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Role of Environmental Cues as Pavlovian-Conditioned Stimuli in Enhancement of Tolerance to Ethanol Effects: 1. Lethal Effects in Mice and Rats

V. L. TSIBULSKY AND Z. AMIT[1]

Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, 1455 de Maisonneuve Boulevard, West, H-1013, Montreal, Quebec H3G 1M8, Canada

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TSIBULSKY, V. L. AND Z. AMIT. *Role of environmental cues as Pavlovian-conditioned stimuli in enhancement of tolerance to ethanol effects: 1. Lethal effects in mice and rats.* PHARMACOL BIOCHEM BEHAV 45(2) 473–479, 1993.— Twice daily for 4 days, Swiss Webster or BALB/c mice were injected with 3.5 g/kg ethanol (20% w/v, IP) immediately after moving their home cages from the colony room to the experimental room. On day 5, half the mice were moved to the same room and the other half to a novel room with different lighting, acoustic, and olfactory stimuli. All mice were injected with ethanol overdoses ranging from 4.5–10.0 g/kg. LD_{50} for ethanol increased following ethanol preexposure as compared to control ethanol-naive mice tested in the same experimental room. However, LD_{50} was lower in both Swiss Webster and BALB/c mice tested in a novel environment than in the familiar environment. Novelty increased sensitivity to the effect of low and moderate but not the highest lethal doses of ethanol. This effect of novelty occurred only in ethanol-experienced, but not ethanol-naive mice. In the following experiments, using a balanced design, Swiss Webster mice and Wistar rats were exposed to ethanol and saline alternatively in two distinct experimental rooms. On the final day, we found that there was no difference between animals tested in the room previously associated with administration of ethanol and animals tested in a saline-associated room in terms of LD_{50} for ethanol. These results suggest that: a) Environmental stimuli do not play a role as Pavlovian conditioning stimuli in the development of tolerance to ethanol-induced lethality; and b) novelty acts as an unconditioned stimulus that increases ethanol's lethal effects by unspecific disruption of conditioned compensatory responses to internal conditioned stimuli, such as irritation of peritoneal cavity, smell, and taste of ethanol.

Tolerance Pavlovian conditioning Novelty Lethal effects Ethanol Mouse strains Rats

CHRONIC intermittent exposure to moderate or high doses of ethanol (EtOH) results in an enhancement of tolerance to the hypothermic, hypnotic (6), and lethal effects of ethanol (3,5,12) in rats and mice. Tolerance enhancement can be induced by drug exposure per se and can be a context-specific consequence of the development of a compensatory classic conditioned response (7). The role of classic conditioning in the development of tolerance to the hypothermic (1) and hypnotic (4,6) effects of ethanol has been previously shown.

The conditioned component of tolerance develops readily for only those effects of the drug that repeatedly manifest themselves during the periods of drug exposure. On the other hand, this component should be seen only in the presence of contextual cues that reliably predict the appearance of the drug effect following repeated drug administrations within the same environment. It is clear that in the case of the lethal effects animals could not have any experience with death, but a variety of effects induced by sublethal doses and tolerance to some of these effects may protect animals against lethality. This hypothesis of "conditioned protection" was supported by experiments with heroin (9), ethanol (5), and pentobarbital (13). Recently, more detailed data was published by Melchior indicating that the LD_{50} for ethanol was significantly higher in an environment previously associated with administration of the drug than in a novel environment (3). However, the conditioning of the lethal effects of heroin and pentobarbital was demonstrated in experiments with a balanced design, while the experimental design employed by Melchior was unbalanced. Thus, animals had unequal exposure to environments that differed in stress and that in turn could affect tolerance. The purpose of the present study was to evaluate a role of Pavlovian conditioning in the development of toler-

[1] To whom requests for reprints should be addressed.

ance to the effects of lethal doses of ethanol in mice and rats using a balanced experimental design.

GENERAL METHOD

BALB/c male mice (18–23 g) and Swiss Webster male mice (19–24 g) (Charles River Canada, Inc.) were housed in groups of five in standard macrolon cages. Wistar male rats (225–275 g) were kept in individual metal grid cages. For 6 days before experiments, all animals were maintained on a 12 L : 12 D cycle (light 0800–2000 h) in the colony room, regulated for constant temperature (22 + 1°C) and humidity. Food and water were available ad lib. Six days were allowed for acclimatization prior to the onset of the experiments.

