Adrenergic mechanisms in rabbit olfactory bulb

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SALMOIRAGHI, G. C., F. E. BLOOM, AND E. COSTA. Adrenergic mechanisms in rabbit olfactory bulb. Am. J. Physiol. 207(6) : 1417-1424. 1964.—Antidromic stimulation of mitral cell axons in the lateral olfactory tract (LOT) produces inhibition of mitral cells in the ipsilateral olfactory bulb, presumably through activation of recurrent axon collaterals. In decerebrate rabbits, electrophoretic administration of norepinephrine (NE), acetylcholine (ACh), or serotonin (5-HT) to individual mitral cells decreases their discharge rate. By means of electrophoretic administration of known antagonists of ACh, NE, and 5-HT as well as of intravenous administration of drugs that deplete brain stores of NE, 5-HT, and dopamine, evidence was obtained favoring a close functional correlation between NE or a related substance and the LOT inhibitory response.

Recurrent axon collaterals of mitral cells appear to exert a complex inhibitory influence upon olfactory bulb neurons (21, 31, 40, 45, 49). From recent extra- and intracellular recordings (21, 26, 31, 39, 40, 45, 46, 47-49) and from available anatomical evidence (1, 2, 8, 9, 19, 22, 32, 33, 42) the following functional organization of the rabbit's olfactory bulb can be formulated. Within the bulb, nerve cells are arranged in concentric layers, and the bodies of the several cell types (glomerular, tufted, mitral, granule) are largely restricted to different layers. Olfactory stimuli excite bipolar receptor cells in the olfactory mucosa. Their axons form the olfactory nerves which synapse within encapsulated glomeruli with primary dendrites of tufted and mitral cells. The axons of tufted cells form the rostral portion of the anterior commissure and end in the contralateral olfactory bulb, presumably within the granule cell layer. The axons of mitral cells form the lateral olfactory tract (LOT), which ends mainly in the prepyriform cortex. However, centrifugal fibers in the LOT also have been described (39, 33). Electrical stimulation of the LOT causes antidromic invasion of mitral cells. Additionally, it brings about a relatively prolonged (40-150-msec) inhibition of the spontaneous firing of many cells in the olfactory bulb (hereafter called LOT inhibitory response), presumably through activation of recurrent axon-collaterals of mitral cells (21, 40, 49). This inhibitory response is accompanied by hyperpolarization of the cell membrane (31, 48, 49) but the precise final step in the transmission is currently unsettled. Some investigators (21) hold that the terminals of recurrent axon-collaterals of mitral cells synapse directly with ipsilateral cells producing some facilitatory, but mainly inhibitory effects (Fig. 1A). Others (31, 39, 40, 49) believe that the axon collaterals synapse in the granule layer with interneurons, which in turn inhibit mitral cells (Fig. 1B).

In a preliminary study (44) we have found that electrophoretic administration of acetylcholine (ACh), norepinephrine (NE), and serotonin (5-HT) to neurons recorded singly in the rabbit olfactory bulb frequently caused a reduction of their spontaneous rate of firing. These amines and/or the enzyme for their synthesis are known to be present in rabbit brain (6, 23, 27, 43). It seemed reasonable, therefore, to suspect that one of these substances might be involved in the LOT inhibitory response. This hypothesis was tested by using drugs which we have shown to be antagonists of ACh, NE, and 5-HT in the olfactory bulb (4) and other chemical agents that deplete brain stores of NE, 5-HT, and dopamine. This paper presents evidence suggesting participation of a catecholamine in the mediation of the LOT inhibitory response.

METHODS

This study was carried out in 53 rabbits (1.5-3.5 kg body wt) electrolytically decerebrated (28) at the midcollicular level during temporary ether anesthesia. Tracheal intubation was done routinely, most animals requiring positive pressure respiration after decerebration. With the head of the animal firmly clamped in a stereotaxic holder, the olfactory bulbs were exposed, the dura removed, and a concentric bipolar stimulating electrode (tip separation 0.5 mm) inserted into one LOT through the frontal cortex (21). Stimuli were single rectangular pulses of 0.05-0.08 msec duration and amplitude between

1 Received for publication 10 June 1964.

* A preliminary report was given at the 48th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1964 (37).
inhibitory synapses of recurrent axon collaterals of mitral cells. Excitatory synapse, B = bipolar cell, G = glomerulus, T = tufted cell, M = mitral cell, CF = commissural fibers.

