ELSEVIER

Pharmacology, Biochemistry and Behavior 68 (2001) 339–346

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

Diazepam during prior ethanol withdrawals does not alter seizure susceptibility during a subsequent withdrawal[1]

Molina C. Mhatre, Stephanie E. McKenzie, Larry P. Gonzalez*

Department of Psychiatry and Behavioral Sciences, University of Oklahoma Health Sciences Center, Room 410, Rogers Building, P.O. Box 26901, Oklahoma City, OK 73190-3000 USA

Received 1 June 2000; received in revised form 15 October 2000; accepted 16 October 2000

Abstract

The number of cycles of alcohol detoxification is suggested to be an important variable in the predisposition to severe withdrawal seizures in alcohol-dependent individuals. Several clinical studies have suggested that exposure to repeated alcohol withdrawals may lead to increased severity of subsequent withdrawal episodes. Consistent with these observations, exposure to multiple cycles of ethanol withdrawal in our previous study significantly increased sensitivity to the convulsive effects of the $GABA_A$ receptor inverse agonist, Ro15-4513, in comparison to continuous ethanol exposure with no intermittent withdrawals. There was also a selective increase in the occurrence of spontaneous spike and sharp wave (SSW) activity in the EEG recorded from hippocampal area CA_3 in proportion to the number of withdrawal episodes experienced. It is hypothesized that during such repeated episodes of ethanol intoxication and withdrawal, changes in neuronal excitation during prior withdrawals could serve as initially subconvulsive kindling stimuli that might eventually result in the increased severity of the withdrawal syndrome. There is some evidence of the successful suppression of such neuronal excitation during acute ethanol withdrawal by positive modulators of the $GABA_A$ receptor. In the present study, the benzodiazepine agonist, diazepam, at a dose (4.0 mg/kg) that suppresses acute withdrawal symptoms, when administered during intermittent withdrawals, did not alter seizure sensitivity during a subsequent nonmedicated withdrawal. Diazepam treatment during prior withdrawals also did not have any effect on the multiple withdrawal-associated increase in SSW activity in hippocampal area CA_3 during an untreated withdrawal. This finding suggests that suppression of acute withdrawal symptoms by diazepam does not prevent long-lasting changes in CNS function resulting from repeated exposures to ethanol withdrawal.

Keywords: Ethanol; Alcohol; Withdrawal; Multiple withdrawal; Repeated withdrawal; Seizures; Ro15-4513; Diazepam; γ-Aminobutyric acid receptor complex; EEG

1. Introduction

Several clinical studies have reported a high correlation between the number of hospital admissions for detoxification from alcohol and the occurrence and severity of withdrawal seizures (Booth and Blow, 1993; Brown et al., 1988; Lechtenberg and Worner, 1990, 1991). Laboratory studies also have reported that animals exhibit an increase in the number, as well as severity of seizures following exposure to repeated cycles of ethanol intoxication and withdrawal (Becker and Hale, 1993; Maier and Pohorecky, 1989; Mhatre and Gonzalez, 1999). In addition to increased severity of the withdrawal syndrome, animals exposed to multiple withdrawals were found to exhibit more intense electrophysiological changes such as increased spiking during subsequent withdrawals compared to animals with no prior withdrawal history (Poldrugo and Snead, 1984; Walker and Zornetzer, 1974). Our laboratory has also previously shown a selective increase in spontaneous EEG spiking in brain regions such as the hippocampal CA_3 area, in proportion to the number of withdrawals experienced (Veatch and Gonzalez, 1996). It has been suggested that the abnormal electrophysiological activity exhibited during prior ethanol withdrawals may

* Corresponding author. Tel.: +1-405-271-2011 ext. 47722; fax: +1-405-271-6236.

E-mail address: larry-gonzalez@ouhsc.edu (L.P. Gonzalez).

[1] This work is supported in part by grants from the Department of Psychiatry and Behavioral Sciences to M.C. Mhatre, PhD, and by NIAAA Grant No. AA09959 and OCAST Contract HR4-063 to L.P. Gonzalez, PhD.

0091-3057/01/$ – see front matter
PII: S0091-3057(00)00481-0

contribute to the increase in severity of subsequent withdrawal episodes (Walker and Zornetzer, 1974).

Findings such as these have led to the speculation that a kindling model may have applicability to the increased severity observed after multiple withdrawal episodes. Kindling refers to a phenomenon wherein repeated electrical or chemical stimulation of low intensity (too low to produce significant behavioral effects) subsequently results in the development of full motor seizures. Ballenger and Post (1978) have theorized that the subcortical EEG abnormalities occurring during alcohol withdrawals in humans are similar in type and location to those described during the electrical kindling process (Goddard et al., 1969). These authors also suggested that during multiple intoxication and withdrawal cycles, withdrawal-induced alterations in neuronal activity could serve as a kindling stimulus and that this kindling might eventually culminate in full motor seizures. If this hypothesis is correct, then suppression of neuronal excitation and acute withdrawal symptoms during prior withdrawals should suppress these kindling stimuli and prevent the enhancement of subsequent withdrawal episodes.

