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Chronic Swim Stress Enhances the Motoric Inhibiting Effects of a Muscarinic Agonist[1]

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DILSAVER, S. C., J. A. PECK, S. H. MILLER, J. HOH, R. S. JAECKLE AND D. TRAUMATA. *Chronic swim stress enhances the motoric inhibiting effects of a muscarinic agonist.* PHARMACOL BIOCHEM BEHAV **37**(2) 213–217, 1990.—The authors previously demonstrated that chronic inescapable swim stress and footshock increase the capacity of a fixed dose of a muscarinic agonist to produce hypothermia in the rat. This project was designed to determine whether chronic inescapable swim stress in cold water would render a low dose of a muscarinic agonist, devoid of an effect on motor behavior in the naive rat (i.e., prior to subjection to the course of swim stress), an inhibitor of mobility. The study involved two groups of rats, an experimental group which received arecoline and a control group which received saline five minutes prior to being placed in an open field. Number of crossings, the dependent variable, was measured in both groups before and after a 14-day course of twice daily inescapable swim stress of 10 minutes duration at 12°C. The arecoline-treated group, as hypothesized, exhibited a significantly greater reduction in number of crossings than the saline-treated groups following the course of swim stress.

Acetylcholine Affective disorders Arecoline Cholingeric Depression Open-field behavior Muscarinic Stress Receptors

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JANOWSKY *et al.* (11) set forth the hypothesis that depression is due to hyperfunction of central muscarinic cholinergic mechanisms. A growing body of data supports this hypothesis (1–5, 14). The possibility that chronic inescapable stressors activate central muscarinic mechanisms in man is now receiving attention by clinical investigators with an interest in affective illness (6–10, 13, 15–17). An effect of chronic stress on critical muscarinic mechanisms could partially account for the increased rate of relapse in patients with affective illness. The literature consistently suggests that stressors are associated with the onset of depressive syndromes in these individuals. One comprehensive review of the literature indicated that the probability of a patient with a depressive diathesis developing the onset of a new episode increases by 500–600% over the six months following a major life stress (19).

Chronic forced swim stress (6, 7, 9) and inescapable footshock (8) produce supersensitivity to the hypothermic effects of the centrally active muscarinic agonist oxotremorine. The hypothermic response to this agonist is thought to be mediated by its action on hypothalamic muscarinic receptors (18). We now report that a dose of a muscarinic agonist which either enhances or has no effect on motor behavior in drug-naive rats produces profound inhibition of crossings in an open field following a course of chronic cold water swim stress.

The objective of this study was to assess the effect of a chronic forced stressor on sensitivity to the motoric inhibiting effects of a centrally active muscarinic agonist, arecoline.

METHOD

Dependent Variable and Experimental Parameters

The dependent variable in this study is the change in number of crossings rats made in an open field after being subjected to chronic inescapable swim stress. The experimental group received arecoline and the control group saline. Crossings were counted for a five-minute interval after an animal was placed in the field. A crossing was scored each time an extremity entered into one of the squares into which the open field was divided.

Hypothesis

This study is designed to test the hypothesis that a dose of arecoline, which is devoid of an effect on motor behavior in naive rats (relative to saline), is associated with a significant reduction in crossings (a measure of motor behavior) following a two-week course of twice daily cold water swim stress.

Design of the Open Field

The open field consisted of an area of $90 \times 90 \times 45$ cm. The field was divided into 16 equal squares of 506.25 cm^2 each. The floor and walls of the field were painted a light gray. Red tape was used to divide the field into squares (the rat cannot perceive the color red) (20,21).

Experimental Parameters

The dose of arecoline used in this study was determined in a preliminary study (10). In this preliminary study the control and experimental groups received an intraperitoneal injection of normal saline (volume = 1 ml/kg) or arecoline 10 minutes prior to being placed in the open field (10). Rats were randomly assigned to groups of 8 animals receiving 0 (saline), 0.125, 0.25, 0.5, 1.0 or 2.0 mg/kg of arecoline (base) IP. Arecoline at a dose of 2.0 mg/kg completely inhibited movement. In contrast, arecoline at a dose of 0.125 mg/kg was associated with increased crossings relative to the saline condition. However, in two other preliminary experiments, arecoline at this dose and saline were associated with the same number of crossings in the naive rat. The general conclusion is arecoline at a dose of 0.125 mg/kg may increase or have no effect upon crossings but that it most certainly does not decrease this measure of motor behavior.

