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Increased Ro15-4513–Induced Seizures Following Multiple Ethanol Withdrawals

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MHATRE, M. C. AND L. P. GONZALEZ. *Increased Ro15-4513–induced seizures following multiple ethanol withdrawals.* PHARMACOL BIOCHEM. BEHAV. **63**(1) 93–99, 1999.—Clinical research into the etiology of ethanol withdrawal seizures has shown an increase in the number and severity of seizures with increasing numbers of withdrawal episodes. The aim of the present study was to determine the effects of multiple ethanol withdrawals on the seizure sensitivity to the $GABA_A$ receptor inverse agonist Ro15-4513. In this study, three groups of laboratory rats received varying amounts of either continuous or intermittent ethanol exposure. A fourth group (Naive) received no ethanol exposure. Eight hours following the last withdrawal from chronic ethanol exposure, animals were tested for sensitivity to Ro15-4513–induced motor convulsions. Seizure sensitivity was significantly increased in all ethanol-treated groups compared to ethanol-naive controls, which did not exhibit any convulsive responses to this dose of Ro15-4513. Furthermore, rats exposed to multiple ethanol withdrawals exhibited significantly higher sensitivity to drug-induced seizures than did animals experiencing only a single ethanol withdrawal. Although the specific mechanism of this enhanced convulsant effect of Ro15-4513 following multiple ethanol withdrawals remains to be determined, these results suggest an involvement of $GABA_A$–benzodiazepine receptors in this multiple withdrawal phenomenon. © 1999 Elsevier Science Inc.

Ethanol Alcohol Withdrawal Multiple withdrawal Repeated withdrawal Seizures Ro15-4513 γ-Aminobutyric acid receptor complex

ALCOHOL withdrawal seizures are serious, potentially fatal, and have been identified as the most common cause of adult-onset seizure disorders. Clinical research into the etiology of these withdrawal seizures has shown an increase in the number and severity of seizures with increasing duration of alcohol abuse and increasing number of withdrawal episodes (6). Several clinical studies have observed a high correlation between the number of hospital admissions for detoxification from alcohol and the occurrence of withdrawal seizures (6,20,21).

Experimental studies also have reported that following repeated cycles of ethanol intoxication and withdrawal, animals exhibit an increase in the number as well as severity of seizures (2,3,9,41). Ballenger and Post (1) and Pinel (36) hypothesized that during multiple intoxication and withdrawal cycles, withdrawal-induced changes in neuronal activity could serve as a kindling stimulus, and that this kindling might eventually culminate in full motor seizures. Animals exposed to multiple cycles of ethanol exposure and withdrawal do show alterations in the development of electrical kindling, although this effect was observed to vary with the site of the kindling stimulus (24,44). Increased spiking has also been observed in the EEG of rats and mice after a second withdrawal episode (37,43,45).

Despite a number of studies reporting an increase in seizure sensitivity following exposure to multiple cycles of ethanol intoxication and withdrawal, the mechanism underlying this multiple withdrawal phenomenon remains to be determined. It is suggested that the CNS hyperexcitability due to withdrawal from previous chronic exposures to alcohol might be responsible for an increase in the occurrence and severity of seizures observed following repeated ethanol withdrawal episodes (1,36).

McCown and Breese (25) have indicated that kindling facilitation observed following multiple withdrawals from chronic ethanol exposures may be caused by a decrease in GABAergic inhibition. In this study, the sensitivity to locally applied bicuculline was found to be increased from 6 to 10 ethanol

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withdrawals, in association with a facilitation of kindling, suggesting a progressive change in $GABA_A$ receptor pharmacology from a single ethanol withdrawal to multiple withdrawals. Kokka and co-workers have also suggested the involvement of $GABA_A$ receptors in kindling facilitation observed following chronic intermittent ethanol treatment based on the similarity between chronic intermittent ethanol-treated and chronic pentylenetetrazole-treated rats (18), and reduced hippocampal $GABA_A$ receptor function has been reported after chronic intermittent ethanol (17).

The neurobiological mechanisms that underlie the behavioral actions of a single episode of chronic exposure to ethanol such as tolerance, physical dependence, and the withdrawal syndrome appear to involve $GABA_A$ receptors as well as NMDA receptors (31,34,46). As shown by us and other workers, a single period of chronic ethanol exposure, with no intermittent withdrawal episodes decreases the functional sensitivity of $GABA_A$ receptors to agonists, but increases sensitivity to inverse agonists of the receptor (4,28,29,31). Our previous studies showed that chronic ethanol exposure produced an upregulation of binding sites for the benzodiazepine inverse agonist and ethanol antagonist Ro15-4513, in cerebral cortex and cerebellum of rat brain and in embryonic cultured neurons, with no change in the binding of benzodiazepine agonists or antagonists (28,29). Correlated with these changes, sensitivity to the convulsant actions of $GABA_A$ receptor inverse agonists also increases following chronic ethanol administration, which suggests a shift in receptor function towards inverse agonism during withdrawal from such chronic exposure (27).

