Original Paper

Journal of Biomedical Science

J Biomed Sci 1997;4:308–314

Received: May 1, 1997
Accepted: June 30, 1997

Bang H. Hwang[a]
Phillip E. Kunkler[a]
Lawrence Lumeng[b–d]

Departments of
[a] Anatomy,
[b] Medicine, and
[c] Biochemistry, Indiana University School of Medicine, and
[d] VAMC, Indianapolis, Ind., USA

Quantitative Autoradiography on [^{35}S]TBPS Binding Sites of Gamma-Aminobutyric Acid$_A$ Receptors in Discrete Brain Regions of High-Alcohol-Drinking and Low-Alcohol-Drinking Rats Selectively Bred for High- and Low-Alcohol Preference

Key Words
γ-Aminobutyric acid$_A$ receptors
t-Butylbicyclophosphorothionate binding sites
Alcohol preference
Nucleus accumbens
Amygdala

Abstract
It has been documented that ethanol can potentiate brain γ-aminobutyric acid (GABA)ergic function, and there is a close link between the GABA$_A$ receptor complex and effects of ethanol, including reinforcement of alcohol which is a fundamental element of alcohol preference. However, it is unknown in what discrete brain regions GABA$_A$ receptors might be associated with alcohol preference. In the present study, [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) was used to localize GABA$_A$ receptors in high-alcohol-drinking (HAD) rats and low-alcohol-drinking (LAD) rats which were selectively bred for high and low alcohol preference, respectively. Initial qualitative observations indicated that [^{35}S]TBPS binding sites were abundant in many brain areas including the cerebral cortex, hypothalamus and amygdala of HAD and LAD rats. Furthermore, the quantitative autoradiographic analysis revealed fewer [^{35}S]TBPS binding sites of GABA$_A$ receptors in the amygdaloid complex, central medial thalamic nucleus, lateral hypothalamic nucleus and anterior hypothalamic nucleus of HAD rats than LAD rats. Collectively, this study has indicated that HAD rats selectively bred for high alcohol preference possess lower [^{35}S]TBPS binding in the brain. Since lower TBPS binding has been proposed to reflect enhanced GABAergic function, as evidenced in rats with seizure or under alcohol withdrawal, the results from the present study suggest that HAD rats might have an enhanced GABAergic function. It is thus likely that enhanced GABAergic function in the brain might be related to high alcohol preference which is characteristic in HAD rats. In addition, the present result showing no difference of [^{35}S]TBPS binding in the nucleus accumbens is also in agreement with a notion that [^{35}S]TBPS binding may represent only a small spectrum of the GABA$_A$ receptor complex which is constituted of a sophisticated subunit combination whose functional compositions are still unknown. In conclusion, the present study supports the working hypothesis that GABA$_A$ receptors are involved in alcohol preference in HAD rats.

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

S. Karger AG, Basel
1021–7770/97/0046–0308$15.00/0
This article is also accessible online at:
http://BioMedNet.com/karger

Bang H. Hwang, PhD
Department of Anatomy (MS-259)
Indiana University School of Medicine
635 Barnhill Drive, Indianapolis, IN 46202 (USA)
Tel. +1 317 274 5016, fax +1 317 278 2040, e-mail hwang@anatomy.iupui.edu

Introduction

It is well known that γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system, and its binding to the $GABA_A$-benzodiazepine receptor complex ($GABA_A$ receptors) activates chloride channels to exert its inhibition. Ethanol has been shown to enhance GABAergic function [4, 35, 55, 57]. For example, ethanol can potentiate $GABA_A$ receptors to mediate $^{36}Cl^-$ flux in isolated brain membrane vesicles [56], whereas extracellular recording studies show that ethanol specifically potentiates GABAergic inhibition of neurons in the brain [4, 35]. The interaction of ethanol with the GABA system can also be appreciated from the facts that chronic ethanol treatment downregulates $GABA_A$ receptors [38] and ethanol potentiates muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes [34]. It has been shown that $GABA_A$ receptors mediate ethanol-induced behavioral changes [26, 40]. In fact, central $GABA_A$ receptors are involved in the modulation of the anxiolytic, sedative and intoxicating properties of ethanol [20, 54, 55]. Conceivably, the $GABA_A$ receptor system plays an important role in actions of ethanol including its reinforcement [18, 20].

It has also been demonstrated that $GABA_A$ receptor antagonists decrease alcohol drinking [14, 37, 43], whereas $GABA_A$ receptor agonists can enhance alcohol intake in Long-Evans rats [49] and also in alcohol-nonpreferring (NP) rats [17]. In addition, $GABA_A$ receptor inverse agonists are known to suppress alcohol drinking in selectively bred alcohol-preferring (P) rats [18, 24, 25, 28]. These observations are consistent with the notion that the central GABA system is involved in regulating alcohol preference [13, 29]. Since the benzodiazepine inverse partial agonist RO 15-4513 interacts with the $GABA_A$ receptor complex to exert its alcohol-antagonistic properties [54], it is conceivable that the reinforcing effect of ethanol [53] is mediated, in part, through $GABA_A$ receptors [34, 55]. Given the role of $GABA_A$ receptors in anxiety [15] and the studies showing an anxiolytic effect of ethanol on anxious P rats [52], it appears that the GABA system is likely to be involved in both alcohol preference and anxiety. In order to understand a possible association of GABA receptors with alcohol preference, this study was designed to examine whether there is an innate difference of $GABA_A$ receptors in the brains of rats selectively bred for high and low alcohol preference.