2 and 15 v, usually about 10 v. Exposed nervous structures were covered with a thick layer of 3% agar in saline to prevent dryness and to minimize movement. Extra-cellular unit recording from the bulb ipsilateral to the stimulating electrode, and simultaneous electrophoretic administration of chemical substances in the immediate vicinity of the neuron under investigation were carried out by means of five-barreled, glass micropipette electrodes, filled with 3 m NaCl solution (d-c resistance of 30–70 megohms) was used to pass a “neutralizing” current, i.e., a current of opposite polarity but magnitude equal to the algebraic sum of the retaining and ejecting currents flowing through the drug-containing barrels (38). This modification of the original iontophoretic technique minimizes electrotonic effects of current flow; in any event, additional controls were carried out routinely by passing currents of appropriate polarity through the 3 m NaCl-filled barrel. Similarly, potential pH effects of drug solutions were excluded by passing currents through NaCl-filled barrels of equivalent pH. Currents were provided by independent d-c sources, each with an output impedance in excess of 1,000 megohms. The magnitude and polarity of the current flowing in each of the three drug-containing barrels and in the NaCl-filled neutralizing barrel were monitored continuously by pen recorders. Standard techniques were used for displaying and recording action potentials. Methods for separating action potentials from base-line noise and for continuously plotting their rate of occurrence have been previously described (5, 34, 38, 44). Unless otherwise indicated, all drugs mentioned in the text were administered electrophoretically.

Statistical analyses comparing the durations of the LOT inhibitory responses of treated and untreated cells were performed by standard methods adapted in the following manner. The filmed records of each of the cells tested were measured as to duration of the LOT inhibitory response in milliseconds and grouped into a frequency distribution histogram of 10-msec classes (from 0 to 169 msec). To avoid bias related to variations in the film record available in each case (25 to 500 responses, avg 150), which was usually longer for cells giving better drug responses, frequency distribution was plotted as percentage of the total number of responses measured. Inspection of these histograms revealed marked skewness of the right tail. Therefore, tests of significance were chosen which did not require the assumption that the observed values arose from a bell-shaped population. The values of probability were calculated by the use of the chi-square method using a 1 X 1 contingency table constructed with a cutoff point of either 0.6% in the 30–39 msec class or 7% in the 40–49 msec class, i.e., the two lowest classes of LOT inhibitory response duration in available records from untreated cells. These values were compared with the percentage values of response duration in the treated cells as short as or shorter than these arbitrary cutoff points, and the most conservative estimate of significance reported. Two groups of data were considered to differ significantly when values of probability were less than 0.01% and to differ in a highly significant fashion when P was less than 0.001%. All values described here as significant recording, until drug ejection was induced by currents (0.05–0.1 μA) of suitable polarity. Difficulties, however, were experienced in controlling the spontaneous outflux of lysergic acid diethylamide (see results, LSD-25 and BOL-148). Routinely, the fourth barrel of the five-barreled glass micropipette electrodes, filled with 3 m NaCl solution (d-c resistance of 30–70 megohms) was used to pass a “neutralizing” current, i.e., a current of opposite polarity but magnitude equal to the algebraic sum of the retaining and ejecting currents flowing through the drug-containing barrels (38). This modification of the original iontophoretic technique minimizes electrotonic effects of current flow; in any event, additional controls were carried out routinely by passing currents of appropriate polarity through the 3 m NaCl-filled barrel. Similarly, potential pH effects of drug solutions were excluded by passing currents through NaCl-filled barrels of equivalent pH. Currents were provided by independent d-c sources, each with an output impedance in excess of 1,000 megohms. The magnitude and polarity of the current flowing in each of the three drug-containing barrels and in the NaCl-filled neutralizing barrel were monitored continuously by pen recorders. Standard techniques were used for displaying and recording action potentials. Methods for separating action potentials from base-line noise and for continuously plotting their rate of occurrence have been previously described (5, 34, 38, 44). Unless otherwise indicated, all drugs mentioned in the text were administered electrophoretically.

