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Neural Activity Changes Correlated with Central Anticholinergic Blockade of Cholinergically-induced Drinking[1]

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SNYDER, J. J. AND R. A. LEVITT. *Neural activity changes correlated with central anticholinergic blockade of cholinergically-induced drinking.* PHARMAC. BIOCHEM. BEHAV. 3(1) 75–79, 1975. – In the rat, microinjections of carbachol into the septal area elicited water ingestion and increased multiple unit activity at this site and also the noninjected lateral hypothalamus. Carbachol injection into the lateral hypothalamus also elicited water ingestion, but multiple unit activity did not increase in this structure, although it did in the noninjected septal area. If carbachol was injected into one of these sites and isotonic saline into the other (conditions comparable to those for which drinking has been previously demonstrated), increased multiple unit activity was still found. However, if carbachol was injected into one of these sites and atropine into the other (conditions comparable to those for which the blockade of drinking has been previously demonstrated), the increases in multiple unit activity were blocked. Carbachol-elicited drinking may result from neural activity changes similar to those recorded in this study, and atropine may inhibit carbachol-elicited drinking by inhibiting such neural firing changes.

Thirst Drinking Multiple unit activity Carbachol Atropine

MICROINJECTIONS of cholinergic drugs into limbic system or hypothalamic sites in the rat have been shown to elicit drinking [3, 6, 7, 10]. Injections of anticholinergic drugs at these sites have been shown to block the drinking response to cholinergic stimulation [8,11]. These data have led to the hypothesis that, in the rat, the neurobiological substrate for water ingestion may consist, in part, of a limbic system circuit that utilizes acetylcholine as its synaptic transmitter [3, 9, 13]. It has been further postulated that cholinergic stimulation elicits drinking by having an excitatory effect on the proposed circuit, and that atropine blocks this effect by causing a temporary inhibition of neural activity in the circuit [9,20].

More recent data have raised a number of questions about the substrate for cholinergically-elicited drinking. There is evidence that when drug solutions are exogenously administered, they may diffuse from the site of injection via axoplasmic flow or via the ventricular system [4, 16, 17]. Thus it may be that the loci at which cholinergic drugs affect water ingestion are not those at which the drugs are injected. Stein and Levitt [20] suggested that if anticholinergic drugs block cholinergic-drinking by inhibiting neural activity at the site of application, then small lesions at positive cholinergic-drinking sites should duplicate the blockade effect. However, they found that small lesions at positive cholinergic-drinking sites in the limbic system did not produce a suppression comparable to that produced by atropine. Therefore, the neurophysiological events mediating cholinergic elicitation and anticholinergic blockade may not be those suggested by the cholinergic circuit hypothesis.

Potential answers about the location and direction of changes in neural activity produced by cholinergic and anticholinergic stimulation may be found by recording neural activity at sites where cholinergic and anticholinergic drugs affect drinking. Grossman [8] found no changes in EEG following cholinergic stimulation of effective drinking sites, and MacPhail [12] found sinusoidal slow waves in the hippocampus, but no change in cortical or amygdalar sites following cholinergic stimulation. A change or lack of change in EEG, however, is not interpretable as neuronal excitation or inhibition.

Changes in hypothalamic single unit activity following electrophoretic application of cholinergic drugs have also been analyzed [14]. Acetylcholine caused an increase in the firing rate of most of the neurons sampled, atropine decreased the firing, and atropine applied simultaneously with acetylcholine eliminated the increases normally

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elicited by acetylcholine. Although these neural changes are consonant with those predicted by the cholinergic circuit hypothesis, no correlative behavioral data were presented.

The recording of multiple unit activity (MUA) allows direct observation of neural excitation and inhibition in cell populations large enough to be meaningfully related to behavioral events [1]. Buerger *et al.* [2] investigated changes in MUA following injections of carbachol, saline or atropine. Carbachol elicited increases in MUA at the stimulated and contralateral homologous sites. Furthermore, the time course of the MUA increase was similar to the time course of carbachol-elicited drinking. Neither atropine nor saline alone had an effect on the baseline level of neural activity. Although these data are only correlational, carbachol increases neural activity in limbic system sites at which it elicits drinking, and the drinking may be related to this increase.