In the present study, an attempt has been made to determine whether exposure to multiple cycles of ethanol intoxications and withdrawals increases sensitivity to the convulsant effects of Ro15-4513 when compared to a single period of chronic ethanol exposure through vapor inhalation with no intermittent withdrawal episodes.

METHOD

Animals

Sixty-four, 60–90-day-old male Sprague–Dawley rats (Harlan, Indianapolis, IN), weighing 303 ± 8 g at the beginning of experimental treatment, were group housed in a temperature- and humidity-controlled vivarium with lights on a 12L:12D cycle. 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.

Drugs

Ro15-4513 (RBI, Natric, MA) was dissolved in dimethyl sulfoxide (DMSO) (final concentration 5.0 mg/ml), diluted as 1:1 in saline, and administered as 5.0 mg/kg, IP. DMSO (1.0 ml/kg, IP) is reported not to have any anticonvulsant or proconvulsant effects per se (27). Vehicle-only injections consisted of DMSO 1:1 in saline, administered as 1.0 ml/kg, IP.

Chronic Ethanol Exposure

One week after delivery, animals were assigned to one of four ethanol-exposure groups. Ethanol was administered through vapor inhalation as described below. Group 1 (naive) received no ethanol exposure, but received similar handling to that of ethanol-exposed groups, including regular collection of tail-vein blood (20 μl). Group 2 (7-day continuous) received one cycle of 7 days of continuous ethanol exposure with no intermittent withdrawal periods. Group 3 (22-day continuous) received one cycle of 22 days of chronic ethanol treatment with no intermittent withdrawal period. Group 4 (multiple withdrawals) received five cycles of 3 days and one last cycle of 7 days chronic ethanol exposure with each exposure cycle followed by a single 24-h period of withdrawal. The total cumulative exposure to ethanol for group 4 was 22 days, with five intermittent 24-h withdrawal periods. Treatment was begun for each group so that animals were the same age at the time of seizure testing.

For each chronic ethanol exposure period, the animals were placed in vapor inhalation chambers as previously described (43). Ethanol vapor, obtained by pumping air through a 1-liter 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 increased systematically throughout each exposure period to accommodate the development of ethanol tolerance and to obtain final blood ethanol levels of 250–350 mg/dl. Flow rate adjustments were varied between groups such that ethanol exposure throughout the final 7-day period of group 4 (multiple withdrawals) was similar to that of group 2 (7-day continuous), and also the total cumulative exposure of groups 3 (22-day continuous) and 4 (multiple withdrawals) were similar. Chronic ethanol exposure was begun at 0800 h on day 1 and ended at 0800 h on the day of withdrawal of the treatment.

Blood Ethanol Levels

Blood ethanol levels were determined periodically during ethanol exposure and at the time of removal from the ethanol vapor inhalation chamber. Samples of blood from the tail vein (20 μl) were collected from animals of all four groups and assayed by gas chromatography as previously described (43).

Assessment of Ethanol Withdrawal Reactions

Ethanol withdrawal hyperexcitability was evaluated by monitoring the susceptibility to Ro15-4513–induced seizures in rats belonging to each of the 4 groups. Susceptibility to Ro15-4513–induced seizures was evaluated 8 to 9 h following the last ethanol withdrawal, when ethanol-exposed rats are reported to exhibit maximum audiogenic seizure susceptibility (14). For these evaluations, each ethanol-exposure group was divided into two subgroups that received a single, acute injection of either vehicle or Ro15-4513. Animals from each group were tested at the same time, and the group identity of individual animals and the type of injection were not revealed to the observer until the completion of testing.

Evaluation of Ro15-4513–Induced Seizures

For the evaluation of drug-induced seizure susceptibility, animals received a single injection of either vehicle or Ro15-4513 (5.0 mg/kg) intraperitoneally, administered between 8 and 9 h after the final withdrawal from chronic treatment. This dose of Ro15-4513 was selected through a preliminary examination of seizure activity induced by injections of 5.0 or 10.0 mg/kg, IP in ethanol-exposed (7-day continuous exposure) and ethanol-naive subjects (six subjects/group). It was observed that 33% of ethanol-exposed rats exhibited seizures during the 10 minutes following 5.0 mg/kg Ro15-4513, while 83% of ethanol-exposed rats exhibited seizures following 10.0 mg/kg Ro15-4513, when administered 8 to 9 h following withdrawal from 7 days of ethanol exposure. Ethanol-naive subjects did not exhibit seizures in response to either dose. The

5.0 mg/kg dose of Ro15-4513 was, therefore, selected for study to allow for the observation of possible increases in sensitivity to Ro15-4513 after different ethanol exposure paradigms in comparison with 7-days of continuous exposure.

For the quantification of Ro15-4513–induced seizure activity, animals were observed for 10 min following drug administration. Behavior of animals following drug injection was rated as follows: 0—no response, 1—muscle tremor, 2—abnormal hindlimb posture, 3—mild limb extension, 4—severe limb extension with severe tremor, and 5—tonic–clonic convulsion.

