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Some Behavioral Effects of Suppressing Choline Transport by Cerebroventricular Injection of Hemicholinium-3[1]

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RUSSELL, R. W. and J. MACRI. *Some behavioral effects of suppressing choline transport by cerebroventricular injection of hemicholinium-3.* PHARMAC. BIOCHEM. BEHAV. 8(4) 399–403, 1978. – Evidence supports the proposition that the high affinity Ch uptake system associated with cholinergic nerve terminals can be the rate limiting step in the synthesis of the neurotransmitter, acetylcholine. The present experiment was designed to test the hypothesis that variations of the system would be reflected selectively in changes of conditioned avoidance behavior. HC-3, which primarily affects the high affinity component of Ch transport thus reducing endogenous levels of ACh and the synthetic capacity of cholinergic nerve terminals, was administered cerebroventricularly at 5 doses ranging from 0.0 (saline control) to 10.0 μg. Whole brain ACh levels determined by GCMS analysis following microwave fixation ranged from 25.0 to 5.0 nmol/g^{-1}. Trend analyses demonstrate a precise dose dependent relation between neurochemical and behavioral variables: median trials to condition increased as ACh level decreased. More detailed analyses of the results lead to the interpretation that suppression of high affinity Ch transport in brain is associated with deficiencies in the use of information and not with sensory input and storage nor with motor output.

Hemicholinium-3 Choline transport Conditioned avoidance Drinking Central information processing

DURING the period since the identification of acetylcholine (ACh) as one of the putative neurotransmitters in the CNS many investigations have been designed to test hypotheses about the involvement of cholinergic mechanisms in behavior. Most of these have employed 2 basic types of pharmacological manipulation of the cholinergic system: interference with normal processes at postjunctional receptor sites using cholinominetic or cholinolytic agents and interference with destruction or dissipation of the transmitter using reversible or irreversible anticholinesterases. Recent developments in knowledge and techniques made it possible to manipulate other features of the system, including some involved in ACh synthesis.

Of special interest is the role which choline (Ch), a precursor of ACh and phospholipids, may play as a pacemaker in the synthetic process. Freeman and Jenden [8] have recently reviewed evidence relevant to the hypothesis that the high affinity Ch uptake system associated with cholinergic nerve terminals [9,17] can be the rate limiting step and have concluded that present research results appear to support the proposition. They have suggested that this system "... may also be a mechanism by which the rate of ACh synthesis is regulated in response to demand."

The present experiment was designed to investigate effects on certain behavior patterns of interfering systematically with the transport of Ch in brain, thereby reducing endogenous levels of ACh and the synthetic capacity of cholinergic nerve terminals. Pharmacological manipulation was carried out by cerebroventricular injections of hemicholinium-3 (HC-3), which primarily affects the high affinity component of Ch transport. Effects on behavior in a conditioned avoidance situation were measured both during acquisition and relearning of the conditioned avoidance response (CAR).

METHOD

Animals

The research design required 5 treatment groups: a saline control and 4 experimental groups treated cerebroventricularly (IVT) with different doses of HC-3. A total of 35 Sprague-Dawley male rats were involved. Ten of these were

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used in preliminary studies to standardize the final procedure. Twenty-five were randomly assigned to 5 replications of the research design. All were housed in individual cages in a temperature and humidity controlled room with continuous light. They were maintained on ad lib laboratory chow and on a water schedule required by the research procedure described below. There were no significant differences among groups in body weights, the range of group means during the experimental period being 225–250 g.

Cannulation

Cerebroventricular cannulation was carried out using standard techniques [1, 10, 13]. A cannula guide was inserted freehand through a hole placed 2 mm lateral to the sagittal suture and 1 mm caudal to the coronal suture. Stainless steel, self-tapping screws were placed in 2 holes nearby as means of anchoring the cannula assembly, which was bonded to the skull with dental acrylic resin. The assembly was constructed by milling the hub of a 21 ga stainless steel hypodermic needle to a dia. on to which a rubber plunger cap from a monoject disposable 1 ml tuberculin syringe was fitted. The needle shaft was cut precisely to a length of 2 mm. When in position the self-sealing assembly provided a guide for multiple injections. Pharmacological agents were injected with a 10 μl syringe (Hamilton 701-SN, 26 ga) fitted with a polyethylene sleeve calibrated to place the tip of the needle 2 mm below the internal end of the cannula guide, i.e., cleanly within the lateral ventricle.

