Biochemical blockade of cholinergic thirst[1]

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The effect of several biochemical agents on thirst induced by cholinergic brain stimulation was studied both (a) when the test chemical was placed in a different site from that receiving carbachol (a cholinergic stimulant) and (b) when the test chemical was used to pretreat the same site that was stimulated with carbachol. Only atropine and scopolamine (antimuscarinic agents) consistently blocked cholinergic thirst. Atropine blocked more consistently when placed at a different site from the carbachol, while scopolamine blocked consistently both at a different and at the same site as the carbachol. Considering all the chemicals, even those which blocked drinking on only a percentage of the trials, the chemicals were more likely to block drinking when placed at a different site than when placed at the same site as the carbachol.

Cholinergic stimulation of several limbic-system/diencephalic structures in the rat brain has been found to induce water ingestion (Fisher & Coury, 1962). Stein & Seifter (1962) have reported a depression of cholinergic drinking with atropine pretreatment of the same site that received cholinergic stimulation. Levitt & Fisher (1966) found that the application of atropine to any "cholinergic drinking site" blocked cholinergic induction of thirst from other limbic-diencephalic structures.

These results suggest the possibility that a group of structures forming a circuit which is normally activated by the synaptic transmitter, acetylcholine, may be the basis for the cholinergic triggering of thirst. Further, anticholinergic stimulation may inhibit the cholinergic induction of thirst by blocking or otherwise disrupting functional transmission in a part of the circuit, and thus, shutting down reverberatory firing patterns responsible for the maintenance of ingestive behavior. One way of testing this model would be to determine the effect of a variety of chemical agents with known pharmacological properties on cholinergic thirst.

In the present experiment the effect of 13 chemical agents on thirst induced by cholinergic brain stimulation is reported, both (a) when the test chemical was placed at a different site from that receiving carbachol (a cholinergic stimulant) and (b) when the test chemical was used to pretreat the same site that was stimulated with carbachol.

METHOD

Data presented are from 94 adult male hooded rats surgically implanted under pentobarbital anesthesia with two hollow guide shafts aimed at one of the following combinations of structures. Data are included from 16 Ss with implants in lateral septal nucleus (LSN) and anterior thalamic nucleus (ATN); 20 implanted in LSN and dorsal fornix (FX); 13 implanted in LSN and anterior medial midbrain (AMM); 10 in FX and AMM; 9 in ATN and AMM; 16 in LSN and contralateral LSN; and 10 in ATN and contralateral ATN (Pellegrino & Cushman, 1968).

The brain implant (guide shaft) consisted of a 23-gg stainless steel cannula encased in a metal holder. The holder was threaded to receive a connector assembly which consisted of a 30-gg stainless steel cannula held by a set screw to an assembly which fitted onto the guide shaft. The guide shaft was cut to end 0.5 mm above the most dorsal extent of the intended structure. The 30-gg cannula could be set using the set screw at any length desired. It was initially set to end even with the dorsal extent of the structure. If a positive drinking response (defined as 4.0 ml or more water consumed during 1 h immediately following stimulation) was not obtained, the cannula was lowered through the structure in 0.5-mm steps in an attempt to induce drinking.

The animals were housed individually and had ad lib access to Purina Lab Chow and tap water in their home cages throughout the experiments. After recovering from surgery (at least 4 days), the Ss were tested two to three times weekly in a test cage containing a calibrated drinking tube (food was not present in the test cage). During the first hour in the test cage (pretest) water intake was measured. At the beginning of the second hour Ss received brain stimulation with carbachol alone or in combination with another chemical, and water intake was recorded during the hour immediately following chemical brain stimulation.

Initial tests in each animal determined whether S drank to carbachol in one or both sites. Following these tests, a trial was given during which S received carbachol combined with another chemical placed either in the same site or in another positive site. When the test chemical was placed in another site, it was always injected first, followed by carbachol within a minute or two. In cases where the test chemical was lowered into the same site that received the carbachol, the test chemical was placed into the brain, then 5 min later its cannula was removed, checked to insure the crystals had dissolved, and then another cannula containing carbachol was lowered into the same site.