[^{35}S]t-butylbicyclophosphorothionate (TBPS) is known to possess a very high affinity to the picrotoxin/convulsant binding sites [16] of the $GABA_A$-benzodiazepine receptor complex ($GABA_A$ receptors) and has been widely used as a pharmacological tool to assess $GABA_A$ receptors [3, 7, 9, 44]. [^{35}S]TBPS has also been used to study the chronic effects of ethanol on the $GABA_A$-receptor-coupled chloride channel [39, 46, 56]. Interestingly, ethanol can inhibit [^{35}S]TBPS binding in vitro [56], but chronic treatment with ethanol does not alter [^{35}S]TBPS binding [39]. This is possibly due to the fact that alcohol treatment alters [^{35}S]TBPS binding affinities without changing its binding densities [44], although in vivo administration of diazepam changes the [^{35}S]TBPS binding density without any effect on its binding affinity. In addition, it is also possible that the effect of ethanol on [^{35}S]TBS binding is associated with endogenous differences in the molecular composition of $GABA_A$ receptors [9, 20, 27] which are formed by different receptor subunits [47, 48] in various parts of the brain. This can be appreciated from the studies showing that the chronic ethanol treatment upregulates GABA receptor β-subunit expression in the cerebral cortex [31], but downregulates α-subunits in the cerebral cortex and on cerebellar granule cells [30, 32, 33].

Because of the high-affinity-binding property of TBPS to the $GABA_A$ receptor-gated chloride channel complex, [^{35}S]TBPS has become one of the most attractive ligands of choice for autoradiographic studies [7] and in vitro studies [27, 39, 46, 48] to assess the function of the $GABA_A$-receptor-coupled chloride channel. To date, the [^{35}S]TBPS binding aspect of the $GABA_A$ receptor complex in rats with a predisposition to high alcohol preference has not been studied. Thus, the present study was carried out to investigate whether there is a unique TBPS binding of the $GABA_A$ receptor complex in discrete brain regions of high-alcohol-drinking (HAD) versus low-alcohol-drinking (LAD) rats.

Materials and Methods

Animals

Six female HAD rats (body weight 237 ± 8 g) and 6 female LAD rats (body weight 234 ± 6 g) of the 19th generation of selective breeding at approximately 6 months of age were studied. They were from the Alcohol Research Center of Indiana University School of Medicine. The drinking scores were determined using the two-bottle (10% ethanol and tap water) free-choice method [24]. The drinking scores for HAD rats were 7.21 ± 0.66 g ethanol/kg body weight/day, and 0.16 ± 0.02 g ethanol/kg body weight/day for LAD rats ($p < 0.001$), indicate of high alcohol preference in HAD rats. HAD and LAD rats were sacrificed for this study at 60 days after alcohol preference testing. Such testing was necesary because there remains some heterogeneity in drinking scores in some HAD and LAD rats. This 2-month interval before sacrifice served to minimize a possible effect of prior ethanol on the $GABA_A$ receptors.

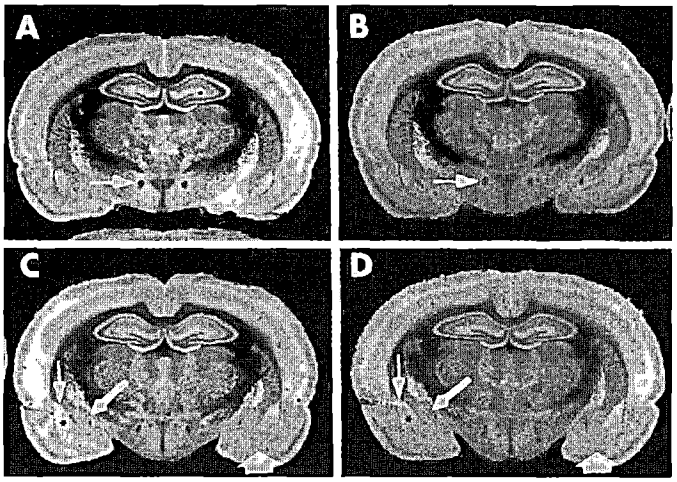

Fig. 1. $GABA_A$ receptor binding in the forebrain of LAD and HAD rats. **A, B** Micrographs are taken approximately at the bregma: 1.9 mm level [36]. The arrows point to the fornix (the dark spot). The paraventricular hypothalamic nucleus is medial to the fornix and contains a low density (darker appearance) of [^{35}S]TBPS binding sites. The LAD rat (**A**) possesses a high density (brighter in their appearance on the micrograph) of [^{35}S]TBPS binding sites in the anterior hypothalamic nucleus which is medial-ventral to the fornix and lateral to the midline 3rd ventricle when compared with the HAD rat (**B**). **C, D** Micrographs are taken at the bregma: 2.3-mm level [36]. The amygdaloid complex is marked with fat arrows at the right lower portion. Marked in the amygdaloid complex are the lateral amygdaloid nucleus (small arrow pointing downward), basolateral amygdala (black asterisks) and CeA (bold arrow). The LAD rat (**C**) possesses significantly more [^{35}S]TBPS binding sites in the basolateral amygdala and CeA than the HAD rat (**D**). **A–D** × 2.7.

