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Original Investigations

Scopolamine Effects on Conditioned Suppression: Influence of Diurnal Cycle and Transitions between Normal and Drugged States*

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Summary. Rats were exposed to either a drug-induced state (1.0 mg/kg scopolamine) or an undrugged state (saline) at each stage of a three-stage procedure. They were first trained to lick a tube, then were exposed to classical fear conditioning in the absence of the tube, and finally were tested for conditioned suppression of licking. Animals tested at the midst of the dark period of the daily light/dark cycle displayed a failure of conditioned suppression if conditioning had occurred under the novel state; suppression was less consistently impaired in animals tested at the midst of the photoperiod. All animals exposed to a consistent drugged (or undrugged) state displayed conditioned suppression. Results suggest that experimental procedures frequently employed in studying drug manipulations of learning and memory may be biased against the transfer of training to the test situation. Scopolamine effects, often attributed to changes in learning or memory, may often represent a weakening in stimulus control of behavior resulting from transitions between drugged and undrugged states. Several factors are suggested as contributing to the diurnal difference in drug effects.

Key-Words: Scopolamine — State-Dependent Learning — Diurnal Cycle — Memory — Conditioned Suppression.

In evaluating the effects of anticholinergic drugs on the processes of learning and memory consolidation, most experimenters have injected the drugs shortly before animals were trained, then later tested performance when the drug state was no longer in effect. With the above experimental design, effects on learning and memory are inferred from comparisons of performance of animals trained under the drug with those trained without the drug. Recently 1.0 mg/kg scopolamine was found to block conditioned suppression of licking ("fear") when conditioning occurred in the drug state and subsequent testing was in the nondrug

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state, but not when both conditioning and testing were under the drug state (Evans and Patton, 1968). These results were not readily attributable to drug effects on learning, memory, or motivation, but were suggestive of dissociation resulting from a transition between normal and drugged states. Berger and Stein (1969a, 1969b) reported similar results which they attributed to both an impairment of neural processes in learning and to an asymmetrical dissociation.

Dissociation of learning between drug and nondrug states has been described for atropine and scopolamine (Overton, 1966; Oliverio, 1968). We proposed that the drug state be considered a stimulus of importance in experiments involving cholinergic manipulations of learning and memory, that drug dissociation may represent one type of stimulus generalization decrement phenomena, and that scopolamine effects on conditioned suppression could be most clearly understood if drug treatments were factorially assigned throughout the three-stage paradigm of pre-training, conditioning, and re-test (Evans and Patton, 1968). Usually, neither drug nor placebo injections are given during the pretraining phase (*e.g.* Evans and Patton, 1968; Vogel *et al.*, 1967; Berger and Stein, 1969a). However, clear evidence of symmetrical dissociation under scopolamine can be obtained only through symmetrical assignments of drug treatments (Overton, 1968). If scopolamine disrupts learning, then *all* groups conditioned under the drug state should show an absence of conditioned suppression in the re-test; if the drug disrupts retention, then *all* groups given the re-test under the drug should display an absence of conditioned suppression; if scopolamine produces an asymmetrical amnesia, then groups conditioned under the drug and re-tested in the nondrug state should display an absence of suppression but those conditioned under the nondrug state and re-tested under the drug should not. Finally, evidence of dissociation or state-dependent learning would be found if the drug and nondrug states were to function as stimuli. In the latter case development of conditioned suppression should be favored by the maintenance of a consistent drug stimulus state throughout pre-training, conditioning and re-test, while the deficit in conditioning would be maximal whenever pretraining and re-test were under the same (drug or nondrug) state while conditioning was under the novel state. Our previous experimental design has therefore been extended to include drug, as well as nondrug, treatments at the pretraining stage of the paradigm.