Ethanol solution was prepared 1–2 days before the beginning of injections by mixing 96% distilled (in the laboratory) ethanol and saline to make a 20% w/v concentration for mice experiments and 10–25% w/v for the experiments with rats. All injections were given IP and the lethal doses were given between 0900–1200 h. Death was defined as absence of breathing and heartbeats for 30 s.

STATISTICAL ANALYSIS

Lethal effects of ethanol were expressed as a percent of dead animals at a given time (mortality). Dose–response data were plotted on logarithmic probability paper and analyzed by the method of Litchfield and Wilcoxon (2) for mortality at 2 h, 24 h, and 10 days after ethanol injections. These time points, as we have shown recently (12), are the most appropriate periods for the assessment of the lethal effects of ethanol in Swiss Webster mice. Significance was set at the $p < 0.05$ level.

EXPERIMENT 1: UNBALANCED DESIGN AND BALB/C MICE

The purpose of the first experiment was to replicate Melchior's experiment with unbalanced design (3).

METHOD

Twice daily at 0900 and 1500 h, cages with BALB/c mice were moved from the colony room (Rm-c) to one of two experimental rooms (small room, one floor downstairs from the Rm-c, with ongoing noise from a radio, Rm-1). Mice were divided into four groups. Experimental mice were moved to Rm-1, where they were given 3.5 g/kg ethanol and left in their home cages. Following 80 min, animals were returned to the colony room. In the morning of day 5, half the mice were moved to Rm-1 and given one of the overdoses (4.5–7.5 g/kg) of ethanol (EtOH/EtOH/same group, $n = 41$) while the other half were moved to a different, unfamiliar room (large, white, brightly lit room with different acoustic and olfactory stimuli, Rm-0; as in Melchior's study, we used a biochemical laboratory) and received the same overdoses of ethanol (EtOH/EtOH/novel group, $n = 49$). Mice of the control groups were moved for 4 days to Rm-1 but instead of ethanol received saline injections (17.5 ml/kg). On day 5, they were also given one of the ethanol overdoses ranging from 4.5–7.0 g/kg in Rm-1 (SAL/EtOH/same group, $n = 32$) or Rm-0 (SAL/EtOH/novel group, $n = 36$). As 93% of deaths occurred within 3.5 h after ethanol injections and only one cluster of survival times (12) was formed, dose–response curves were statistically analyzed at the 3.5-h time point.

RESULTS AND DISCUSSION

Ethanol overdoses resulted in deaths in a dose-dependent manner ($p < 0.01$, Fig. 1). The graphic method of LD_{50} estimation revealed a value of 6.7 g/kg (with $p = 0.05$, confidence interval 6.5–6.9 g/kg) in the SAL/EtOH/same group of mice. Repeated injections of ethanol resulted in an enhancement of LD_{50} to 7.15 (6.9–7.3) g/kg in the EtOH/EtOH/same group of mice ($p < 0.05$). Moving animals to a novel environment resulted in mortality enhancement after injection of the lower doses of ethanol but not after injection of the highest dose (Fig. 1). This effect of novelty in ethanol-experienced mice resulted in a considerable decrease in the LD_{50} (6.9 g/kg, $p = 0.05$) and in a deviation of dose–response curves of EtOH/EtOH/same and EtOH/EtOH/novel groups from parallelism ($p = 0.05$). This type of deviation was observed also in the SAL/EtOH/novel group ($LD_{50} = 6.5$ g/kg) but the difference was insignificant ($p < 0.1$).

The results of this experiment confirmed both main findings of Melchior's study (3). Tolerance to the lethal effects of ethanol developed following eight injections of 3.5 g/kg ethanol for 4 days and an increase in lethality was observed in a new environment in ethanol-experienced BALB/c mice. Besides, we have shown that the loss of tolerance was observed in a novel environment only at low lethal doses of ethanol but the tolerance to the highest challenge doses of ethanol on day 5 was not affected by novelty.

EXPERIMENT 2: UNBALANCED DESIGN AND SWISS WEBSTER MICE

The purposes for the next experiment were the following. First, it seemed interesting to repeat the previous experiment but using another strain, that is, Swiss Webster mice, which are less sensitive to the lethal effects of ethanol (12). Second, the enhancement in ethanol-induced lethality in Rm-0 (Experiment 1) might be a result of novelty or a result of the presence of some specific stimuli in Rm-0 that could promote ethanol-induced lethality. Moreover, the above-mentioned trend to deviation from parallelism had been observed in the SAL/EtOH/novel group of BALB/c mice compared to the SAL/EtOH/same group.