Tissue Processing

All animals were decapitated, and the brains were removed quickly, frozen with dry ice powder and stored in a deep freezer until sectioning. Coronal sections (14 μm) for each discrete brain region including the nucleus accumbens (NA), central amygdaloid nucleus (CeA), basolateral amygdaloid nucleus, basomedial amygdaloid nucleus, cingulate gyrus cortex, the caudate putamen, CA3 of the hippocampus and hypothalamic nuclei were cut with a cryostat microtome and identified according to Paxinos and Watson [36]. Frozen sections were collected and thaw-mounted onto gelatin-coated slides and stored in a deep freezer.

In situ Receptor Binding Method

The protocol of Edgar and Schwartz [7] was applied for localizing $GABA_A$ receptor binding sites. In brief, tissue sections without fixation were preincubated with 50 m*M* K_2HPO_4/NaH_2PO_4, 200 m*M* NaCl and 1 m*M* EDTA, pH 7.4, at room temperature for 10 min and then incubated with 2 n*M* [^{35}S]TBPS (DuPont; specific activity = 2,000 Ci/mmol) in the above-mentioned buffer without EDTA in a humidified chamber for 2 h at room temperature for total binding study. For nonspecific binding, tissue sections were incubated with the above-mentioned radioactive ligand in the presence of 10 μ*M* unlabeled picrotoxin. Tissue sections were then rinsed 3 times (10 min for each rinse) at room temperature with 50 m*M* K_2HPO_4/NaH_2PO_4, pH 7.4, and 200 m*M* NaCl. After a brief rinse for 3 s in deionized water, tissue sections were dried quickly, apposed to Ultrofilms (LKB) and processed for an autoradiographic exposure for 2 days.

Quantitative Autoradiography and Statistical Analysis

The [^{35}S]TBPS binding capacity in discrete brain regions was determined by quantitative autoradiography routinely used in our laboratory [12, 21]. In brief, ^{35}S-labeled brain paste standards were used to generate a standard curve in 1,000 cpm/mg protein unit. The relative quantity of $GABA_A$ receptor binding sites in a discrete brain region was obtained by reading their respective autoradiograms with a computer-assisted image analysis system run by the JAVA program of Jandel Scientific [12, 21]. Then, tissue sections were counterstained with cresyl violet stain for an anatomical reference to identify each brain region. The optical density from the autoradiograms for each structure was electronically compared with the standard curve [12, 21]. The specific binding was determined by subtracting the nonspecific binding from the total binding of each section. Readings from eight autoradiograms were made to represent each structure of 1 animal. Six animals per group were studied. The two-tailed Student's t test was used for statistical analysis to compare specific [^{35}S]TBPS binding sites in each discrete region between HAD versus LAD rats.

Results

Specific [^{35}]TBPS binding sits were localized in numerous regions of HAD and LAD rats. For example, the cerebral cortex (fig. 1A–D), globus pallidus and lateral amygdala (fig. 1C, D) were among those containing high binding densities in the forebrain. In fact, the lateral amygdala (fig. 1C) had the highest density of $GABA_A$ receptors (table 1) within the amygdaloid complex (fig. 1C, D). However, the discrete brain regions such as the central caudate putamen and NA possessed relatively low binding levels (table 2). Among all areas examined in this study, the globus pallidus contained the highest binding capacity of [^{35}S]TBPS binding sites (table 2). The cerebral cortex also contained a high density of receptors (table 2). A high binding capacity of [^{35}S]TBPS binding sites was also seen in the lateral hypothalamic nucleus, central medial thalamic nucleus and CA3 of the hippocampus (table 1), whereas the lowest binding capacity was determined in the paraventricular hypothalamic nucleus (fig. 1C, D, table 1).

The quantitative analysis showed that [^{35}S]TBPS binding sites for $GABA_A$ receptors in the central, basolateral and basomedial nuclei of the amygdaloid complex of HAD rats were significantly fewer than those of LAD rats

(table 1). In the diencephalic areas, a significantly lower binding was seen in the lateral and anterior hypothalamic nucleus (fig. 1A, B) of HAD rats than LAD rats (table 1). The binding of the CA3 of the hippocampus was also not different between HAD and LAD rats. However, although the globus pallidus possessed a high density of receptor binding, the [^{35}S]TBPS binding sites in the globus pallidus were not significantly different between HAD and LAD rats (table 2). The binding capacity in the NA was also not statistically different between HAD and LAD rats (table 2). Similarly, the binding capacity in the caudate putamen, cingulate gyrus cortex and the layer 4 of the parietal cerebral cortex was also not greatly different between HAD and LAD rats (table 2).