Although the technique had been used and cannula placements verified in several of our previous studies, checks were made on a random sample of the present animals. Gentian violet, 5 μl, was injected via the cannula guide in the same manner as HC-3 had been administered earlier. The animals were sacrificed using pentobarbital anesthesia, their brains removed and sectioned along the line of the cannula guide. In all cases staining of the ventricle was clearly visible. Balbian Verster *et al.* [1] earlier reported results of a large number of histological verifications of cannulations using the freehand technique which showed misplacement in less than 2% of the cases.

These authors have also described the distribution of ^{14}C-Ch and ^{3}H-Ch injected via cannulae so implanted. Autoradiographic determinations showed that the radioactivity migrated rapidly, with fairly even distribution on both sides of the brain. There was concentration in such paraventricular structures as the hippocampus, basal ganglia and structures around the 3rd and 4th ventricles. Particularly relevant to the present research is the report by Domino *et al.* [4] of the autoradiographic distribution of ^{14}C-HC-3 following IVT administration in dogs. The ^{14}C-HC-3 was present during a period of 5 min to 4 hr after injection, first along the walls of the ventricles accumulating soon thereafter in the caudate nucleus and hippocampus.

Hemicolinium-3

HC-3 was obtained from Aldrich Chemical Co., Inc. and was recrystallized from absolute ethanol/methanol (1:1) in order to remove possible contaminants [11]. The research design required administration of HC-3 at four dose levels, using saline as a control. The doses were: 0.01, 0.1, 1.0 and 10.0 μg. All were injected in a volume of 2 μl.

Behavioral Measures

Behavior was observed in two test situations. One involved performance of the simple operant response of licking a tube of water under conditions of 23.5 hr water deprivation. On arrival in the laboratory all animals were placed in cages where water could only be obtained through metal drinking tubes, but was available ad lib. Ten days prior to the experimental treatment drinking time was reduced, first to 1 hr per day and 5 days later, to a 30 min test period which continued during the remaining days of the study. Drinking behavior was measured in ml of water intake.

The second test situation provided measures for the acquisition and retention of a discrete trial conditioned avoidance response (CAR). It consisted of an alley 122 cm long, 21 cm deep and 15 cm at its maximum width. The sides of the alley were molded from 2 sheets of stainless steel to provide a U-shaped trough, separated at the bottom and divided into 3 sections. This arrangement permitted 2 kinds of flexibility: when placed in the alley an animal completed an electrical circuit, the shock intensity of which could be varied; division into 3 sections enabled the experimenter to select the end of the straightway to be activated on any particular trial. Clear plastic doors could be rotated to contain an animal in a 20 cm compartment at either end. Rotation upward released the animal, activated a Bell Audiolarm (Model AL 100) with a frequency of 2600 Hz at 68 dB which served as the conditioned stimulus (CS), and started a digital timer. The latter was terminated automatically when the animal entered the compartment at the opposite end of the alley. A timing unit permitted regulation of the onset of the CS and of a shock circuit, which provided the unconditioned stimulus (UCS). In the present experiment the CS sounded 10 sec preceding the UCS and continued until the latter was terminated. A shock control unit was set to deliver 0.5 mA. During acquisition and relearning trials an animal was taken from the home cage, placed in the starting compartment of the straightaway and time unit zeroed. Raising the compartment door started the CS and opened the alley for the animal's response. The mean intertrial interval (ITI) was 20 sec, ample time for the experimenter to transfer an animal to the starting compartment, record the response time and set the apparatus for the following trial. Six consecutive avoidance responses were selected as the criterion for acquisition.

