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Short communication

Chronic ethanol exposure selectively increases diazepam-insensitive [^{3}H]RO15-4513 binding in mouse cerebellum

Howard C. Becker [a,*], Michael F. Jarvis [b]

[a] *Center for Drug and Alcohol Programs, Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina and VAMC, Charleston, SC 29425, USA*

[b] *Rhone-Poulenc Rorer Central Research, Collegeville, PA 19426, USA*

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Abstract

The effect of chronic ethanol exposure and withdrawal on [^{3}H]RO15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate) binding to diazepam-sensitive and diazepam-insensitive binding sites was determined in mouse brain. Neither chronic ethanol treatment nor withdrawal significantly altered total [^{3}H]RO15-4513 binding in mouse cortex or cerebellum. However, diazepam-insensitive [^{3}H]RO15-4513 binding density (B_{max}) in cerebellum was significantly increased immediately following chronic ethanol treatment (60%) and at 8 h following withdrawal (75%). [^{3}H]RO15-4513 binding affinity was not significantly influenced by chronic ethanol exposure or withdrawal. These results indicate that chronic ethanol treatment and withdrawal can selectively up-regulate diazepam-insensitive [^{3}H]RO15-4513 binding sites and suggest that this unique $GABA_A$ receptor subtype may play some role in ethanol dependence and withdrawal.

Keywords: Ethanol; RO15-4513; $GABA_A$/benzodiazepine receptor complex; Ethanol dependence/withdrawal

1. Introduction

It is generally recognized that $GABA_A$ receptors are involved in many pharmacologic effects of ethanol and chronic exposure to ethanol results in neuroadaptive changes in $GABA_A$ receptor function. The imidazobenzodiazepine RO15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate) is a partial benzodiazepine inverse agonist that has been shown to antagonize some (but not all) of the pharmacological and physiological actions of ethanol (for review see Ticku and Kulkarni, 1988). Chronic ethanol exposure has been demonstrated to enhance behavioral sensitivity to the proconvulsant actions of RO15-4513 (Becker and Anton, 1989), as well as increase the ability of RO15-4513 to inhibit GABA-operated $^{36}Cl^-$ flux (Buck and Harris, 1990). In contrast, the effects of chronic ethanol exposure and withdrawal on [^{3}H]RO15-4513 binding have been equivocal. For example, Ticku and his colleagues reported an increase in the density of [^{3}H]RO15-4513 binding sites in rat cortex and cerebellum, as well as in mouse cultured spinal neurons following chronic ethanol exposure and withdrawal (Mhatre and Ticku, 1989; Mhatre et al., 1988). However, Buck and Harris (1990) did not observe a change in the number of [^{3}H]RO15-4513 recognition sites following chronic ethanol exposure in mice.

The pharmacology of [^{3}H]RO15-4513 binding in mammalian brain is complex in that both diazepam-sensitive and diazepam-insensitive sites are labeled by this ligand with roughly equivalent affinity (Luddens et al., 1990; Sieghart et al., 1987; Turner et al., 1991). However, a distinction between diazepam-sensitive and diazepam-insensitive sites was not assessed in these earlier studies. Korpi et al. (1992) found no differences in the number of diazepam-sensitive or diazepam-insensitive [^{3}H]RO15-4513 binding sites following chronic ethanol exposure in rats. In contrast, analysis of post-mortem cerebellar tissue from human alcoholics revealed an increased affinity of [^{3}H]RO15-4513 binding in alcoholics compared to controls, but no significant difference in diazepam-insensitive [^{3}H]RO15-4513

* Corresponding author.

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binding (Korpi et al., 1992). Thus, the influence of chronic ethanol exposure on [^{3}H]RO15-4513 binding and in particular, the unique diazepam-insensitive binding sites, is unclear at present.

The present study was conducted to examine the effects of chronic ethanol exposure and withdrawal on both diazepam-sensitive and diazepam-insensitive [^{3}H]RO15-4513 binding in mouse cerebellum. Mice were chronically exposed to ethanol via the inhalation route. This model of ethanol dependence has been previously shown to yield relatively stable blood ethanol levels during the course of the treatment, withdrawal-related seizures, and enhanced behavioral sensitivity to the proconvulsant properties of RO15-4513 (Becker and Hale, 1992).

2. Materials and methods

Adult male C3H/He mice (80–100 days of age) were purchased from Charles River Laboratories (Portage, MI, USA). The animals were housed 3–4 per cage under a 12 h light/dark cycle (lights on at 06.00 h). Food and water were continuously available.