Data Analysis

Differences in drug-induced seizure scores following ethanol withdrawal were tested for significance using nonparametric contingency table tests for categorical data (42). Analysis of variance (ANOVA) was used to determine the significance of group differences in weight change during the experimental period and in blood ethanol levels. Data were analyzed to determine the significance of between group differences in response to ethanol exposure and to test drug administration (vehicle or Ro15-4513).

RESULTS

During ethanol exposure, the rats exhibited sedation and ataxia. After withdrawal from ethanol exposure, animals exhibited mild muscle tremor, rigid posture, and splaying of the hindlimbs. Rats belonging to group 4 (multiple withdrawals) did not show obvious signs of withdrawal during the 24 h between 3-day ethanol exposure cycles, but did show symptoms similar to those of the continuous exposure groups after the final 7-day exposure period. These observations were not quantified, however, and group differences were not evaluated statistically.

Blood Ethanol Levels

Blood ethanol levels at the time of final withdrawal from ethanol exposure did not differ significantly, [$F(2, 43) = 0.03$, $p > 0.05$, between ethanol exposure groups (groups 2, 3, and 4). The mean blood ethanol levels for groups 2, 3, and 4 were 316 ± 20 mg/dl ($n = 18$), 331 ± 30 mg/dl ($n = 15$), and 325 ± 30 mg/dl ($n = 16$), respectively. Although blood ethanol levels were not measured at the time of testing in these animals, ethanol was not detectable in the blood of any animals in a similarly treated group of subjects 8 h postwithdrawal (mean blood level at the time of withdrawal = 358 ± 23 mg/dl, $n = 5$).

Effect of Chronic Ethanol Treatment on Body Weight

All four groups of rats were monitored for changes in body weight. Naive rats showed a $17 \pm 1\%$ ($n = 12$) increase in total body weight during the experimental period, whereas rats in group 2 (7-day continuous) gained $3 \pm 2\%$ ($n = 18$), rats in group 3 (22-day continuous) gained $4 \pm 2\%$ ($n = 15$), and rats in group 4 (multiple withdrawals) gained $7 \pm 3\%$ ($n = 16$) of their initial body weight. These differences were statistically significant, $F(3, 47) = 6.98$, $p < 0.001$, but reflect a difference in weight gain of less than 12% between ethanol-naive and ethanol-exposed animals. The mean weight ($\pm$SEM) of animals in each group at the end of the experiment was as follows: group 1 (naive): 325 ± 1 g; group 2 (7-day continuous): 304 ± 5 g; group 3 (22-day continuous): 310 ± 5 g and group 4 (multiple withdrawals): 309 ± 9 g.

Drug-Induced Seizures

The administration of Ro15-4513 (5.0 mg/kg, IP) resulted in mild behavioral responses in ethanol-naive animals (seizure ratings = 2 or less). However, the same dose of Ro15-4513 induced a variety of symptoms in ethanol-exposed animals, including muscle tremor, hindlimb extension, and mild tonic–clonic convulsions of short duration. Naive and ethanol-treated groups differed significantly in the observed seizure ratings ($p < 0.001$).

In addition, seizure ratings after Ro15-4513 administration were significantly affected by the pattern of ethanol exposure, with group 4 animals (multiple withdrawals) found to be more sensitive to the convulsant effects of Ro15-4513 (see Fig. 1A). Seizure ratings were significantly higher in this group in comparison to group 1 (naive) ($p < 0.001$), group 2 (7-day continuous) ($p < 0.01$), and group 3 (22-day continuous) ($p < 0.05$). 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 2 ($p < 0.01$) or group 3 ($p < 0.05$), suggesting that previous exposures to ethanol withdrawals enhanced the sensitivity to Ro15-4513 (see Fig. 2).

There were no significant differences ($p > 0.05$) observed between group 2 (7-day continuous) and group 3 (22-day continuous) in seizure ratings or in the number of animals undergoing full motor convulsions following the administration of Ro15-4513.

Vehicle injections were not observed to elicit seizure symptoms in any of the groups (see Fig. 1B), although some ethanol-exposed animals continued to show muscle tremor and abnormal hindlimb posture after vehicle injection (ratings = 1 or 2). Groups did not differ significantly in seizure ratings following vehicle injection ($p > 0.05$).

DISCUSSION

Clinical research has shown an increased propensity for alcohol-withdrawal seizures in patients who have previously undergone alcohol-withdrawal seizures (6,20,21). These studies, as well as several studies of animal models, support the hypothesis of Ballenger and Post (1), suggesting an enhancement of withdrawal signs and symptoms with subsequent withdrawals from chronic alcohol exposure. However, despite several studies reporting increased sensitivity to seizures following repeated withdrawal episodes, the neural mechanisms underlying this phenomenon have not been elucidated.

The results from the present study show a significant increase in seizure ratings following Ro15-4513 administration in all ethanol-treated groups in comparison to control animals (Fig. 1A). This supports the previous observation by Mehta and Ticku (27), suggesting increased behavioral sensitivity to Ro15-4513 during withdrawal from chronic ethanol exposure. These results are also consistent with previous findings of an increase in Ro15-4513 binding sites in rat brain, as well as in cultured spinal cord neurons, following chronic ethanol treatment (28,29).