The observation of an increased occurrence and severity of seizures following exposure to multiple withdrawal episodes has important implications for the evaluation of current clinical treatments of the alcohol withdrawal syndrome. Thus, an effective treatment should prevent acute withdrawal symptoms, but it should also prevent repeated withdrawal enhancement of the withdrawal syndrome. There is some evidence of the suppression of withdrawal-induced neuronal excitation and acute ethanol withdrawal symptoms by positive modulators of the $GABA_A$ receptor such as benzodiazepines (Gonzalez and Veatch, 1994). Benzodiazepines, which have been widely used for alcohol detoxification, have been found to be effective in reducing hyperexcitability and the incidence of motor convulsions during acute alcohol withdrawal (Gonzalez and Veatch, 1994). However, there is little information available regarding the effect of benzodiazepine treatment during prior withdrawals on seizure sensitivity during a subsequent untreated withdrawal. One investigator (Ulrichsen et al., 1995) has reported results suggesting that diazepam treatment may be effective in preventing the effects of repeated withdrawals. The method of ethanol exposure used in this study, however, resulted in a very high level of mortality (50%), so that measures of withdrawal were only obtained from a highly selected population of surviving subjects, and these results may not be representative of the effects of more moderate ethanol exposure.

In the present study, we determined the effect of diazepam treatment during prior, repeated withdrawal episodes on Ro15-4513-induced seizures during a subsequent untreated withdrawal. The use of ethanol vapor inhalation as the method of ethanol administration in this study permitted moderate levels of chronic ethanol exposure without the confound of high mortality. The present study also examined the effect of diazepam treatment during prior withdrawal episodes on the spontaneous spike and sharp wave (SSW) activity observed in the EEG at various brain sites during a subsequent untreated withdrawal. The hypothesis to be tested in this study was that suppression of acute withdrawal symptoms during prior, repeated ethanol withdrawal episodes by diazepam would reduce the multiple withdrawal-associated increase in severity of the ethanol withdrawal syndrome.

2. Methods

2.1. Animals

Forty-seven 60–90-day-old male Sprague–Dawley rats (Harlan, Indianapolis, IN), weighing 305 ± 6 g, were group-housed in a temperature- and humidity-controlled vivarium with lights on a 12-h light/dark cycle (6:00 a.m./6:00 p.m.). Animals were maintained for 1 week prior to the start of any experimental treatment and had free access to food and water throughout this study.

2.2. Drugs

Diazepam has been reported to suppress symptoms of ethanol withdrawal in doses ranging from 2 to 15 mg/kg or higher, using various routes of administration (Adams and Hirst, 1982; Riihioja et al., 2000; Ulrichsen et al., 1995). In preliminary studies (Gonzalez and Veatch, 1994; Mhatre et al., 1999), diazepam, in doses of 1.25, 2.50, and 4.0 mg/kg ip, was observed to block acute withdrawal symptoms after chronic ethanol exposure, but only the higher doses (2.50 and 4.0 mg/kg) were found to reduce EEG SSW activity. In the present study, 4.0 mg/kg ip was selected for use, as this dose blocked peripheral and central symptoms of acute withdrawal but produced only mild sedation. Since diazepam and ethanol produce additive effects on sedation (Weller and Preskorn, 1984), diazepam was administered 2 h after animals were removed from vapor inhalation chambers, when blood ethanol levels had begun to decrease. Administration at this dose and time avoided excessive sedation but provided treatment before the onset of acute withdrawal symptoms (Riihioja et al., 2000).

Ro15-4513 and diazepam were purchased from Research Biochemicals International (Natick, MA). Ro15-4513 (5.0 mg/ml) was dissolved in 50:50 dimethyl sulfoxide (DMSO) and saline. DMSO (1 ml/kg ip) alone was not found to have any anticonvulsant or proconvulsant effects per se (Mhatre and Gonzalez, 1999). Diazepam (50.0 mg/ml) was dissolved in 50:50 DMSO/Tween 20 and diluted as 1:50 in saline (final concentration 1 mg/ml), administered at a dose of 4.0 mg/kg ip.

2.3. Experimental plan

One week after delivery, animals were assigned to one of five groups. *Group 1* (ethanol-naive diazepam) received no ethanol exposure, but received similar handling to that of the ethanol-exposed groups described below, including the regular collection of tail-vein blood (20 μl). These rats received diazepam (4.0 mg/kg ip, one dose per day) on Days 4, 8, 12, 16, and 20 (at 10:00 a.m.) of the experiment, and they were tested for seizure sensitivity with Ro15-4513 on Day 28, between 4:00 and 5:00 p.m. *Group 2* (7 days of continuous ethanol exposure) received one cycle of 7 days of continuous ethanol exposure with no intermittent withdrawal periods. *Group 3* (22 days of continuous ethanol exposure) received one cycle of 22 days of chronic ethanol exposure with no intermittent withdrawal period. *Group 4* (multiple withdrawals) received five cycles of 3 days of ethanol exposure and one last cycle of 7 days chronic ethanol exposure, with each exposure cycle followed by a single 24-h period of withdrawal. These rats received vehicle injections (1.0 ml/kg ip; DMSO/Tween 20:saline, 1:50) on Days 4, 8, 12, 16, and 20 (2 h following withdrawal, at 10 a.m.), and they were tested for seizure sensitivity with Ro15-4513 on Day 28, between 4:00 and 5:00 p.m. *Group 5* (multiple withdrawals with intermittent diazepam) received diazepam (4.0 mg/kg ip) on Days 4, 8, 12, 16, and 20 (one dose per day, 2 h after withdrawal from ethanol exposure) during each of the five intermittent withdrawal episodes, but not during the final withdrawal on Day 28.