2.1. Chronic ethanol administration

Mice were chronically exposed to ethanol vapor in inhalation chambers as described by Becker and Hale (1992). The ethanol chamber concentration was maintained at 10–12 mg/l air, which produced a blood ethanol concentration of 165–185 mg/dl (36–40 mM). One group of mice ($n = 28$) received 64 h continuous exposure to ethanol vapor while a control group of mice ($n = 28$) received no treatment. One half of each group of mice were killed at the end of the ethanol exposure (0 h) while the remaining mice were killed following an 8 h period of abstinence (8 h). Brains were rapidly removed and dissected to yield samples of cerebral cortex and cerebellum. Brain regions from 3–4 mice were pooled and homogenized in 20 vols. of ice-cold 50 mM Tris-HCl (pH 7.4) using a Brinkman polytron (setting 6, 20 s). This membrane homogenate was then centrifuged at $48\,000 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in buffer and recentrifuged at $48\,000 \times g$ for 10 min at 4°C. This washing procedure was repeated three times. The final pellet was frozen at −70°C until the time of assay.

2.2. [^{3}H]RO15-4513 binding

The binding of [^{3}H]RO15-4513 (29 Ci/mmol, Dupont NEN Products, Boston, MA, USA) to diazepam-sensitive and diazepam-insensitive sites was measured in the absence or presence of 10 μM diazepam (Sigma, St. Louis, MO, USA) as described by Turner et al. (1991). Routine ligand saturation assays were carried out, in triplicate, in 12 × 75 mm polypropylene test tubes containing an aliquot of cerebellar (or cortical) membranes (75–150 mg protein/ml) in incubation buffer (50 mM Tris-HCl, pH 7.4, 4°C) with 6–8 concentrations of [^{3}H]RO15-4513 (0.1–50 nM) in a final volume of 0.5 ml. All assays were conducted at 4°C for 60 min. Non-specific binding was defined in the presence of 10 μM unlabeled RO15-4513 (Research Biochemicals, Natick, MA, USA).

All binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using an M-48 Brandel Cell Harvester (Gaithersburg, MD, USA). Filters were washed twice with ice-cold buffer (5 ml) and placed in scintillation vials, and bound radioactivity was determined using conventional liquid scintillation spectroscopy techniques at an efficiency of 40–50%. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the reference standard. Saturation

Table 1
Effect of chronic ethanol treatment on saturation binding parameters for [^{3}H]RO15-4513 in mice

		K_D (nM)	B_{max} (pmol/mg protein)
Cortex	Control	2.36 ± 0.32	4.92 ± 0.16
	Ethanol (0 h)	2.20 ± 0.20	4.53 ± 0.44
	Ethanol (8 h)	2.53 ± 0.25	5.32 ± 0.43
Cerebellum	Control	3.91 ± 1.10	3.22 ± 0.30
	Ethanol (0 h)	4.42 ± 0.15	3.71 ± 0.43
	Ethanol (8 h)	4.05 ± 0.51	3.71 ± 0.41
(+) 10 μM diazepam			
	Control	2.98 ± 0.42	0.95 ± 0.11
	Ethanol (0 h)	5.25 ± 1.20	1.53 ± 0.24 [a]
	Ethanol (8 h)	5.55 ± 0.98	1.69 ± 0.18 [a]

Mice were chronically exposed to ethanol vapor in inhalation chambers as described in Materials and methods, and killed either immediately upon removal from chamber (0 h) or 8 h following withdrawal (8 h). Saturation isotherms were analyzed by Scatchard analysis to determine K_D and B_{max} values. In cerebellum, [^{3}H]RO15-4513 binding parameters were determined in the absence and presence of 10 μM diazepam. Values represent means ± S.E.M. from four separate experiments. [a] Significant difference from Control, $P < 0.05$.

binding parameters (K_D and apparent B_{max}) were determined using the non-linear regression curve-fitting program Accufit (Beckman Instruments, Fullerton, CA, USA). Statistical differences between the experimental groups were compared using analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post-hoc test.

3. Results

Preliminary binding studies revealed that [^{3}H]-RO15-4513 bound to mouse cortical and cerebellar membranes with high specificity (specific binding = 80–90% of total binding). In mouse cerebellar membranes, diazepam (10 μM) was found to inhibit only 75–80% of the specific [^{3}H]RO15-4513 binding in a fashion consistent with labeling both diazepam-sensitive and diazepam-insensitive sites (Turner et al., 1991).

Ligand saturation studies indicated that [^{3}H]RO15-4513 bound with high affinity and limited capacity in mouse cortex and cerebellum (Table 1). The affinity of [^{3}H]RO15-4513 binding was similar in both cortex and cerebellum, and total specific [^{3}H]RO15-4513 binding was approximately 30% greater in cortex as compared to cerebellum. Chronic ethanol exposure did not significantly alter the [^{3}H]RO15-4513 affinity or density in mouse cortex when the mice were still intoxicated (0 h) or after an 8 h withdrawal period (8 h) (Table 1). Similar results were obtained in cerebellum (total [^{3}H]RO15-4513 binding) regardless of whether the mice were killed while intoxicated or after the withdrawal period.

