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Behavioral Analysis of PTZ-Kindled Rats After Acute and Chronic Ethanol Treatments

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DAVIDSON, M., W. CHEN AND P. A. WILCE. *Behavioral analysis of PTZ-kindled rats after acute and chronic ethanol treatments.* PHARMACOL BIOCHEM BEHAV **64**(1) 7–13, 1999.—The present study was designed to examine the response of PTZ-kindled and saline-injected animals to both acute and chronic ethanol treatment. Acute injection of ethanol (3.0 g/kg; IP) resulted in a rapid onset of loss of righting reflex (LORR) in both PTZ-kindled and saline-injected animals. However, the PTZ-kindled animals recovered from LORR significantly more quickly than control animals. Using a tilt-plane test as a measure of motor incoordination, the PTZ-kindled animals had significantly less motor incoordination compared to controls. Blood alcohol levels (BAL) were not significantly different between the groups. We also compared the degree of tolerance and dependence in chronic ethanol-treated, PTZ-kindled, and control animals. PTZ-kindled, saline-injected and naive control animals were chronically treated with ethanol vapor. The PTZ-kindled group tolerated high vapor concentrations (in terms of food consumed/rat) and, at the end of the treatment, displayed intoxication characteristics different from those of the control groups despite having similar blood alcohol levels. The PTZ-kindled group also displayed withdrawal behavior that was similar to a group of ethanol-treated animals that had experienced a prior cycle of dependency and withdrawal. These data show many intriguing similarities between animals that are PTZ-kindled and chronically treated with ethanol and suggest the use of PTZ-kindled animals as a model for alcohol withdrawal kindling. © 1999 Elsevier Science Inc.

Pentylenetetrazol Kindling Ethanol dependence Withdrawal GABA NMDA

KINDLING is a process by which repeated administration of initially subconvulsive stimuli leads to a long-lasting progressive intensification of behavioral convulsive activity (17). Models include repeated electrical or chemical stimulation of the amygdala and hippocampus or repeated administration of subconvulsant doses of convulsants such as pentylenetetrazol (PTZ). PTZ interacts competitively with the picrotoxin binding site of the $GABA_A$ receptor, thereby decreasing Cl^- flux across the membrane (44) and inducing generalized tonic–clonic seizures. This property is used as an experimental animal model of epilepsy and epileptogenesis (17).

Repeated systemic administration of subconvulsive doses of PTZ results in a progressive increase in seizure intensity (33) that is long lasting and irreversible. The development of PTZ kindling may be related to functional alterations in various neurotransmitter systems, including a gradual reduction in $GABA_A$ receptor function and an enhancement of glutamatergic transmission that together result in hyperexcitability and seizure activity (10,39). The $GABA_A$ receptors are the major inhibitory neurotransmitter system in the mammalian central nervous system. Decreased GABA-mediated inhibitory synaptic transmission is considered a major factor contributing to the pathophysiology of alcoholism and epilepsy; thus, GABA receptor agonists such as diazepam are used to enhance GABAergic transmission during ethanol withdrawal. In addition, the *N*-methyl-D-aspartic acid (NMDA) receptor complex is involved in the development of PTZ kindling and kindling expression (13).

Ballenger and Post (4) originally suggested that episodes of ethanol intoxication and withdrawal could serve as a kindling stimulus, and thereby enhance the severity of the withdrawal response. Subsequently, this concept has been supported using several models of repeated alcohol dependence and withdrawal (7,26). Prior exposure to kindling regimes, including PTZ, increased the severity of symptoms associated with chronic ethanol withdrawal (40). Kokka et al. (26) determined if the sensitivity to ethanol, in terms of temperature response to a challenge dose of ethanol (1.5 g/kg), had been altered by kindling with PTZ, and found no significant changes after eight injections of PTZ but significant changes after 16

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injections. PTZ-kindled rats (22 injections) that had never been exposed to ethanol were also tested for temperature change after a 4.5 g/kg ethanol challenge and found to exhibit tolerance (26). These observations, along with the extensive data implicating GABA and NMDA receptor systems in the effects of ethanol [(49), for review], prompted us to study in more depth the behavioral interactions of ethanol and PTZ-kindled animals.

