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Glutamic Acid and Ethanol Dependence

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FREED, W. J. AND E. K. MICHAELIS. *Glutamic acid and ethanol dependence.* PHARMAC. BIOCHEM. BEHAV. 8(5) 509–514, 1978. – Glutamate diethyl ester, a specific glutamate antagonist, attenuated the seizures and decreases in behavioral activity that were observed in mice during withdrawal. Prior to withdrawal, ethanol-dependent animals were supersensitive to kainic acid, a potent glutamate agonist, but they were not supersensitive to the convulsant drug pentylenetetrazol. These findings suggest that supersensitivity to glutamate develops during ethanol dependence, and that this phenomenon contributes to the signs of ethanol withdrawal.

Ethanol dependence Ethanol withdrawal Alcohol addiction Glutamic acid Glutamic acid diethyl ester
Kainic acid Central denervation supersensitivity Mechanisms of drug dependence Pentylenetetrazol

GOLDSTEIN [16, 17, 18] made the observation that the convulsions that result from experimentally-induced ethanol withdrawal in animals did not seem to be the result of overactivity of either the acetylcholine, the serotonin, or of the catecholamine brain neuronal systems, and suggested [16] that "the withdrawal reaction reflects overactivity of an endogenous brain excitatory system of unknown nature (p. 9)". We propose that the brain glutamic acid system could be this unknown excitatory element, for the following reasons: (1) glutamic acid, a putative neurotransmitter, is excitatory for almost all central nervous system (CNS) neurons [7,26]; (2) γ-aminobutyric acid (GABA) is an effective antagonist of the excitation that is produced by glutamic acid when it is applied iontophoretically to central neurons [8]; (3) agents that elevate brain GABA levels can greatly alleviate ethanol-withdrawal convulsions [16, 17, 18, 22, 35], while picrotoxin, a GABA antagonist, aggravates such convulsions [16, 17, 18].

We chose the inhalation model of ethanol dependence developed by Goldstein [15, 17, 25] to explore the involvement of the brain glutamic acid system in ethanol dependence. The fact that it is necessary to administer pyrazole, an alcohol dehydrogenase inhibitor, when using this model has somewhat limited its generalizability, as pyrazole itself has a number of troublesome side effects (cf. [17]). For example, one study reported a change in brain norepinephrine levels as a result of pyrazole treatment [30], although a later investigation failed to confirm this finding [2]. Nonetheless, the profile of drugs that are effective in alleviating the withdrawal reaction using the inhalation model closely parallels the drugs that are effective in treating human ethanol withdrawal [13,17].

In the present study we report that glutamate diethyl ester, a glutamic acid antagonist, greatly attenuates the ethanol withdrawal reaction. In a second series of experiments we found that ethanol-dependent animals are supersensitive to kainic acid, a putative glutamate agonist, but not to the convulsive drug pentylenetetrazol. These findings support the hypothesis that an increase in glutamate-mediated CNS excitation is a cause of at least some of the signs of ethanol withdrawal, and suggest the possibility that the primary adaptation of the central nervous system to chronic ethanol administration involves supersensitivity to the physiological effects of glutamate.

EXPERIMENT 1

If excess activity of brain glutamic acid systems is indeed responsible for the ethanol-withdrawal reaction, then this reaction should be lessened by glutamate antagonists. Glutamate diethyl ester (GDEE) is a specific antagonist of neuronal excitation induced by excitatory amino acids but causes no direct neuronal suppression of its own [6, 21, 28, 31, 42] and penetrates the blood-brain-barrier [9]. We administered GDEE at various times following the withdrawal of animals from chronic ethanol explosure and observed two manifestations of the withdrawal reaction, seizures elicited by handling and decreases in behavioral activity. For purposes of comparison, the effects of imidazole acetic acid (IMA), a putative GABA agonist [14,43], on alcohol-withdrawal seizures and on the activity of normal animals was also examined.