METHOD

Swiss Webster mice were divided into four groups and received the same treatment as BALB/c mice in Experiment 1, with one exception. Half of each group was moved into the same small room with ongoing noise (Rm-1) and received eight injections of ethanol or saline; meanwhile, the other half were moved for injections into another small room (Rm-2), somewhat more brightly illuminated, with six black wooden boxes normally used as "open-field" apparatuses. Two home cages with mice were placed in each open black box. On day 5, all animals were given overdoses of ethanol ranging from 7.5–10.0 g/kg in the same room or in a novel room. Rm-1 was a new room for animals that received repeated injections in Rm-2 and vice versa.

RESULTS AND DISCUSSION

The lethal effects of ethanol on day 5 were observed to be dose dependent ($p < 0.01$, Fig. 2). There was no difference between groups tested in Rm-1 or Rm-2 as a novel environment; therefore, the data were collapsed and analyzed together for groups SAL/EtOH/same ($n = 50$), SAL/EtOH/novel ($n = 50$), EtOH/EtOH/same ($n = 51$), and EtOH/EtOH/novel ($n = 61$).

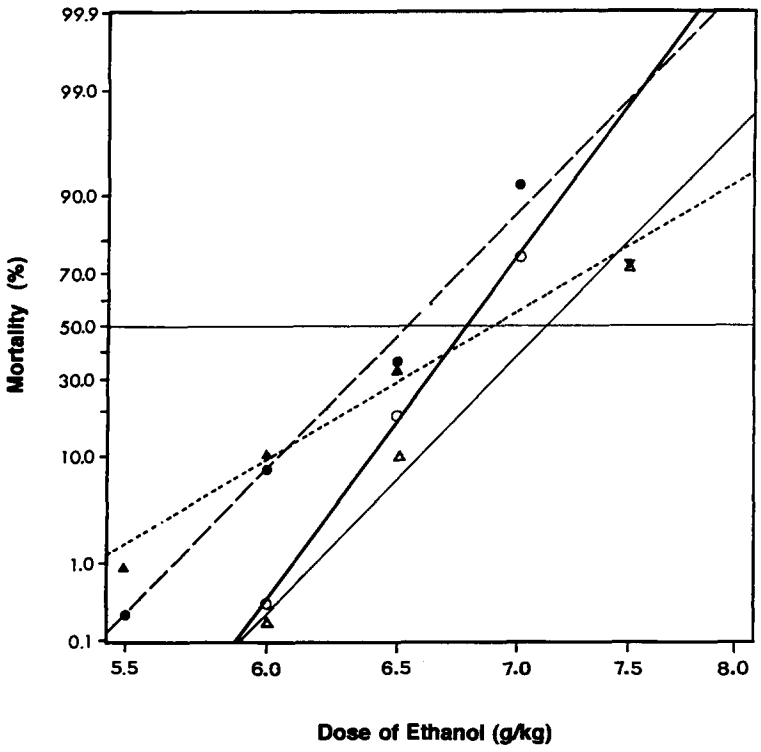

FIG. 1. Dose-response curves at 24 h following injections of ethanol in BALB/c mice. (—○—), SAL/EtOH/same; (--●--), SAL/EtOH/novel; (—△—), EtOH/EtOH/same; (--▲--), EtOH/EtOH/novel. The Litchfield and Wilcoxon method revealed significant differences ($p < 0.05$) between LD_{50} for SAL/EtOH/same groups and EtOH/EtOH/same groups and between EtOH/EtOH/same and EtOH/EtOH/novel groups.

LD_{50} for the SAL/EtOH/same group was 8.6 g/kg at 2 h, 8.0 g/kg at 24 h (with 95% confidence interval 7.8–8.2 g/kg), and 7.4 g/kg at 10 days. Mice in the EtOH/EtOH/same group demonstrated increased tolerance to the lethal effects of ethanol (LD_{50}s were 9.4, 8.9, and 8.7 g/kg, $p < 0.05$ when compared with the SAL/EtOH/same group at corresponding times). On the final day, day 5, moving ethanol-naive mice of the SAL/EtOH/novel group into a novel environment had no influence on mortality and survival time. However, ethanol-experienced mice were more vulnerable to the lethal effects of low and moderate doses of ethanol in a novel environment compared to the familiar environment. LD_{50} decreased to 9.0, 8.5, and 8.1 g/kg ($p < 0.05$). While dose-response curves for saline- as well as for ethanol-preexposed animals tested in the familiar and novel environments deviated from parallelism, this effect did not reach significance. As the effects of novelty did not depend upon time passed after injection of a lethal dose, Fig. 2 represents dose-response curves for all groups at 24 h.