Rats trained during the light phase of the daily light/dark cycle reportedly acquire an avoidance response more rapidly than those trained during the dark (Gordon and Scheving, 1968). Krieger *et al.*, 1968 have presented evidence of a diurnal cycle in the action of atropine on the hypothalamic-pituitary system of cats. Variations in the prevailing

light/dark cycle have not been employed in previous studies of scopolamine effects on learning and memory. The present article reports the effects of all eight possible sequences of drug treatments on a one-trial test of fear conditioning for rats tested during the photoperiod as well as for those tested during the dark phase of the diurnal cycle.

Method

Subjects. Subjects were 224 naive male Holtzman rats[1] weighing 225—325 gm at the start of the experiment and housed individually in one of two adjacent colony rooms having photoperiods from 0600—1800 hours (Colony L) or from 1800—0600 hours (Colony D). Photoperiod illumination varied between 16 ft-c and 0.26 ft-c at the level of the rat cages, and dark period illumination was 0.016 ft-c or less, as measured by a Lunasix-Gossen electronic exposure meter. Room temperature varied between 22—24° C during the course of the experiment. Animals had free access to food and water except as described in the Procedure. At least two weeks were allowed for adaptation to the Colony lighting schedule. Red lights were used whenever it was necessary to enter the Colony rooms during the dark period. Rats were randomly assigned to one of 32 treatment groups, 16 in each Colony. All testing was done between 1200—1330 hrs.

Apparatus. Test equipment was similar to that used previously (Evans and Patton). Six boxes, 17.5 × 15 × 16 cm high, were equipped with detachable Grason-Stadler (E4690A) drinkometers and stainless steel drinking tubes. Output from the drinkometer circiut was channelled through high-speed pulseformers (Scientific Prototype 4069-J) instead of the electromechanical ones that we had used previously. Footshock was presented through the wire floors of the boxes by a Lehigh Valley Electronics 1531 constant current generator. Tones were presented by a Condover CPO-4 audio oscillator through a speaker mounted in the rear wall of each box (90 db S.P.L.). At other times random noise was presented at 68 db as measured by a General Radio 1565-A sound level meter. The test boxes were opaque on all sides with the exception of the front wall, which was made of clear Plexiglas and upon which was mounted the water tube. Room lights provided diffuse illumination of less than 0.02 ft-c inside the test boxes. Programming equipment was located in an adjacent room from which the subjects could be observed through a one-way mirror.

Procedure. Procedures were similar to those of Vogel *et al.* On Day 1 water was removed from the animals' home cages. On Day 2, at the 24th hour of deprivation, each animal was placed in a covered metal box,

1 Two animals were replaced because of equipment malfunction.

carried to an adjacent room, placed in a test chamber and given 5 min access to the water tube. Animals were then returned to *ad lib.* water in the home cage. This initial exposure to the test box will be refered to as pre-training. On Day 3 fear conditioning trials were given in the same test boxes but in the absence of the drinking tube. All animals received five 15 sec tone presentations with an interval of 60 sec between presentations. The tone presentations began 30 sec after the animals had been placed in the boxes. Half of the animals received 0.8 mA footshock for the final 2 sec of each tone presentation; the others received only the tone. Total session length was 345 sec. Water was again removed from the home cages on Day 4. On Day 5 each 24 hr-deprived rat was again given access to the water tube while the sequence of tones was repeated without shock. Rate of licking during the Day 5 re-test was used to estimate conditioned suppression ("fear").

Initial response rates (licks/seconds), calculated from the time to complete the first 100 licks at the drinking tube, are assumed to reflect the generally disruptive effects of fear elicited by the stimuli of the test situation. Total response rates were also recorded. Suppression ratios were calculated according to the formula $\frac{B}{A+B}$ in which B is the response rate during the 15 sec tone presentation and A is the response rate during a 15 sec period immediately before the tone presentation. The suppression ratio is assumed to indicate the degree to which suppression is elicited by a specific, distinctive stimulus (the tone).