METHOD

Animals

Adult male Swiss-Webster mice (CF1 strain, obtained from Midwest Research Animals, Shawnee Mission, KS) weighing 22 to 43 g were used in the experiments. These animals had no previous experimental history.

Drugs and Injections

All drugs were dissolved in distilled water and injected IP in a volume of 10 ml/kg. L–glutamic acid diethyl ester and imidazole acetic acid were obtained from Sigma Biochemicals and pyrazole was obtained from Eastman Chemical Company.

Apparatus

Mice were exposed to ethanol vapor in a 38 cm × 25 cm × 26 cm high Plexiglas chamber similar to that described by Goldstein [15,20], except that ethanol was introduced into the chamber by bubbling between one-sixth and one-seventh of the incoming air through absolute ethanol as described by Patel and Lal [37]. Behavioral activity was measured in a Lehigh Valley photocell activity cage as has been described in a previous study from this laboratory [11].

Ethanol Determinations

An enzymatic assay was used for the measurement of both blood and chamber ethanol concentrations [29]. Blood ethanol was determined by removing 0.2 ml of blood from selected mice by heart puncture, diluting with four volumes of 2% perchloric acid solution, centrifuging at 1000 g for 10 min, and measuring the concentration of ethanol in the supernatant. Chamber ethanol was measured by withdrawing 1.0 and 0.5 ml of air from the inhalation chamber with a Hamilton gas-tight syringe and injecting it into cuvettes which contained the assay mixture and which had been fitted with serum stoppers. Since the results from the two volumes did not at any time differ substantially, the datum used was the average of the two (0.5 and 1.0 ml) determinations.

The concentration of ethanol in the chamber atmosphere was maintained at ($\bar{X}$ ± SD) 8.6 ± 1.3 mg/l for the first 24 hr, 12.3 ± 1.0 mg/l from 24–48 hr, and 13.7 ± 1.1 mg/l from 48–72 hr.

Procedure

Groups of 10–20 mice were exposed to ethanol vapor in the Plexiglas chamber for 3 days, using the methods of Goldstein [15, 16, 20]. Each mouse was injected IP with 1.67 g/kg of ethanol just prior to his introduction into the test chamber, and with 68 mg/kg pyrazole at the same time and after 24 and 48 hr. Pyrazole does not interfere with the enzymatic assay for blood ethanol used here [15]. After 72 hr of exposure the mice were removed from the inhalation chamber.

Convulsions elicited by lifting the mice by their tails were rated on a 1–4 scale, at the start of the inhalation sessions, once per hr for the first 14–15 hr after removal from ethanol exposure, then every few hours until 27 hr had elapsed, and thereafter once per day for the next three days. The convulsion rating scale was modified from that used by Goldstein [15, 16, 20] as follows: A score of 1 was assigned for intermittent or mildly severe tonic flexion of the limbs, a score of 2 for continuous and severe tonic flexion of the limbs possibly accompanied by a very mild, brief tonic-clonic episode, a score of 3 for a single distinct tonic-clonic episode, and a score of 4 was assigned for multiple tonic-clonic episodes.

Behavioral activity was measured in four groups of mice, 10 mice per group, given either saline (2 groups) or 480 mg/kg GDEE (2 groups). Half of the animals were exposed to ethanol for three days and injected upon removal from the ethanol-inhalation chamber; the other animals (normal animals) were never exposed to ethanol or to pyrazole. Activity was measured for 1 min at 1 and 3 hr after the injections of GDEE or saline.

The activity of three additional groups of 10 animals was tested as controls for the effects of pyrazole injections. One of these groups was treated chronically with pyrazole and tested after injection of 480 mg/kg GDEE, a second group was treated chronically with pyrazole and tested after injections of saline, and a third group was treated chronically with saline and tested after injections of saline.

Four additional groups of normal mice were given injections of either saline (N = 9) or IMA in one of the three doses, 41 mg/kg (N = 8), 82 mg/kg (N = 7) and 164 mg/kg (N = 6) and their activity was measured after 1 and 3 hr. IMA (82 mg/kg) was also administered 5 hr after withdrawal from ethanol to test for its effects on withdrawal seixures.