The present experiments attempted to extend the work of Buerger *et al.* [2]. Other neural structure combinations were sampled, and the effect of atropine on the carbachol-elicited increase in MUA was studied. Specifically, the experiments attempted to answer the following questions: (a) Does carbachol produce changes in MUA at neural sited where drinking is elicited by this drug? (b) Do the changes in MUA at the site of carbachol stimulation differ from those at contralateral non-homologous drinking sites? (c) Does an injection of atropine at a positive cholinergic site affect the change in MUA produced by carbachol injected at a contralateral non-homologous site? (d) Is the effect of atropine on MUA following carbachol stimulation different from that of a control injection of isotonic saline?

METHOD

Animals

The animals were 54 adult male Long-Evans rats, weighing from 300 to 400 g at the beginning of the experiment. Rats were individually housed with Purina lab pellets and water constantly available, except during drinking tests, when only water was available. A normal 12 hours-light, 12 hours-dark light-dark cycle was maintained and all experimental procedures occured during the daylight hours.

Surgery

Two hollow guide shafts, made from 18 ga hypodermic needles, were stereotaxically implanted under pentobarbital anesthesia (50 mg/kg). The implants were aimed either at the right or left lateral septal nucleus (LSN: AP 7.8, L .80, D +2.0) and the contralateral lateral hypothalamic area (LHA: AP 5.4, L 1.5, D −2.5) [15]. Due to their size, the guide shafts were lowered only 2 mm below the top of the skull to minimize the destruction of brain tissue.

Drugs

The drugs used for intracranial stimulation were carbachol (CARB; choline chlorine carbamate, a cholinomimetic), atropine sulfate (AT; an antimuscarinic cholinergic blocking agent), and sterile isotonic saline (NS). Both the carbachol and the atropine were dissolved in isotonic saline in a concentration of 2.0 μg/μl.

Apparatus and Procedure

After a minimum of 5 days following surgery, the animals were anesthetized with pentobarbital (55 mg/kg), and were placed inside an electrically shielded enclosure. Two 30 ga cannulae, each separately insulated with epoxylite except for their tips and then joined with another coat of epoxylite, were then lowered through each of the previously implanted guide shafts. The cannulae were set to reach the previously mentioned dorsal-ventral coordinates. A short length of PE 10 polyethylene tubing was connected to one of the 30 ga cannulae at each site, and attached to a 702 Hamilton microsyringe operated by a Hamilton PB-600-1 repeating dispenser, set to deliver 1 μl of solution. Each of the injection systems was prefilled with one of three solutions: (a) carbachol (2.0 μg/μl); (b) atropine sulfate (2.0 μg/μl); (c) sterile isotonic saline; or left empty. These doses of carbachol and atropine are comparable to those shown to be effective in previous studies of the elicitation of drinking by carbachol and its blockade by atropine [9,11].

Recordings of MUA were obtained from both brain sites using the two 30 ga cannulae at each site as bipolar electrodes. Activity from each brain site was carried to a Grass P-15 a.c. preamplifier by two shielded wires, with a 100 ohm resistor in series with one of the wires. The preamplifier was adjusted for a 1/2 amplitude band pass of 300–3000 Hz and an amplification factor of 100. The output of the P-15 was fed to an a.c. combination preamplifier and activity integrator (Grass 7P3B), a d.c. driver amplifier (Grass 7DAE) and was displayed by an ink writing oscillograph (Grass 7WC16PA). The preamplifier's time constant was set at 0.2, its threshold at 4, its sensitivity at maximum, and its rectification on full wave. The driver amplifier's sensitivity was set at maximum, its 1/2 amp high frequency at 15, and its 60 Hz filter engaged.

Before the recording period began, each channel was calibrated by introducing a 1000 Hz sine wave of a known amplitude (between 1 and 10 μV) across the 100 ohm resistor. After 15 min of baseline recording, the experimenter operated the drug dispenser for each site. Recording then continued for another 30 min. At the end of the post-stimulation recording period, each channel was recalibrated to verify the stability of the recording.

After a 5 day recovery period, the animal was tested for the behavioral elicitation of drinking by carbachol (2.0 μg in 1.0 μl) at one site, and after another 5 days, at the other site (in counterbalanced order). If the animal drank at least 4.0 ml more during the 30 min test period than during the preceding 30 min control period, the stimulation site was considered positive.

Histology

After completion of the experiment, the brain of each rat was perfused, frozen sections were stained with cresyl violet, and stimulation and recording loci were verified.