The total cumulative exposure to ethanol was 22 days for Groups 3–5, with Groups 4 and 5 also receiving five intermittent 24-h withdrawal periods. Animals were tested for seizure sensitivity with Ro15-4513 (5.0 mg/kg ip) on Day 28 of the experiment, 8 h following final withdrawal for animals receiving chronic ethanol exposure. During the course of the experiment, treatment was begun for each group so that all animals were of the same age at the time of testing seizure sensitivity.

2.4. Chronic ethanol exposure

For each chronic ethanol exposure period, the animals were placed in ethanol vapor inhalation chambers. Ethanol vapor, obtained by pumping air through a 1-l aspirator bottle containing 1000 ml of 95% ethanol, was added to the fresh air flow, at the rate of 0–2 l/min. The ethanol flow rate was adjusted systematically throughout ethanol exposure periods to obtain final blood ethanol levels of approximately 250 mg/dl. Chronic ethanol exposure began at 8:00 a.m. on Day 1 and ended at 8:00 a.m. on the day of withdrawal of the treatment. The ethanol flow was gradually increased in all ethanol exposure paradigms to accommodate ethanol tolerance in these rats. During chronic ethanol exposure, rats were monitored for changes in body weight every fourth day, and stages of intoxication were observed three times each day. Blood ethanol levels were determined every 2–3 days during chronic exposure as noted below. Highly intoxicated rats were provided with a nutritional supplement (Ensure) and sweet fruits to reduce their weight loss during periods of ethanol intoxication. Ethanol-naive control rats were also given small amounts of these as well, and blood samples were collected on the same schedule as ethanol-treated rats.

2.5. Blood ethanol levels

Blood ethanol levels were determined periodically during ethanol exposure (every 2–3 days) and at the time of removal from ethanol vapor inhalation chambers. Blood samples were also obtained 4 and 8 h after removal of the animals from the chamber following the final exposure period. Samples of blood (20 μl) were collected from the tail vein of animals in each experimental group and assayed using an Analox Instruments alcohol analyzer (Lunenburg, MA).

2.6. Electrode implantation and histological verification

After a 7-day habituation period, each animal was surgically implanted with cortical and subcortical electrodes in motor cortex, amygdala, and hippocampal areas CA_1 and CA_3 as described previously (Veatch and Gonzalez, 1996). Briefly, after anesthetization with sodium pentobarbital and chloral hydrate, each animal received stereotaxically implanted monopolar, stainless-steel, semi-microelectrodes (120 μm) in hippocampal area CA_1 (anterior–posterior (AP), −3.8 mm; lateral (L), −3.5 mm; ventral (V), −2.8 mm), hippocampal area CA_3 (AP, −3.8 mm; L, 3.5 mm; V, −4.0 mm), and amygdala (AP, −0.8 mm; L, +3.4 mm; V, −9.0 mm). Two stainless-steel screw electrodes (0–80 × 1/8 in.) were used for cortical recordings. These were positioned over the motor cortex (AP, 2.0 mm; L, ±2.5) of all animals. A screw electrode was also placed in the skull over the frontal sinus (AP, 11.0 mm; L, −1.0 mm) as an animal ground and as a reference for monopolar recordings. Subcortical electrode placements were according to the atlas of Paxinos and Watson (1986). The electrodes were connected to a miniature amphenol plug that was fixed to the skull with dental acrylic cement for subsequent connection to electrophysiological recording equipment. After surgery, animals were allowed at least 7 days for recovery before any further experimental treatment.

Following the completion of all experimental procedures, animals were deeply anesthetized with sodium pentobarbital (85.0 mg/kg ip), and subcortical electrode placements were marked by applying a 50-μA DC current through each electrode for 20 s. The animals were then perfused with saline followed by 10% formalin, after which the brains were carefully removed, cut into 40-μm slices, and stained in neutral red for the evaluation of electrode placements by the investigator.

2.7. General electrophysiological recording procedures

For recording EEG activity, each animal was placed in a sound-attenuating, electrically shielded recording chamber. Electrophysiological activity was observed by attaching the animal to a recording cable containing field-effects transistors to reduce movement artifacts. The cable was connected to Grass 7P511 amplifiers through a slip-ring assembly, which permitted the animal freedom of movement within the chamber. The signals were then digitized with a CED 1401 data acquisition computer and stored for subsequent analysis. Fifteen minutes were allowed for the habituation of the animal to the recording chamber, after which a 15-min recording of spontaneous EEG activity was obtained. During the recording period, the behavior of the animal was also monitored. Recordings were obtained 8 h after the animal was removed from the ethanol vapor chamber. The recordings of spontaneous electrophysiological activity were then computer-analyzed for the occurrence of SSW activity by a modified version of the method reported by Frost (1979, 1985), as described previously (Veatch and Gonzalez, 1996).