Double-blind procedures were used throughout; the nature of the treatment was not known either to the person giving injections or to the person making behavioral observations.

RESULTS

The schedule of ethanol inhalation that was used resulted in blood ethanol concentrations of ($\bar{X}$ ± SD) 0.71 ± 0.10 mg/ml after 3 hr, 0.77 ± 0.08 mg/ml after 25 hr, 1.28 ± 0.16 mg/ml after 49 hr, 1.43 ± 0.07 mg/ml after 72 hr of exposure, and 0.57 ± 0.11 mg/ml 2 hr after removal from ethanol exposure.

The temporal pattern of withdrawal scores observed in this study (see Fig. 1) was similar to that observed by Goldstein [16]. It is noteworthy that Goldstein observed peak scores 8–10 hr after removal from exposure to ethanol, and we also observed distinct peaks after 9–10 hr for the control animals in most experiments. However, in a few experiments (such as that shown in Fig. 1) there was only a broad peak between 8 and 14 hr. Mean inter-observer agreement for seizure scores plus or minus one point was 95%.

GDEE caused a marked decrease in the seizure scores observed during ethanol withdrawal (Fig. 1). When injected 5 hr after the termination of ethanol inhalation, 480 mg/kg GDEE caused a 61% decrease in seizure scores ($p<0.002$, two-tailed Mann Whitney U test). This attenuation of seizure scores by this dose of GDEE became apparent within 2 hr after the injection of GDEE and persisted for approximately 11 hr (Fig. 1). In general, the maximal effect of the drug appeared to begin about 2 or 3 hr after the injection, and lasted for at least 6 hr. Thus the effects of GDEE on withdrawal seizures were evaluated in terms of the sum of the scores obtained in the determinations made 2, 3 and 4 hr after the injections.

The effect of GDEE was found to be dose-related and

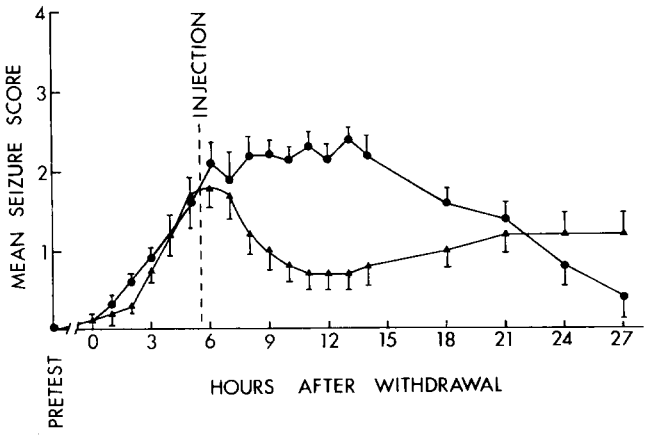

FIG. 1. The time course of ethanol-withdrawal seizures for 10 mice that were given IP injections of 480 mg/kg GDEE 5 hr after removal from ethanol exposure (triangles) as compared to 10 controls given saline (circles). Vertical bars indicate standard errors of the mean.