In addition, there was a significant increase in Ro15-4513–induced seizure ratings and in the incidence of tonic–clonic seizures in animals exposed to multiple cycles of ethanol withdrawal in comparison to animals receiving the same amount of ethanol exposure but no intermittent withdrawal (Figs. 1 and 2). This was observed for groups equated with the multiple withdrawal group (group 4: multiple withdrawals) on amount of ethanol exposure during the last exposure cycle (group 2: 7-day continuous) or on the total amount of ethanol

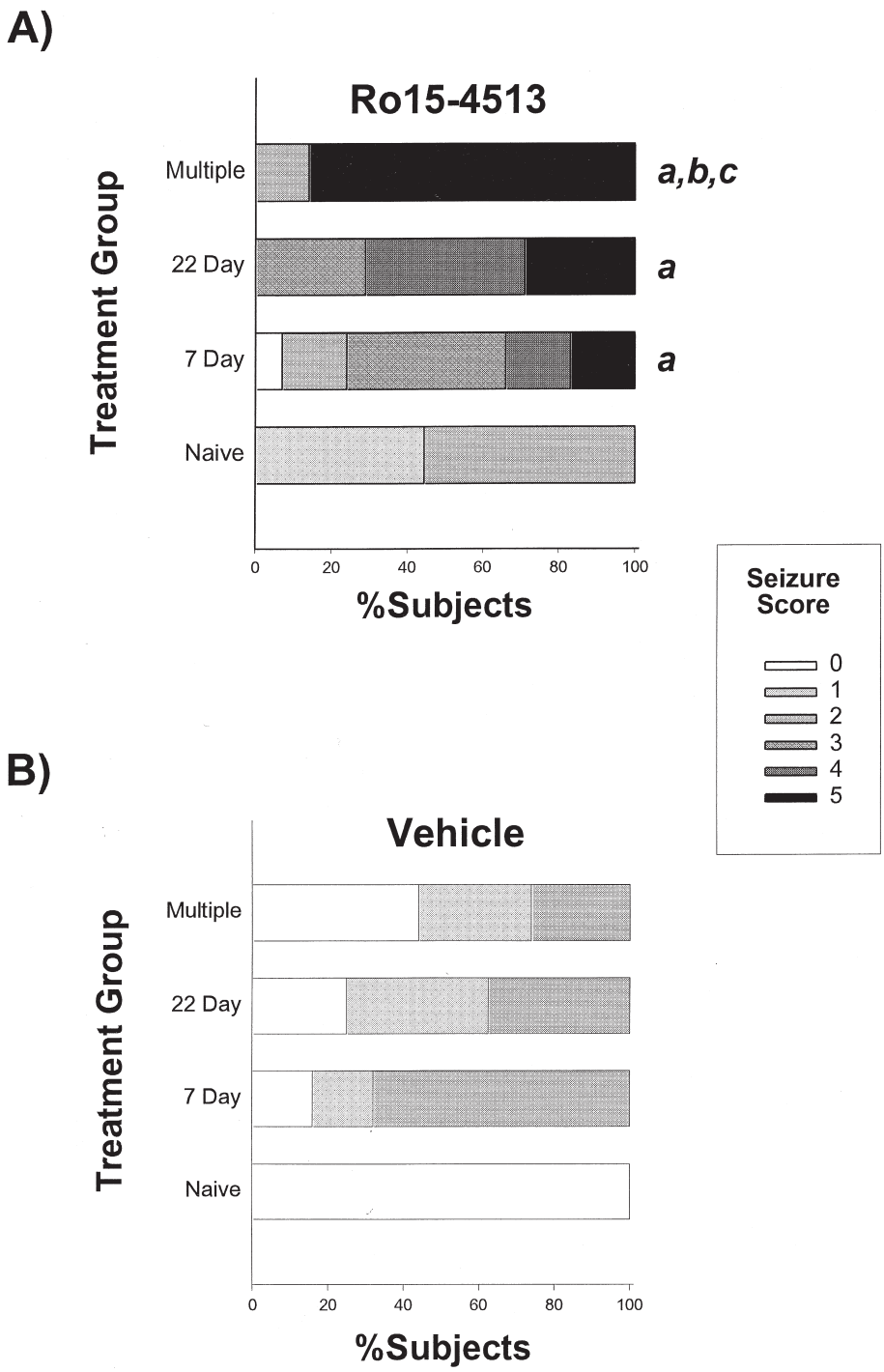

FIG. 1. Effects of chronic ethanol exposure and withdrawal on (A) Ro15-4513–induced seizure ratings, and (B) vehicle-induced ratings in rats. Bars represent the percentage of animals from each group receiving the given seizure rating during a 10-min period following the acute administration of Ro15-4513 or vehicle. (A) Ethanol-exposed rats were significantly more sensitive to the seizure-inducing effects of Ro15-4513 than were ethanol-naive rats ($p < 0.001$). [a]$p < 0.001$ compared to group 1 (naive); [b]$p < 0.01$ compared to group 2 (7 day continuous ethanol), and [c]$p < 0.05$ compared to group 3 (22 day continuous ethanol). (B) Groups did not differ significantly in their responses following vehicle injection.