Table 1. Specific [^{35}S]TBPS binding (1,000 cpm/mg protein) for $GABA_A$ receptors in the amygdaloid, diencephalic and hippocampal regions of HAD versus LAD rats

	LAD (n = 6)	HAD (n = 6)
LaA	70.9±2.2	66.1±1.1
CeA	49.0±1.4	44.3±1.0*
BLA	64.3±2.1	58.3±1.2*
BMA	55.8±1.8	49.0±1.3*
CA3	44.9±1.4	42.7±0.9
CMT	58.6±1.7	52.1±1.6*
PVN	17.2±0.6	15.4±1.0
VMH	43.8±1.1	40.3±1.2
LH	41.5±0.9	36.8±0.9**
AH	47.0±1.0	42.8±0.5**

Eight readings of autoradiograms for each structure were made to represent 1 animal. Values are expressed as means ± MSE.

LaA = Lateral amygdaloid nucleus; BLA = basolateral amygdaloid nucleus; BMA = basomedial amygdaloid nucleus; CMT = central medial thalamic nucleus; PVN = paraventricular hypothalamic nucleus; VMH = ventromedial hypothalamic nucleus; LH = lateral hypothalamic nucleus; AH = anterior hypothalamic nucleus; n = number of animals studied. * $p < 0.05$, ** $p < 0.01$ by the two-tailed Student's *t* test.

Table 2. Specific [^{35}S]TBPS binding (1,000 cpm/mg protein) for $GABA_A$ receptors in the basal ganglia, forebrain and cerebral cortex of HAD and LAD rats

	LAD (n = 6)	HAD (n = 6)
NA core	24.6±0.7	24.8±0.7
NA shell	27.4±0.6	25.5±1.0
CPc	22.2±0.4	21.6±0.7
CPv	39.1±0.9	36.4±1.1
GP	70.8±0.5	69.3±1.0
CGC	40.8±1.3	42.6±1.4
PC4	52.0±0.7	53.0±1.3

The numbers of autoradiograms read and animals studied were similar to those indicated in table 1.

CPc = Central caudate putamen; CPv = ventral caudate putamen; GP = globus pallidus; CGC = cingulate gyrus cortex; PC4 = layer 4 of the parietal cerebral cortex. No statistical differences were found between HAD and LAD rats by the two-tailed Student's t test.

Discussion

In agreement with the existing literature on outbred rats [7, 9, 45, 58], the present study confirms that there is a high density of [^{35}S]TBPS binding sites in numerous brain regions including the cerebral cortex, anterior hypothalamic nucleus and amygdaloid complex. As compared with LAD rats, HAD rat brains contain fewer [^{35}S]TBPS binding sites of $GABA_A$ receptors in the central medial thalamic, lateral and anterior hypothalamic nuclei and amygdaloid nuclei (the central, basolateral and basomedial amygdaloid nuclei). In fact, the present study is the first of its kind showing that [^{35}]TBPS binding sites of the $GABA_A$ receptor complex are fewer in certain brain regions of HAD rats which exhibit a high alcohol preference when compared with those of LAD rats which exhibit a low alcohol preference. Since HAD rats are selectively bred for high alcohol preference [24], the above result suggests that $GABA_A$ receptors may be involved in alcohol preference and possibly also in anxiety. An association between alcohol preference and anxiety is supported by recent studies showing that (1) Sardinian P rats are a genetic animal model for anxiety [1]; (2) anxiety is a potential predictor of vulnerability to ethanol self-administration [51], and (3) P rats with a high alcohol preference are more anxious than NP rats [52].

Since an experimentally induced increase in [^{35}]TBPS binding has been linked to a decreased GABAergic capacity [2], it has been suggested by Concas et al. [3] and Sanne et al. [44, 46] that a lower binding capacity of [^{35}]TBPS ligand reflects an enhancement of GABAergic function. In fact, there is concomitant decreased [^{35}S]TBPS and increased [^{3}H]muscimol in the cerebral cortex of rats with stress [6], indicating that lower [^{35}S]TBPS binding is associated with enhanced GABAergic function. Recently, Hawkinson et al. [10] reported that neuroactive steroids potentiate GABA-evoked chloride current, enhance [^{3}H]flunitrazepam binding, but significantly decrease [^{35}S]TBPS binding. The latter again suggests an inverse