2.8. Assessment of ethanol withdrawal reactions

Following each ethanol exposure period, ethanol-treated animals were observed to quantify the severity of the ethanol withdrawal syndrome. Animals were monitored at 2-h intervals during the first 8 h after removal from the vapor inhalation chamber. Withdrawal severity was rated according to the following scale: 1 (no withdrawal symptoms), 2 (sedate with abnormal posture), 3 (muscle rigidity and tremors with abnormal posture), and 4 (occurrence of spontaneous seizures). The highest rating received during these observation periods was used as the final withdrawal score. Ethanol withdrawal hyperexcitability during the final, nonmedicated withdrawal period was also evaluated by observing susceptibility to Ro15-4513-induced seizures in animals belonging to each of the experimental groups as described below.

2.9. Evaluation of Ro15-4513-induced seizures

Susceptibility to Ro15-4513-induced seizures was evaluated after behavioral and electrophysiological observations of withdrawal severity were complete, between 8 and 9 h following the final withdrawal from ethanol exposure. Ethanol-exposed animals have been reported (Frye and Ellis, 1977; Gonzalez et al., 1989) to exhibit maximum audiogenic seizure susceptibility at this time postwithdrawal. For the evaluation of drug-induced seizure susceptibility, animals received a single injection of Ro15-4513 (5.0 mg/kg ip) as described previously (Mhatre and Gonzalez, 1999). This dose was selected to give an appropriate threshold to evaluate differences in Ro15-4513-induced seizure susceptibility in different ethanol exposure groups. A seizure rating was determined for each animal individually by observing the animal for 30 min following drug administration. Behavior of animals following the drug injection was rated as follows:

0:	no response
1:	muscle tremors
2:	abnormal hindlimb posture
3:	mild limb extension
4:	severe limb extension with severe muscle tremors
5:	tonic–clonic convulsion

2.10. Data analysis

Data to be analyzed consisted of the behavioral ratings of withdrawal severity and RO15-4513 seizures, and the frequency of occurrence of SSW events in the EEG during each recording period. Behavioral ratings were analyzed using a nonparametric Contingency Table Test for Categorical Data (Veatch and Gonzalez, 1995) and EEG SSW activity was analyzed using a general linear model (SAS: Proc GLM) for the computation of analysis of variance (ANOVA). ANOVA was also used to determine the significance of group differences in weight change during the experimental period and in blood ethanol levels. Where ANOVA indicated significant overall effects, Duncan's Multiple Range Test was used in the evaluation of post-hoc group comparisons. Data were analyzed to determine the significance of between-group differences in response to diazepam treatment, as well as to different patterns of ethanol exposure.

3. Results

3.1. Body weight

All the groups of rats were monitored for changes in body weight. The mean weight of all subjects at the beginning of this study was 305 ± 6 g. Average weights of animals in individual groups at the end of the experiment were as follows: Group 1 (424 ± 3 g), Group 2 (393 ± 9 g), Group 3 (363 ± 9 g), Group 4 (360 ± 12 g), and Group 5 (367 ± 6 g). Ethanol-treated animals (Groups 2–5) gained significantly less weight ($P < .05$) over the course of the experiment than did ethanol-naive animals (Group 1), but the average weight of ethanol-treated groups was within 15% of the weight of the ethanol-naive group at the end of the experiment. The different ethanol-treated groups (Groups 2–5) did not differ significantly [$F(3,26) = 2.89$, $P > .05$] from one another in body weight at the time of the final withdrawal period.

3.2. Blood ethanol levels

Blood ethanol levels at the time of final withdrawal from ethanol exposure did not differ significantly [$F(3,26) = 0.25$, $P > .05$] between ethanol exposure groups (Groups 2–5). Mean blood ethanol levels for these groups are listed in Table 1. For all of the animals exposed to ethanol, mean blood ethanol level at the time of final withdrawal was 278 ± 18 mg/dl, and this decreased to 78 ± 6 mg/dl by 4 h, after the time of withdrawal. Ethanol concentrations were below the level of detection at 8 h postwithdrawal. Ethanol-exposed groups did not differ significantly in this change in blood ethanol level with time after withdrawal [$F(6,52) = 0.44$, $P > .05$]. In addition, the multiple ethanol exposure groups (Groups 3 and 4) did not differ significantly from one another in blood ethanol level during the intermittent exposure periods [$F(4,48) = 0.95$, $P > .05$].

3.3. Ethanol and diazepam effects on behavioral ratings

Animals from each of the ethanol groups exhibited mild sedation and ataxia during periods of ethanol exposure. Withdrawal severity was relatively mild during each of the repeated periods of 3 days of ethanol exposure in the multiple exposure groups (Groups 4 and 5). Treatment with diazepam (4.0 mg/kg ip), however, significantly reduced ($P < .03$) withdrawal ratings during these acute, intermittent withdrawal periods (Group 4: multiple withdrawals, rating $= 1.25 \pm 0.08$, vs. Group 5: multiple withdrawals with diazepam, rating $= 1.05 \pm 0.03$). After the final, non-medicated withdrawal from 7 days of ethanol exposure, animals exhibited ethanol withdrawal symptoms including mild muscle tremor, abnormal gait and posture, vocalization upon handling, and spontaneous seizures in some animals. Analysis of the withdrawal ratings observed after this final withdrawal from chronic ethanol exposure indicated that ethanol-exposed groups did not differ significantly from one another ($P > .05$). Mean ratings during this time were 2.8 ± 0.3, 3.5 ± 0.4, 3.4 ± 0.3, and 3.3 ± 0.3 for Groups 2, 3, 4, and 5, respectively. Withdrawal severity following this final, 7-day period of ethanol exposure was considerably more severe than that observed after the shorter, 3-day intermittent periods of exposure.