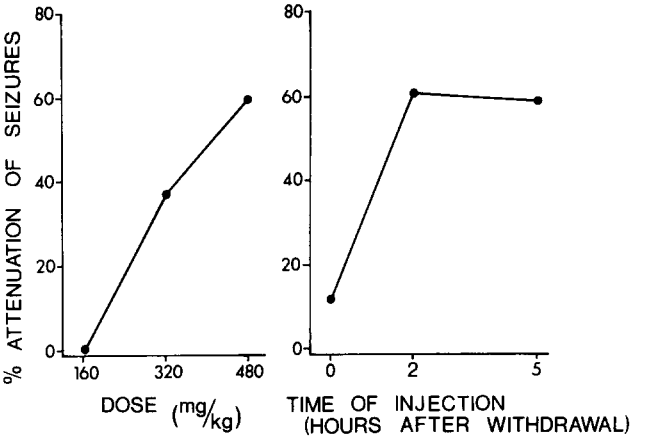

FIG. 2. The left-hand graph shows the percentage attenuation of ethanol-withdrawal seizures by different doses of GDEE administered 5 hr after removal from ethanol exposure. The right-hand graph shows the percentage attenuation of ethanol-withdrawal seizures by 480 mg/kg GDEE when administered simultaneously with removal from ethanol exposure, or 2 hr or 5 hr thereafter. Each point represents a single experiment for which 8–10 control animals and 8–10 GDEE-injected animals were used. The score from which these percent-attenuation figures were derived is the sum of the third through fifth seizure determinations after injections were given.

dependent upon the time of drug administration (Fig. 2). It is notable that 480 mg/kg GDEE was not very effective when administered at the time of removal from ethanol exposure, before the withdrawal reaction was underway. It is also interesting that in two experiments (480 mg/kg, 2 hr and 480 mg/kg, 5 hr) the mean seizure score for the GDEE group from 24–100 hr after removal from ethanol exposure was slightly (but not significantly, $p>0.2$) greater than that for the controls. Nonetheless, the withdrawal reaction in these GDEE-treated animals never reached a maximum severity approaching that observed in controls.

Chronic exposure to ethanol in combination with pyrazole was found to cause a 62% decrease in spontaneous behavioral activity scores as compared to normal controls ($p<0.002$, two-tailed) (Fig. 3), while exposure to pyrazole alone caused only an 8% decrease in activity ($p>0.2$). Such activity decreases could be correlated with the severity of withdrawal signs. For the ethanol-exposed animals whose data are depicted in Fig. 3, there was in fact a small (Kendall's *Tau* of −0.48) but statistically significant ($p = 0.0412$, two-tailed) negative correlation between behavioral activity and seizure scores after 3 hr of withdrawal.

GDEE (480 mg/kg) was found to cause a 32% decrease ($p<0.02$) in the spontaneous behavioral activity of normal mice 1 hr after injection (Fig. 3). Decreases in activity were also observed after the injection of 480 mg/kg of GDEE in mice pretreated with pyrazole alone; these effects (39% decrease after 1 hr, $p<0.02$; 7.5% decrease after 3 hr, $p>0.2$) were similar to those observed in normal animals treated with neither pyrazole nor ethanol. However, when GDEE was administered at the time of removal of the animals from ethanol exposure it did not significantly decrease activity after 1 hr (11% less, $p>0.2$). After 3 hr of withdrawal, the mice treated with GDEE showed an increase in behavioral activity (31%, $p = 0.064$).

IMA also decreased behavioral activity in a dose-dependent fashion. One hr after injection the smallest dose, 41 mg/kg decreased mean activity by 9.4% as compared to controls, 82 mg/kg decreased activity by 36.5% and 164 mg/kg decreased activity by 93.2%. This effect of IMA had disappeared by 3 hr after the injections. In terms of causing activity decreases, a dose of 82 mg/kg IMA was