relationship between the [^{35}S]TBPS binding capacity and GABAergic function. This is further supported by the fact that GABA inhibits [^{35}S]TBPS binding [58] and acute stress increases [^{35}S]TBPS binding, which is believed to be a consequence of decreased GABAergic function [45]. Therefore, the lower binding level of [^{35}S]TBPS ligand seen in HAD rats may indicate an enhancement of GABAergic function in the brain of these animals. In short, this study supports the hypothesis that an enhanced GABAergic function is associated with a high alcohol preference or anxiety in HAD rats. This is in accord with our previous study showing that there are increased GABAergic terminals in the NA of P and HAD rats [13]. These data support the role of the GABA system in alcohol preference [20, 57]. Recently, it has been demonstrated that (1) the CeA is associated with fear and anxiety [5], and (2) alcohol can produce anxiolytic effects [52]. The finding of a significant difference in [^{35}S]TBPS binding in the CeA between HAD and LAD rats suggests that $GABA_A$ receptors in the amygdala may play a role in alcohol preference and/or anxiety. This notion is consistent with the work of Hyytia and Koob [14] who have demonstrated that microinjections of $GABA_A$ receptor antagonists into the extended amygdaloid regions selectively reduce operant responding for ethanol in outbred rats.

However, it should also be mentioned that other receptors in the amygdala are likely involved in alcohol preference or anxiety. For example, we have reported that the CeA has a lower binding capacity of calcitonin-gene-related peptide receptors not only in HAD rats, but also in P rats, as compared with their control LAD and NP rats, respectively [12]. This suggests that calcitonin-gene-related peptide receptors in the CeA may also participate in alcohol preference and/or anxiety in HAD and P rats. It is generally believed that alcoholism is a multi-gene-related disorder. Thus, different genetic influences are most likely to mediate alcohol preference and/or anxiety in HAD and P rats [25]. Although HAD and P rats have been selectively bred for alcohol preference [24, 41, 42] and are useful animal models for studying alcoholism [22, 23], alcohol reinforcement between HAD versus LAD rats and P versus NP rats is not the same as pointed out by Lankford et al. [22, 23] and Ritz et al. [41]. This may be related to the fact that HAD/LAD rats and P/NP rats were selectively bred from different foundation stocks [24, 25].

Although [^{3}H]muscimol, [^{3}H]flunitrazepam and [^{35}S]TBPS have all been widely used to study $GABA_A$ receptors, their binding patterns and capacities are not identical in the brain [50]. For example, the [^{35}S]TBPS binding pattern is similar to [^{3}H]flunitrazepam, but not to [^{3}H]muscimol [50]. However, the heterogeneity of $GABA_A$ receptors in different ligand bindings may be associated with their differences in receptor subtypes and receptor subunit combinations [47]. Although the present study demonstrated no differences in [^{35}S]TBPS in the NA between HAD and LAD rats, it should be noted that microinjections of muscimol into the NA reduce ethanol self-administration [11], suggesting that GABAergic transmission in the NA is involved in the termination of ethanol self-administration. However, it is unknown whether there is a difference of $GABA_A$ receptor binding using [^{3}H]muscimol in the NA between HAD/LAD rats. Since $GABA_A$ receptors are formed by an unknown combination of receptor subunits [9, 47, 57], the different binding properties for each of the above-mentioned ligands for $GABA_A$ receptors may be due to their various affinities to different $GABA_A$ receptor subunits. For instance, chronic alcohol treatment affects $GABA_A$ receptor subunits and their mRNA. It has also shown that prolonged ethanol treatment decreases $GABA_A$ receptor α-subunit [32, 33], but upregulates $GABA_A$ receptor β-subunit mRNA including β_1-, β_2- and β_3-subunit mRNA [31].

In conjunction with the above literature, the present study suggests that it is worthwhile to include an exploration of $GABA_A$ receptor subunit mRNA for future studies. For instance, greater levels of α_1-, α_6- and β_2-subunit mRNA were detected in the cerebellum of naive ethanol-withdrawal seizure-resistant mice, compared with naive ethanol-withdawal seizure-prone mice [19], although there is no difference in [^{35}S]TBPS binding between naive ethanol-withdrawal seizure-resistant and seizure-prone mice [8]. It should be mentioned that [^{35}S]TBPS is known to bind to the convulsant sites of picrotoxin of the $GABA_A$ receptor complex [16] associated with seizure. Therefore, [^{35}S]TBPS may not be able to interact with all of the convulsant binding sites without a convulsive condition or treatment, suggesting that some of the convulsant sites of picratoxin may be 'masked' in naive animals without seizure. Interestingly, other studies [6, 9] have also indicated that [^{35}S]TBPS binding is increased in rats treated with the convulsant (isoniazid) or exposed to foot shock stress which can potentiate the convulsant activity of isoniazid [2, 3, 44]. This notion is further supported by other studies [44, 46] showing that (1) seizure increases [^{35}S]TBPS binding, and (2) increased TBPS binding takes place in intoxicated ethanol-dependent rats.