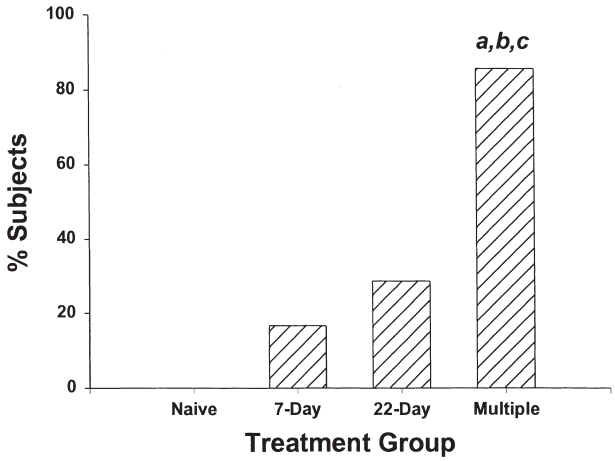

FIG. 2. Effect of chronic ethanol exposure and withdrawal on Ro15-4513–induced tonic–clonic seizure incidence in rats. Each bar represents the percentage of animals from each group undergoing tonic–clonic motor seizures during the 10-min period following administration of Ro15-4513. Significantly more animals from the multiple ethanol withdrawals group underwent full tonic–clonic convulsions following administration of Ro15-4513 than any other group. None of the animals receiving vehicle alone exhibited any tonic–clonic seizures (Fig. 1B) $^{a}p < 0.001$ compared to group 1 (naive), $^{b}p < 0.01$ compared to group 2 (7 day continuous ethanol), and $^{c}p < 0.05$ compared to group 3 (22 day continuous ethanol).

exposure (group 3: 22-day continuous). This indicates that the sensitivity to the behavioral effects of the $GABA_A$ receptor inverse agonist Ro15-4513 is increased due to previous exposures to ethanol withdrawal. Although the current study included observations of responses to just a single dose of Ro15-4513 (5.0 mg/kg, IP) at one time postwithdrawal (8–9 h), the results are consistent with our preliminary observations of the effects of 5.0 and 10.0 mg/kg Ro15-4513, IP, in ethanol-naive and 7-day continuous ethanol-exposed animals. There it was observed that ethanol-naive animals showed little sensitivity to these doses of Ro15-4513, but that animals withdrawn from chronic ethanol exposure exhibited a dose-related increase in drug-induced seizure ratings. Thus, although the use of a single dose and time point in the current study prevents a complete assessment of treatment-induced changes, the significant increase in seizure ratings observed in animals exposed to multiple withdrawal episodes following 5.0 mg/kg Ro15-4513, IP, in the present study is suggestive of an increased sensitivity to Ro15-4513 at a time after withdrawal when animals show the highest sensitivity to other seizure-inducing treatments (14).

The imidazodiazepine Ro15-4513 is a structural analogue of the benzodiazepine receptor antagonist Ro15-1788, and is a partial inverse agonist of the $GABA_A$-benzodiazepine receptor (5,39). This ligand binds to the classical benzodiazepine recognition site, as well as to a "diazepam-insensitive" site associated with the $GABA_A$ receptor complex in the cerebellar granule cell layer (α6-subtype) as well as in cerebral cortex (α4-subtype) (22,47). It has been reported to antagonize the effects of pharmacologically relevant concentrations of ethanol on the GABA-gated chloride channel (26). Furthermore, behavioral studies have also demonstrated the selective ability of Ro15-4513 to antagonize some of the behavioral effects following acute administration of ethanol in experimental animals (5,10,40). A single episode of chronic ethanol treatment (7-days continuous exposure) has been found to increase the number of recognition sites for this compound in discrete brain areas of the rat (28). Also, the affinity for the cerebellar binding of [^{3}H]Ro15-4513 was found to be higher in the tissue from human alcoholics than in tissues from control subjects (19), although changes in Ro15-4513 binding site density were not observed in postmortem tissue from human alcoholics or in brain membranes of alcohol drinking (AA) rats. These investigators, however, suggested that the lack of effect in their animal study may have been the result of the low levels of ethanol exposure in that study. Buck and Harris (8) observed that chronic ethanol exposure through a liquid diet in mice significantly enhanced the ability of the benzodiazepine inverse agonists Ro15-4513 and DMCM to reduce chloride flux following the withdrawal of ethanol, but this effect was not accompanied by changes in receptor density or affinity.

In a previous study, however, the enhanced sensitivity to behavioral effects (seizure sensitivity) of Ro15-4513 observed during withdrawal from a single episode of chronic ethanol treatment in a similar paradigm (27) was correlated with an increase in Ro15-4513 binding sites. Interestingly, in another study increased sensitivity to the actions of inverse agonists (DMCM and Ro15-4513) following chronic ethanol intake was seen only in a genetically selected line of mice prone to severe ethanol withdrawal seizures, and was not seen in those selected for resistance to withdrawal seizures (7). This finding suggests a possible link between the sensitivity to inverse agonists and the susceptibility to severe seizures (7).