On Days 2, 3 and 5 all animals were weighed and given i.p. injections of either 1.0 mg/kg scopolamine HBr dissolved in normal saline at a concentration of 1.0 mg/ml or an equivalent volume of the saline. Injections were given 35—40 min prior to testing. All data were initially subjected to computer analysis of variance program BMD O2V (Dixon, 1961) for a $2 \times 2 \times 2 \times 2 \times 2$ factorial design. The five variables were: Day 2 drug state, Day 3 drug state, Day 3 shock, Day 5 drug state, and Diurnal condition (Colony D *vs.* Colony L). There was an appropriate control group of unshocked animals for every sequence of drug treatments.

Results

Response Baselines. The mean licking rates recorded from saline-treated animals of the present experiment (Day 2, Colony D, 3.09 licks/sec) are double the baseline rates we had observed previously (Evans and Patton, mean of all Day 2 scores = 1.52 licks/sec). The increased response sensitivity of the present experiment was the result of the improved drinkometer circuit which is capable of recording rates up to 25 per sec. The present higher baseline rates resemble the normal average licking rate of 24 hr-deprived rats (Stellar and Hill, 1952). Day 2 mean total

licking rates for saline-treated animals were 2.63 licks/sec for Colony L and 3.09 licks/sec for Colony D, indicating a diurnal difference in response baselines ($t = 2.40$, $df = 110$, $p < .05$).

Scopolamine reduced Day 2 licking to 1.06 licks/sec for Colony L and to 1.22 licks/sec for Colony D. The interaction of drug effect with diurnal condition was not significant but, in contrast to our previous observations, total response rate was reduced in all scopolamine animals on Day 2 ($F = 113.67$, $df = 1/220$, $p < .01$). Groups receiving scopolamine on Day 5 also had lower initial response rates ($F = 11.01$ for Colony D, $F = 22.85$ for Colony L, $df = 1/96$, $p < .01$). It was upon the high baseline rates of the present study that this significant reduction in drinking was observed. Day 5 initial rates ranged from 0.25 to 6.60 licks/sec. The diurnal effect for Day 5 initial response rate was also significant ($F = 3.96$, $df = 1/192$, $p < .05$). The data for the Day 5 re-test was therefore analysed separately for each Colony.

Effects of Drug and Nondrug States on Measures of Conditioned Suppression. Evidence of conditioned fear is found in a suppression of Day 5 response rates of those animals given tone-shock pairings on Day 3, in comparison with rates of their non-shocked controls. All animals having received tone + shock showed a marked suppression of licking during tone presentations ($F = 20.20$ for Colony D, $F = 42.78$ for Colony L, $df = 1/96$, $p < .01$). Response suppression during the tone was not significantly affected by drug sequence, indicating that the specific tone stimulus exerted strong control over behavior. The remainder of the results represent effects on initial licking rates which are taken to present suppression to various unspecified stimuli of the entire test situation. These unspecified stimuli may include tactile, visual and olfactory stimuli of the test procedure that were not associated exclusively with the shock presentation.

The effect of Day 3 shock on Day 5 initial rates was significant for both Colony D ($F = 6.70$, $df = 1/96$, $p < .05$) and Colony L ($F = 7.99$, $df = 1/96$, $p < .01$) indicating a disruption of drinking pattern by the stimuli of the test situation. The Table contains the Day 5 initial response rates for all groups. For Colony D, Day 5 initial rates failed to show suppression only in groups for which conditioning and re-testing were under different states (Day 3 state $\times$ Shock $\times$ Day 5 state interaction, $F = 5.74$, $df = 1/96$, $p < .05$), indicating that the effect of the general test apparatus stimuli was disrupted by the change in state. There was good evidence of conditioned fear (suppression of initial rates) in animals experiencing a consistent state throughout the experiment, *i.e.*, Groups S-S-S and D-D-D. Suppression was not severely disrupted when conditioning and re-test were under the same state but training under the other state (Groups D-S-S and S-D-D). Animals having their initial drug