RESULTS

The data presented are from 54 animals (9 per group) that drank to stimulation with carbachol at both the LSN and the LHA during the behavioral tests that followed MUA recording. Data from 48 animals were discarded because of early death, plugged guide shafts, or negative testing to carbachol at one or both of the drinking sites.

There were no significant differences in the volume of water consumed following stimulation of the LSN or LHA

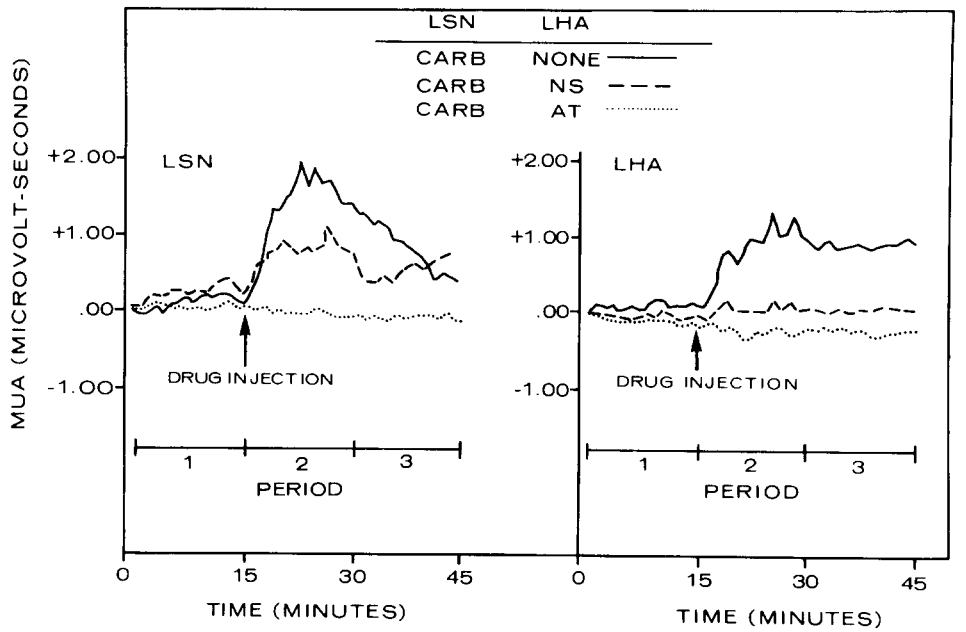

FIG. 1. Chemical brain stimulation and MUA: Carbachol in the LSN. At the beginning of Period 2 carbachol (CARB) was injected into the LSN and either no injection was made into the LHA (NONE), or isotonic sodium chloride (NS) or atropine (AT) was injected into the LHA. The data presented are mean levels of MUA for each group (see text for details).

