate the consumption of alcohol as substrate, rendering the stimulation of GABA-mediation.

We observed that chronic exposure to ethanol causes deep and prolonged neuroadaptive changes of BzDR in cerebral cortex of "heavy drinkers" rats. This may be due to changes in expression and

composition components of $GABA_A$ receptor's subunits related with their different functional contribution to the inhibitory processes in the CNS and play a significant role in adaptation to chronic exposure to ethanol and the development of tolerance [18]. Neuroplastic adaptive changes of $GABA_A$/BzDR in the brain is a common mechanism underlying the changes in neuronal excitability and behavior associated with reduced sensitivity to BzD caused ductility $GABA_A$ receptor populations with different pharmacological and biophysical properties. Chronic exposure to ethanol can decrease subunits α1, α3, α5, and increase subunit α2 [19,20] and subunits α4 and α6 in the structure of the $GABA_A$/BzDR insensitive to benzodiazepines, and cause a decrease GABAA receptor function [11,21,22]. Reduced expression $GABA_AR$ containing β2 and β3 subunit leads to disruption of inhibitory processes in the brain and may cause the hyper excitability in the development of AWS [23]. Alcohol abuse causes induction of neuroplasticity in the CNS [16,20,23,24] that may underlie differences in susceptibility to alcohol and lead to the emergence of compulsive behaviour in alcohol addiction.

Exposure to ethanol causes a greatest changes of BzDR "peripheral type" localized in external mitochondrial membranes mainly in the glial cells and not associated with $GABA_AR$ compared to synaptically localized BzDR "central type" that was consistent with physiological and defensive functions of mitochondrial BzDR under the influence of toxic substances. Besides that BzDR "peripheral type" provides the transfer of cholesterol into the mitochondria [25] thus influencing the regulation of the synthesis of neurosteroids that are endogenous modulators of the $GABA_A$/BzDR in the CNS [26]. It can be considered our findings that indicate deeper changes of the BzDR "peripheral type" as expressed reaction to prolonged exposure to alcohol. These neuroplastic changes may lead to the development of severe alcohol motivation and formation of alcohol dependence.

We have found that administration of m-ch-BHU to "heavy drinkers" rats largely modulates the properties of BzDR "central" and "peripheral" types increasing their affinity and reducing their density that indicates a decrease of receptor expression under the influence of therapy.

The prospect of the treatment of alcohol dependence with the use of new anticonvulsants, have a modulating effect on neurotransmitter processes in the brain, are widely discussed in modern literature [20,27,28]. So, new original pharmacological agent m-ch-BHU that modulates properties of BzDR may be promising in the treatment of alcohol addiction (Figure 3). Accordingly we suggest that m-ch-BHU have modulatory action on the benzodiazepine receptor system of the brain of "heavy drinkers" rats, which leads to increased neuromediation GABA in the brain of these animals and causes a reduction in alcohol consumption [29] that may provide a novel pharmacotherapeutic approach to the treatment of alcohol addiction and prevention of recurrence of the disease without causing negative effects after prolonged use of the BzD [30].

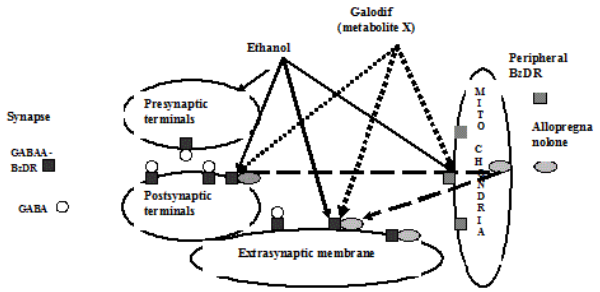

Figure 3: $GABA_A$/benzodiazepine receptor system of the rat brain during alcohol addiction: molecular targets of ethanol and Galodif (GABA – gamma aminobutyric acid; $GABA_A$ -BzDR - synaptical benzodiazepine receptor; Peripheral BzDR – mitochondrial benzodiazepine receptor; Allopregnanolone – neuroactive steroid, synthesized in mitochondria.

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