METHOD

Adult male Wistar rats (approximately 200 g) were purchased from the Central Animals Breeding House (The University of Queensland). Animals had free access to a commercial rodent diet (18% protein) and water. Environmental conditions were a 12 L:12 D cycle at 22 ± 2°C.

Pentylenetetrazol Kindling

Over a period of 6 weeks animals were injected with a subconvulsive dose of PTZ (25 mg/kg, IP) in saline, two to three times a week. After each injection the behavior of the rat was scored (43) for a period of 30 min as follows: score 0—no response; score 1—ear and facial twitching; score 2—convulsive waves axially through body; score 3—myoclonic jerks and rearing; score 4—clonic forelimb convulsions; score 5—generalized tonic–clonic seizure. Animals considered kindled exhibited at least three consecutive stages 4–5 seizures. Controls received multiple injections of saline in parallel to PTZ injections. To confirm the kindled state, a number of animals were injected with a subconvulsive dose of PTZ, 2 weeks after the last injection in the kindling regime. All these animals exhibited a response similar to that elicited by the last kindling injection.

Loss of Righting Reflex (LORR)

An anesthetic dose of ethanol [3.0 g/kg, 25% (w/v) solution in saline] was administered IP and the onset and duration of LORR was determined. LORR was defined as the inability of the animal to right itself within 30 s from a supine position. The duration of LORR was used as an index of CNS depression. The time between ethanol injection and LORR was scored in seconds and referred to as "time to onset of LORR." The interval between initial LORR and subsequent gain of righting reflex was scored in minutes and referred as the "duration of LORR."

Tilt-Plane Test

The motor impairment induced by ethanol (3.0 g/kg, IP) was quantitated by the tilt-plane test (3,25). A percentage of maximal impairment was measured at each time interval by placing the animal on a slightly roughened surface on a plane that was tilted (range of 0–90°, marked at 2° intervals) until the animal began to slide from its original position. The degree of ethanol-induced ataxia was expressed as the percentage change in sliding-angle compared to preinjection value for the same animal. The sliding angle was measured at 0.5-h intervals to 3 h.

Chronic Ethanol Treatment

Three groups were used: PTZ-kindled, saline-injected, with a further group acting as an uninjected control. Chronic ethanol administration via ethanol inhalation was used as previously described (14,21,34). The rats were made physiologically dependent by exposure over a period of 15 days to an increasing ethanol vapor concentration (range 10–25 mg ethanol/l air) with airflow being maintained at 12 l/min. Ethanol (96% beverage grade) was delivered via a peristaltic pump (Gilson Minipuls 2) using silicone tubing (grn/grn—0.073 i.d., Technicon) into an air-tight flask held in a 42°C water bath. The pump speed was adjusted to vary the ethanol flow (0.4–0.75 ml/min) and create different levels of vapor. A constant stream of air (12 l air/min) was passed through the flask, and the resultant ethanol vapor flowed into a 450-l chamber into which the rats were placed. Over the period of treatment blood alcohol levels (BAL) were maintained such that approximately 2 mg/ml was achieved within a week, and was maintained for at least 5 days before the animals were used for experiments.

Degree of Tolerance

At the end of the chronic ethanol treatment, individual rats were assigned a tolerance score. Tolerance scores were based upon the degree of intoxication using the signs and responses described by Majchrowicz (31,32) and used successfully by other groups to assess intoxication after ethanol exposure (27,48): score 0 (neutrality,—no overt signs; score 1 (sedation)—slow locomotor activity) reduced muscle tone, no gait impairment or motor incoordination; score 2 (ataxia 1)—abdomen and pelvis markedly elevated, lowest degree of gait impairment and motor incoordination; score 3 (ataxia 2)—accentuated staggering gait; score 4 (ataxia 3)—slowed righting reflex, heavily impaired motor incoordination, absence of pelvic or abdominal elevation; score 5 (LORR)—loss of righting reflex, little or no spontaneous motor activity; score 6 (coma)—no signs of movement, completely unresponsive.

