*Pharmacology Biochemistry & Behavior*, Vol. 25, pp. 1111–1115, 1986. © Ankho International Inc. Printed in the U.S.A. 0091-3057/86 $3.00 + .00

BRIEF COMMUNICATION

# Effects of Pavlovian Conditioning on the Ethanol Withdrawal Syndrome in Rats

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Received 18 April 1986

NUMAN, R. *Effects of Pavlovian conditioning on the ethanol withdrawal syndrome in rats.* PHARMACOL BIOCHEM BEHAV **25**(5) 1111–1115, 1986.—Male, hooded rats were made physically dependent upon ethanol using intravenous infusions. Following this induction procedure, physical dependence was maintained, but now a tone (CS) was associated with ethanol infusions (US) that reduced withdrawal distress. A pretest-posttest, counterbalanced, repeated measures design was used to assess the effects of three treatments (ethanol, tone, none) on withdrawal reactions (withdrawal signs, body temperature, open-field activity) measured under blind conditions. Only the ethanol treatment reduced withdrawal distress, suggesting that classical conditioning did not occur. The results are discussed in terms of recent conditioning theories of drug responses, and the potential role of stress in these reactions.

Ethanol dependence Intravenous infusions Pavlovian conditioning Rats

SEVERAL recent studies have focused on the contribution of Pavlovian conditioning to drug tolerance [7–9, 16, 19] and drug abuse [4, 6, 10, 12, 14, 21], and many excellent reviews are available [2, 3, 5, 17, 21]. Some of these studies have suggested that conditioned drug responses are compensatory [7, 8, 10, 16, 17], while others conclude that the conditioned response reflects the direct effects of the drug [3, 5, 14, 20]; these different views, however, are not necessarily antagonistic [1–3]. My focus has been on conditioned responses which mimic the direct effects of the drug. For example, in one study [14], physical dependence upon morphine was induced in rats. Dependence was then maintained by daily injections of morphine, and each injection was paired with a conditioned stimulus. Following this conditioning phase the drug treatment was withdrawn, and withdrawal symptoms were rated. The rats were then exposed to either morphine, no-treatment, or the conditioned stimulus. Morphine, of course, reduced the withdrawal symptoms, while withdrawal symptoms were somewhat increased under the no-treatment condition. The conditioned stimulus mimicked the morphine effect and also reduced withdrawal distress. This procedure is advantageous because it allows the measurement of many dependent variables, and the within-group design decreases variability and holds important stress variables [19, 22, 23] constant across experimental conditions. In the current experiment, we apply a similar procedure to study the role of classical conditioning in ethanol dependent rats.

## METHOD

### *Animals and Apparatus*

Eighteen male hooded rats of the Long-Evans strain (Charles River, Wilmington, MA) were used. The rats were acclimated to laboratory conditions for at least one week prior to surgery; during this time they were housed in pairs in solid bottom box cages with a contact bedding. Food (Wayne Lab Blox) and water were freely available, and temperature and lighting conditions were controlled as described below. On the day of surgery the rats weighed between 283–389 g (mean 329 g), and each was implanted with a chronic indwelling jugular cannula while under Nembutal anesthesia (50 mg/kg). The cannula was passed from the jugular vein, subcutaneously, to exit at the dorsal region of the animal's neck. The rat was then placed in a harness which had a 40 cm length of spring attached to it, and the cannula was passed through this protective spring. Each rat was then individually housed in an operant chamber (30×25×27 cm) that was enclosed in a sound attenuating cubicle (both from BRS/LVE, Laurel, MD). The spring and cannula tubing were attached to a Small Animal Infusion Swivel (Harvard Apparatus, Ealing Co., South Natick, MA) positioned above the center of the sound attenuating cubicle. The swivel, in turn, was connected by polyethylene tubing to an injection system (Harvard Apparatus Compact Syringe Pump, model 975) located outside of the sound attenuating cubicle. The one-way cannula was constructed of polyethylene tubing

(PE 50) with a silastic tubing tip (0.037 in. o.d.). A more detailed description of the surgical procedures, and directions for cannula and harness construction can be found elsewhere [18].

The animals remained in the behavioral chambers 24 hr a day (except where noted) throughout the entire experiment. Food (granulated Wayne Lab Blox) was supplied in spill-proof jars, and water was available in calibrated drinking tubes. The chambers were well ventilated, temperature controlled (23±1°C) and internal lighting alternated on a 12 hr day-night (0800–2000 hr) cycle. The scheduling of infusions, and stimulus presentations was automatically programmed with electromechanical circuitry.