Table 1

Ethanol exposure group	Blood ethanol levels at final withdrawal (in mg/dl ± S.E.)
Group 2: 7 days of continuous ethanol exposure	304 ± 67
Group 3: 22 days of continuous ethanol exposure	264 ± 23
Group 4: multiple withdrawals	242 ± 73
Group 5: multiple with diazepam	245 ± 63

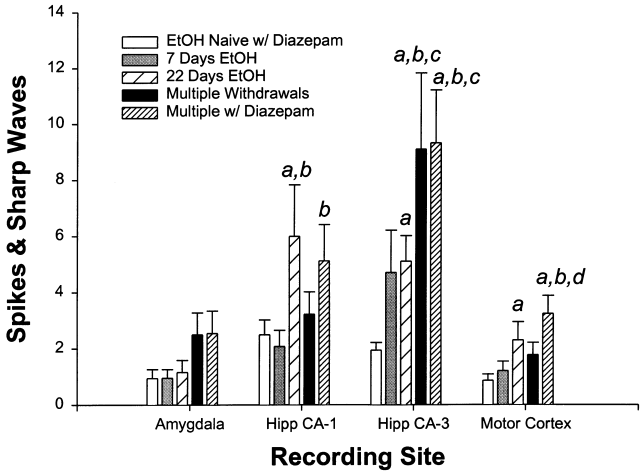

Fig. 1. Effects of ethanol exposure and withdrawal on spontaneous SSW activity recorded from cortical and subcortical sites in freely moving animals. Each bar represents the mean (±S.E.) amount of SSW activity observed during a 15-min recording period at the eighth hour following the final withdrawal from chronic ethanol exposure. Bars are grouped by brain site. The effects of the length of ethanol exposure and the number of withdrawal cycles differed significantly by brain site. Multiple withdrawal and multiple withdrawal with diazepam groups (Groups 4 and 5) differed significantly from naive, 7-day ethanol, and 22-day ethanol groups ($P < .05$). (a) $P < .05$, compared to Group 1 (ethanol-naive with diazepam), (b) $P < .05$, compared to Group 2 (7 days of continuous ethanol exposure), (c) $P < .05$, compared to Group 3 (22 days of continuous ethanol exposure), and (d) $P < .05$, compared to Group 4 (multiple withdrawals).

3.4. Effect of ethanol exposure and withdrawal on SSW activity

SSW activity was observed in the EEG of all brain sites examined following the final withdrawal from ethanol exposure (Fig. 1). Statistical analysis indicated significant region-specific differences in SSW activity that depended upon the pattern of ethanol exposure. The SSW activity was significantly higher in hippocampal area CA_1 of animals after 22 days of continuous ethanol exposure (Group 3) in comparison to ethanol-naive/diazepam-treated animals (Group 1, $P < .03$) and to animals exposed to 7 days of continuous ethanol (Group 2, $P < .01$). Animals exposed to multiple withdrawal episodes (Group 4), however, showed significantly higher levels of SSW activity in hippocampal area CA_3 than did animals in the ethanol-naive group (Group 1, $P < .0001$) or animals exposed to continuous ethanol (Groups 2 and 3, $P < .005$). The SSW activity in the amygdala also showed a similar trend toward higher SSW activity after multiple withdrawal episodes, but the group differences were not statistically significant ($P > .05$). The SSW activity in the hippocampal CA_1 region and in the motor cortex appeared to be less affected by the number of withdrawal episodes than to the length of ethanol exposure, while activity in hippocampal area CA_3 appeared most sensitive to exposure to repeated withdrawal episodes.

3.5. Diazepam and multiple withdrawal episodes

Animals that received diazepam treatment during prior repeated ethanol withdrawal episodes (Group 5) exhibited levels of SSW activity similar to that of animals treated with vehicle during repeated withdrawal episodes (Group 4). Thus, during a final, nonmedicated withdrawal episode, SSW activity was significantly higher in the hippocampal CA_3 region of animals exposed to repeated withdrawal episodes, treated with either diazepam (Group 5, $P<.005$) or vehicle (Group 4, $P<.02$), than in any other group. Diazepam treatment during prior withdrawals did not reduce withdrawal-related SSW activity at any brain site and did not reduce or prevent the multiple withdrawal-associated increase in SSW activity during the final, nonmedicated withdrawal in any of the areas studied. The SSW activity observed following multiple withdrawals with intermittent diazepam treatment was not significantly different in any region from that shown by rats from the multiple withdrawal vehicle group ($P>.05$) except in the motor cortex, where significantly higher levels of SSW activity were observed in diazepam-treated animals ($P<.05$).