Ethanol Withdrawal Behaviors

The scoring system for withdrawal behaviors was modified after Karanian et al. (23). Animals were scored as follows: score 0, no reaction, normal behavior; score 1–4, tail stiffening (2) tail tremor (2), body tremor (2), and whole body rigidity (2); score 5, handling-induced brief seizure following gentle rotation through a 180° arc before being placed back in the cage; score 6, as above with a handling-induced continuous seizure; score 7, spontaneous seizure (tonic–clonic, no handling involved) without death. No rat achieved a score of 5 without showing the previous four behaviors. Scores were monitored at intervals of either 1 or 2 h up to 24 h after cessation of ethanol treatment by at least two investigators "blind" to the treatment of animals. Repeated handling of animals at different times had no effect on subsequent responses.

Blood Alcohol Determination

Blood samples (0.2 ml) were obtained from the tail 1 h after acute injection of ethanol and at the time of onset of withdrawal (0 h) with the chronic treated rats. Blood was assayed for alcohol content using ADH-NAD enzyme-spectrophotometer method according to Lundquist (29).

Statistical Analysis

Significant differences between two groups were determined by Student's *t*-test. Experiments involving two variables (group and time) were assessed using two-way analysis of variance, while experiments involving three groups were assessed using one-way analysis of variance followed by a post hoc test, as indicated. The linear regression analysis and com-

parison of slope and intercept were determined using GraphPad Prism 2.01 for Windows 95 (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± SEM.

RESULTS

Kindling

Initial body weights were 282 ± 7 and 289 ± 5 g for the saline and PTZ groups, respectively. At the end of the chronic injections, body weights were 430 ± 11 and 446 ± 11 g, respectively ($p = 0.33$), which suggested that PTZ-induced seizures had no effect on the general health or nutrition of the animals. During the course of the 15 consecutive injections, some rats with seizures terminated in exitus due to respiratory arrest (mortality 6 of 41). Aside from their hypersensitivity to PTZ, kindled rats stayed in good condition and could not be distinguished in appearance and behavior from saline-injected or naive controls. Figure 1 demonstrates the progressive increment in the susceptibly to seizures after repeated PTZ administration.

Acute injections of ethanol

LORR. Injection of a sedative dose of ethanol resulted in rapid onset of LORR in seven of the eight animals from both saline-injected and the PTZ-kindled groups [Fig. 2, $t(12) = 0.47$, $p = 0.65$]. The duration of LORR was significantly different between the groups [Fig. 2, $t(12) = -2.85$, $p = 0.015$], although, 1 h postinjection, the BALs were not significantly different (saline, 2.49 ± 0.19 mg/ml; PTZ, 2.41 ± 0.06 mg/ml; mean ± SEM, $n = 8$ per group, $p = 0.665$).

The Tilt-Plane Test

Ethanol (3.0 g/kg; IP) impairs motor performance in a tilt-plane test. Maximum impairment by ethanol was recorded 30 min after injection in both the PTZ-kindled group and saline-injected group [Fig. 3; $t(14) = 0.789$, $p = 0.44$] and at the end of the test period (3 h) animals had almost recovered their motor performance (Fig. 3). The PTZ-kindled group recovered from the motor incoordinating effects of ethanol faster than the saline control group [Fig. 3; two-way ANOVA (group × time), $F(1, 95) = 28.9$, $P < 0.0001$]. No significant difference in BAL between groups was observed.

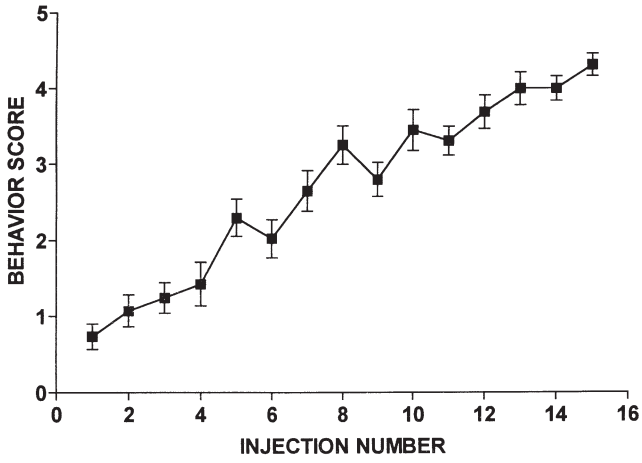

FIG. 1. Development of the susceptibility of rats to PTZ (25 mg/kg, IP) in the course of kindling. Data are mean ± SEM of 35–41 animals.