Motor behavior was measured in a room illuminated with red light. The rat cannot detect red light (20,21). Consequently, the illumination of a room with red light allows an investigator to perform all necessary manipulations but leaves the rats "in the dark." The animals used in Experiment 1 were allowed 10 minutes to adapt to the red light condition prior to being placed in the open field. Those used in Experiment 2 were allowed at least one hour to adapt to this condition.

Animals

Adult, male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were housed in The Ohio State University's vivarium. The ambient temperature was maintained at 21°C. The animals were maintained on a twelve-hour day/night cycle (lights on at 6:00 a.m. and off at 6:00 p.m.). Rat chow and water were available ad lib.

Pharmaceuticals

Arecoline hydrobromide was purchased from Sigma Chemical Company (St. Louis, MO). Doses of arecoline used in this study consistently refer to base form. Arecoline was chosen in lieu of oxotremorine because oxotremorine produces tremor at low doses. The tremor contributes to extraneous movement which is distracting when one attempts to measure motor behavior.

Forced Swim Stress

The animals were subjected to forced swim stress for 10 minutes twice daily at 12°C between 7:00 and 10:00 a.m. and 4:00 and 7:00 p.m. for 14 days. The depth of the water was adjusted so that the animals could not balance themselves with their tails. Forced swim stress sessions began the morning following the initial (baseline) challenges with arecoline and saline. The course of swim stress concluded two days before remeasurement of motor behavior.

STATISTICAL ANALYSIS

Distribution of Dependent Variables

The Shapiro-Wilk's test was performed using SAS in order to determine whether the dependent variables used in our analyses were normally distributed (25). Number of crossings before and after subjection to forced swim stress and change in number of crossings were normally distributed in both experiments. The Shapiro-Wilk's test statistic (W:normal) ranged from 0.89 to 0.96. The probability that the actual value of these test statistics would be less than W:normal ranged from 0.24 to 0.75. This probability statement indicates that it is highly probable that crossings were normally distributed in both experiments before and after forced swim stress.

Statistical Tests

Student's *t*-test for independent samples was used to determine whether sample means between the saline controls and arecoline-treated animals differed before and following a course of chronic forced swim stress. Student's paired *t*-test was also employed to

determine the probability of change in crossings within the control and experimental groups before and following a course of forced swim stress.

The mean mass of each animal and its number of crossings before and after courses of forced swim stress are presented for descriptive purposes.

All measures of variance in the text refer to the standard error of the mean (SEM). The critical value of α was set at $p<0.05$, two-tailed.

RESULTS

Experiment 1

Crossings were measured in groups of rats injected with either saline (1 ml/kg IP) or arecoline before and after 14 days of twice daily forced swim stress at 12°C for 10 minutes (by J.H. and D.T.). Motor behavior was measured after allowing the animals 10 minutes to adapt to the red lighting condition. Saline or arecoline was injected 5 minutes before the animals were placed in the open field. The mean masses of the saline and arecoline samples were 249.7 ± 3.5 (n = 19) and 246.6 ± 6.2 (n = 13) g, respectively. The saline sample crossed 133.1 ± 6.7 and 105.3 ± 14.0 times before and after the course of chronic inescapable swim stress. The arecoline group averaged 122.4 ± 10.9 crossings prior to and 70.6 ± 11.7 crossings following the course of swim stress. The decrement in crossings in the arecoline group was not significantly greater than that in the saline sample.

Visual inspection of the data suggested that an effect of arecoline relative to saline occurred in those rats which crossed ≥ 100 at baseline. One of the arecoline-treated rats exhibited an increase from 107 to 160 crossings following forced stress. This placed this animal >5 standard deviations from the mean change in crossings exhibited by the 5 other rats with ≥ 100 prior to subjection to forced swim. The analysis with the inclusion of this rat did not disclose a difference in the decrement in crossings between the saline and arecoline groups. A reanalysis included animals crossing ≥ 100 times and excluded the outlier.