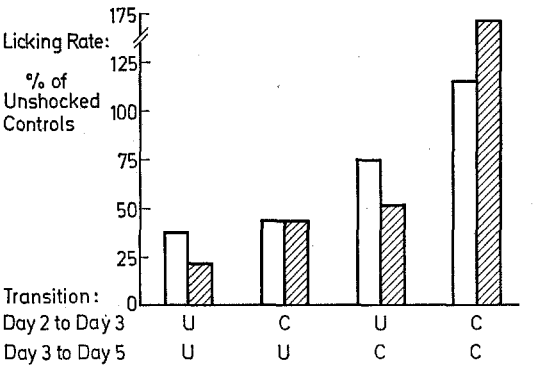

Fig. 1. Conditioned suppression of rats tested during the dark phase. Open bars represent groups from Colony D of the present experiment; shaded bars have been calculated from data of Evans and Patton (1968). *C* Change of state. *U* Unchanged state. See text for details

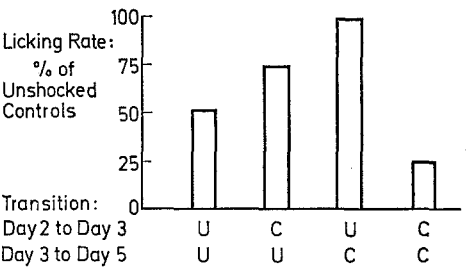

Fig. 2. Conditioned suppression of rats tested during the photoperiod (Colony L). *C* Change of state. *U* Unchanged state

experience on the re-test (Group S-S-D) showed no evidence of suppression while the complementary group (D-D-S) did show suppression. The difference in performance of these two groups is unexplained. As predicted, there was no suppression when conditioning was administered in the novel state (Groups S-D-S and D-S-D).

The influence of change of state on conditioned suppression is summarized in Figs. 1 and 2. The quantitative estimate of suppression represents the quotient of the mean Day 5 initial response rate of shocked animals divided by the comparable rate of the appropriate unshocked control group, expressed as a percentage. Symmetrical pairs of treatment groups have been combined; *e.g.*, the "unchanged-unchanged" condition represents Groups S-S-S and D-D-D while "changed-changed" represents Groups D-S-D and S-D-S. Data from a previous experiment (Evans and Patton) has been recalculated to demonstrate consistency of the trend.

Among animals tested at the midst of the photoperiod (Colony L) there was good evidence of conditioned suppression in groups exposed to an unchanging state. However, reversal of state did not significantly disrupt suppression. Suppression appeared to be blocked only in Group D-D-S. The results for Colony L are shown in Fig. 2.

Discussion

There has been continuing interest in the effects of anticholinergic drugs on animal behavior (Carlton, 1963; Reeves, 1966; Deutsch and Deutsch, 1966) including numerous reports of anticholinergic disruption of the processes of learning and memory consolidation (Buresova *et al.*, 1964; Bohdanecky and Jarvik, 1967; Carlton, 1968; Daly, 1968; Dilts and Berry, 1967; Meyers, 1965; Wiener and Deutsch, 1968). In the above reports, the range of effective doses of scopolamine HBr was 0.3 to 2.0 mg/kg.

In the present experiment a one-trial conditioned suppression technique was employed to evaluate the effects of 1.0 mg/kg scopolamine HBr. It was found that rats tested without drug during the dark period of their diurnal cycle had higher baseline licking rates than those tested during the photoperiod. Gutman *et al.* (1969) recently reported similar effects. Licking rates of all animals were significantly reduced by 1.0 mg/kg scopolamine, thus reversing our earlier failure to detect a drug effect on licking baselines (Evans and Patton, 1968). Others have reported scopolamine, in doses equivalent to those used here, to reduce food and water intake (Stein, 1963; Gerald and Maickel, 1969) although Berger and Stein (1969a) reported 1.0 mg/kg scopolamine to have no effect on liquid food consumption. The reduction of licking rates by scopolamine in the present experiment may be an indication of a disruptive photophobia resulting from mydriasis (Oliverio, 1968), of anticholinergic blockade of thirst mechanisms (Stein, 1963), or the general hyperactivity observed in scopolamine-treated rats (Calhoun and Smith, 1968; Carlton, 1968). The effect of 1.0 mg/kg scopolamine on water consumption of rats is gone within 24 hours after injection (Gerald and Maickel, 1969).