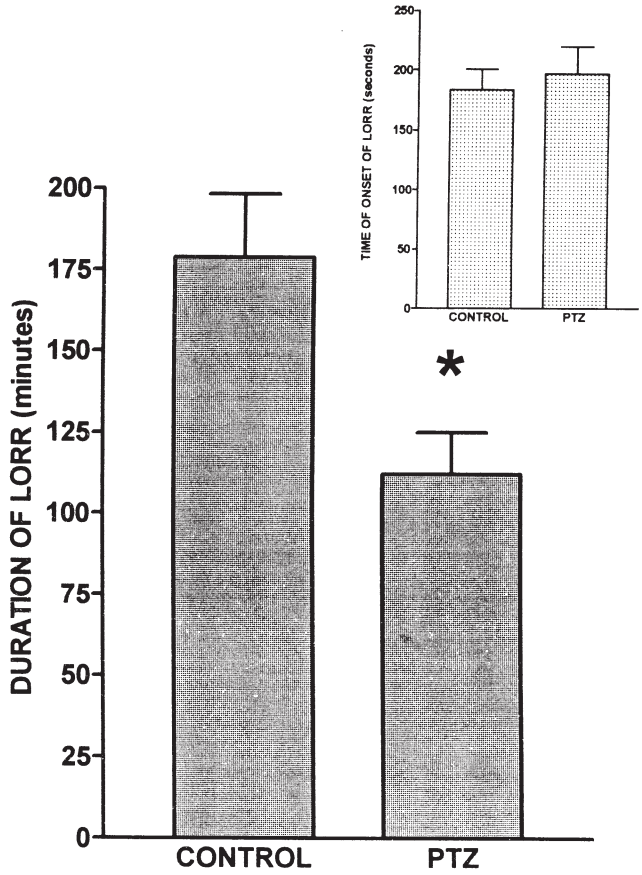

FIG. 2. Effect of administration of ethanol (3.0 g/kg, IP) on the sedative properties of ethanol in naive PTZ-kindled and saline-injected animals. The time taken for onset of the LORR (insert) was measured in seconds (mean ± SEM, $n = 7$) and duration of LORR was measured in minutes (mean ± SEM, $n = 7$). *$p < 0.05$ Student's t-test.

Chronic Ethanol Treatment

Three groups (PTZ-kindled, saline-injected, and uninjected controls) were used to assess the degree of tolerance and dependence on chronic ethanol. Early on in the treatment it was noted that one group was tolerating the intoxication, in terms of appetite and sedation, better than the other two. This was confirmed when food consumption was measured from day 5 onwards. The PTZ-kindled animals ate more than both control groups, which suggested they were tolerating the effects of the increasing concentrations of ethanol vapor better than controls [Fig. 4; two-way ANOVA (group × time), $F(2, 29) = 34.32$, $p < 0.0001$]. This was not related to BAL, which was similar between groups (data not shown).

Tolerance in PTZ-Kindled and Control Rats

A blood sample was taken from the tail vein at the end of the ethanol vapor treatment. The animals were assessed for degree of sedation and their behaviors were scored during withdrawal. The data in Fig. 5 show clear differences in the degree of tolerance between the PTZ-kindled and control groups. As the saline group and the control group were not