3.6. Ro15-4513-induced seizures

The administration of Ro15-4513 (5.0 mg/kg ip) resulted in mild behavioral responses in ethanol-naive animals (seizure rating = 2 or less). However, the same dose of Ro15-4513 induced a number of additional symptoms in ethanol-exposed animals including muscle tremor, hindlimb extension, and mild tonic–clonic convulsions of short duration. Seizure ratings following Ro15-4513 were significantly higher in each of the ethanol-treated groups compared to the ethanol-naive group (Fig. 2).

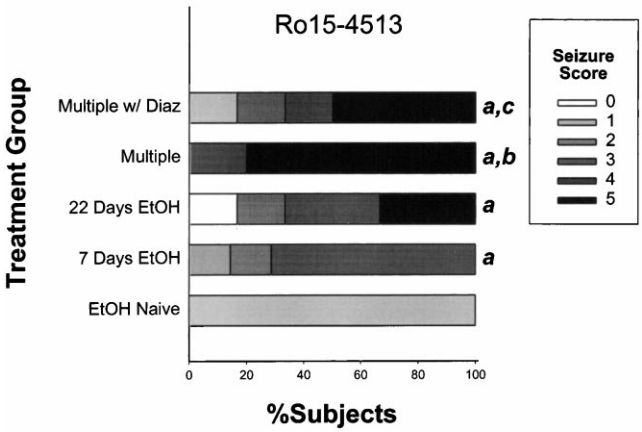

Fig. 2. Effects of chronic ethanol exposure and withdrawal on Ro15-4513-induced seizure ratings. Bars represent the percentage of animals from each group receiving the given seizure rating, as indicated by the shading within the bar, during a 30-min period following the acute administration of Ro15-4513. Ethanol-exposed rats were significantly more sensitive to the seizure-inducing effects of Ro15-4513 following ethanol withdrawal than were ethanol-naive rats. Group 4 (multiple withdrawals) and Group 5 (multiple with diazepam) did not differ significantly from one another in their response to the Ro15-4513 injection. Group 1 (ethanol-naive with diazepam) showed mild behavioral responses following Ro15-4513 administration (seizure rating = 2 or less). (a) $P<.02$, compared to Group 1 (ethanol-naive with diazepam), (b) $P<.01$, compared to Group 2 (7 days of continuous ethanol exposure), (c) $P<.05$, compared to Group 2 (7 days of continuous ethanol exposure).

In addition, consistent with our previous study (Mhatre and Gonzalez, 1999), seizure ratings after Ro15-4513 administration were significantly affected by the pattern of ethanol exposure, with Group 4 (multiple withdrawals) and Group 5 (multiple withdrawals with diazepam) found to be more sensitive to the convulsive effects of Ro15-4513. Seizure ratings after Ro15-4513 were significantly higher in these groups in comparison to both the ethanol-naive group ($P<.02$) and the 7-day, continuous ethanol-exposure group (Group 2 vs. Group 4, $P<.01$; and Group 2 vs. Group 5, $P<.05$). Animals exposed to 22 days of continuous ethanol (Group 3) did not differ significantly from Group 2 ($P>.05$). Also, significantly more animals in the multiple ethanol withdrawal group (Group 4) exhibited full tonic–clonic convulsions following administration of Ro15-4513 than did animals in Group 1 ($P<.02$) or Group 2 ($P<.01$), further suggesting that previous exposures to ethanol withdrawals enhanced sensitivity to Ro15-4513 (see Fig. 2).

There were no significant differences ($P>.05$) in seizure ratings (Fig. 2) or in the number of animals undergoing tonic–clonic convulsions, between the multiple withdrawal group (Group 4) and the multiple withdrawal group with diazepam (Group 5). Diazepam treatment during previous ethanol withdrawals, therefore, did not alter the multiple withdrawal-associated increase in the sensitivity to the convulsive effect of Ro15-4513 following the final withdrawal period.

4. Discussion

Various clinical and laboratory studies have reported an increase in the occurrence and severity of seizures, with increasing numbers of withdrawal episodes (Becker, 1994; Becker and Hale, 1993; Brown et al., 1988; Lechtenberg and Worner, 1990, 1991; Maier and Pohorecky, 1989). Consistent with these observations, rats exposed to multiple cycles of ethanol withdrawal in our previous study showed increased sensitivity to the convulsive effects of the $GABA_A$ receptor inverse agonist, Ro15-4513, in comparison to rats exposed to continuous ethanol treatment with no intermittent withdrawals (Mhatre and Gonzalez, 1999). Rats exposed to multiple withdrawals also showed a selective increase in the occurrence of spontaneous SSW activity in hippocampal area CA_3 in comparison to rats exposed to continuous ethanol or to ethanol-naive rats (Veatch and Gonzalez, 1996).