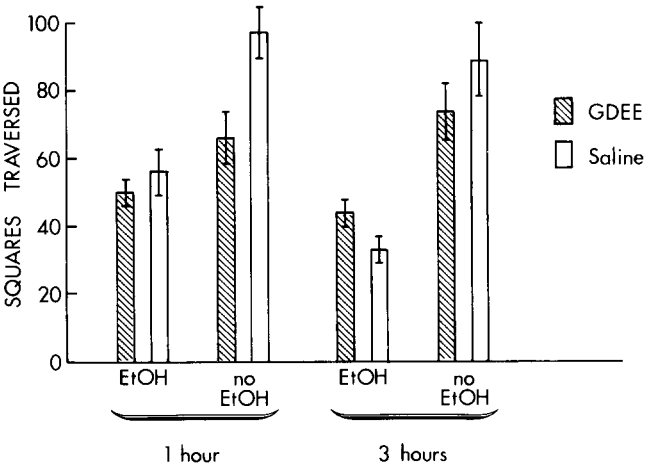

FIG. 3. Behavioral activity of mice that had been removed from ethanol exposure (EtOH) and simultaneously given IP injections of 480 mg/kg glutamic acid diethyl ester (GDEE) or saline, or of normal mice given the same injections (No EtOH), measured for 1 min, 1 and 3 hr after the injection. Each entry represents the mean and standard error of the mean for the scores of ten animals.

roughly equivalent to a dose of 480 mg/kg of GDEE (36.5% and 32% decreases in activity, respectively). However, when tested for effects on ethanol-withdrawal seizures, 82 mg/kg IMA caused a 31% decrease ($p<0.002$), which was only about half as great a decrease as that obtained for 480 mg/kg GDEE (61%).

DISCUSSION

GDEE, in doses that resulted in only moderate and short lived decreases in behavioral activity, caused large and long

lasting decreases in withdrawal seizure scores. Since similar decreases in behavioral activity were not observed in animals administered GDEE during ethanol withdrawal (a tendency for activity to increase was in fact observed 3 hr after injection), GDEE could not have been attenuating seizure scores merely by causing general sedation. This conclusion is also supported by the observation that doses of IMA (a GABA agonist) that were similar to GDEE in terms of causing activity decreases were much less effective than GDEE in diminishing withdrawal seizures.

GDEE, when administered simultaneously with removal from ethanol exposure, seemed to cause a partial attenuation of the withdrawal-associated decreases in activity. Since activity decreases correlated with seizure scores these decreases may be considered as an alternative measure of the severity of withdrawal. Thus, GDEE was somewhat effective in ameliorating withdrawal symptoms by this measure also. However, since the statistical significance of results relating to behavioral activity was marginal, conclusions here should be considered to be only tentative.

GDEE was not greatly effective in decreasing seizure scores when administered at the time of removal from ethanol exposure. It is suspected that, in this case, GDEE was partially metabolized before the withdrawal scores had reached significant levels: Desi and his colleagues [9] observed that the effects of GDEE on EEG began to diminish about 3 hr after IP injections. The short duration of action of GDEE is also attested to by our finding that most of the effects of GDEE on behavioral activity had disappeared by 3 hr after injection. Therefore it is interesting that the same dose of GDEE was effective in decreasing seizure scores for as long as 11 hr when administered 5 hr after removal from ethanol exposure. A plausible mechanism for such an anomalously long activity of GDEE is the presence of a critical period of CNS hyperexcitability in the ethanol withdrawal phase and that if an agent is capable of blocking this increased excitability at that critical period, then it allows for the recovery of neurobiological mechanisms that reduce excitability. Thus the duration of the GDEE effect, as well as its magnitude, was dependent on the time of drug administration. Whether this observation is unique to GDEE or would be shared by other drugs in the ethanol withdrawal situation has yet to be determined.

EXPERIMENT 2

The phenomenon of postsynaptic supersensitivity has been observed primarily as a sequel to interruptions in the innervation of peripheral excitable tissues [10]. It has been suggested that drugs for which chronic administration brings about physical dependence have the ability to functionally denervate at least some central neurons [3, 4, 24, 39] thus causing them to develop supersensitivity to certain chemical agents. There is some evidence that both opiate and ethanol dependence involve supersensitivity phenomena [5, 12, 33, 40, 44]. In fact, at the cellular level, changes in synaptosomal glutamate-binding activity have been observed in our laboratory as a result of chronic ethanol ingestion [34]. Based on these findings and the effectiveness of GDEE on ethanol withdrawal we decided to test the hypothesis that supersensitivity to the CNS excitatory effects of glutamate develops during states of ethanol dependence.