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The response was observed in 33 ACh-sensitive units before which were NE sensitive. In unit spontaneous discharge rate (3). Reported here were those provided by all relevant tests could be completed. The views cause of technical failures of various nature, The views judging from the absence of spontaneous discharge during penetration, and, if active, seldom exhibited a clear LOT inhibitory response. Presumably these findings reflected accidental trauma to the dorsal layers of the bulb during exposure and/or variations in the relative position of the stimulating electrode. Granule cells seldom yielded action potentials of sufficient amplitude to permit their study through five-barreled micropipette electrodes. The data presented here, therefore, refer to units in the mitral layer (presumably mitral cells) which yielded action potentials of not less than 150–200 μV and had a spontaneous rate of discharge sufficiently high (> 20/sec) to show a clear LOT inhibitory response for stimuli suprathreshold for the production of maximal field response. Once adjusted, all stimulus parameters were kept constant throughout the duration of the pharmacological tests on a particular cell. In many cases all desired tests could not be completed as programmed because of technical failures of various nature. The views reported here were those provided by 175 units on which all relevant tests could be completed.

RESULTS

ACh antagonists. The duration of the LOT inhibitory response was observed in 33 ACh-sensitive units before and during the electrophoretic administration of the acetylcholine blocking agents atropine, hexamethonium, chlorisondamine (Ecolid), and dihydro-β-erythroidine. In all cases, the cholinolytic agent blocked the cell’s responsiveness to ACh without influencing the duration of the LOT inhibitory response.

NE and adrenergic blockers. NE administration decreased the spontaneous discharge rate of most (80–90%) of the units studied in this series of unanesthetized rabbits. None responded to NE with an increase in rate of activity, as observable in anesthetized preparations (44). Electrophoretic administration of N-(2-chloroethyl)-dibenzylamine hydrochloride (Dibenamine) for 1–15 min was ineffective against ACh and 5-HT responses but frequently reduced or completely blocked the response of olfactory neurons to NE (4). NE blockade was at times accompanied by a moderate increase (10–20%) in the unit spontaneous discharge rate (3).

The effect of Dibenamine on the duration of the LOT inhibitory response was investigated on 69 units, 54 of which were NE sensitive. In 20 of these cells Dibenamine reduced or blocked the effect of NE and in all these it concomitantly shortened the duration of the LOT inhibitory response. Data sufficient for statistical evaluation could be obtained from 15 of these units, illustrated in Fig. 2. Thus, all units in which Dibenamine blocked NE response also showed shortening of the LOT inhibitory response. In some cases, however, Dibenamine reduced the LOT inhibitory response when either the cell was not sensitive to NE (9 units) or the NE effect was not reduced (15 units). An interpretation for the latter observations is offered in the discussion.

The LOT inhibitory response was also reduced by electrophoretic administration of phentolamine (Regitine), another sympatholytic agent which blocks alpha receptors. However, because of its poor solubility, this substance was found to be difficult to handle experimentally. On the other hand, in nine NE-sensitive units, administration of the beta receptor blocker dichloroisoproterenol (DCI), which has no effect upon sensitivity of olfactory neurons to NE (4), was ineffective on the duration of the LOT inhibitory response.

**DISCUSSION.**

The LOT inhibitory response was also reduced by electrophoretic administration of phentolamine (Regitine), another sympatholytic agent which blocks alpha receptors. However, because of its poor solubility, this substance was found to be difficult to handle experimentally. On the other hand, in nine NE-sensitive units, administration of the beta receptor blocker dichloroisoproterenol (DCI), which has no effect upon sensitivity of olfactory neurons to NE (4), was ineffective on the duration of the LOT inhibitory response.

**FIG. 2.** Percentage frequency histogram of duration of LOT inhibitory responses of 15 neurons before and after electrophoretic administration of Dibenamine. For this and subsequent histograms: in ordinate, percent of occurrence of LOT inhibitory responses; in abscissa, duration of LOT inhibitory responses grouped in 10-msec classes.
LOT inhibitory response within the usual range and in these four cases it was promptly reduced by LSD electrophoresis (Fig. 3). It was also noted that when one of the barrels of the electrode contained LSD-25, the likelihood of encountering NE-sensitive units was reduced by about two-thirds while that of encountering 5-HT-sensitive units was unchanged. These effects were attributed to the diffusion of LSD from the electrode’s tip in spite of the large retaining currents (up to 0.1 μA) applied to the LSD barrel. As reported elsewhere (4), electrophoretic administration of LSD-25 was found to be a more effective antagonist of NE than of 5-HT responses of olfactory neurons.

Similar effects were obtained with BOL-148, but its diffusion from the electrode’s tip appeared better controlled than that of LSD-25, as judged from the duration of the LOT inhibitory responses exhibited by the nine units investigated with it. Like LSD-25, it significantly shortened their LOT inhibitory responses when administered for 1–3 min by cationic currents of 0.04–0.1 μA. BOL-148 blocked both 5-HT and NE responses, the latter more efficiently than the former (Fig. 4; see also ref. 4).