In general, these results are in agreement with the results of Experiment 1. In Swiss Webster mice, eight injections of 3.5 g/kg ethanol produced nearly an equal decrease in sensitivity to the lethal effects (9.3% at 2 h after injection) as seen in BALB/c mice (7.5%) using the same regimen of exposure to ethanol. The effect of moving into a novel environment produced precisely the same tolerance increase in LD_{50} of 4.3%. In contrast to the findings of Experiment 1, there was no divergence between dose-response curves for SAL/EtOH/same and SAL/EtOH/novel groups of Swiss Webster mice. It would appear that novelty per se did not enhance the lethal effects of ethanol in ethanol-naive animals.

EXPERIMENT 3: BALANCED DESIGN AND SWISS WEBSTER MICE

This experiment was designed to examine whether an association between ethanol-induced lethality and environmental stimuli during repeated injections is important for the development of tolerance to ethanol's lethal effects in Swiss Webster mice, in other words, whether environmental stimuli play a role as conditioned stimuli. Mice were matched for experience with the two environments to exclude influences of nonassociative factors.

METHOD

At 0900 h for 8 consecutive days, the cages with experimental mice were moved to Rm-1 (with ongoing noise). Mice were injected with 3.5 g/kg ethanol. After 50 min, the cages were returned to the colony room. At 1500 h, the same mice were moved to Rm-2 (black boxes) and given saline injections. After 50 min, they were also returned to the colony room. On day 9 at 0800 h, half the mice were moved to Rm-1 and other half to Rm-2, where all mice received overdoses of ethanol (8.5–10.5 g/kg). The same number of mice were treated by a counterbalanced schedule, that is, for 8 days in the morning ethanol injections were paired with moving to Rm-2 while in the evening mice received saline injections in Rm-1. The type

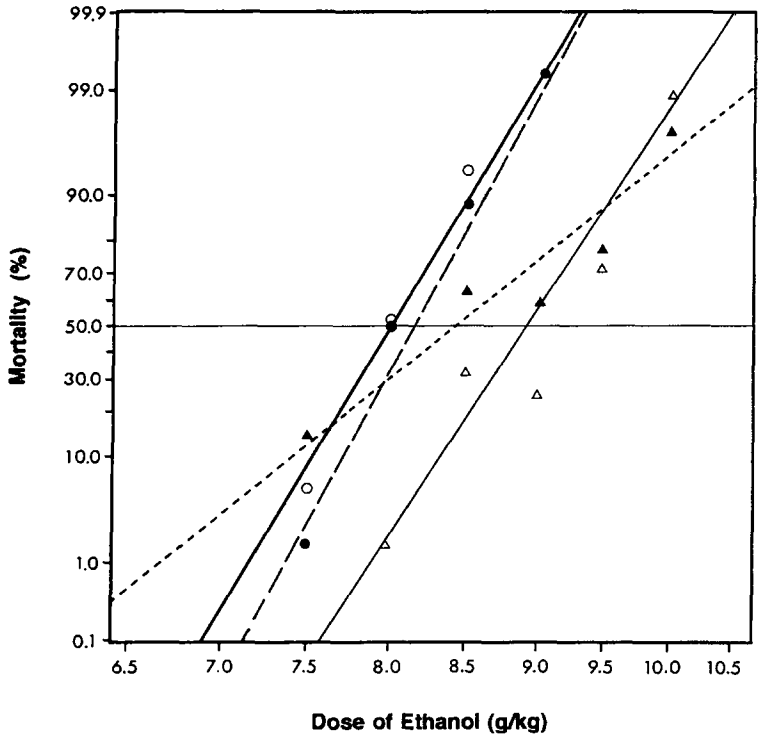

FIG. 2. Dose-response curves at 24 h following injections of ethanol in Swiss Webster mice. (—○—), SAL/EtOH/same; (--●--), SAL/EtOH/novel; (—△—), EtOH/EtOH/same; (--▲--), EtOH/EtOH/novel. The Litchfield and Wilcoxon method revealed significant differences ($p < 0.05$) between LD_{50} for SAL/EtOH/same groups and EtOH/EtOH/same groups and between EtOH/EtOH/same and EtOH/EtOH/novel groups.