Results of earlier studies from this and other laboratories have indicated that a single episode of chronic ethanol exposure and withdrawal leads to hyperexcitability and increased seizure susceptibility (Gonzalez et al., 1989; Gon-

zalez and Sun, 1992; Walker and Zornetzer, 1974). It is hypothesized that this withdrawal hyperexcitability may be due in part to a decrease in GABA-mediated inhibitory transmission, which may occur as an adaptation to ethanol's acute potentiation of GABA-induced chloride influx. In support of this hypothesis, a reduction in basal $GABA_A$ receptor function has been observed in synaptosomes and primary cultured neurons (Mhatre and Ticku, 1993; Morrow et al., 1988) following chronic ethanol treatment. Exposure to a single episode of chronic ethanol treatment has been found to result in significant region-specific alterations in $GABA_A$ receptor gene and polypeptide expression, suggesting that chronic ethanol exposure and withdrawal might cause a change in the conformation of the receptor, which may result in the hyperexcitability observed during ethanol withdrawal (Buck and Harris, 1990; Mhatre and Ticku, 1994; Mhatre et al., 1993).

Benzodiazepines, positive modulators of the $GABA_A$ receptor, have been found to be quite efficacious in the clinical suppression of acute ethanol withdrawal symptoms. However, little information is available on the effect of benzodiazepine treatment administered during prior withdrawals on seizure activity observed during a subsequent untreated withdrawal. In the present study, diazepam treatment suppressed symptoms during repeated acute withdrawal episodes, but this suppression of acute withdrawal symptoms did not have any protective effect on the sensitivity of animals to Ro15-4513-induced seizures during a subsequent nonmedicated withdrawal. This suggests that the suppression of acute withdrawal symptoms by benzodiazepines is not sufficient to prevent important changes in CNS function following withdrawal from chronic ethanol exposure. These changes in neuronal activity may act as subconvulsive, kindling-like stimuli that induce increased sensitivity and severity of withdrawal symptoms during a subsequent withdrawal. These findings are in contrast to a report (Ulrichsen et al., 1995) that diazepam treatment during nine repeated withdrawal episodes reduced the incidence of spontaneous motor convulsions in rats during four subsequent nonmedicated withdrawal episodes. This study, however, used a high-dose binge model of ethanol exposure that resulted in a very high rate of mortality during ethanol exposure and, thus, a highly selected subject population that may limit the generality of the observed results.

The present study also demonstrated site-dependent effects of ethanol exposure on spontaneous EEG activity. Consistent with a previous study from the authors' laboratory (Veatch and Gonzalez, 1996), the EEG from the hippocampal CA_3 area displayed significantly higher levels of SSW activity in animals that underwent multiple cycles of ethanol detoxification and withdrawal than in any other group. It was also observed that treatment with diazepam did not prevent the multiple withdrawal-associated, selective increase in SSW activity at this site. No other brain areas exhibited a selective increase in SSW activity as a result of exposure to multiple withdrawal episodes. This finding is similar to our previous report (Veatch and Gonzalez, 1996) that animals exposed to two 10-day periods of ethanol exposure and two withdrawal periods exhibited significantly more SSW activity in the hippocampal CA_3 area than did animals receiving a single continuous ethanol exposure of 20 days. These observations may suggest the specific involvement of the hippocampal CA_3 area in the mechanisms underlying enhancement of the ethanol withdrawal syndrome after repeated withdrawal episodes.

In contrast to this observation in the hippocampal CA_3 region, SSW activity in the hippocampal CA_1 area was significantly higher in the 22-day, continuous ethanol group (Group 3) in comparison to ethanol-naive and 7-day ethanol groups ($P < .05$). This suggests that the SSW activity in the hippocampal CA_1 area maybe more directly related to the length of ethanol exposure, rather than to the number of withdrawal episodes. These differences in the responses of the hippocampal CA_1 and CA_3 sites to single or to repeated cycles of ethanol intoxication and withdrawal in the present and previous study (Veatch and Gonzalez, 1996) suggest different roles for these areas in the ethanol withdrawal syndrome.

In conclusion, chronic ethanol exposure and withdrawal increased the incidence of SSW activity in several brain sites and increased seizure sensitivity to the inverse GABA agonist, Ro15-4513. These effects were potentiated by exposure to repeated withdrawal episodes. Diazepam treatment during intermittent withdrawal episodes reduced behavioral measures of acute withdrawal severity, but did not have any protective effect on measures of withdrawal severity observed during a subsequent untreated withdrawal episode. These findings suggest that current pharmacotherapy involving the use of benzodiazepines for acute alcohol detoxification may not prevent persistent changes in CNS function that contribute to the potentiation of withdrawal symptoms following exposure to repeated withdrawal episodes. Studies such as this emphasize the importance of further characterizing the significance of withdrawal history on subsequent withdrawal episodes and the importance of evaluating the effects of drug treatments after repeated withdrawal episodes.

References

Adams MA, Hirst M. The ethanol withdrawal syndrome in the rat: effects of drug treatment on adrenal gland and urinary catecholamines. Subst Alcohol Actions/Misuse 1982;3:287–98.

Ballenger JC, Post RM. Kindling as a model for alcohol withdrawal syndromes. Br J Psychiatry 1978;133:1–14.

Becker HC. Positive relationship between the number of prior ethanol withdrawal episodes and the severity of subsequent withdrawal seizures. Psychopharmacology 1994;116:26–32.

Becker HC, Hale RL. Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures — an animal model of alcohol withdrawal kindling. Alcohol: Clin Exp Res 1993;17:94–8.