When the above-mentioned differences in response baselines are taken into consideration, the following conclusions emerge. Scopolamine treatments did not interfere with manifestation of fear in the presence of a distinctive conditioned stimulus. This observation confirms other experiments which indicate that behavior under strong stimulus control is resistant to drug effects (Weiss *et al.*, 1969; Laties and Weiss, 1966; Wagman and Maxey, 1969). The administration of scopolamine at the time of conditioning also failed to consistently block suppression in the presence of stimuli of the general test situation, hence there was no

Table. *Day 5 mean initial licking rates. (N = 7 for each mean. "S" = Saline, "D" = Drug)*

State			Colony D		Colony L	
Pre-Test (Day 2)	Conditioning (Day 3)	Re-Test (Day 5)	Shocked	Unshocked	Shocked	Unshocked
S	S	S	1.43	4.10	1.80	2.83
S	S	D	1.97	1.59	0.94	1.61
S	D	S	4.40	4.29	0.89	3.82
S	D	D	0.93	2.94	0.97	1.36
D	S	S	1.40	2.44	2.42	3.20
D	S	D	2.08	1.41	0.37	1.04
D	D	S	1.05	2.47	2.58	1.94
D	D	D	0.61	1.39	0.34	1.32

evidence that the drug blocks learning of fear. Similarly, there was no evidence that the drug affects retention because the administration of the drug at the time of re-test did not consistently block suppression. As can be seen from the Table, animals receiving the drug on *both* Day 3 and Day 5 displayed the same degree of suppression as animals receiving saline throughout.

The present evidence does not favor the notion that scopolamine produces an asymmetrical effect or a one-way amnesia for fear. Reversal of states between Day 3 and Day 5 was no more disruptive when the change was from D to S than when the change was from S to D. The effects of several drugs thought to produce "amnesia" have been re-evaluated in terms of dissociation or state-dependent learning (Bindra and Reichert, 1967; Goodwin *et al.*, 1969). Dissociation of learning under scopolamine has been described by Overton (1966, 1968) and by Oliverio (1968) and may be attributed to a stimulus generalization decrement resulting from changes in drug states. The present results may be best interpreted in similar terms, *i.e.*, of the stimuli available to the animal in the behavioral test situation. The term stimulus "generalization decrement" has been used to describe a weakening in control of behavior resulting from an alteration in environmental stimuli. Rats trained to drink in a constant environment display a decrement in drinking in proportion to the extent to which the stimulus characteristics of the test apparatus are altered (Fink and Patton, 1953). In a test of the effects of conditioned fear upon drinking, rats receiving both drinking experience and fear conditioning in the same apparatus displayed suppression of drinking but those receiving conditioning in a novel test apparatus did not show suppression when re-tested in the drinking apparatus (Amsel, 1950; Amsel and Maltzman, 1950). Stimulus generali-

zation decrements in conditioned fear are not peculiar to tests involving the licking response (*e.g.*, King, 1969). In experiments on the pharmacology of learning and memory, as in the present case, the stimuli of the test apparatus are typically held constant but the drug states are shifted during the training and test procedures. Brown *et al.* (1968) indicated that drug dissociation may be a special type of generalization decrement in which drug-induced internal conditions are the major stimuli. Barry (1968) reviewed evidence that animals can learn a discrimination task with drug state serving as the only cue, and the internal effects induced by scopolamine doses as low as 0.06 mg/kg have been found to serve as a discriminative stimulus (Kubena and Barry, 1969). In the present paradigm the absence of the drinking tube and water-deprivation during the administration of fear conditioning (Day 3) do not permit a complete uniformity of stimuli throughout the three-stage procedure.