In summary, there are high densities of $GABA_A$ receptors in numerous discrete brain regions of HAD/LAD rats using [^{35}S]TBPS as a binding ligand. With quantitative

autoradiographic analysis, this study has also shown that there are fewer [^{35}S]TBPS binding sites of $GABA_A$ receptors in the amygdaloid complex of HAD rats compared to LAD rats. Fewer [^{35}S]TBPS binding sites of $GABA_A$ receptors are also detected in the lateral hypothalamic nucleus, anterior hypothalamic nucleus and central medial thalamic nucleus of HAD rats than LAD rats. Since lower [^{35}S]TBPS binding for $GABA_A$ receptors reflects an enhanced GABAergic function, the present study supports the hypothesis that facilitatory GABAergic function in the brain is associated with high alcohol preference in HAD rats. In conclusion, this study, together with the existing literature, points to the fact that future studies should include an effort to study how alcohol affects $GABA_A$ receptor subunits and their mRNA expression in conjunction with the use of [^{35}S]TBPS in rats, particularly with a seizure treatment, to further understand the role of $GABA_A$ receptors in alcoholism.

Acknowledgments

We would like to thank Drs. T.-K. Ki and Harry L. June for their comments and suggestions and Ms. Sherrilyn E. Guthrie for her technical assistance with the alcohol preference test. This work was in part supported by PHS grant AA08553 and an IUPUI faculty development fund.

References

1 Colombo G, Agabio R, Lobina C, Reali R, Zocchi A, Fadda F, Gesa GL. Sardinian alcohol-preferring rats: A genetic animal model of anxiety. Physiol Behav 57:1181–1185;1995.
2 Concas A, Mascia MP, Sanna E, Sautoro G, Serra M, Biggio G. 'In vivo' administration of valproate decreases t-[^{35}S]butylbicyclophosphorothionate binding in the rat brain. Naunyn Schmiedebergs Arch Pharmacol 343:296–300; 1991.
3 Concas A, Sanna E, Serra M, Mascia MP, Santoro V, Biggio G. 'Ex vivo' binding of [^{35}S]-TBPS as a tool to study the pharmacology of $GABA_A$ receptors. In: Biggio G, Costa E, eds. GABA and Benzodiazepine Receptor Subtypes. New York, Raven Press, 89–108;1990.
4 Criswell HE, Simson PE, Duncan GE, McCown TJ, Herbert JS, Morrow AL, Breese GR. Molecular basis for regionally specific action of ethanol on $GABA_A$ receptors: Generalization to other ligand-gated ion channels. J Pharmacol Exp Ther 267:522–536;1993.
5 Davis M, Rainnie D, Cassell M. Neurotransmission in the rat amygdala related to fear and anxiety. Trends Neurosci 17:208–214;1994.
6 Drugan RC, Paul SM, Crawley JN. Decreased forebrain [^{35}S]TBPS binding and increased [^{3}H]muscimol binding in rats that do not develop stress-induced behavioral depression. Brain Res 631:270–275;1993.
7 Edgar PP, Schwartz RD. Localization and characterization of ^{35}S-t-butylbicyclophosphorothionate binding in rat brain: An autoradiographic study. J Neurosci 10:603–612;1990.
8 Feller DJ, Harris RA, Crabbe JC. Differences in GABA activity between ethanol withdrawal seizure prone and resistant mice. Eur J Pharmacol 157:147–154;1988.
9 Giorgi O, Cancedda E, Lecca D, Orlandi M, Corda MG. Allosteric modulation of [^{35}S]TBPS-binding in the cerebral cortex of the rat during postnatal development. Dev Brain Res 80:73–80;1994.