In cases where two sites were stimulated, the data were used only when it was found through retesting that carbachol would still elicit thirst from each site following the double stimulation trial. In cases where a single site was stimulated with both chemicals, if the animal drank, the data were used. If S did not drink, the data were used only if S drank to carbachol alone on the next trial following the double stimulation. These control procedures reduced the likelihood of blocking data being due simply to one or both structures having lost the capacity to elicit cholinergic drinking. It has been found that following the elicitation of drinking to carbachol, the S will drink to carbachol on the next trial only about 70 to 85% of the time.

The cholinergic stimulant used was carbachol (choline chlorine carbamate). The chemicals tested for their ability to inhibit the cholinergic induction of thirst (test chemicals) are listed in Table 1. All chemicals were administered in only one dose, four tamps of the crystalline chemical into the tip of a 30-gg cannula. This technique delivers about 2 to 3 micrograms of chemical.

Histological verification of the intended placements was made for most Ss.

RESULTS

The number of combined stimulation tests varied from animal to animal. Order effects were also not controlled. Each drug was tested until enough trials were obtained to determine if it blocked cholinergic thirst as consistently as atropine had in an earlier study (Levitt & Fisher, 1966). In this earlier study atropine blocked cholinergic thirst without regard to the particular structure receiving either the carbachol or the atropine. The only critical variable was that both chemicals were placed in positive drinking sites. Atropine also blocked drinking without regard to the number of previous stimulations the animal had received. In the present study no effect (a) of the particular structure or combination of structures stimulated or (b) of the order of testing the chemicals was evident on the blocking of cholinergic thirst.

Drinking scores during the 1-h pretests averaged 1.0 ml (range 0.0-2.9 ml). The mean water intake during 24 1-h tests, during which an empty cannula was lowered into a positive drinking site, was 1.1 ml.

Rather than treating water intake as a continuous variable, it has been found useful to consider carbachol-induced drinking on a test trial as a dichotomy. If S drinks 4.0 ml or more, it is scored as having been induced to drink (no blockade). If S drinks 3.9 ml or less, it is scored as not having been induced to drink (blockade). Table 2 presents data

Table 1
The Inhibition of Carbachol-Induced Thirst by Pharmacological Agents

Test Chemicals	Number of Instances and Percentage of Blockade and Failure to Block with Blocking Chemical at: Different Site Block/No Block	% Block	Same Site Block/No Block	% Block
Atropine Sulfate (muscarinic blocking agent)	101/4	96%	11/7	61%
Scopolamine Hydrobromide (muscarinic blocking agent)	12/0	100	9/0	100
Dihydro-B-Erythroidine (neuro-muscular blocking agent)	6/15	28	1/15	6
Hexamethonium Chloride (ganglionic blocking agent)	6/18	25	3/11	21
Acetylcholinesterase (enzyme which deactivates acetylcholine)	8/12	40	3/15	17
Hemicholinium-3 (inhibitor of acetylcholine synthesis)	9/13	41	8/16	33
Phenoxybenzamine Hydrochloride (adrenergic blocking agent)	12/25	32	0/9	0
Pentobarbital Sodium (general anesthetic)	9/18	33	4/6	40
Procaine Hydrochloride (local anesthetic)	15/26	36	4/16	20
Sodium Chloride ("non-specific" stimulant)	0/8	0	2/9	18
Potassium Chloride (agent used to produce "Spreading Cortical Depression")	3/18	14	2/10	17
Strychnine Sulfate (central nervous system stimulant)	16/15	52	0/10	0
Picrotoxin (central nervous system stimulant)	6/10	38	3/6	33

Table 2
Water Intake Following Cholinergic Stimulation Alone or in Combination with a Test Chemical

Structure Receiving Carbachol	Carbachol Alone	Carbachol in Combination with a Test Chemical in A Different Site Block	No Block	The Same Site Block	No Block
LSN	13.2 ml	1.0 ml	14.6 ml	2.9 ml	13.2 ml
ATN	13.7	1.6	12.0	1.3	12.9
FX	13.3	1.5	12.6	1.4	16.3
AMM	12.9	1.5	12.4	1.1	13.2

on water intake following cholinergic stimulation alone or in combination with a test chemical. When this dichotomy is used, water intake on test trials that S drinks at least 4.0 ml is very similar to drinking scores to carbachol alone. Also, drinking scores on trials during which S drinks less than 4.0 ml (the test chemical is said to have "blocked" cholinergic thirst) are quite similar to pretest or empty cannula scores.