In the present experiment animals tested in the midst of the daily period of darkness exhibited a clear absence of conditioned suppression in three of the four groups undergoing a change in state between conditioning and testing. These results are in close agreement with those observed previously under comparable conditions (Evans and Patton, 1968). Group S-D-S represents the treatment sequence most frequently employed in demonstrating disruption of learning by scopolamine. The failure of *both* Group S-D-S and Group D-S-D to show the effects of conditioning administered in a similar test environment suggests that the effects of conditioning do not generalize well from stimuli of the novel state to those of the alternate state and invites comparison with the results of Amsel in which a constant non-drugged state was maintained but fear conditioning was administered in a novel test environment. In each case a failure of conditioned suppression was observed following alteration of the stimulus situation. Groups S-S-D and D-D-S both had pretraining and conditioning in the same state but were tested in the novel state. Our results suggest that a shift of this type may also produce a moderate disruption of performance. The conclusion that disruption of performance on this one-trial test is not the result of specific anti-muscarinic activity of scopolamine is supported by observations of similar disruptions following administration of a variety of drugs (Dilts and Berry, 1967; Berger and Stein, 1969a; Bohdanecky and Jarvik, 1967; Chiappetta and Jarvik, 1969).

While the present results for rats tested in the dark indicate that the drug-stimuli dissociation hypothesis is better able to account for scopolamine effects on conditioned suppression than other hypothesis thus far proposed, the generality of the dissociation hypothesis remains to be determined under controlled circadian variation. In a test similar to the

present procedure, Vogel *et al.* (1967) found 0.6 mg/kg scopolamine HBr to have no effect on conditioning suppression in rats housed and tested under constant illumination (P. L. Carlton, personal communication). The present observation that rats tested at the midst of the photoperiod do not show a consistent state-dependency of conditioned suppression is in close agreement with the data of Vogel *et al.* These results also indicate that the different outcomes of the Evans and Patton (1968) and the Vogel (1967) experiments are not attributable to the difference in drug doses but rather to the diurnal condition of the subjects of the two experiments.

The diurnal effect of the present results may reflect several factors. Perhaps the rats tested during the dark (Colony D) were less sensitive to the effects of footshock than those tested during the light. Berger and Stein (1969a) demonstrated that a decrease in shock intensity is accompanied by increased susceptibility to the disruptive effects of scopolamine. Rats are reported to learn a shock avoidance task best when trained during the photoperiod (Gordon and Scheving, 1968). Another possibility is that the animals of Colony L showed more consistent suppression of licking because they were less severely water-deprived than animals of Colony D. Rats normally drink 78% of their daily water intake during the dark (Stellar and Hill, 1952). After being deprived of water for 24 hours, rats drink less if tested in the light than if tested in the dark (Gutman *et al.*, 1969). In addition to the above, the diurnal effect may also reflect specific neurochemical phenomena. Mice are most susceptible to the toxic effects of scopolamine during the midst of the daily photoperiod (A. H. Friedman, personal communication). If the present results were an indication that the rats of Colony L had suffered greater toxic effects of scopolamine, then one would have expected scopolamine to have a greater effect on the drinking of unshocked rats of Colony L than on the drinking of Colony D. Such was not the case. Rats' adrenocortical response to stress varies with the diurnal cycle (Ader and Friedman, 1968) and degree of conditioned suppression is related to adrenocortical activity (King, 1969). A small dose of atropine can block elevation of plasma corticosteroids only if given within a critical period of the diurnal cycle (Krieger and Krieger, 1967; Krieger *et al.*, 1968).

In the present experiment rats tested in the photoperiod appeared more resistant to disruption of conditioned suppression by scopolamine than rats tested in the dark. Additional evidence is required to determine the extent to which this effect involves diurnal differences in sensitivity to shock, in water-deprivation and in specific neurochemical systems. Weiss and Heller (1969) have discussed methodological problems in evaluating the role of cholinergic mechanisms in behavior. An additional problem may involve the failure to report and control diurnal factors.

The present results suggest that diurnal factors may account for apparent discrepancies in reports of scopolamine effects on conditioned suppression.

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