**Strychnine.** Electrophoretic administration of strychnine to three units for 1–3 min with cationic currents of 0.04–

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**FIG. 3.** Percentage frequency histogram of duration of LOT inhibitory responses of four neurons before and after electrophoretic administration of LSD-25.

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**FIG. 4.** Polygraph record of discharge rate of one olfactory neuron during electrophoretic administrations of norepinephrine (NE), serotonin (5-HT), and brom-lysergic acid diethylamide (BOL) by cationic currents of indicated value. Each vertical pen deflection is proportional to total number of action potentials recorded in successive 1-sec periods. BOL administration (0.08 μA) begins 2 min after end of record A. B begins 4 min after end of A. C begins 7.5 min after end of B. D begins 5 min after end of C.
0.1 μA did not shorten, and in one case prolonged the duration of the LOT inhibitory response (for difficulties experienced in the control of spontaneous diffusion of strychnine from the electrode's tip, see 11, 15).

Effects of intravenous administration of reserpine and of α-methyl-metatyrosine (α-MMT). Intravenous administration of these two drugs was used as an alternative approach to clarify further the data on the shortening of the LOT inhibitory response by Dibenamine, Regitine, LSD-25, and BOL-148, and to try to distinguish further between catecholamines and 5-HT participation in the mediation of the LOT inhibitory response. It is well established that intravenously injected reserpine (1-3 mg/kg) completely depletes brain stores of NE, 5-HT, and dopamine within a few hours (7). Intravenous injection of α-MMT (100 mg/kg) initially depletes brain stores of NE and dopamine and reduces those of 5-HT, but recovery of 5-HT and dopamine is complete within 24 and 48 hr, respectively. NE stores, on the other hand, remain depleted for nearly 2 weeks (10, 20).

In eight rabbits, once a cell yielding clear responses to NE and 5-HT had been found and its response to supra-maximal LOT stimuli photographed, reserpine (1 mg/kg) was injected intravenously. Tests were then repeated at 5- to 30-min intervals for 1-4 hr, i.e., until electrical contact with the unit was lost. All 8 units showed progressive shortening of the LOT inhibitory response (Fig. 5), which was significant within 1 hr of reserpine injection. An additional group of 16 units was studied in these reserpine-treated animals after the cell originally investigated had been lost. When tested with identical LOT stimuli, they exhibited LOT inhibitory responses which were significantly shorter than those exhibited by cells in untreated animals. In one case, reserpine was successfully administered by pressure (100-110 mm Hg) from a reserpine-filled barrel of the electrode without losing electrical contact with the unit. In this case also, the LOT inhibitory response became progressively less (Fig. 6), while the unit's response to electrophoretically administered NE remained unchanged.

It is not technically possible to record the response of the same cell before and after α-MMT effects, since this compound requires several days to produce the desired selective biochemical change (20). LOT inhibitory responses recorded from 31 units in 3 rabbits which had received 100 mg/kg of α-MMT intravenously 7-11 days
were loaded with NE. NE administration (0.1 μA) consistently reduced the number of spikes and the burst duration elicited by LOT stimulation.

In this instance, all three drug barrels of the electrode were loaded with NE. NE administration (0.1 μA) consistently reduced the number of spikes and the burst duration elicited by LOT stimulation.

DISCUSSION

These results suggest the participation of an adrenergic mechanism in the mediation of the LOT inhibitory response. Such a possibility arises from the following observations. a) In unanesthetized rabbits, electrophoretic administration of NE to mitral cells decreased their spontaneous rate of discharge by 80-90% of cells tested. b) When electrophoretic administration of the sympatholytic Dibenamine shortened the duration of the LOT inhibitory response, the cell's response to NE electrophoresis was also blocked. c) LSD-25 and BOL-148 blocked the LOT inhibitory response when given electrophoretically to olfactory bulb neurons and proved to be effective antagonists of NE at these central sites (4). It should be noted that inability of electrophoretically administered LSD-25 to block responsiveness to 5-HT of lateral geniculate and cortical neurons has also been reported (12, 25). d) Finally, depletion of NE, 5-HT, and dopamine brain content by reserpine (7) and of NE content by α-MMT (10, 26) also dramatically shortened the duration of the LOT inhibitory response.