Procedure

The research design was organized as a 5 x 5 latin square, i.e. 5 replications of 5 treatments, balanced so that no treatment appeared more than once in any row or column. One of the latin squares provided by Fisher and Yates [5] was used as a basis for assigning doses to the 25 cells. The assignment was made independently of the experiments in order that the behavioral tests could be administered without their knowledge of the dosage group to which any animal belonged. The coding was not broken until completion of the experiment.

HC-3 was injected IVT 2 hr prior to the start of behavioral testing. Previous studies in our laboratory [7], using microwave fixation [2] and a procedure for assaying ACh content in brain based upon gas chromotagraphy/mass spectrometry [6,12], had provided basic information about

the effect of HC-3 IVT at the dose levels administered in the present experiment. Time-response data indicated that peak effects were attained over a period between 2 and 6 hr after injection.

Behavior in both test situations was observed during this period: measurement of drinking was followed by acquisition of the CAR. The latter was limited to a total of 50 trials. Animals were returned to their home cages after achieving the criterion for acquisition or after the 50 trial limit, which ever came first. Measurements of both behaviors were repeated 24 hr later, when ACh levels in brain had returned to approximately 75% normal [7].

Statistical analyses were carried out using nonparametric methods [3] and following the accepted procedure of testing first by analysis of variance, then by 2-way comparisons when such testing indicated significance at or better than the 0.05 level of confidence. The Kruskal-Wallis statistic was used in analyzing $k \geq 2$ independent samples and the Friedman test in analyzing $k \geq 2$ related samples.

RESULTS

Drinking Behavior

Analyses of drinking behavior showed no significant effect of the experimental treatments. Pretreatment baselines, taken as the means of tests for the 5 days immediately preceding drug administration, did not differ among the 5 groups, H(4) = 4.4166, $p>0.05$. Analysis of variance of intragroup measures during this period also showed no significant trends, H(4) = 2.6382, $p>0.05$.

CAR: Dose-Response Analysis

The relation between the experimental treatments and the median trials to reach the criterion during acquisition of the CAR is shown graphically in Fig. 1. A trend analysis using Spearman's rank order correlation between the medial trials and the various dose levels of HC-3 provided an r = + .90, which is significant at $p = 0.01$.

Kruskal-Wallis analyses of variance showed that there were no significant differences among the treatment groups in times of either escape or avoidance responses (R_e: H(4) = 3.9240, $p>0.05$; R_a: H(4) = 7.1182, $p>0.05$).

Behavior During Conditioning Trials

A more detailed analysis of behavior during conditioning trials revealed a basic feature of individual performances: all control animals were successful in achieving the conditioning criterion within the 50 trial limit; 35% of the HC-3 animals failed to do so, the percentage increasing as dose increased. The Mann-Whitney statistic was used to compare the response times of those who learned with those who did not.

An escape response to an electric shock involves a stimulus-response connection which appears without learning, i.e. is innate. Comparison of the mean R_e times showed no significant difference between animals who conditioned and those who did not (U = 54, $p>0.05$).

In contrast, an avoidance response must be acquired, i.e. is not innate. In this case the mean R_a times for the 2 groupings of animals were significantly different at a