Table 1 presents the data on the blockade of cholinergic thirst by the various chemicals used. The data on the effect of atropine when placed in a different site from the carbachol are from an earlier study (Levitt & Fisher, 1966). There is a significant tendency for the chemicals (all the chemicals considered as a whole) to block more frequently when placed at a different site than when placed at the same site as the carbachol (chi square = 9.8, $p < .01$, df = 1).

A recent study examined the possibility that some percentage of blockade might be due to "random" or uncontrolled factors (Levitt & Krikstone, 1968). A 16% blockade score was obtained in the absence of any treatment during the test trial. A chi square test was made between the blockade effectiveness of each pair of test chemicals if the total number of observations in the comparison was at least 40 or, if between 20 and 40, the minimum expected cell frequency was five. If the chi square test was not appropriate because of too small a number of observations in the comparison, the Fisher Exact Probability Test was used. Differences at the .05 level or greater were considered statistically significant. Scopolamine when placed either in the same or a different site from the carbachol and atropine when placed in a different site blocked from 96 to 100% of the time and these percentages were significantly higher than any of the other conditions. Atropine in the same site as the carbachol and strychnine in a different site blocked less reliably than the first three treatments, but more reliably than the "random block" or the other treatments that blocked between 0 and 25% of the time. None of the other treatments significantly differed from each other or from the random-block condition.

DISCUSSION

It is rather interesting that the various test chemicals, considered as a whole, blocked cholinergic thirst more consistently when placed at a site different from the carbachol than when the two chemicals were placed at the same site. This result is consistent with the cholinergic thirst circuit hypothesis since when placed at a different site, the test chemical would have to inhibit, block, or otherwise disturb the action of the naturally occurring acetylcholine (perhaps secreted as a result of input from the cholinergically stimulated site). In contrast, when the test chemical is placed at the same site it would have to block the carbachol artificially present at that site in addition to any naturally occurring acetylcholine.

A purpose of this experiment was to determine if there were differences in the actions of the various sites from which cholinergic thirst is elicitable. In this study no difference in the blockade of cholinergic thirst by the various chemicals used could be attributed to the particular structure or combination of structures tested.

Several major faults which minimize any detailed consideration of the apparent lack of blockade by most of the tested chemicals are (a) the lack of any dose-response data, (b) the relatively small number of trials for each condition, and (c) the post hoc nature of any attempt to explain the data. However, the finding that antimuscarinic agents, but no others, consistently blocked cholinergic thirst is striking and of major interest. Atropine seems to be less potent than scopolamine in the blockade of cholinergic thirst when carbachol is present at the same site. These data are consistent with the findings that atropine is a less potent antimuscarinic agent in other physiological systems (Cullumbine, 1967). The finding that atropine and scopolamine (antimuscarinic agents), but neither a ganglionic blocking agent nor a neuromuscular blocking agent, significantly inhibited cholinergic thirst is consistent with the conclusion of Stein & Seifter (1962) that the cholinergic thirst system is a muscarinic one.

The lack of blockade by several chemicals that might have been expected to block cholinergic thirst, if the cholinergic thirst circuit model were correct, may be given some import, especially acetylcholinesterase and also hemicholinium-3. If the antimuscarinic blockade were equivalent to a

"temporary functional lesion," then procaine also should have blocked cholinergic thirst. This local anesthetic agent has been shown to mimic lesion effects in the hypothalamus (Epstein, 1960). However, many of these agents surely were not administered at an optimum dose; the dose used may not even have been within the range of effective doses for a number of compounds. Any final answer to the question of effectiveness of blockade or the selectivity of blockade must await the proper dose-response data and a comparison of the effects of these agents on cholinergic thirst with their effects on other relevant behaviors.