10 Hawkinson JE, Kimbrough CL, Belelli D, Lambert JJ, Purdy RH, Lan NC. Correlation of neuroactive steroid modulation of [^{35}S]-t-butylbicyclophosphorothionate and [^{3}H]-flunitrazepam binding and γ-aminobutyric $acid_A$ receptor function. Mol Pharmacol 46:977–985; 1994.
11 Hodge CW, Chappelle AM, Samson HH. GABAergic transmission in the nucleus accumbens is involved in the termination of ethanol self-administration in rats. Alcoholism: Clin Exp Res 19:1486–1493;1995.
12 Hwang BH, Kunkler PE, Lumeng L, Li T. Calcitonin gene-related peptide (CGRP) content and CGRP receptor binding sites in discrete forebrain regions of alcohol-preferring vs. nonpreferring rats and high alcohol-drinking vs. low alcohol-drinking rats. Brain Res 690: 249–253;1995.
13 Hwang BH, Lumeng L, Wu JY, Li TK. Increased number of GABAergic terminals in the nucleus accumbens is associated with alcohol preference in rats. Alcohol Clin Exp Res 14: 503–507;1990.
14 Hyytia P, Koob GF. $GABA_A$ receptor antagonism in the extended amygdala decreases ethanol self-administration in rats. Eur J Pharmacol 283:151–159;1995.
15 Ishihara S, Hiramatsu M, Kameyama T, Nabeshima T. Development of tolerance to anxiolytic effects of chlordiazepoxide in elevated plus-maze test and decrease of $GABA_A$ receptors. J Neural Transm 91:27–37;1993.
16 Ito Y, Ho IK. Studies on picrotoxin binding sites of $GABA_A$ receptors in rat cortical synaptoneurosomes. Brain Res 33:373–378;1994.
17 June HL, Murphy JM, Heywitt RL, Greene TL, Lin M, Mellor-Burke JJ, Lumeng L, Li TK. Benzodiazepine receptor ligands with different intrinsic efficacies alter ethanol intake in alcohol-nonpreferring (NP) rats. Neuropsychopharmacology 14:55–66;1996.
18 June HL, Murphy JM, Mellor-Burke JJ, Lumeng L, Li TK. The benzodiazepine inverse agonist Ro19-4603 exerts prolonged and selective suppression of ethanol intake in alcohol-preferring (P) rats. Psychopharmacology 115: 325–331;1994.
19 Keir WJ, Morrow AL. Differential expression of $GABA_A$ receptor subunit mRNAs in ethanol-naive withdrawal seizure resistant (WSR) vs. withdrawal seizure prone (WSP) mouse brain. Mol Brain Res 25:200–208;1994.
20 Korpi ER. Role of $GABA_A$ receptors in the actions of ethanol and in alcoholism: Recent advances. Alcohol Alcohol 29:115–129;1994.
21 Kunkler PE, Hwang BH. Lower $GABA_A$ receptor binding in the amygdala and hypothalamus of spontaneously hypertensive rats. Brain Res Bull 36:57–61;1195.
22 Lankford MF, Myers RD. Genetics of alcoholism: Simultaneous presentation of a chocolate drink diminishes alcohol preference in high drinking HAD rats. Pharmacol Biochem Behav 49:417–425;1194.
23 Lankford MF, Roscoe AK, Pennington SN, Myers RD. Drinking of high concentrations of ethanol versus palatable fluids in alcohol-preferring (P) rats: Valid animal model of alcoholism. Alcohol 8:293–299;1991.
24 Li TK, Lumeng L, Doolittle DP. Selective breeding for alcohol preference and associated responses. Behav Genet 23:163–170;1993.
25 Li TK, Lumeng L, Froehlich JC, Murphy JM, McBride WJ. Genetic influence on response to the reinforcing properties of ethanol in rats. In: Kuriyama K, Takada A, Ishii H, eds. Biomedical and social aspects of alcohol and alcoholism. New York, Elsevier, 487–490;1988.
26 Liljequist S, Engel J. Effect of GABAergic agonists and antagonists on ethanol-induced behavioral changes. Psychopharmacology 78:71–75;1982.