Withdrawal from chronic ethanol exposure leads to increased sensitivity to the convulsant action of agents such as pentylenetetrazol [23,38] whereas acute ethanol injections have an anticonvulsant effect [1,32]. Normally, one would not expect a general hypersensitivity to convulsants to appear while an organism is still receiving ethanol. However, if supersensitivity only to certain excitatory CNS transmitters does develop during the appearance of physical tolerance to ethanol, then it would be possible that even under the condition of continuous ethanol exposure these organisms will be hypersensitive to agonists of those particular transmitters. We tested the prediction that supersensitivity to glutamate develops during ethanol dependence by looking for a change in the sensitivity of the CNS to kainic acid, a putative glutamate receptor agonist [25, 36, 41] in ethanol-dependent animals which were still inhaling ethanol. This procedure was selected so that if the animals showed supersensitivity to kainic acid it could not be attributed to general hyperexcitability of the CNS due to withdrawal of ethanol, but rather due to an effect on receptors that we have hypothesized to be supersensitive even in the presence of ethanol. For comparison, we also tested the possibility of a generalized state of supersensitivity to excitatory agents by determining thresholds for the convulsive drug pentylenetetrazol [27] in ethanol-dependent (while still inhaling ethanol) as compared to control animals.

METHOD

Animals

Experimentally naive adult male Swiss-Webster mice, similar to those used in Experiment 1, were employed.

Drugs and Injections

Kainic acid was dissolved in distilled water and the pH was adjusted to 7.0 with NaOH. Pentylenetetrazol was dissolved in physiological saline solution. All injections were IP, in a volume of 10 ml/kg. Kainic acid and pentylenetetrazol were obtained from Sigma Biochemicals.

Apparatus

Mice were exposed to ethanol vapor in the same apparatus described in Experiment 1. Mice not exposed to ethanol were observed in a 30 cm dia. Plexiglas cylinder.

Procedure

Groups of animals (dependent animals) were exposed to ethanol vapor as in Experiment 1, except that ethanol exposure was continued until 99 hr and an additional pyrazole injection was given after 72 hr of exposure. Control animals were not exposed to ethanol vapor, but were simultaneously deprived of food and water to mimic the weight losses that occurred in the ethanol-dependent animals. Drugs were injected beginning after 96 hr of ethanol exposure, while the dependent mice were briefly removed from the inhalation chamber, and all observations were made while the dependent animals (but not the controls) continued to inhale ethanol vapor. Any noticeably lethargic or sick animals were not used.

At 96 hr, we injected 40 mg/kg of neutralized kainic acid, a dose that we found to be subconvulsive for the majority of animals in preliminary testing. In the first of two identical experiments ten dependent animals and fifteen controls were used, and in the second experiment

seven dependent animals and ten controls were used. The animals were observed continuously for 3 hr, and the occurrence of seizures and any other unusual behavior was noted.

In a third experiment, thresholds for the induction of seizures by the convulsive drug pentylenetetrazole were determined in seven ethanol-dependent (while still inhaling ethanol) and eight control animals. The method used [27] involves determination of the number of 15 mg/kg IP injections, equally spaced at 15 min intervals, until the onset of generalized convulsions. These injections were initiated after 96 hr of exposure to ethanol vapor and were continued until each of the animals had generalized convulsions.

Finally, a control experiment was performed to determine whether chronic pyrazole treatment alone would have any effect on the convulsive response to kainic acid. This experiment was performed at a later date and, for an unknown reason, the animals were more sensitive to kainic acid than previously as 5 of 7 normal animals had convulsions after treatment with 40 mg/kg. Thus a smaller dose, 30 mg/kg, was used. Eleven controls and 11 pyrazole-treated animals were tested according to exactly the same regime as that used for the first two experiments except that no ethanol was administered.