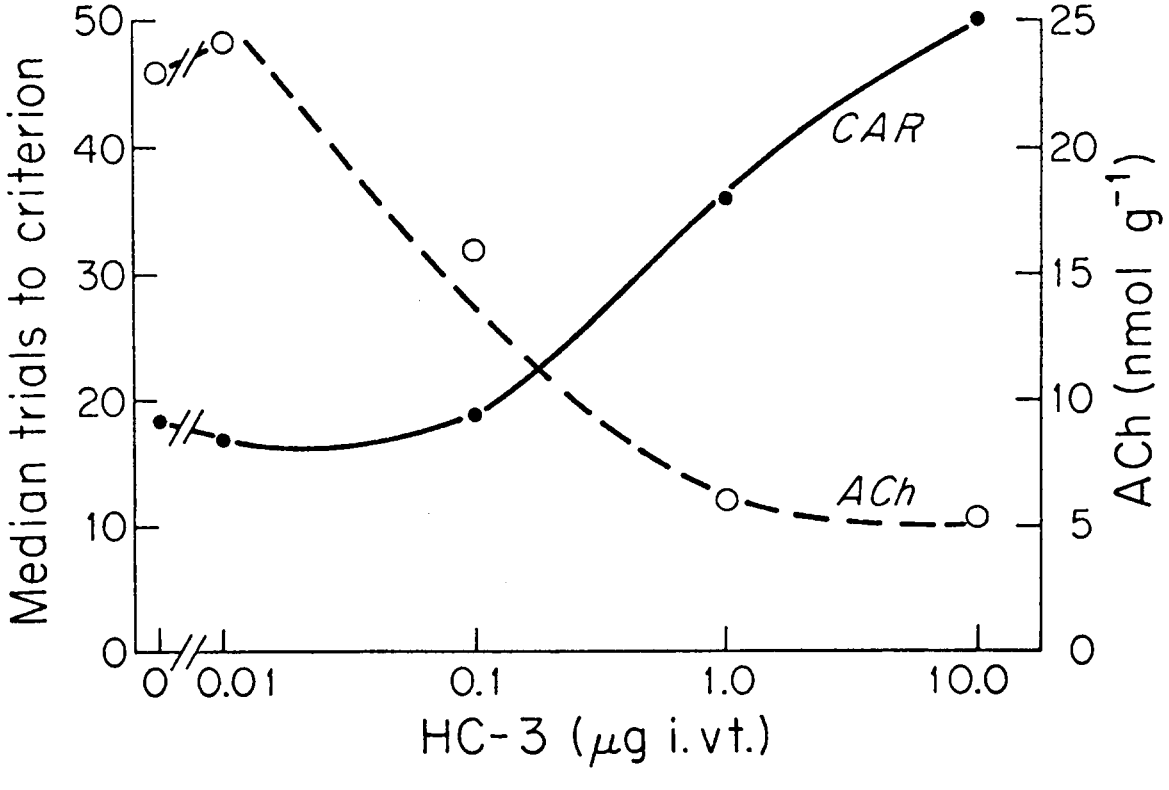

FIG. 1. Dose-response effects of hemicholinium-3 administered cerebroventricularly on acquisition of a conditioned avoidance response and on levels of acetylcholine in whole brain.

$p<0.001$ level of confidence (U = 6). Animals who did not condition responded more slowly to the conditioned auditory stimulus, there being only one case of overlap between the two groupings.

Behavior Without Drug Treatment During Relearning Trials

All animals were given relearning trials 24 hr after their original period of conditioning. No drug treatments had been given since injections the preceding day. Trials to reach the same criterion for learning, i.e. 6 consecutive CARs, varied from 1 to 25 with a mean of 6.25 for the group which had learned on Day 1 and of 11.86 for the group which had not. It is important to emphasize that, although the latter group took significantly more trials than the former to reach the criterion ($U = 27.50; p<0.025$), all animals succeeded. Clearly effects of experience on Day 1 had transferred to Day 2 in both groups.

Classical methods [16] may be used to estimate the transfer effects by calculating the savings in performance on Day 2, following trials on Day 1. The transfer effect may be expressed as follows:

$$\frac{\bar{X}L_1 - \bar{X}N_2}{\bar{X}L_1 - \bar{X}L_2} \times 100$$

where

$\bar{X}L_1$ = mean trials to criterion of animals who learned on Day 1

$\bar{X}L_2$ = mean trials to criterion of these animals on Day 2

$\bar{X}N_2$ = mean trials to criterion on Day 2 of animals who did not learn on Day 1

Applying this analysis to the present data show that savings from 50 trials on Day 1, even when conditioning did not occur, was 57.7%.

Relation Between CAR and ACh Content

Dose-response data for effects of HC-3 on ACh content in whole brain are plotted in Fig. 1. Comparison of the plots for ACh and CAR as shown in the figure suggests a highly inverse relation between the 2 variables. A trend analysis for the HC-3 group using Spearman's rho statistic shows a correlation of -1.00, which is significant at $p<0.01$. These results demonstrate a very precise dose-dependent relation between the neurochemical and behavioral variables in which, as ACh level decreased, median trials to learn increased.