Temperature measurements were made with a Yellow Springs Instruments Digital Thermometer (model 49TA) supplied with a 402 temperature probe.

The open-field was a 93×93 cm slab of plywood divided into 36 equal sized squares (15.5×15.5 cm each). The open-field rested on a stool (48 cm high) in the center of the room housing the operant chambers.

*Procedure*

The eighteen rats were received from the supplier in groups of 6 rats each at approximately 1.5 month intervals. Each group of rats was exposed to the entire experimental procedure (approximately 30 days duration) prior to the testing of the next group. Since we used a within-group design, all subjects received both experimental and control treatments, and the staggered delivery of animals allowed age and weight variables to be held relatively constant across the three groups. Hence, we had 3 groups of 6 rats each (henceforth designated as Groups 1, 2 and 3 respectively) tested successively at approximately 1.5 month intervals. Each group was treated identically *except* for the order of treatment application (see below), which was determined prior to the onset of the study, and randomly assigned to each group.

Below we describe the various stages of the experimental protocol along with the procedures employed to quantify the dependent measures. Throughout all phases of the experiment, food and water intake, and body weights were recorded daily.

*Habituation.* The 7 days immediately following surgery served as a surgical recovery and habituation period. During this time, sterile isotonic (0.9%) saline was infused intravenously, to each rat, every 4 hr around the clock (6 infusions/24 hr). Each infusion was administered at a rate of 0.3 ml/min over a duration of 5 min. On days 4 and 6 of this habituation period we obtained baseline rectal temperatures and open-field activity measurements (see below). On each of these days, in order to habituate the animals to subsequent procedures, we obtained, for both the temperature and activity measures, a preinfusion recording (30 minutes prior to the onset of the 12 noon infusion) and a postinfusion recording (30 minutes following the 12 noon infusion).

*Dependence induction.* Following habituation, the rats were made dependent upon ethanol using procedures previously described [11,13]. Ethanol (30% v/v, prepared from 95% ethanol and sterile saline) was infused intravenously every 4 hr around the clock at a rate of 0.3 ml/min (6 infusions/day). The dependence induction period continued for 7–9 days in the different groups (8, 7, and 9 days for Groups 1, 2 and 3 respectively). This small variability in days of exposure to ethanol, between the groups, was necessitated by weekend schedule conflicts, and did not influence the results of this within-group design (see Table 1).

The mean daily dose of ethanol administered during the dependence induction period was 9.38 g/kg/day (range: 8.22–10.67). As described previously [13], each animal's dose was regulated to maintain moderate levels of intoxication throughout the dependence induction period. Hence, the small variability in the dose delivered to each animal was due to slight differences in their sensitivity to ethanol. The conditioned stimulus (CS) was *not* presented at any time during this dependence induction phase.

While we did not directly verify physical dependence in this phase of the experiment, we did observe withdrawal signs in the conditioning phase (see below), and our prior work [13] has shown that this procedure is effective in inducing mild to moderate levels of physical dependence (as measured by withdrawal signs) in all rats tested.

*Conditioning.* Our objective was to begin conditioning only after dependence was firmly established so that the CS would be associated with ethanol infusions (unconditioned stimulus, or US) that reduced withdrawal distress [13,14]. Conditioning procedures and testing were carried out over 3 phases employing a repeated measures counterbalanced design. In the first phase, ethanol dependence was maintained by intravenous infusions of ethanol (30% v/v, 0.3 ml/min) delivered every 6 *hr* around the clock (4 infusions/day), and each infusion was associated with a tone CS (Mallory Sonalert, 2.8 kHz). The tone was initiated 1 minute prior to the onset of the infusion and continued for the duration of the infusion (8–15 minutes, depending on animal weight and alcohol dose). The mean daily dose of ethanol administered during the first conditioning phase was 8.92 g/kg/day (S.E. ±0.27). We chose a 6-hr interinfusion interval so that the tone would be associated with alcohol infusions that reduced the mild withdrawal symptoms that we observed at this time (data not shown, but see [13]). Since the daily dose of ethanol infused was comparable to that administered during dependence induction, physical dependence was maintained. The first conditioning phase continued for 7–8 days and thus allowed 28–32 pairings of the CS (tone) with the US (ethanol). Following conditioning phase 1, a test day was administered (see below). This first test day was followed by conditioning phase 2 which was identical to conditioning phase 1 except that between 11–19 pairings of CS and US were administered, and the mean daily dose of ethanol infused was 9.84 g/kg/day (S.E. ±0.09). This second conditioning phase was followed by a second test day. The third and last conditioning phase was then initiated during which 11–15 pairings of CS and US were administered, and the mean daily ethanol dose was 10.02 g/kg/day (S.E. ±0.11). The third conditioning phase was followed by a third and final test day.