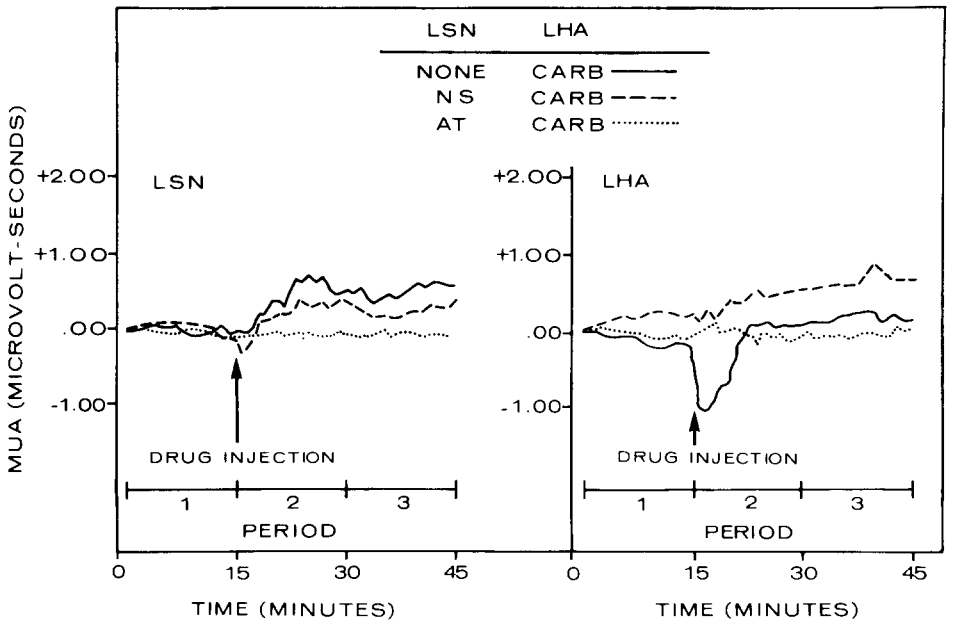

FIG. 2. Chemical brain stimulation and MUA: Carbachol in the LHA. At the beginning of Period 2 carbachol was injected into the LHA and either no injection was made into the LSN, or isotonic sodium chloride or atropine was injected into the LSN. The data presented are mean levels of MUA for each group (see text for details, and the legend to Fig. 1).

on the behavioral tests for any of the groups. Group water intake averages ranged between 5.1 and 9.2 ml (average of 7.2 ml).

The minimal level of activity during each minute of the recording was referenced to the first minute (which was arbitrarily set at zero), and the difference was quantified in microvolt-seconds by comparison to the calibration signal. Frequently, a large transient change in voltage occured during injection of the solutions. Activity from this short period (less than 5 sec) was not included in the data analysis.

The recording session was divided into three 15 min time blocks (Periods 1, 2, and 3; Figs. 1 and 2), and a mean level of MUA was computed for each time block to facilitate

statistical analysis. Comparisons were then made between the mean levels of MUA recorded during each time block using an analysis of variance and the Tukey HSD test for post-anova comparisons [5]. Figures 1 and 2 show the mean level of MUA for each minute of the 45 min recording sessions for each drug treatment group and each recording site. During the 15 prestimulation minutes (Period 1), the level of MUA was quite stable.

When CARB was injected into 1 of the 2 recording sites, in general, MUA was increased (compared to Period 1) during all or part of the 30 minute post-drug recording (Period 2 = first 15 min postdrug; Period 3 = second 15 min postdrug). The increase in MUA produced by CARB was significant during both Periods 2 and 3 when CARB was injected into the LSN and activity was recorded from the LSN ($F = 8.27$, $p<0.01$; $F = 4.49$, $p<0.05$) or from the LHA ($F = 5.91$, $p<0.01$; $F = 6.46$, $p<0.01$). Similarly, when CARB was injected into the LHA, the level of MUA was greater during both Periods 2 and 3 than Period 1 when recording from the LSN ($F = 3.86$, $p<0.05$; $F = 5.67$, $p<0.01$), but not when recording from the LHA.

When CARB was injected into the LSN and NS into the LHA, the level of MUA recorded from the LSN during Periods 2 and 3 did not differ from Period 1, but the level of MUA recorded from the LHA during Period 2 (but not Period 3) was greater than that during Period 1 ($F = 3.78$, $p<0.05$). For the group stimulated with CARB in the LHA and NS in the LSN, the MUA level was higher during Period 2 (but not 3) than during Period 1 when recording from the LSN ($F = 3.70$, $p<0.05$), but there was no difference in the level of MUA recorded during any of the periods from the LHA.

The increased MUA sometimes found during Periods 2 and 3 when CARB was injected into the LSN or the LHA, or when CARB was injected into one of these sites and NS into the other, was not found when the combination of CARB and AT was used.

Another way of evaluating these data is by comparing the drug treatment groups to each other. During Period 1 there was no difference in the level of MUA between any of the drug treatment groups. In contrast, during Periods 2 and 3, several of the drug treatment groups did differ from each other in MUA. When CARB was injected into the LSN and recordings were made from the LSN, all 3 treatment groups differed in MUA during Period 2 (CARB vs NS: $F = 4.02$, $p<0.05$; CARB vs AT: $F = 9.61$, $p<0.01$; NS vs AT: $F = 5.67$, $p<0.01$). During Period 3 the CARB and NS groups differed from the AT group ($F = 5.43$, $p<0.01$; $F = 4.57$, $p<0.05$), but these two groups did not differ from each other (CARB vs NS). For these same treatment groups (CARB in the LSN), MUA in the LHA during both Periods 2 and 3 differed for the CARB vs NS and the CARB vs AT comparisons (Period 2 – CARB vs NS: $F = 6.69$, $p<0.01$; CARB vs AT: $F = 8.90$, $p<0.01$; Period 3 – CARB vs NS: $F = 7.08$, $p<0.01$; CARB vs AT: $F = 9.67$, $p<0.01$), but the NS and AT groups were not different from each other.