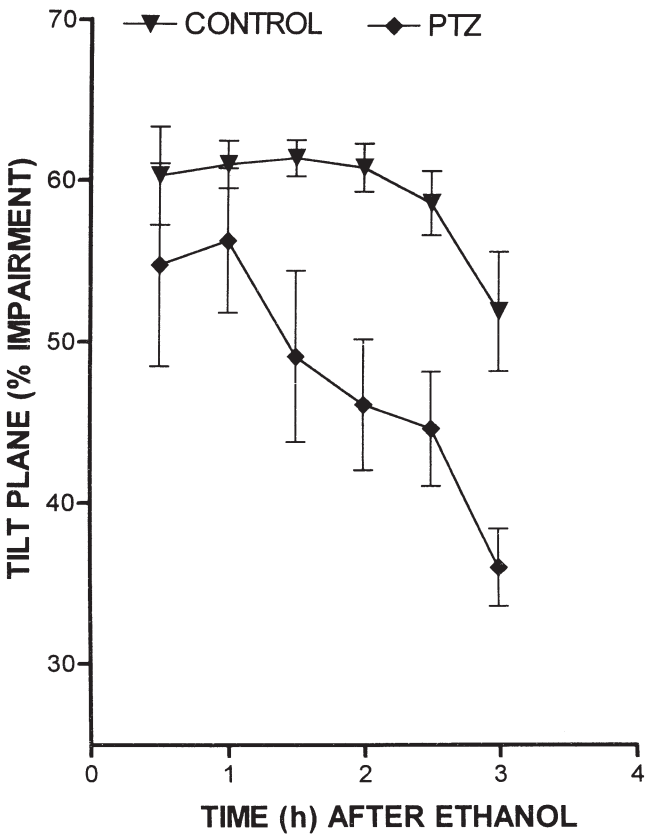

FIG. 3. Effect of administration of ethanol (3.0 g/kg, IP) on motor incoordination with the tilt-plane test. Motor incoordination was assessed as % maximal impairment at different times points in ethanol-naive PTZ-kindled rats and saline-injected control rats. Results are mean ± SEM of eight animals per group. Two-way analysis of variance shows a significant difference of group, $F(1, 95) = 28.87, p < 0.0001$.

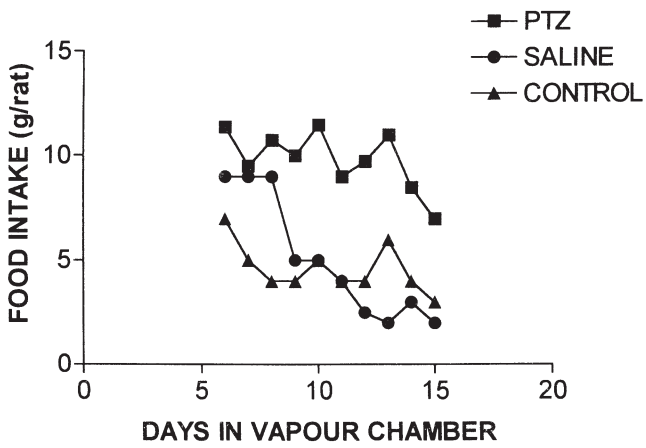

FIG. 4. Food consumption (g/rat) in the PTZ-kindled, saline injected, and control groups over the time of chronic ethanol vapor inhalation. Rats were placed in ethanol vapor chamber and made dependent by gradual increases of the ethanol vapor concentration as described in the Method section. Food consumption was measured in each cage and recorded as average food intake/rat in each group. Two-way analysis of variance (group and time) showed that the PTZ-kindled group tolerated the effects of increasing ethanol vapor to a significant greater degree than the control groups, $F(2, 29) = 34.32, p < 0.001$. Post hoc test (Bonferroni's method) showed signigicant ($p < 0.05$) differences between PTZ-kindled rats and both saline-injected and naive control groups.

different in either BAL or level of intoxication, both groups were combined for linear regression analysis. Analysis showed that although the differences in the slopes of the lines approached significance, $F(1, 26) = 3.44$, $p = 0.075$, the degree of elevation/intercept differed significantly, $F(1, 27) = 10.9$, $p = 0.002$. The BAL of the three groups were not significantly different [Fig.5 insert; $F(2, 27) = 1.74$, $p = 0.19$].