The enhanced convulsant effect of Ro15-4513 during withdrawal from a single period of chronic ethanol treatment has been found to be mediated through $GABA_A$–benzodiazepine receptors, as it could be blocked by the $GABA_A$ receptor antagonist Ro15-1788 (27). A marked increase in the mRNA levels of $GABA_A$ receptor α_4, α_6, and β_2 subunits has also been reported following chronic ethanol exposure and withdrawal (11,12,30,32). Because α_4 and α_6 subunits uniquely form subtypes that encode diazepam-insensitive Ro15-4513 recognition sites (22,47), these data support previous findings that chronic ethanol exposure results in an increase in these sites (4,28,29). This conclusion should be considered with caution, however, because it has been observed that $GABA_A$ receptors immunoprecipitated with γ_1 or δ antibodies do not bind [^{3}H]Ro15-4513 (38).

In relation to the above findings, the observations from the present study suggest the possibility that previous withdrawals from chronic ethanol exposures may further upregulate $GABA_A$ receptor subunits encoding the Ro15-4513 recognition sites. Such an upregulation could contribute to the enhanced sensitivity to the convulsive actions of Ro15-4513, in correlation to the number of withdrawals from chronic ethanol exposures. In support of this hypothesis, chronic intermittent ethanol treatment has recently been shown to produce a significant and long-lasting increase in diazepam-insensitive binding sites in cultured cortical neurons (16).

Exposure to a single episode of chronic ethanol treatment alters the pharmacology of $GABA_A$ receptors (31), and produces significant region-specific alterations in $GABA_A$ receptor gene expression suggesting that chronic ethanol exposure and withdrawal might cause a change in the conformation of the receptor that could mediate the behavioral effects of chronic exposure to ethanol such as tolerance, physical dependence, and the withdrawal syndrome (11,12,23,30–33). Also, as shown by us and other workers, a single episode of

chronic ethanol exposure decreases the functional sensitivity of the $GABA_A$ receptor to its various modulators (13,15,31,35) and increases sensitivity to inverse agonists of the receptor (4,8,28,29), suggesting a shift in receptor function towards inverse, agonism. From the present observations it seems possible that the sensitization to withdrawal symptoms following multiple ethanol withdrawals could involve a further decrease in $GABA_A$ receptor-mediated inhibitory neurotransmission and an increased behavioral response to $GABA_A$ receptor inverse agonists.

This argument is further supported by the previous study of McCown and Breese (25), where sensitivity to locally applied bicuculline (administered directly into the inferior colliculus) was found to increase from 6 to 10 ethanol withdrawals in association with a facilitation of electrical kindling, suggesting a progressive change in $GABA_A$ receptor pharmacology with increasing numbers of withdrawal episodes. A shift of the receptor to inverse agonism as stated above, could be associated with an increased sensitivity to the convulsant effects of $GABA_A$ receptor inverse agonists, such as Ro15-4513, and antagonists, such as bicuculline, as indicated in the present study and that of McCown and Breese (25).

In conclusion, exposure to multiple cycles of ethanol intoxication and withdrawal increases sensitivity to the convulsant effects of the $GABA_A$ receptor inverse agonist Ro15-4513. The specific mechanism of this enhanced convulsant effect of Ro15-4513 following multiple ethanol withdrawals remains to be determined. This increased sensitivity to a $GABA_A$ receptor inverse agonist following repeated withdrawals from chronic ethanol exposure suggests a possible mechanism for the increased severity of the alcohol-withdrawal syndrome sometimes seen in human alcoholics after repeated withdrawal episodes, and may have clinical significance for treatment of the alcohol-withdrawal syndrome.