RESULTS

In the first kainic acid experiment, 8 of 10 dependent animals but only 3 of 25 controls had seizures during the period from 80–100 min after the injections, when the number of animals having seizures had reached asymptotic levels (p = 0.00483, two-tailed Fisher exact test). In the second kainic acid experiment, 5 of 7 dependent animals and 2 of 10 controls had seizures during this period (p = 0.0522). For the grouped data from the two experiments the differences were highly significant (p = 0.00076, two-tailed). The time course of seizure activity after the injections for the grouped data from these two experiments are shown in the left half of Fig. 4. In contrast, 2 of 11 animals treated chronically with pyrazole only and 2 of 11 saline-injected controls had convulsions after injections of 30 mg/kg of kainic acid.

The pentylenetetrazol threshold determinations revealed no differences between the groups; the time course of the onset of seizures is also shown in Fig. 4. The dependent animals began to have seizures after a mean of 5.8 injections, and the control animals after a mean of 6.0 injections.

DISCUSSION

In two experiments, a striking difference in the sensitivity to kainic acid was demonstrated between ethanol dependent and control animals; Ethanol dependent animals are supersensitive to kainic acid, a putative glutamate agonist. That observation alone does not necessarily indicate a significant role for glutamic acid in the ethanol-dependence process, since ethanol dependence might be accompanied by a non-selective supersensitivity to many or all central excitatory agents. However, no differences in the pentylenetetrazol seizure thresholds between ethanol dependent and control animals were noted. Therefore, ethanol dependent animals are supersensitive to kainic acid

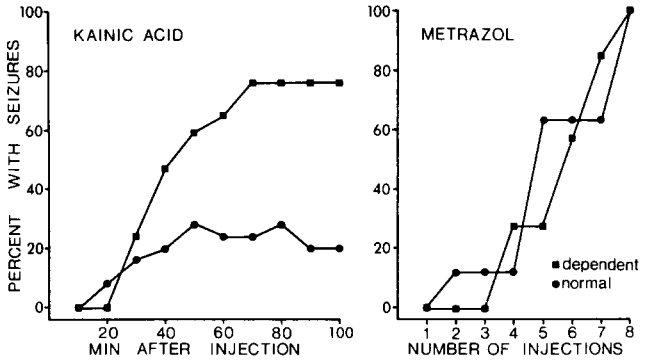

FIG. 4. The percent of animals having seizures as a function of time elapsed since a single 40 mg/kg injection of kainic acid (left-hand graph) or as a function of the number of 15 mg/kg injections of pentylenetetrazol given at equal 15-min intervals (right-hand graph). The squares indicate the data for 17 (left-hand graph) or 7 (right-hand graph) animals that had been inhaling ethanol vapor for 4 days, while the circles indicate the data for 25 (left-hand graph) or 8 (right-hand graph) animals that were never exposed to ethanol.

while they continue to be exposed to ethanol, even though they are not supersensitive to pentylenetetrazol under the same conditions.

GENERAL DISCUSSION

The results obtained in this study seem to be consistent with a role for glutamic acid in the ethanol dependence process, although there are other possible explanations for the present findings. One is that GDEE might impede the metabolism of ethanol. However, if this were true, one would expect GDEE to be less effective once the withdrawal reaction was well underway. This is contrary to the experimental findings described in this paper. Another possibility is that the results could be due to some as yet undiscovered nonspecific effect of the drugs employed. A final possibility to be considered is that the effects obtained could be peculiar to the use of pyrazole, since pyrazole injections accompanied ethanol administration in all of the experiments.

The observation of supersensitivity to kainic acid, however, may be a reflection of an increase in the sensitivity of the central nervous system to the action of glutamate. We suggest that this change occurs as a compensatory adaptation to continuous ethanol exposure. It thus follows that when ethanol exposure is terminated, withdrawal seizures result as a consequence of the return to normal activity at an excess number of glutamate receptor sites. This is in agreement with our findings that excess CNS excitation in ethanol dependent animals was more easily produced by a putative glutamate agonist and that a glutamate antagonist was effective in attenuating an experimental ethanol-withdrawn reaction.

ACKNOWLEDGEMENTS

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