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NOTE

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The toxic effects of three different dosages of pentobarbital sodium on the Long-Evans rat

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Male Long-Evans rats were statistically superior to females in recovering from intraperitoneal injections of pentobarbital sodium. The data also provide support for a stock difference previously reported.

An earlier paper (Collins & Lott, 1968) reported a difference in tolerance level between Long-Evans and Wistar rats following intraperitoneal injections of pentobarbital sodium. A sex by strain comparison revealed that the males were more resistant than the females of their respective strains which confirmed earlier findings (Holck & Kannan, 1934; Homburger, Etsten, & Himwich, 1947). The Long-Evans stock was more resistant than the Wistar. The experiment has been replicated using only Long-Evans animals to equalize the number of males and females and to provide a larger sample of this stock to control for the variability often found in drug research.

METHOD

The Ss were 180 Long-Evans rats, 90 male and 90 female, which were derived from stock purchased from Simonsen Laboratories, Gilroy, California, approximately 2-3 years ago. This is the same source as for the animals in the first experiment.

The Ss' ages ranged from 3 to 13 months and the mean age was 7 months. None of the Ss had received anesthetic prior to the experiment. They were maintained on an ad lib diet of Purina rat chow and water. The Ss were housed in one of three conditions: individually, in groups of 3 to 6, and in groups of 7 to 14. No difference in the Ss' responses to the drug was correlated with age or housing group.

The Ss were randomly assigned to one of three dosage groups, weighed, and injected intraperitoneally with 48, 72, or 96 mg/kg of pentobarbital sodium, 60 mg/ml in a solution of 10% alcohol and 20% propylene glycol (Diabutal, Diamond Laboratories, Des Moines, Iowa). Postinjection, the Ss were placed in individual cages.

The effect of the anesthetic was determined by lightly touching the cornea with a blunt instrument and by tightly clamping a hemostat on one of the hind paws. Recovery (or failure to recover) following the anesthetic was recorded.

RESULTS

No S in any group responded by blinking when touched on the cornea or withdrew its leg when its paw was squeezed. The absence of these reflexes indicates that a satisfactory state of anesthesia was reached by all Ss.

The recover (no-recover) data by sex and dosage group are shown in Table 1. The percentage of Ss failing to recover for that sex and group is shown in parentheses in the table. When these data are examined by means of the chi square test, no significant difference exists between males and females at the 48 mg/kg dosage level ($p > .10$). There is, however, a statistically significant difference between males and females at the 72 mg/kg ($p < .001$) and 96 mg/kg ($p < .001$) levels with more males recovering than females.

DISCUSSION

The results of this study are similar to those reported earlier for the male Long-Evans rat. The only discrepancy is that 23% of males in the 96 mg/kg group failed to recover compared with 33% in this study.

There were large differences between the percentages of Long-Evans females recovering as reported earlier and these findings. Nineteen female Ss were used in the first study. The 48 and 72 mg/kg groups each had six, all of whom recovered. There were seven females in the 96 mg/kg group and three failed to recover. The larger number of Ss in the present study presumably yields a more realistic picture of the Long-Evans stock's normal response to pentobarbital sodium.

These findings support the stock difference in the response of Wistar and Long-Evans animals reported earlier.

Table 1
Mortality of Long-Evans Rats Following Injections of Graded Doses of Pentobarbital Sodium

Sex	Mean Weight (in g)	48 mg/kg	72 mg/kg	96 mg/kg
Male	423	29 (1) (3.33%)+	29 (1)* (3.33%)	20 (10)* (33.33%)
Female	235	25 (5) (16.67%)	13 (17) (56.67%)	7 (23) (76.67%)

+ Lived/(Died) (% Died)
** $p < .001$*

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