The slight increase in the ethanol dose, over the conditioning phases, was due to the development of tolerance [11,13]. As in the dependence induction period, the ethanol dose was adjusted daily to maintain moderate levels of intoxication following each infusion. Hence, as tolerance developed an increase in dose was necessary (see [13]).

It should also be noted that in each conditioning phase there is some variability in the number of CS-US pairings. As in the dependence induction phase (for days of ethanol exposure), this variability was due to weekend schedule conflicts. However, the number of CS-US parings was held constant within each group and only varied between the groups of this within-group design. All Group 1 rats received 32, 11, and 15 CS-US pairings during conditioning phases 1, 2 and 3, respectively. Group 2 received 31, 19, and 11 pair-

TABLE 1

EFFECTS OF PAVLOVIAN CONDITIONING ON THE ETHANOL WITHDRAWAL SYNDROME

| | Pretest Scores, mean ± S.E. | | | Posttest Change Scores, mean ± S.E. | | |
|---|---|---|---|---|---|---|
| Treatment | Temperature (° C) | Activity | Withdrawal* Score | Temperature (°C) | Activity† Ratio | Withdrawal Score |
| Tone+Saline | 37.58 ± 0.18 | 9.50 ±1.69 | 4.50 ±0.40 | +0.32 ±0.17 | 1.45 ±0.20 | −0.08 ±0.23 |
| Tone+ETOH§ | 37.24 ± 0.11 | 8.75 ±1.97 | 5.25 ±0.45 | −0.18 ±0.17 | 8.54 ±2.52 | −5.25 ±0.45 |
| None | 37.28 ± 0.14 | 9.33 ±2.08 | 4.67 ±0.36 | +0.52 ±0.18 | 1.95 ±0.92 | −0.17 ±0.24 |
| Statistical‡ | NS | NS | NS | $p<0.01$ | $p<0.01$ | $p<0.05$ |

*Withdrawal scores were based on the analysis of 3 signs: tremor, rigidity, and tail stiffening. Each sign was rated on a scale from 0 (absent) to 3 (severe); these individual values were then summated to obtain a withdrawal score for each rat.

†Ratio of postactivity score/preactivity score. Activity indicates number of grid crossings, in an open-field, in a 2-minute period.

§2.51 ± 0.03 g/kg/IV.

‡Statistical analysis involved first an ANOVA with repeated measures for the temperature and activity data or the Friedman ANOVA by Ranks for the withdrawal scores. These tests were followed by the Newman-Keuls test or the Wilcoxon Signed-Ranks Test. NS indicates not significant. The same 12 rats were used for all treatments in a counterbalanced repeated measures design. The Table shows the $p$ values obtained with the overall ANOVA tests, $p$ values for the subsequent multiple comparisons can be found in the text.

ings during the three conditioning phases, respectively; and Group 3 received 28, 15, and 11 pairings, respectively. These small differences did not influence the results of this counterbalanced design (see below).

*Testing.* On test days, programmed infusions of ethanol were terminated at 7 a.m., and withdrawal was maintained for 10 hr (until 5 p.m.), the time of peak withdrawal symptomatology under this paradigm [13]. At this time, one of three test treatments was administered: (A) tone + saline infusion (duration of infusion identical to that received during the last ethanol infusion of the conditioning phase), (B) tone + ethanol infusion (duration and dose of infusion identical to that received during the last ethanol infusion of the conditioning phase), or (C) no treatment (duration as above, except no treatment administered). If the tone acquired ethanol-like properties via classical conditioning, one would expect treatment 'A' to reduce withdrawal symptoms. In order to determine such an effect, a pretest-posttest design was employed. The pretest was conducted 30 minutes prior to the onset of treatment (at 10 hr of withdrawal) and the posttest was conducted 30 minutes following treatment (treatment refers to treatment A, B, or C noted above). For both the pretest and the posttest we measured, in order, (1) signs of withdrawal, (2) body temperature, and (3) open-field activity. These measurements were carried out under blind conditions. The order of presentation of the treatment conditions, on test days, was counterbalanced across subjects. Group 1 received treatment order A-B-C on test days 1, 2 and 3, respectively. Group 2 received treatment order C-A-B, and Group 3 received treatment order B-C-A.