When CARB was injected into the LHA and recordings were also made from the LHA, during Period 2 the CARB group differed from the NS group ($F = 5.35$, $p<0.01$) and the NS and AT groups also differed ($F = 3.62$, $p<0.05$), but the CARB and AT groups did not differ from each other. During Period 3 (with CARB injections into the LHA and recordings from the LHA) the NS and AT groups differed n MUA ($F = 3.78$, $p<0.05$), but the CARB group did not differ from the NS or AT groups. For these same treatment groups (CARB in the LHA), MUA in the LSN during Period 2 differed between the CARB and AT groups ($F = 4.96$, $p<0.01$), but not between the NS and either the CARB or AT groups. During Period 3 for these same conditions, the AT group differed in MUA from both the CARB ($F = 6.69$, $p<0.01$) and NS ($F = 4.17$, $p<0.05$) groups, but the CARB and NS groups did not differ from each other.

Data were also collected from those animals that drank to carbachol injections at only the LSN, only the LHA, or at neither site. Comparisons were made between the mean levels of MUA recorded during each time block for all three of these groups. Comparisons were also made between these groups and those that drank to carbachol at both sites [19]. These data are equivocal; group sizes are low; however, approximately half of the comparisons are consistent with the main data, half are not.

Histology

Tables showing the stimulation and recording sites are available elsewhere [19]. The LSN placements were between 7.0 and 7.8 mm AP, .25 and .75 mm L, and between +2.0 and +2.75 mm in depth. These placements were located in the dorsal portion of the LSN. The LHA placements were between 4.8 and 5.4 mm AP, 1.25 and 1.75 mm L, and between −2.0 and −3.0 mm in depth [15]. Most of the LHA placements were just lateral to the fornix and ventral to the zona incerta.

DISCUSSION

Brain loci where carbachol elicits drinking also usually show increased MUA after cholinergic stimulation. Increases in MUA are found in both the carbachol injected sites and in contralateral nonhomologous noninjected cholinergic drinking sites. These data are similar to those of Buerger *et al.* [2] in that there was a simultaneous short latency fast rise in activity at both sites after cholinergic stimulation. This simultaneous fast increase suggests that carbachol elicits increased MUA in a circuit by acting at its site of injection rather than diffusing to the contralateral site or some other more distal site. These data suggest a parallel between neurophysiological and behavioral events consistent with the hypothesis that carbachol elicits drinking by causing an increase in the frequency of neural firing, the number of neurons fired, or both, in a circuit whose activity facilitates water ingestion.

The blockade of the increases in MUA by atropine at both the cholinergically stimulated and the contralateral nonhomologous atropine injected sites parallels the behavioral demonstration of atropine blockade of cholinergic drinking [11]. Again, these data are consistent with the hypothesis that atropine blocks cholinergic-drinking by inhibiting the neural firing increases elicited by cholinergic stimulation [11].

The findings are not totally consistent, however, While injection of carbachol into the LSN produced a clear increase in MUA at both the LSN and the LHA, injection of carbachol into the LHA produced an increase in the LSN, but not the LHA. It is possible that the LHA contains pools of neurons that both increase and decrease in firing in response to carbachol while the septal area contains neurons that generally increase in firing. Atropine in either

site returns MUA to baseline. There is also a general tendency toward depression of MUA with injection of saline into either site. Reductions of MUA have previously been found to occur following injections of isotonic sodium chloride [2], and a nonspecific or ionic cause seems likely. These data are also confounded by the use of an anesthetic; different results may be found if MUA was recorded from unanesthetized animals.

Although much of these data are consistent with the hypothesis of a limbic system cholinergic thirst circuit in the rat, others have suggested that diffusion plays a major role in the wide distribution of sites at which cholinergic stimulation elicits drinking [16,17]. Recently, the subfornical organ has been suggested as a critical focus for chemically elicited drinking [18]. It is conceivable that the increases in MUA found at sites at which carbachol is a dipsogen are not directly related to the induction of drinking. However, since these increases are selectively blocked by atropine, they seem to be cholinergically mediated and not related to some noncholinergic property of carbachol. Further research utilizing multiple techniques will be required to clarify this controversy.

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