Booth BM, Blow FC. The kindling hypothesis: further evidence from a US National Study of alcoholic men. Alcohol Alcohol 1993;28:593–8.

Brown ME, Anton RF, Malcolm R, Ballenger JC. Alcohol detoxification and withdrawal seizures: clinical support for a kindling hypothesis. Biol Psychiatry 1988;23:507–14.

Buck KJ, Harris RA. Benzodiazepine agonist and inverse agonist actions on $GABA_A$ receptor-operated chloride channels: II. Chronic effects of ethanol. J Pharmacol Exp Ther 1990;253:713–9.

Frost JD. Microprocessor-based EEG spike detection and quantification. Int J Biomed Comput 1979;10:357–73.

Frost JD. Automatic recognition and characterization of epileptiform discharges in the human EEG. J Clin Neurophysiol 1985;2(3):231–49.

Frye GD, Ellis FW. Effects of 6-hydroxydopamine or 5,7-dihydroxytryptamine on the development of physical dependence on ethanol. Drug Alcohol Depend 1977;2:349–59.

Goddard GV, McIntyre DC, Leech CK. A permanent change in brain function resulting from daily electrical stimulation. Exp Neurol 1969; 25:295–330.

Gonzalez LP, Sun R. Electrophysiological and neurochemical effects of ethanol on hippocampal CA3 neurons. In: Watson R, editor. Alcohol and neurobiology: brain development and hormone regulation. New York: CRC Press, 1992. pp. 201–20.

Gonzalez LP, Veatch LM. Differential behavioral and electrophysiological responses to diazepam after chronic ethanol. Alcohol: Clin Exp Res 1994;18:522.

Gonzalez LP, Czachura JF, Brewer KW. Spontaneous versus elicited seizures following ethanol withdrawal: differential time course. Alcohol 1989;6:481–7.

Lechtenberg R, Worner TM. Seizure risk with recurrent alcohol detoxification. Arch Neurol 1990;47:535–8.

Lechtenberg R, Worner TM. Relative kindling effect of detoxification and non-detoxification admissions in alcoholics. Alcohol Alcohol 1991;26: 221–5.

Maier DM, Pohorecky LA. The effect of repeated withdrawal episodes on subsequent withdrawal severity in ethanol-treated rats. Drug Alcohol Depend 1989;23:103–10.

Mhatre M, Ticku MK. Alcohol: effects on $GABA_A$ receptor function and gene expression. Alcohol Alcohol 1993;2:331–5 (Supplement).

Mhatre M, Ticku MK. Chronic ethanol treatment upregulates the $GABA_A$ receptor beta subunit expression. Mol Brain Res 1994;23:246–52.

Mhatre MC, Gonzalez LP. Increased Ro15-4513-induced seizures following multiple ethanol withdrawals. Pharmacol, Biochem Behav 1999; 63:93–9.

Mhatre MC, Pena G, Sieghart W, Ticku MK. Antibodies specific for $GABA_A$ receptor alpha-subunits reveal that chronic alcohol treatment down-regulates alpha-subunit expression in rat brain regions. J Neurochem 1993;61:1620–5.

Mhatre MC, McKenzie S, Gonzalez LP. Effects of diazepam or MK-801 during prior ethanol withdrawals on the seizure susceptibility during a subsequent withdrawal. Soc Neurosci Abstr 1999;25:1578.

Morrow AL, Suzdak PD, Karanian JW, Paul SM. Chronic ethanol administration alters gamma-aminobutyric acid, pentobarbital and ethanol-mediated ^{36}Cl-uptake in cerebral cortical synaptoneurosomes. J Pharmacol Exp Ther 1988;246:158–64.

Paxinos G, Watson C. The rat brain in stereotaxic coordinates. New York: Academic Press, 1986.

Poldrugo F, Snead IOC. Electroencephalographic and behavioral correlates in rats during repeated ethanol withdrawal syndromes. Psychopharmacology 1984;83:140–6.

Riihioja P, Jaatinen P, Oksanen H, Haapalinna A, Heinonen E, Hervonen A. Dexmedetomidine, diazepam, and propranolol in the treatment of ethanol withdrawal symptoms in the rat. Alcohol: Clin Exp Res 2000;21: 804–8.

Ulrichsen J, Bech B, Allerup P, Hemmingsen R. Diazepam prevents progression of kindled alcohol withdrawal behaviour. Psychopharmacology 1995;121:451–60.

Veatch LM, Gonzalez LP. Contingency table testing for categorical data: SAS implementation. Behav Res Methods, Instrum Comput 1995;27: 404–7.

Veatch LM, Gonzalez LP. Repeated ethanol withdrawal produces site-dependent increases in EEG spiking. Alcohol: Clin Exp Res 1996;20: 262–7.

Walker DW, Zornetzer SF. Alcohol withdrawal in mice: electroencephalographic and behavioral correlates. Electroencephalogr Clin Neurophysiol 1974;36:233–43.

Weller RA, Preskorn SH. Psychotropic drugs and alcohol: pharmacokinetic and pharmacodynamic interactions. Psychosomatics 1984;25: 301–6, 309.