Experiment 1 (Reanalysis)

Eight (8) saline- and 6 arecoline-treated animals were included in the reanalysis. The former group averaged 139.6 ± 6.6 and 115.0 ± 14.1 crossings before and after being subjected to chronic swim stress. These means do not differ, $t(7) = -1.67$, $p = 0.14$. However, the arecoline group averaged 155.4 ± 8.5 crossings at baseline compared to 53.4 ± 3.3 following the course of swim stress. The change in absolute number of crossings in the arecoline cell is highly significant, $t(4) = -12.65$, $p = 0.0002$. The saline (139.6 ± 6.6) and arecoline (155.4 ± 8.5) groups did not differ in crossings at baseline, $t(11) = 1.47$, $p = 0.17$. Following the two-week course of forced swim stress the saline and arecoline animals exhibited 115.0 ± 14.1 and 53.4 ± 3.3 crossings, respectively. These means differ significantly, $t(11) = -3.37$, $p = 0.0063$. The decrement in crossings in the arecoline group (-102.0 ± 8.1) was 420% greater than that exhibited by the saline control group (-24.6 ± 14.8). This difference is significant, $t(11) = -3.88$, $p = 0.0026$.

The low frequency of animals crossing ≥ 100 times could be due to the limited time the animals were allowed to adapt to the darkness, a mere 10 minutes. This may leave the animals in the process of adaptation rather than actually adapted to the red light condition. Second, the experiment was done by trained medical students who nonetheless had limited experience in handling laboratory animals. There is a substantial stress of mere handling which can alter the behavior of the rat.

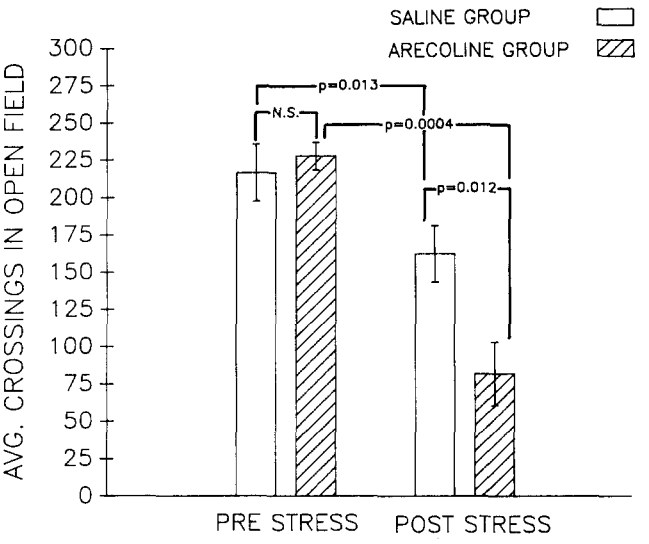

FIG. 1. This is a pictorial presentation of crossings both within and between the saline and arecoline samples before and following a 14-day course of forced swim stress of 10 minutes duration at 12°C.

Experiment 2

Experiment 2 (conducted by the PI) excluded any animal with <100 crossings at baseline (one animal was excluded). The rats used in this particular experiment were allowed one hour to adapt to the red light condition. Once the animals were placed in the "darkroom" they were not removed until the movement of all animals had been scored.

Crossings were measured in rats injected with saline (n = 10, 1 ml/kg IP) or arecoline (n = 9, 0.125 mk/kg IP) between 12:00 p.m. and 3:00 p.m. following at least one hour of adaption to the red light condition. One animal in the arecoline group was excluded due to failure to cross >100 times. The control and arecoline cells exhibited a mean of 227.9 ± 9.3 (n = 10) and 216.8 ± 19.0 (n = 9) crossings at baseline. These means do not differ, $t(17) = -0.54$, $p = 0.59$.

The saline-treated animals exhibited fewer crossings (162.6 ± 21.3 after versus 227.9 ± 9.3 before) following the 14-day course of twice daily swim stress, $t(9) = -3.11$, $p = 0.013$. The arecoline group demonstrated a profound reduction in crossings (82.0 ± 19.0 after versus 216.8 ± 19.0 before) following this 14-day course, $t(8) = -5.8$, $p = 0.0004$. Absolute number of crossings was significantly greater in the saline animals poststress, $t(17) = -2.80$, $p = 0.01$.