27 Luddens H, Korpi ER. GABA antagonists differentiate between recombinant $GABA_A$/benzodiazepine receptor subtypes. J Neurosci 15: 6957–6962;1995.
28 McBride WJ, Murphy JM, Lumeng L, Li TK. Effects of Ro15-4513, fluoxetine and desipramine on the intake of ethanol, water and food by the alcohol-preferring (P) and nonpreferring (NP) lines of rats. Pharmacol Biochem Behav 30:1045–1050;1988.
29 McBride WJ, Murphy JM, Lumeng L, Li TK. Serotonin, dopamine and GABA involvement in alcohol drinking of selectively bred rats. Alcohol 7:199–205;1990.
30 Mhatre MC, Pena G, Sieghart W, Ticku MK. Antibodies specific for $GABA_A$ receptor α subunits reveal that chronic alcohol treatment down-regulates α-subunit expression in rat brain regions. J Neurochem 61:1620–1625; 1993.
31 Mhatre M, Ticku MK. Chronic ethanol treatment upregulates the GABA receptor beta subunit expression. Mol Brain Res 23:246–252; 1994.
32 Montpied P, Morrow AL, Karanian JW, Ginns EI, Martin BM, Paul SM. Prolonged ethanol inhalation decreases gamma-aminobutyric $acid_A$ receptor subunit mRNAs in the rat cerebral cortex. Mol Pharmacol 39:157–163;1991.
33 Morrow AL, Montpied P, Lingford-Hughes A, Paul SM. Chronic ethanol and pentobarbital administration in the rat: Effects on $GABA_A$ receptor function and expression in brain. Alcohol 7:237–244;1990.
34 Morrow AL, Suzdak PD, Karanian JW, Paul SM. Chronic ethanol administration alters gamma-aminobutyric acid, pentabarbital and ethanol-mediated ^{36}Cl uptake in cerebral cortical synapto-neurosomes. Pharmacol Exp Ther 246:158–164;1988.
35 Nestoro JN. Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. Science 209:708–710;1980.
36 Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Sydney, Academic Press, 1986.
37 Rassnick S, D'Amico E, Riley E, Koob GF. GABA antagonist and benzodiazepine partial inverse agonist reduce motivational responding for ethanol. Alcohol Clin Exp Res 17: 124–130;1993.
38 Rassnick S, Krechman J, Koob GF. Chronic ethanol produces a decreased sensitivity to the response-disruptive effects of GABA receptor complex antagonists. Pharmacol Biochem Behav 44:943–950;1993.
39 Rastogi SK, Thyagarajan R, Clothier J, Ticku MK. Effect of chronic treatment of ethanol on benzodiazepine and picrotoxin sites on the GABA receptor complex in regions of the brain of the rat. Neuropharmacology 25:1179–1184; 1986.
40 Rastogi SK, Ticku MK. Involvement of a GABAergic mechanism in the anticonvulsant effect of pentobarbital agonist maximal electroshock-induced seizures in rats. Pharmacol Biochem Behav 22:141–146;1981.
41 Ritz MC, Garcia JM, Protz D, George FR. Operant ethanol-reinforced behavior in PP, NP, HAD and LAD rats bred for high versus low ethanol preference. Alcohol Clin Exp Res 198:1406–1415;1994.
42 Ritz MC, Garcia JM, Protz D, Rael AM, George FR. Ethanol-reinforced behavior in P, NP, HAD and LAD rats: Differential genetic regulation of reinforcement and motivation. Behav Pharmacol 5:521–531;1994.
43 Samson HH, Tolliver GA, Pfeffer AO, Sadeghi KG, Mills FG. Oral ethanol reinforcement in the rat: Effect of the partial inverse benzodiazepine agonist Ro15-4513. Pharmacol Biochem Behav 27:517–519;1987.
44 Sanna E, Concas A, Serra M, Santoro G, Biggio G. Ex vivo binding of t-[^{35}S]butylbicocylophosphorothionate: A biochemical tool to study the pharmacology of ethanol at the gamma-aminobutyric acid-coupled chloride channel. J Pharmacol Exp Ther 256:922–928;1991.
45 Sanna E, Cuccheddu T, Serra M, Conca A, Biggio G. Carbon dioxide inhalation reduces the function of $GABA_A$ receptors in the rat brain. Eur J Pharmacol 216:457–458;1992.
46 Sanna E, Serra M, Cossu A, Colombo G, Follesa P, Cuccheddu T, Concas A, Biggio G. Chronic ethanol intoxication induces differential effects on $GABA_A$ and NMDA receptor function in the rat brain. Alcohol Clin Exp Res 17:115–122;1993.
47 Sieghart W. Structure and pharmacology of γ-$aminobutyric\ acid_A$ receptor subtypes. Pharmacol Rev 47:181–234;1995.
48 Slany A, Zezula J, Tretler V, Sieghart W. Rat β3 subunit expressed in human embryonic kidney 293 cells form high affinity [^{35}S]-butylbicyclophosphorothionate binding sites modulated by several allosteric ligands of γ-aminobutyric acid type A receptors. Mol Pharmacol 48:385–391;1995.
49 Smith BR, Robidoux J, Amit Z. GABAergic involvement in the acquisition of voluntary ethanol intake in laboratory rats. Alcohol Alcohol 27:227–231;1992.
50 Sola C, Martinez E, Camon L, Pazos A, Rodriguez-Farre E. Lindane administration to the rat induces modifications in the regional cerebral binding of [^3H]-muscimol, [^3H]-flunitrazepam and t-[^{35}S]-butylbicyclophosphorothionate: An autoradiographic study. J Neurochem 60: 1821–1834;1993.
51 Spanagel R, Montkowski A, Allingham K, Stohr T, Shoaib M, Holsboer F, Landgraf R. Anxiety: A potential predictor of vulnerability to the initiation of ethanol self-administration in rats. Psychopharmacology 122:369–373; 1995.
52 Stewart RB, Gatto GJ, Lumeng L, Li TK, Murphy JM. Comparison of alcohol-preferring (P) and nonpreferring (NP) rats on tests of anxiety and for the anxiolytic effects of ethanol. Alcohol 10:1–10;1993.
53 Stewart RB, Perlanski E, Grupp LA. Ethanol as a reinforcer for rats: Factors of facilitation and constraint. Alcohol Clin Exp Res 12:599–608; 1988.
54 Suzdak PD, Glowa JR, Crawley JN, Schwartz RD, Skolnick P, Paul SM. A selecting imidazobenzodiazepine antagonist of ethanol in the rat. Science 234:1243–1246;1986.
55 Suzdak PD, Schwartz RD, Skolnick P, Paul SM. Alcohols stimulate gamma-aminobutyric acid receptor-mediated chloride uptake in brain vesicles: Correlation with intoxication potency. Brain Res 444:340–345;1988.
56 Thyagarajan R, Ticku MK. The effect of in vitro and in vivo ethanol administration on [^{35}S]t-butylbicyclophosphorothionate binding in C57 mice. Brain Res Bull 15:343–345;1985.
57 Ticku MK, Mhatre M, Mehta AK. Modulation of GABAergic transmission by ethanol. In: Biggio G, Concas A, Costa E, eds. GABAergic Synaptic Transmission. New York, Raven Press, 255–268;1992.
58 Vincens M, Dartois E, Moyse E, Haour F, Fillion G. An autoradiographic study comparing the interactions of 3α-OH-5α-prognan-20-one pregnenolone sulfate and pentobarbital with [^{35}S]-TBPS binding sites and their modulation by GABA in different structures of the rat brain. Naunyn Schmidebergs Arch Pharmacol 351:356–361;1995.