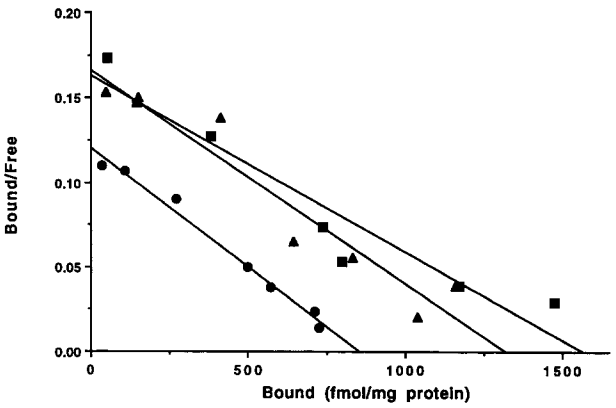

Fig. 1. Representative Scatchard plots of saturation binding studies to examine the effects of chronic ethanol exposure on diazepam-insensitive [^{3}H]RO15-4513 binding. Binding studies were conducted using cerebellar membranes from mice that had received no ethanol exposure (Control, ●), mice that were killed immediately following chronic ethanol treatment (0 h, ▲), and mice that were killed 8 h following withdrawal from chronic ethanol exposure (8 h, ■). Mean ± S.E.M. binding parameters for these groups (K_D and B_{max} values) are contained in Table 1.

When ligand saturation experiments were conducted in the presence of 10 μM diazepam to measure diazepam-insensitive [^{3}H]RO15-4513 binding, a different pattern of results was observed. Chronic exposure to ethanol resulted in a significant ($F(2,9) = 4.29$, $P < 0.05$) increase in the density of diazepam-insensitive binding sites in cerebellum. This effect was present both in mice that were killed while still intoxicated with ethanol (0 h) and in mice that were killed 8 h (8 h) after the ethanol exposure (Table 1). Ethanol exposure or withdrawal did not significantly influence the apparent affinity of [^{3}H]RO15-4513 for diazepam-insensitive binding sites in cerebellum (Fig. 1).

4. Discussion

A substantial body of evidence suggests that chronic exposure to ethanol alters $GABA_A$/benzodiazepine receptor activity, and such neuradaptive changes underlie, at least in part, the development of physical dependence and expression of a characteristic withdrawal syndrome (Ticku and Mehta, 1995). Behavioral and biochemical studies have demonstrated enhanced sensitivity to the partial benzodiazepine inverse agonist RO15-4513 (and other related compounds) following chronic ethanol exposure and withdrawal (Becker and Anton, 1989; Buck and Harris, 1990; Ticku and Mehta, 1995). However, studies examining the effects of chronic ethanol exposure on [^{3}H]RO15-4513 binding in brain have not yielded consistent results (Buck and Harris, 1990; Korpi et al., 1992; Mhatre et al., 1988). These equivocal findings are not surprising given the wide variation in ethanol treatments used in these studies and the fact that [^{3}H]RO15-4513 labels both diazepam-sensitive and diazepam-insensitive recognition sites in mammalian brain (Sieghart et al., 1987; Turner et al., 1991). Since diazepam-insensitive [^{3}H]RO15-4513 binding sites are predominately localized in cerebellum (Turner et al., 1991), the present study examined the effects of chronic ethanol exposure and withdrawal on both total [^{3}H]RO15-4513 binding in mouse cortex and cerebellum and on the diazepam-insensitive binding in cerebellum.

Consistent with some previous studies (Buck and Harris, 1990; Korpi et al., 1992), the present data indicate that chronic ethanol exposure did not produce any alterations in the binding parameters for [^{3}H]RO15-4513 in mouse cortex or cerebellum. However, an ethanol-induced increase in the density of diazepam-insensitive [^{3}H]RO15-4513 binding was found in cerebellum. These results indicate that chronic ethanol treatment can selectively up-regulate the diazepam-insensitive component of [^{3}H]RO15-4513 binding. Congruent with these findings are reports indicating that chronic ethanol treatment produced an in-

crease in mRNA levels for the α_6 subunit in cerebellum, while the same chronic ethanol treatment resulted in decreased mRNA levels and polypeptide expression of the α_1 subunit in cerebellum (Mhatre and Ticku, 1992; Mhatre et al., 1993). Diazepam-insensitive [^{3}H]RO15-4513 binding in cerebellar tissue is dependent on the presence of the α_6 subunit in $GABA_A$ receptors (Luddens et al., 1990). These results offer the possibility that this $GABA_A$ receptor subtype may be particularly sensitive to chronic ethanol exposure. Although the α_6 subunit has not been detected in other brain regions, diazepam-insensitive [^{3}H]RO15-4513 binding sites have been identified in other areas (Turner et al., 1991). Whether chronic ethanol exposure alters [^{3}H]RO15-4513 binding to these sites, and the role of these $GABA_A$ receptors in ethanol dependence/withdrawal, remains to be determined.

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