Ethanol Withdrawal

In the first experiment, ethanol-naive animals that had been either PTZ kindled, saline-injected or uninjected were subjected to chronic ethanol exposure and withdrawal. Figure 6A depicts ethanol-withdrawal behaviors with time upon cessation of the ethanol vapor (0 h) for the PTZ-kindled group and the combined control groups. As the control groups (saline-injected and uninjected) were indistinguishable in terms of either BAL or withdrawal behaviors they were combined for analysis. The BALs at time of withdrawal were not significantly different between PTZ-kindled or control groups (PTZ 3.6 ± 0.14; control 4.1 ± 0.25). An examination of the withdrawal profiles showed that the PTZ-kindled rats had an accelerated withdrawal reaction, that is, a greater peak withdrawal at an earlier time point than the control rats. Two-way ANOVA (group and time) showed significant differences between the PTZ-kindled animals and control groups ($F(1, 367) = 69.1$, $p < 0.0001$ (Fig. 6A) for this withdrawal profile. In a second experiment control animals were subjected to chronic vapor treatment and withdrawal twice with 2 weeks separating the cycles. There was no difference in BAL between the two groups (withdrawal 1 3.5 ± 1.26; withdrawal 2 3.23 ± 0.56). The withdrawal behaviors were compared (Fig. 6B). There were significant differences (two-way ANOVA: group and time) between groups undergoing their first and second withdrawal, $F(1, 149) = 9.2$, $p < 0.01$. Similarities between the withdrawal profiles of PTZ-kindled animals and twice withdrawan animals vs. their controls (Fig. 6A and B) were noted.

DISCUSSION

In an animal model developed for the bioassay of anxiogenic and axiolytic properties of drugs, Lal et al. (27) initially observed that a PTZ-like interoceptive stimulus was elicited during withdrawal from ethanol that correlated with occurrence of overt behavioral and physical signs of withdrawal (27). The elicitation of this PTZ stimulus in rats naive to ethanol may partially represent decreased GABAergic function. Idemudia et al. (22) showed antagonism of the GABA system by bicuculline and picrotoxin increased the withdrawal stimulus in an additive manner. Rats exhibited a PTZ-like stimulus as soon as the blood ethanol and ethanol intoxication declined after cessation of chronic ethanol treatment (22). These initial findings suggest some common mechanisms in PTZ-kindled and ethanol-dependent rats. Therefore, we compared chronic ethanol-treated and PTZ-kindled animals to provide additional clues on the neuronal mechanisms that mediate development and persistence of ethanol dependence. Kokka et al. (26) showed those PTZ-kindled rats, without prior exposure, exhibited tolerance to the hypothermic effects of a challenge dose of ethanol (4.5 g/kg) similar to the tolerance exhibited

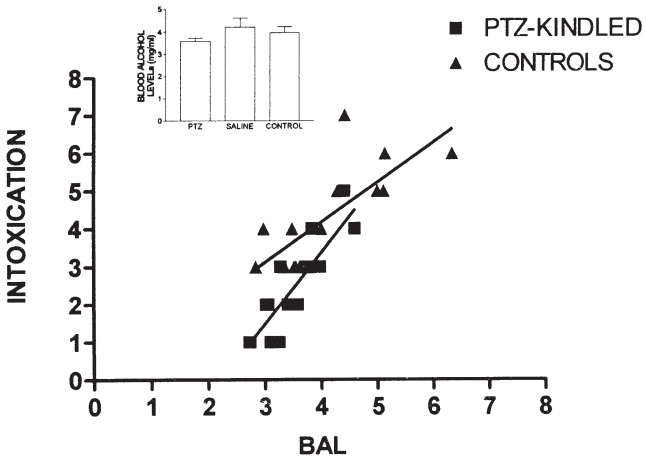

FIG. 5. Levels of ethanol-induced intoxication in PTZ-kindled and control animals after chronic ethanol treatment. Behavioral intoxication was scored using the rating scale of Majchrowicz as described in the Method section. Values represent individual scores vs. actual BAL for that animal and are plotted as linear regressions ($n = 15$ per group). Saline-injected and control groups were combined for the regression analysis. The differences between slopes was not significant, $F(1, 26) = 3.44$, $p = 0.075$, but there was a significant difference between the elevations, $F(1, 27) = 10.91$, $p = 0.0027$).

by rats with 5 and 12 chronic intermittent ethanol administrations. We extended these observations to show that naive PTZ-kindled rats have increased tolerance to both the sedative and motor incoordinating effects of acute ethanol. Further, these animals had increased tolerance to chronic ethanol, and showed an initial heightened withdrawal score similar to that displayed by animals undergoing withdrawal for the second time.