DISCUSSION

Central Mechanisms

The cerebroventricular route for HC-3 administration was chosen in order to involve the brain selectively in the experimental treatment. HC-3 does not readily penetrate the blood-brain barrier when administered by a peripheral route. Evidence presented above indicates that, by IVT injection, HC-3 reaches the walls of the ventricles and accumulates in the caudate nucleus and the hippocampus [4]. Furthermore, results of experiments designed to study the intracellular distribution of ^{14}C-HC-3 suggest that it becomes associated with nerve endings in both these neuroanatomical regions [14]. Effects of the doses given cover a range of ACh levels in whole brain from normal to 20% of normal when the rate of decrease approaches an asymptote [7]. Peak effects are obtained at 2 hr after injection, the time at which behavioral measurements were made in the present study.

Present results, when analyzed as relations between ACh levels and parameters of behavioral variables, indicate that efficiency of behavior decreased as availability of ACh decreased. The latter was, however, a consequence of changes at an earlier step in ACh synthesis. The locus of action of HC-3 in the cholinergic system is predominantly in the high affinity transport of Ch. Under conditions identical with those of the present experiment, total endogenous Ch levels as determined after microwave fixation were not affected by HC-3 [7] and, therefore, not related to the systematic changes in behavior observed in this study.

Such changes can be seen as consequences of a specific series of dynamic events initiated by the experimental treatment: suppression of the high affinity transport of Ch resulting in reduction of ACh synthesis and, hence, a decrease in the availability of the transmitter for normal neural activity. It also appears reasonable to hypothesize that these events occurred within neurons in one or both of two major neuroanatomical sites: the caudate nucleus and the hippocampus. Of the two the hippocampus has already been implicated as being significantly involved in behaviors related to those studied in the present experiments.

Analysis of Behavior

The results also provide some groups upon which to differentiate among the several hypotheses which have been proposed to encompass empirical observations about interactions between the cholinergic system and behavior. The literature relevant to these hypotheses is vast, but has recently been succinctly summarized [15] in three main categories: response inhibition, stimulus control and stimulus selection. There are results in research reports to support hypotheses of all these kinds, yet no single hypothesis is adequate to account for all the evidence.

Analysis of the responses measured suggests that the behavioral effects observed were not likely to be related to differences in motivational states. Responses, R_e, to shock, the CS, did not differ significantly between animals who conditioned during the period of HC-3 treatment and those who did not. Furthermore, measures of drinking behavior, motivated by water deprivation and independent of the CAR conditioning, also showed no significant dose-related trends and did not differ from pretreatment baselines. Thus, neither changes in the specific motivational state involved nor generalized changes in motivation appear to underlie the behavioral effects of HC-3.

The possibility should be considered that behavioral effects observed in the present study could be accounted for by changes induced outside the CNS, i.e. peripheral sensory or motor effects. Because of the central route of administration, the small doses of HC-3 injected and the properties of the agent vis-a-vis the blood-brain barrier, this possibility seems very unlikely. However, there are behavioral data from the experiment which, although not eliminating the hypothesis completely, make it less tenable. First, there were no significant differences among dosage groups in locomotor capabilities as measured by their

escape responses. Second, the very considerable transfer effect to performance on Day 2, which was evident in animals from all dosage groups who did not learn on Day 1, can only be interpreted as indicating that there was information input to the CNS during HC-3 trials which was being stored in a form that could be used later.

Thus, the results of the present experiment support the hypothesis that events affected by suppressing high affinity Ch transport in brain influence the central processing of information input in a selective manner. Incoming information appears to arrive and to be stored; whether in a fully normal manner cannot be judged from the present results. While the cholinergic system is not functioning adequately deficiencies seem to occur in the use to which the information is put.

A brief word of caution is needed in concluding this discussion. It is all too easy to become impressed with effects which manipulating one neurotransmitter system may have on particular observations of behavior. But it is essential to keep in mind that the reactions of a living organism within its biosphere involve the integration of a multitude of events occurring in many bodily sites. Although the present results implicate the cholinergic system in certain behaviors, it must be the case that other events, even within the CNS, are also involved.

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