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REFERENCES

1. Ballenger, J. C.; Post, R. M.: Kindling as a model for alcohol withdrawal syndromes. Br. J. Psychiatry 133:1–14; 1978.
2. Becker, H. C.: Positive relationship between the number of prior ethanol withdrawal episodes and the severity of subsequent withdrawal seizures. Psychopharmacology (Berlin) 116:26–32; 1994.
3. Becker, H. C.; Hale, R. L: Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: An animal model of alcohol withdrawal kindling. Alcohol. Clin. Exp. Res. 17:94–98; 1993.
4. Becker, H. C.; Jarvis, M. F.: Chronic ethanol exposure selectively increases diazepam-insensitive [^{3}H]Ro15-4513 binding in mouse cerebellum. Eur. J. Pharmacol. 296:43–46; 1996.
5. Bonetti, E. P.; Burkard, W. P.; Gabl, M.; Hunkeler, W.; Lorez, H. P.; Martin, J. R.; Moehler, H.; Osterrieder, W.; Pieri, L.; Polc, P.; Richards, J. G.; Schaffner, R.; Scherschlicht, R.; Schoch, P.; Haefely, W. E.: Ro15-4513: Partial inverse agonism at the BZR and interaction with ethanol. Pharmacol. Biochem. Behav. 31:733–749; 1989.
6. Brown, M. E.; Anton, R. F.; Malcolm, R.; Ballenger, J. C.: Alcohol detoxification and withdrawal seizures: Clinical support for a kindling hypothesis. Biol. Psychiatry 23:507–514; 1988.
7. Buck, K. J.; Hahner, L.; Sikela, J.; Harris, R. A.: Chronic ethanol treatment alters brain levels of gamma-aminobutyric acid$_A$ receptor subunit messenger RNAs—relationship to genetic differences in ethanol withdrawal seizure severity. J. Neurochem. 57:1452–1455; 1991.
8. Buck, K. J.; Harris, R. A.: Benzodiazepine agonist and inverse agonist actions on $GABA_A$ receptor-operated chloride channels. II. Chronic effects of ethanol. J. Pharmacol. Exp Ther. 253:713–719; 1990.
9. Clemmesen, L.; Hemmingsen, R.: Physical dependence on ethanol during multiple intoxication and withdrawal episodes in the rat: evidence of a potentiation. Acta Pharmacol. Toxicol. 55:345–350; 1984.
10. Dar, M. S.: Antagonism by intracellular Ro15-4513 of acute ethanol-induced motor incoordination in mice. Pharmacol. Biochem. Behav. 52:217–223; 1995.
11. Devaud, L. L.; Fritschy, J. M.; Sieghart, W.; Morrow, A. L.: Bidirectional alterations of $GABA_A$ receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. J. Neurochem. 69:126–130; 1997.
12. Devaud, L. L.; Smith, F. D.; Grayson, D. R.; Morrow, A. L.: Chronic ethanol consumption differentially alters the expression of gamma-amino butyric acid$_A$ receptor subunit mRNAs in rat cerebral cortex: Competitive, quantitative reverse transcriptase-polymerase chain reaction analysis. Mol. Pharmacol. 48:861–868; 1995.
13. Gonzalez, L. P.; Czachura, J. F.: Reduced behavioral responses to intranigral muscimol following chronic ethanol. Physiol. Behav. 46:473–477; 1989.
14. Gonzalez, L. P.; Czachura, J. F.; Brewer, K. W.: Spontaneous versus elicited seizures following ethanol withdrawal: differential time course. Alcohol 6:481–487; 1989.
15. Harris, R. A.; Valenzuela, C. F.; Brozwski, S.; Chuang, L.; Hadinham, K.; Whiting, P. J.: Adaptation of gamma-aminobutyric acid type A receptors to alcohol exposure: Studies with stably transfected cells. J. Pharmacol. Exp. Ther. 284:180–188; 1998.
16. Hu, X. J.; Ticku, M. K.: Functional characterization of a kindling-like model of ethanol withdrawal in cortical cultured neurons after chronic intermittent ethanol exposure. Brain Res. 767:228–234; 1997.
17. Kang, M.; Spigelman, I.; Sapp, D. W.; Olsen, R. W.: Persistent reduction of $GABA_A$ receptor mediated inhibition in rat hippocampus after chronic intermittent ethanol treatment. Brain Res. 709:221–228; 1996.
18. Kokka, N.; Sapp, D. W.; Taylor, A. M.; Olsen, R. W.: The kindling model of alcohol dependence: Similar persistent reduction in seizure threshold to pentylenetetrazol in animals receiving chronic ethanol or chronic pentylenetetrazole. Alcohol. Clin. Exp. Res. 17:525–531; 1993.
19. Korpi, E. R.; Uusi-Oukari, M.; Wegelius, K.; Casanova, M.; Zito, M. A. K. J. E.: Cerebellar and frontal cortical benzodiazepine receptors in human alcoholics and chronically alcohol-drinking rats. Biol. Psychiatry 31:774–786; 1992.
20. Lechtenberg, R.; Worner, T. M.: Seizure risk with recurrent alcohol detoxification. Arch. Neurol. 47:535–538; 1990.
21. Lechtenberg, R.; Worner, T. M.: Relative kindling effect of detoxification and non-detoxification admissions in alcoholics. Alcohol Alcohol. 26:221–225; 1991.
22. Luddens, H.; Pritchett, D. B.; Kohler, M.; Killisch, I. I.; Keinan, K.; Monyer, H.; Sprengel, R.; Seeburg, P. H.: Cerebellar $GABA_A$ receptor selective behavioral alcohol antagonist. Nature 346:648–651; 1990.
23. Matthews, D. B.; Devaud, L. L.; Fritschy, J. M.; Sieghart, W.; Morrow, A. L.: Differential regulation of $GABA_A$ receptor gene expression by ethanol in rat hippocampus versus cerebral cortex. J. Neurochem. 70:1160–1166; 1998.
24. McCown, T. J.; Breese, G. R.: Multiple withdrawals from chronic ethanol kindles inferior collicular seizure activity—Evidence for kindling of seizures associated with alcoholism. Alcohol. Clin. Exp. Res. 14:394–399; 1990.
25. McCown, T. J.; Breese, G. R.: A potential contribution to ethanol withdrawal kindling-reduced GABA function in the inferior collicular cortex. Alcohol. Clin. Exp. Res.17:1290–1294; 1993.