Kindling is associated with trans-synaptic changes, synaptic reorganization and morphology, and changes in protein synthesis (38). The development of kindling is related to alterations in glutamate and/or GABA receptors (39). A majority of studies have shown increased glutamate ligand binding (28,46,47), including presynaptic metabotropic receptors (45), although possibly accompanied by a downregulation of glutamate receptor gene expression in several areas of the kindled brain (28,30). The results have been interpreted as an enhancement of glutamategic neurotransmission during the kindling process. In contrast, GABAergic receptor function may be decreased, although this may be secondary to the changes in glutamatergic neurotransmission. Both chronic ethanol and PTZ kindling results in reduction in ^{35}S-TBPS binding and GABA-stimulated $^{36}Cl^-$ uptake (1,10,11,21). There are other parallels between PTZ kindling and ethanol dependence and withdrawal (Table 1). These include the action of MK801, which prevents ethanol withdrawal behavior and development of PTZ kindling (16,19). MK801 also prevents the effect of chronic PTZ administration on ^{35}S-TBPS binding (16), suggesting that blockade of the NMDA receptor channel prevents alterations in the biochemical parameters of GABAergic transmission elicited by PTZ kindling. MK801 also prevents the development of electrical kindling (36), suggesting a common mechanism with all kindling-like phenomenon. The NMDA type of glutamate receptor is a prime target for ethanol action (49). Thus, the adaptive changes associated with the kindled state may decrease the sensitivity of the