of environment was found not to have any influence on ethanol's lethal effects; therefore, the data were collapsed and analyzed together for EtOH-SAL/EtOH/cued ($n = 46$) and EtOH-SAL/EtOH/uncued groups ($n = 45$). Control groups of mice received saline injections in both rooms for 8 days. On day 9 at 0800 h, they received an overdose of ethanol in the room to which they used to be moved in the morning (SAL-SAL/EtOH/cued group, $n = 45$) or in the evening (SAL-SAL/EtOH/uncued group, $n = 35$) for the 8 previous days.

RESULTS AND DISCUSSION

The two ethanol-naive groups showed similar sensitivity to the lethal effects of ethanol (Fig. 3). The two ethanol-experienced groups did not differ significantly from each other but were more tolerant ($p < 0.05$) than ethanol-naive mice to the lethal effects of ethanol in terms of mortality. LD_{50}s for the combined control groups were 9.0, 8.0, and 7.9 g/kg and for the combined ethanol-experienced groups were 9.6, 9.1, and 8.9 g/kg at 2 h, 24 h, and 10 days, respectively.

In this experiment, all mice had equal exposure to both environments. The dissociation between injection time and environment in the SAL-SAL/EtOH/uncued group was the only difference between cued and uncued ethanol-naive mice. Moving control mice into the familiar environment with the exception of the unexpected injection time as a cue did not affect ethanol-induced lethality. The different schedule of ethanol preexposure used in this experiment produced nearly the same tolerance enhancement as in Experiments 1 and 2. However, injections of lethal doses of ethanol in the environment that was familiar but not previously associated with ethanol injections did not significantly affect ethanol-induced lethality. The failure to demonstrate an influence of nonassociated with ethanol injections environment on the lethal effects of ethanol may not be explained by insufficiency of the differences between the two environments because Experiment 2 had shown that the differences were large enough to increase mortality in the EtOH/EtOH/novel group. It is suggested on the basis of the present findings that environmental cues may not play a significant role in the development of tolerance to ethanol's lethal effects in Swiss Webster mice.

EXPERIMENT 4: BALANCED DESIGN AND WISTAR RATS

The purpose of this experiment was to expand the previous finding with mice and determine a possible role for Pavlovian conditioning in the development of tolerance to the lethal effects of ethanol in Wistar rats.

METHOD

Fifty-two male Wistar rats were divided into three groups. The first group received daily alternating IP injections of ethanol and saline over a period of 20 days. One to 2 min following ethanol injections, rats were moved from the colony room to Rm-1. Following saline injections, they were moved to Rm-2.