*Withdrawal signs.* For these observations, the rats remained in the operant chamber attached to the tethering system. The doors to the sound attenuating and operant chambers were opened and signs of withdrawal were rated, over a 1-minute period, on a scale of 0 (absent) to 3 (severe). The signs rated were body rigidity, tremor, and tail stiffening. A detailed characterization of these signs, and the scaling of their severity have been previously reported by us [13]. We did not attempt to induce audiogenic seizure activity because of its potentially disruptive effects on conditioning, and because we did not want to lose animals to seizure-related death. Therefore, based on our observations and scaling procedure, an overall withdrawal score of 9 would reflect maximal withdrawal severity (3 signs rated × severity score of 3).

*Body temperature.* For these measurements, the rats remained in the operant chamber attached to the tethering system. The rat was gently restrained, by hand, and the temperature probe was inserted 5 cm into the rectum, and maintained in that position until the thermometer indicated a stable temperature value (after about 30 sec). Temperature was recorded to the nearest 0.01°C.

*Open-field activity.* Each rat was separately removed from its operant chamber and placed in the center of the open-field. Activity was indicated by the number of squares the rat entered (both hind and forelegs) in a 2-minute period. The rat was then returned to its operant chamber, and the open-field was cleaned prior to testing the next rat.

*Statistical analysis.* Analysis of the data for the test days consisted of a one-way ANOVA with repeated measures followed by the Newman-Keuls Test for temperature and activity measures, or the Friedman ANOVA by Ranks followed by the Wilcoxon Signed-Ranks Test for withdrawal scores.

## RESULTS AND DISCUSSION

Six rats were lost prior to the completion of the experiment, and their data were therefore excluded from the subsequent analysis of this repeated measures design. One rat died from ethanol overdose, and 5 destroyed their catheters. The

total N was therefore reduced to 12, leaving 4 rats in Group 1, 5 rats in Group 2, and 3 rats in Group 3.

The 12 rats that completed the study tolerated the procedures well, and remained healthy throughout all stages of the experiment. However, as was expected from our earlier work [11,13], there was some weight loss during the initial dependence induction period. The mean weight (±S.E.) for all animals during dependence induction was 320±7 g (compared to an initial mean weight of 329±10 g). This weight loss, however, was not statistically significant, $t(11)=1.872$, $p>0.05$. In contrast to this initial weight loss, the animals regained weight during the conditioning phases of the experiment. The mean (±S.E.) weights during conditioning phases 1–3 were 323 (±7), 327 (±6) and 333 (±6) g, respectively. The weight increase from the dependence induction period through conditioning phase 3 was statistically significant, $F(3,33)=8.68$, $p<0.01$. This weight increase was not due to changes in food intake, which varied between a mean (±S.E.) of 13.3 (±0.7) g/day during dependence induction and 14.6 (±0.8) g/day during conditioning phase 3, $F(3,33)=1.12$, $p>0.05$, but rather to a statistically significant increase in water intake throughout this same period, from a mean of 23.8 (±1.7) ml/day during dependence induction to 38.0 (±2.9) ml/day during conditioning phase 3, $F(3,33)=15.87$, $p<0.01$.

Table 1 shows the results obtained on test days. The left side of the table shows the pretest averages for body temperature, activity, and withdrawal score. The right side of the table shows the changes from these pretest values following the application of treatment A, B, or C (posttest change scores). Temperature and withdrawal score changes were derived, for each subject, by subtracting the pretest value from the posttest value. Activity changes are indicated by the ratio of postactivity score/preactivity score.

As the pretest scores show, all pretest measures were similar prior to the application of the different treatments; there were no statistically significant differences between the different treatment conditions for pretest values (all $p>0.05$). This stability of pretest scores enhances the meaningfulness of the posttest changes, and also shows that the rats were in a similar state of alcohol withdrawal prior to the application of each treatment.