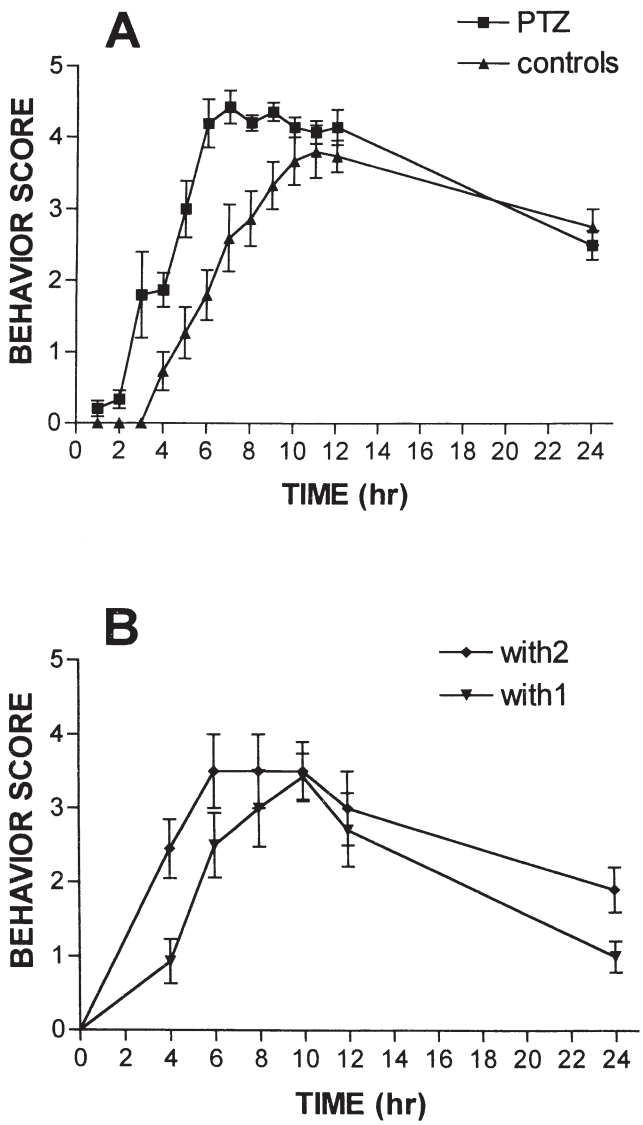

FIG. 6. Ethanol withdrawal profiles from ethanol-dependent PTZ-kindled rats (A) and twice-withdrawn ethanol-dependent rats (B). Animals were made ethanol dependent as described in the Method section, and allowed to withdraw so their withdrawal behaviors could be measured over time. Both PTZ-kindled (A) and twice-withdrawn rats (B) displayed an accelerated withdrawal profile over their respective control groups. Two-way ANOVA (group and time) showed a significant difference in this profile between PTZ-kindled rats and controls, $F(1, 367) = 69.1$, $p < F(1, 149) = 9.2$, $p < 0.01$; (B). Data are mean ± SEM of 15 animals per group. Control group in (A) consisted of both saline-injected and uninjected controls.

glutamatergic transmitter system to the effects of ethanol. Predictably, as a consequence, PTZ-kindled rats would demonstrate tolerance to the sedative and motor incoordinating effects of acute ethanol. Our data would support this conclusion.

Early work by Pinel and colleagues clearly showed a strong relationship between prior electrical kindling and the subsequent intensification of the ethanol withdrawal syndrome (40–42). Carrington et al. (9) observed that chronic ethanol treatment of rats reduced the number of amygdala stimulations required to kindle seizure activity, and McCown

TABLE 1

SIMILARITIES/DIFFERENCES IN RESPONSE TOWARDS DIFFERENT ASSAY SYSTEMS BETWEEN PTZ-KINDLED ANIMALS AND ETHANOL-DEPENDENT/ETHANOL WITHDRAWING ANIMALS

Receptor System	Assay or Compound	PTZ-Kindled	Ethanol Dependence and Ethanol Withdrawal	
GABA	PTZ	lowered seizure threshold (12)	lowered seizure threshold (26);	
	Cl^- flux	Reduced (10)	Reduced (1)	
	Diazepam	inhibits kindling (6);		inhibits withdrawal (2)
	^{35}S-TBPS	decreased binding (16);	decreased binding (21)	
	Picrotoxin	lowered seizure threshold (13)	no change (24)	
Glutamate	NMDA	Decreased sensitivity (24)		increased sensitivity (15)
	Glutamate Binding	increased binding (46),	increased binding (19)	
	MK801	inhibits kindling (13)		inhibits withdrawal (19)
Other	Polyamines	Increased polyamines (20)	increased polyamines (14)	
	ODC	increased ODC-ir (8)	increased ODC activity (14)	
	Hippocampal Abalation	decreased kindling (5)		decreased withdrawal (34)
	Strychnine	no effect on seizures (12)		no effect on seizures (18)

Number in parentheses is reference citation.

and Breese (35) showed prior withdrawals from chronic ethanol treatment resulted in a decreased number of stimuli required for generation of seizures in the inferior colliculus. Other studies have also shown that ethanol's anticonvulsant properties could impede kindling (37). Our data demonstrates an altered pattern of behaviors early in withdrawal after prior exposure to PTZ kindling, although the intensity of behavior activity at the peak of withdrawal was not enhanced. This may be a reflection of the subjective and nonlinear nature of the withdrawal behavior scoring system that is biased towards quantification of low-intensity behaviors. In addition, the vapor dependence paradigm results in maximal withdrawal behaviors. Thus, we have demonstrated subtle alterations in the pattern of onset of withdrawal after both treatments, but a possible enhancement of maximal withdrawal by either kindling or by multiple withdrawals remains equivocal.

In summary, naive PTZ-kindled animals show increased tolerance to acute ethanol, as assessed by LORR and tilt-plane paradigms. This increased tolerance was also apparent as reduced intoxication during chronic ethanol treatment. However, the development of physical dependence, manifested by withdrawal, was not impeded by PTZ kindling, but the withdrawal profiles in the PTZ-kindled rats and rats undergoing two withdrawals were similar. Although these results may suggest that experimental approaches using PTZ-kindled animals may be of significant value for the elucidation of the neurochemical and molecular aspects of ethanol dependence, withdrawal, and ethanol withdrawal kindling, more research into understanding basic mechanisms for both kindling and withdrawal are needed before a PTZ-kindling model can be successfully used in ethanol withdrawal studies.

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