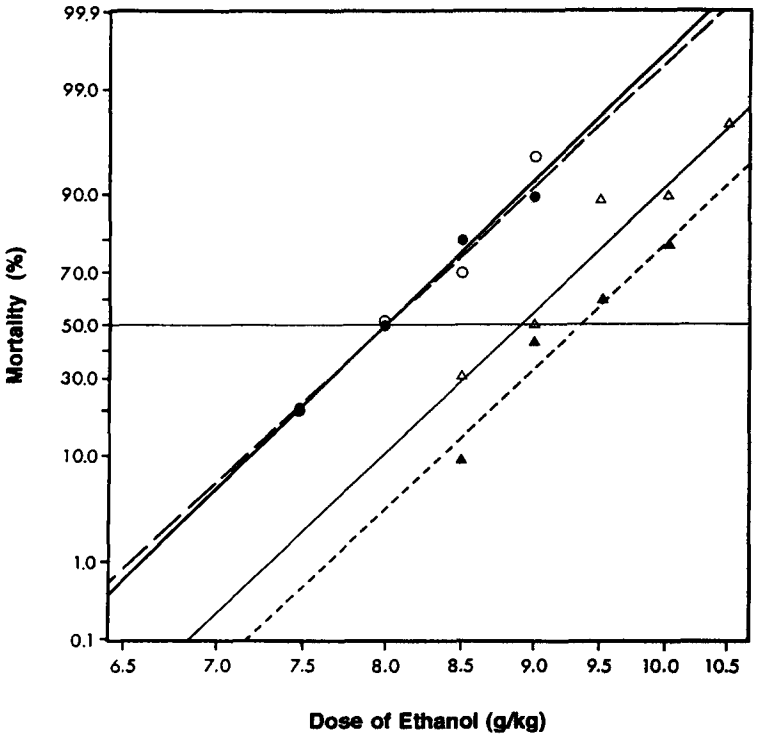

FIG. 3. Dose-response curves at 24 h following injections of ethanol in Swiss Webster mice. (—○—), SAL/EtOH/cued; (--●--), SAL/EtOH/uncued; (—△—), EtOH/EtOH/cued; (--▲--), EtOH/EtOH/uncued. The Litchfield and Wilcoxon method revealed significant differences ($p < 0.05$) between LD_{50} for both SAL/EtOH/groups and EtOH/EtOH/groups.

In Rm-1, animals were individually placed in small Plexiglas cylinders (diam. 30 cm) with pine shavings on the floor. In Rm-2, they were individually placed into black wooden boxes. After 3 h, rats were returned to their home cages. On day 21, rats were given overdoses of ethanol (25% w/v) ranging from 7.0–9.0 g/kg and moved to Rm-1 (EtOH-SAL/EtOH/cued group, $n = 10$) or Rm-2 (EtOH-SAL/EtOH/uncued group, $n = 12$). Control animals were handled but given no injections for 20 days. On day 21, they received ethanol overdoses (6.0–9.0 g/kg) and were moved to one of the two rooms. Because the type of environment was found not to have any influence on lethality, data for control animals were collapsed (HAND/EtOH/novel group, $n = 30$) across environments.

The dose and concentration of ethanol solution were increased from 0.4 g/kg (10% w/v) on day 1 to 4.0 g/kg (25% w/v) on day 20 by 0.4-g/kg steps. On day 21, an overdose of ethanol was given in two injections of equal volume within a 25- to 30-min interval. This was done to avoid rapid administration of a large volume of solution (11 ml/300 gbw for 9.0 g/kg ethanol). The concentration of solution was increased to 25% w/v for the same reason.

RESULTS AND DISCUSSION

On day 21, ethanol injections produced a dose-dependent ($p < 0.01$) lethality in rats of all groups (Fig. 4). The graphic method of analysis revealed the LD_{50} to be 6.1 g/kg (95% confident interval was 5.7–6.5 g/kg) for the HAND/EtOH/novel group at the end of the observation period (14 days). Repeated injections of ethanol increased ($p < 0.05$) tolerance to ethanol's lethal effects in EtOH-SAL/EtOH/cued and EtOH-SAL/EtOH/uncued groups (LD_{50} was 7.9 g/kg in both groups).

These findings are consistent with the results of Experiment 3. First, intermittent subchronic injections of ethanol resulted in the development of tolerance to its lethal effects in Wistar rats. Second, environmental stimuli did not appear to play any role in the development of this tolerance in rats as well as in mice.

GENERAL DISCUSSION

The ethanol exposure schedules used in the present investigation resulted in an increase in tolerance to the lethal effects of ethanol in terms of percentage of dead animals. Ethanol-tolerant, but not ethanol-naive, animals displayed higher sensitivity to the lethal effects of ethanol when they were given overdoses of ethanol within a novel environment. These results are consistent with the previous literature (3). The data obtained in Experiments 2 and 3 with a balanced design, when animals were equally exposed to different environments, showed that environmental stimuli did not play a role as signals but rather appeared to serve as "stressful" stimuli. It remains unclear, however, why ethanol-experienced BALB/c and Swiss Webster mice were found to be not tolerant in a novel environment as much as in the familiar environment, particularly with regard to the lethal effects of low and moderate doses of ethanol. One possible explanation is that it might be due to the appearance of novel and potentially stressful stimuli (from a novel environment) that may interfere with