The posttest change scores show that only the 'ethanol' condition significantly affected behavior. Both the 'tone' and 'no treatment' conditions led to slight increases in both temperature and activity, but virtually no change in the withdrawal score. In contrast, the 'ethanol' condition led to a slight decrease in body temperature, a dramatic (eight-fold) increase in activity, and a complete reversal of withdrawal distress (the average dose of ethanol administered across all 'ethanol' test conditions was 2.51±0.03 g/kg). The overall F, comparing these change scores across conditions, was statistically significant for each behavioral measure [temperature, $F(2,22)=6.579$, $p<0.01$; activity, $F(2,22)=6.080$, $p<0.01$; withdrawal score, chi-square $(2)=18.0$, $p<0.05$]. Subsequent comparisons with the Newman-Keuls Test (for temperature and activity) or the Wilcoxon Signed-Ranks Test (for withdrawal scores) showed that the behavioral changes following the 'ethanol' treatment were significantly different ($p<0.05$) from both the 'tone' and 'no treatment' conditions, but that these latter two treatment conditions did not significantly differ from each other ($p>0.05$).

These results show, at least for the current paradigm, that conditioning did not occur. In this regard, ethanol seems to differ from the opiates. In a prior study [14], we have shown that when intravenous morphine infusions are paired with a predictive tone stimulus using the same paradigm employed here, the tone does acquire the ability to mimic the direct effects of morphine, and reduce withdrawal symptomatology.

In addition, the temperature measurements recorded here do not provide evidence for a Pavlovian mediated compensatory response which has previously been reported for ethanol in rats [7,8]. While the comparison between the 'ethanol' condition and the 'tone' condition does suggest a compensatory response (ethanol produced a mild hypothermia, while the tone produced hyperthermia), this interpretation is contradicted by the effect of the 'no treatment' condition, which, like the tone, also led to hyperthermia. These data suggest that a non-specific factor, perhaps stress induced by the extensive handling of the ethanol withdrawn rats during the test periods, was responsible for the hyperthermia and not a Pavlovian mediated compensatory response. A similar role for stress factors, rather than conditioning, has recently been suggested to mediate morphine induced hyperthermia [19,23].

Ethanol infusions in the rats, at 10 hr of withdrawal, also produced a dramatic increase in activity. Since this effect was so large, and activity is easily quantified, one might expect even a small conditioning effect to be detected here, but it was not. In contrast to these data, it has been shown that environments associated with morphine administration do acquire the ability to mimic the direct stimulatory effects of morphine on motor activity [9,20]. However, these morphine studies, unlike ours, did not test dependent animals undergoing withdrawal.

The fact that the current paradigm differs in many respects from those employed in other studies may account, at least in part, for the current findings. First, most studies pair the CS with drug from the outset of training. This is obviously different from our design, where the CS was introduced only after dependence upon ethanol was established. Our rationale was to associate the CS with ethanol infusions that reduced withdrawal distress. One can argue, however, that other contextual stimuli may have served as CSs for drug infusions during dependence induction, and that these CSs blocked the subsequent conditioning to the tone. While this argument has merit, it is not supported by our earlier work [6,14] which employed procedures virtually identical to those used here to effectively reduce morphine withdrawal signs.

Secondly, the ethanol test condition in the current experiment is actually a tone + ethanol condition. Therefore, while we found that the tone + saline condition did not influence withdrawal behavior, one cannot rule out the possibility that the tone did modify the effects of ethanol, since the effects of ethanol alone were not assessed. Lastly, our findings may differ because we used drug dependent rats, while most other studies have used non-dependent subjects. While only future research will determine if conditioning effects are more difficult to obtain in dependent animals, our earlier work with morphine [5, 6, 14] suggests that such conditioning is, in fact, possible.

These remarks considered, it should nonetheless be clear from this study, as well as others [1, 15, 19, 22, 23], that much additional work is necessary before we will be able to determine those drug-related responses most susceptible to Pavlovian conditioning (both direct and compensatory), and the potential role of non-specific factors, such as stress, in mediating these putative effects.

ACKNOWLEDGEMENTS

Supported by NIAAA Grant AA05666. Thanks are extended to A. Naparzewska for assistance with the conduct of this experiment. Portions of this paper were presented at the 94th Annual Convention of the American Psychological Association, Washington, DC, August, 1986.

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