26. Mehta, A. K.; Ticku, M. K.: Ethanol potentiation of GABAergic transmission in cultured spinal cord neurons involves gamma-aminobutyric $acid_A$ gated chloride channels. J. Pharmacol. Exp. Ther. 246:558–564; 1988.
27. Mehta, A. K.; Ticku, M. K.: Chronic ethanol treatment alters the behavioral effects of Ro15-4513, a partially negative ligand for benzodiazepine binding sites. Brain Res. 489:93–100; 1989.
28. Mhatre, M.; Mehta, A. K.; Ticku, M. K.: Chronic ethanol administration increases the binding of the benzodiazepine inverse agonist and alcohol antagonist [^{3}H]Ro15-4513 in rat brain. Eur. J. Pharmacol. 153:141–145; 1988.
29. Mhatre, M.; Ticku, M. K.: Chronic ethanol treatment selectively increases the binding of inverse agonists for benzodiazepine binding sites in cultured spinal cord neurons. J. Pharmacol. Exp. Ther. 251:164–168; 1989.
30. Mhatre, M.; Ticku, M. K.: Chronic ethanol administration alters gamma-aminobutyric $acid_A$ receptor gene expression. Mol. Pharmacol. 42:415–422; 1992.
31. Mhatre, M.; Ticku, M. K.: Alcohol: effects on $GABA_A$ receptor function and gene expression. Alcohol Alcohol. 2:331–335; 1993.
32. Mhatre, M.; Ticku, M. K.: Chronic ethanol treatment upregulates the $GABA_A$ receptor beta subunit expression. Mol. Brain Res. 23:246–252; 1994.
33. Mhatre, M. C.; Pena, G.; Sieghart, W.; Ticku, M. K.: Antibodies specific for $GABA_A$ receptor alpha-subunits reveal that chronic alcohol treatment down-regulates alpha-subunit expression in rat brain regions. J. Neurochem. 61:1620–1625; 1993.
34. Mihic, S. J.; Ye, Q.; Wick, M. J.; Koltchine, W.; Krasowski, M. D.; Finn, S. E.; Mascia, M. P.; Valenzuela, C. F.; Hanson, K. K.; Greenblatt, E. P.; Harris, R. A.; Harrison, N.: Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. Nature 25:389; 1997.
35. Morrow, A. L.; Suzdak, P. D.; Karanian, J. W.; Paul, S. M.: Chronic ethanol administration alters gamma-aminobutyric acid, pentobarbital and ethanol-mediated [^{36}Cl]-uptake in cerebral cortical synaptoneurosomes. J. Pharmacol. Exp. Ther. 246:158–164; 1988.
36. Pinel, J. P. J.: Alcohol withdrawal seizures: Implications of kindling. Pharmacol. Biochem. Behav. 13:225–231; 1980.
37. Poldrugo, F.; Snead, I. O. C.: Electroencephalographic and behavioral correlates in rats during repeated ethanol withdrawal syndromes. Psychopharmacology (Berlin) 83:140–146; 1984.
38. Quirk, K.; Gillard, N. P.; Ragan, C. I.; Whiting, P. J.; McKernan, R. M.: Model of subunit composition of γ-aminobutyric acid A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. J. Biol. Chem. 269:16020–16028; 1994.
39. Suzdak, P. D.; Glowa, J. R.; Crawley, J. N.; Schwartz, R. D.; Skolnick, P.; Paul, S. M.: A selective imidazobenzodiazepine antagonist of ethanol in the rat. Science 234:1243–1247; 1986.
40. Suzdak, P. D.; Paul, S. M.; Crawley, J. N.: Effects of Ro15-4513 and other benzodiazepine receptor inverse agonists on alcohol-induced intoxication in the rat. J. Pharmacol. Exp. Ther. 245: 880–886; 1988.
41. Ulrichsen, J.; Clemmesen, L.; Hemmingsen, R.: Convulsive behaviour during alcohol dependence: Discrimination between the role of intoxication and withdrawal. Psychopharmacology (Berlin) 107:97–102; 1992.
42. Veatch, L. M.; Gonzalez, L. P.: Contingency table testing for categorical data: SAS implementation. Behav. Res. Methods, Instrum. Comput. 27:404–407; 1995.
43. Veatch, L. M.; Gonzalez, L. P.: Repeated ethanol withdrawal produces site-dependent increases in EEG spiking. Alcohol. Clin. Exp. Res. 20:262–267; 1996.
44. Veatch, L. M.; Gonzalez, L. P.: Chronic ethanol retards kindling of hippocampal area CA_3. Neuroreport 8:1903–1906; 1997.
45. Walker, D. W.; Zornetzer, S. F.: Alcohol withdrawal in mice: Electroencephalographic and behavioral correlates. Electroencephalogr. Clin. Neurol. 36:233–243; 1974.
46. Whittington, M. A.; Lambert, J. D. C.; Little, H. J.: Increased NMDA receptor and calcium channel activity underlying ethanol withdrawal hyperexcitability. Alcohol Alcohol. 30:105–114; 1995.
47. Yang, W.; Drewe, J. A.; Lan, N. C.: Cloning and characterization of the human $GABA_A$ receptor $alpha_4$ subunit: Identification of a unique diazepam-insensitive binding site. Eur. J. Pharmacol. 291:319–325; 1995.