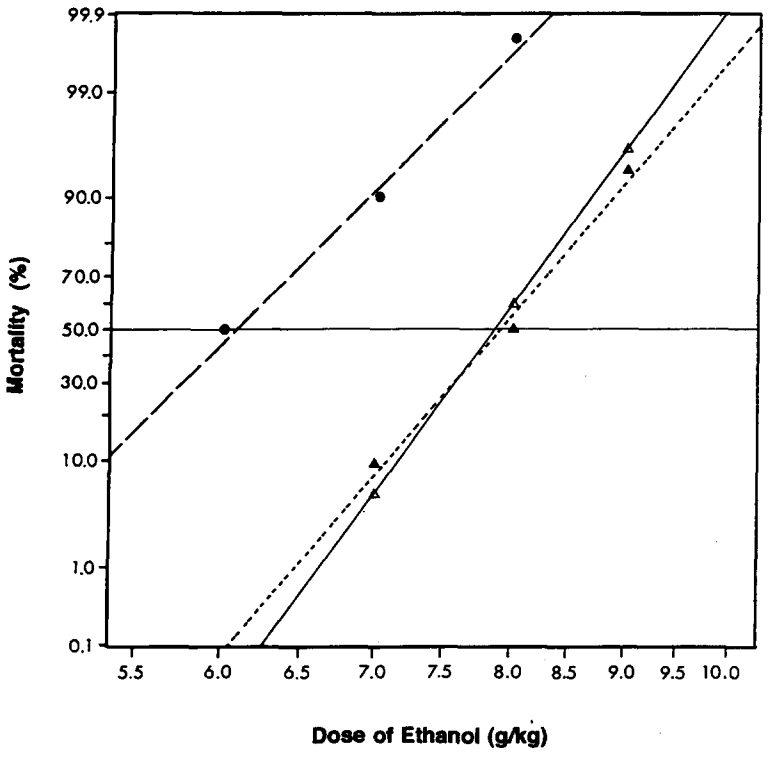

FIG. 4. Dose-response curves at 14 days following injections of ethanol in Wistar rats. (--●--), HAND/EtOH/novel; (△), EtOH/EtOH/cued; (--▲--), EtOH/EtOH/uncued. The Litchfield and Wilcoxon method revealed significant differences ($p < 0.05$) between LD_{50} for HAND/EtOH/novel and both EtOH/EtOH/groups.

internal conditioned stimuli (if they developed during exposure to ethanol), but it cannot be explained by the absence of external conditioned stimuli. In this way, stressful stimuli may interfere with the development of compensatory responses that protect animals against the lethal effects of ethanol. Such external inhibition has been shown to eliminate tolerance to the hypothermic effect of ethanol (10).

Two other studies, employing balanced experimental designs, have shown that tolerance to the lethal effects of heroin and pentobarbital is, in part, under the control of environmental conditioned stimuli that is context sensitive (9,13). Tolerance to the lethal effects of ethanol appears, on the basis of the present findings, not to be under the control of environmental conditioned stimuli. It would seem reasonable to assume that the lack of Pavlovian conditioning of environmental stimuli would result in low (<15%) development of tolerance to ethanol-induced lethal effects (12) compared to the lethal effects of heroin, pentobarbital, and other drugs.

Sklar and Amit (11) were the first to show resistance to tolerance of the lethal effects of morphine using an active extinction procedure provided by saline injections in the environment earlier paired with morphine administration in rats. They concluded that learning does not mediate the increase in tolerance to morphine's lethal effects. Using the same experimental design, Siegel and colleagues (8) confirmed that, in terms of mortality, tolerance to the lethal effects of morphine was not diminished by saline injections in the same environment. In terms of survival time, they found that increased tolerance was extinguished. It was concluded that drug-associated environmental cues contribute to the development of morphine tolerance. However, both conclusions seem to be somewhat unconvincing. First, extinction is not an essential attribute of a classically conditioned response, at least theoretically. One could assume that, once developed, tolerance to the lethal effects may be extremely important to the animal and its sensitivity to extinction procedures may not be as great as to other conditioned reflexes. Second, in their next study Siegel and colleagues (9) employed another paradigm but the same measure of the lethal effects (survival time), yet failed to demonstrate significant differences between tolerance to the lethal effects of heroin in cued and uncued environments.

In summary, the present series of experiments has shown that administration of ethanol in novel environments significantly increased sensitivity to the effect of low (near threshold) and, to a lesser degree, the effect of moderate (near LD_{50}) but not the highest lethal doses of ethanol in Swiss Webster and BALB/c mice. Environmental stimuli did not play a role as conditioned stimuli in the protection against ethanol-induced lethality in ethanol-experienced Swiss Webster mice and Wistar rats. One of the possible explanations is that novelty as a powerful unconditioned stimulus increased ethanol's lethal effects by unspecific disruption of conditioned compensatory responses to internal conditioned stimuli such as irritation of peritoneal cavity, smell, and taste of ethanol. If so, this may explain why lethality following injection of the highest doses of ethanol was resistant to the effect of novelty. An alternative explanation may be that tolerance to ethanol-induced lethality does not have any Pavlovian components.

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