Admittedly none of these observations individually constitutes unequivocal proof of NE participation in the LOT inhibitory response. With reference to accepted criteria for the identification of neurohumoral transmitters (17, 18, 27, 30), it should be noted that NE has been shown to be present in the rabbit brain (6, 23, 27, 43) although its presence within axon terminals mediating the LOT inhibitory response and its release following LOT stimulation have not been demonstrated. Regrettably, available techniques do not yet permit pursuit of this approach. Nevertheless, the sum total of the evidence that we have presented as well as the inner consistency of the data favor a close functional correlation between NE or a related substance and the LOT inhibitory response.

Perfect coincidence between a neuron's response to a suspected transmitter, its antagonists, and nerve stimulation cannot be expected with the microelectrophoretic technique. Access of a substance to active sites may be unpredictably limited by barriers, "synaptic" (14) or enzymatic, which might not prove equally effective toward another chemical substance released from the same electrode. Poor solubility of a compound, rectification, or accumulation of tissue debris around the tip of the electrode may equally interfere by preventing ejection of a substance in sufficient amounts to affect equally all pertinent receptive sites of the cell membrane. These technical limitations may account for the few instances in which the neuron did not show a clear response to electrophoretically administered NE, although subsequent ejection of Dibenamine shortened the LOT inhibitory response. An additional complication may be introduced by the time course of the Dibenamine effect, which in the periphery produces first a reversible and then an irreversible block (29). This biphasic effect may explain the few cases where reduction of the LOT inhibitory response occurred in the absence of blockade of a unit's response to electrophoretically administered NE. In the absence of reliable methods for the estimation of the concentration of ejected substance achieved at potentially reactive sites, it would seem that undue reliance upon "negative" findings (i.e., absence of observable effects) is not justifiable at present (35, 36).

None of our data provides direct evidence for the site of the postulated adrenergic mechanism. Our attempts to gain insight into this problem through the study of the effects of electrophoretically administered substances upon the cell's response to testing and conditioning stimuli were frustrated by technical shortcomings. These were chiefly due to the difficulty of recognizing driven action potentials (usually of small size when recorded with five-barreled micropipette electrodes) within the large complex field response evoked by stimulation of the LOT or of the olfactory nerve, and the fact that the latter in rabbits is made up of many separate twigs, each with a discrete pattern of distribution, requiring...
ADRENERGIC MECHANISMS IN OLFAC TORY BULB

Fig. 8. Response of a neuron encountered in granule layer to LOT stimulation. Plots on left show relationship of burst duration (upper) and number of spikes per burst (lower) with stimulus intensity. At right, oscilloscope records of A: spontaneous rate; B-F: its identification and selective stimulation for reliable activation of a given olfactory neuron (39). Nevertheless, it seems probable that the postulated adrenergic mechanism may involve structures very near the sites at which action potentials of LOT inhibited neurons were recorded. Distant mediation of the effects of electrophoretically administered NE and adrenergic drugs, e.g., through their actions on interneurons of the type postulated in Fig. 1B, seems unlikely because of the distance involved and the observation that when NE was administered to a granule layer neuron that behaved in Renshaw cell-like fashion (Fig. 8), NE did not facilitate it, but rather somewhat depressed its response to the LOT stimulus. It should be noted, however, that the functional role of this unit is difficult to assess: it may represent an interneuron of the type postulated in Fig. 1B, but it may also be a granule cell driven by afferent fibers known to be present in the LOT together with the axons of mitral cells (32, 33).

It is conceivable, then, that a catecholamine may be an inhibitory transmitter for mitral cells or cause presynaptic inhibition of mitral cells. It is also possible, although unlikely, that it may have excitatory functions for a neighboring unit functionally related to the one under observation. In anesthetized rabbits, we have occasionally found olfactory bulb neurons that were facilitated by NE electrophoresis (44), but have never seen this effect in the unanesthetized rabbit. Clearly, we cannot yet distinguish between these alternatives, nor can we yet explain the functional significance of olfactory neuron responses to electrophoretically administered ACh and 5 HT.

We are indebted to Dr. D. H. Tedeschi, Smith, Kline & French, Philadelphia, Pa., for generous gifts of Dibenamine and Dibenzyline; Drs. A. J. Frey, J. A. Harris, and J. DerHovanesian, Sandox Pharmaceuticals, Hanover, N. J., for LSD-25 and BOL-148; Dr. L. McClelland, Merck Sharp & Dohme, for dihydro-P-erythroidine; and Dr. A. J. Plummer, Ciba Pharmaceutical Co., Summit, N. J., for lyophilized reserpine phosphate. The technical assistance of A. P. Oliver was invaluable to the completion of these experiments.

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