Figure 1 pictorially presents the average crossings $\pm$SEM in the control and experimental groups both before and following the 14-day course of twice daily forced cold-water swim stress.

The most important finding was a meager decrease in mean change in crossings in the saline (-65.3 ± 21.0) compared to the arecoline group (-134.8 ± 23.2). This difference was, as hypothesized, significant, $t(17) = -2.22$, $p = 0.04$. The relative difference in the decrement in crossings in the saline and arecoline samples is illustrated in Fig. 2.

DISCUSSION

This series of experiments strongly indicate that the conditions

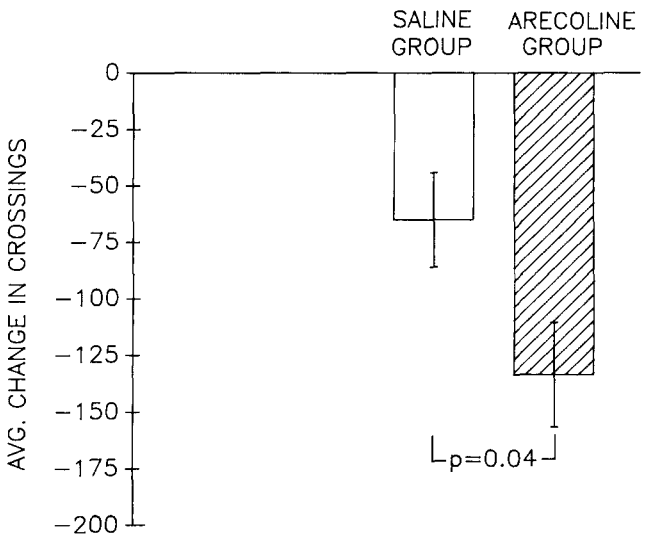

FIG. 2. This figure illustrates the magnitude of the difference in crossings between the saline and arecoline groups following the course of forced swim stress.

under which motor behavior is measured can be critical. First, it is helpful to select rats with a high frequency of crossings (≥100 crossings/5 minutes) at baseline if one desires to demonstrate that forced stress enhances the motoric inhibiting effect of a muscarinic agonist. This may prevent a floor effect. It is sometimes difficult to demonstrate a treatment-induced reduction in a given parameter if the sample or population mean of that parameter is below a threshold. Second, crossings per unit time are greatly increased when the rat is allowed to acclimate to a darkened environment before receiving a pharmacologic challenge. The rat cannot perceive red light (20,21). The use of red light allows the investigator to perform the necessary tasks of handling, weighing, and injecting a drug. Finally, both the experiments presented here and four preliminary experiments in our laboratory conducted over the two-year period prior to performing Experiments 1 and 2 suggest that it is helpful to use a dose of agonist devoid of an inhibitory effect on motor behavior if one's objective is to test the hypothesis that a given experimental manipulation (in this case chronic forced swim stress) renders the rat supersensitive to a given agonist.

The finding that crossings decreased in the animals challenged with saline may seem curious. However, this is expected (26–29). Overstreet *et al.* (22–24) showed that rats bred to maximize sensitivity of central muscarinic mechanisms to diisopropylfluorophosphonate exhibit greater stress-induced immobility when subjected to a single session of forced swim stress relative to rats with "normal" muscarinic cholinergic systems. Swim stress itself enhances sensitivity of central muscarinic mechanisms (6). It is, therefore, expected that the saline-challenged sample would exhibit a decrease in crossings.

Documentation that a two-week course of twice daily cold-water inescapable swim stress greatly decreased crossings in the rats challenged with arecoline relative to saline is the critical finding in this study. This suggests that a chronic forced stressor increases the sensitivity of a muscarinic cholinergic mechanism involved in the regulation of motor behavior to a muscarinic agonist. This finding is consistent with previous reports that multiple chronic forced swim stress protocols (6,7) and inescapable footshock (9) augment the hypothermic response of the rat to oxotremorine. Thus, forced stress enhances the responsiveness to a muscarinic agonist using two endpoints regulated by distinct neuronal pathways. These preclinical findings are consistent with the hypothesis (first advanced by clinical investigators) that chronic stress can activate muscarinicc mechanisms.

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