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ANALYSIS OF INDIVIDUAL RABBIT OLFACTORY BULB NEURON RESPONSES TO THE MICROELECTROPHORESIS OF ACETYLCHOLINE, NOREPINEPHRINE AND SEROTONIN SYNERGISTS AND ANTAGONISTS[1]

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Recently, neurons of the olfactory bulb were found to reduce their spontaneous discharge rates in response to microelectrophoretically administered norepinephrine (NE), serotonin (5-HT) and acetylcholine (ACh) (von Baumgarten *et al.*, 1963). These locally elicited chemical responses suggested that the three endogenous amines might participate in the function of an olfactory inhibitory synaptic pathway (Green *et al.*, 1962; Yamamoto *et al.*, 1963; Phillips *et al.*, 1963). In order to evaluate this possibility, a more complete pharmacological analysis of these inhibitory responses was undertaken and preliminary results have been reported (Bloom *et al.*, 1963b, 1964). The objective of these studies has been to establish certain pharmacological points of reference from which characterization of olfactory bulb inhibitory transmitters might be initiated.

Five-barreled glass micropipette electrodes (Curtis and Eccles, 1958a,b) have been used for the microelectrophoresis of desired drugs and amines into the immediate environment of a neuron while simultaneously recording its activity extracellularly. This approach excludes many of the problems of response interpretation related to blood-brain barrier permeability. In addition, it helps to rule out drug effects due to fluctuations in systemic blood pressure, to changes in the composition of the extracellular fluid (*e.g.*, pH, electrolytes, or metabolites) or to primary drug effects upon neurons many synapses removed.

METHODS. Experiments were completed in 55 young adult pigmented rabbits acutely decerebrated with electrolytic (Magoun, 1956) lesions at the midcollicular level during temporary light ether anesthesia. Ether was initially administered by nasal cone and continued briefly through a tracheal cannula. This method of decerebration produces nonreactivity to pain. Occasionally, preparations showed thrashing movements of the extremities or cessation of spontaneous respiratory movements which were possibly related to variations in the extent of the lesion. When required, animals were artificially respired with mixtures of oxygen and room air. Successfully decerebrated animals were mounted in a stereotaxic head-holder and the olfactory bulbs and frontal cortex were cautiously exposed. The lateral olfactory tract (LOT) was impaled with a bipolar concentric stimulating needle electrode (tip diameter 0.5 mm) inserted through the frontal cortex just posterior to the neck of the bulb (Green *et al.*, 1962). Stimuli of variable amplitude and duration were delivered from a stimulus isolation unit.

Properly filled 5-barreled glass micropipette electrodes (see below) were inserted into the bulb with a precision micromanipulator (Debroske *et al.*, 1960). Field potentials and extracellular action potentials of spontaneously active neurons were recorded with the central barrel of the pipette filled with 4.7 M NaCl solution and connected to a high impedance unity gain amplifier (Bak, 1958). These potentials were displayed on dual-beam oscilloscopes and in addition, were fed into a voltage gating circuit from which action potentials could be separated from background noise. The output of the gating circuit was coupled to a linear counting device adjusted to recycle every 0.5 or 1.0 second (reset time: less than 3 msec). The output of this circuit was displayed on one channel of an ink-writing polygraph yielding a continuous record of discharge rate.

Five-barreled glass micropipette electrodes with overall external tip diameters of 3 to 8 micra were prepared as previously described (Salmoiraghi and Steiner, 1963; Bloom *et al.*, 1963a). Briefly this

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method consists of prefilling pipettes by boiling in methanol, replacing methanol by diffusion against water, and finally aspirating the water and transferring into three of the four outer barrels freshly prepared concentrated (0.5 to 1.5 M) aqueous solutions of the following chemicals: acetylcholine chloride (ACh), atropine sulfate, dihydro-β-erythroidine hydrobromide, chlorisondamine dimethochloride (Ecolid), hexamethonium chloride, L-norepinephrine bitartrate (NE), dimethylphenylpiperazinium iodide (DMPP), guanethidine sulfate, bretylium tosylate, tyramine hydrochloride, L-metaraminol D-bitartrate, phentolamine hydrochloride (Regitine), Dibenamine chloride, Dibenzyline chloride, lysergic acid diethylamide (LSD-25) and 2-brom-lysergic acid diethylamide (BOL-148). Both lysergic acid derivatives were acidified to pH 3.5. Because of their relative insolubility, 5-hydroxytryptamine oxalate or creatinine sulfate (5-HT) was used at a concentration of 0.05 M. Drug barrels filled with these compounds regularly exhibited electrical resistances of the order of 20 to 150 megohms. By means of centrifugation, pipettes could usually be filled and ready for use within 45 minutes after preparation of the solution from fresh crystalline sources. The fourth outer barrel of the 5-barreled glass micropipette electrode was filled with 3 M NaCl and served as the source of neutralizing current (Salmoiraghi and Steiner, 1963) used to balance the retaining and ejecting currents (del Castillo and Katz, 1955). Moreover, this outer barrel could also be purposely imbalanced in either polarity to exclude the possible electrotonic effects of current flow. All the currents (positive, negative, and the net difference at the pipette tip) were continuously monitored on separate channels of the polygraph. All tests in which unintentional current imbalance at the tip exceeded 10% of the total ejecting current were discarded. Possible effects of pH were controlled by electrophoresis of equally acidified saline or water. Drugs were ejected from the pipette with cationic currents (0.005 to 0.15 microamp) and retained with anionic currents (0.02 to 0.10 microamp). Relating these current values to Faraday's law (Falkenhagen and Kelbg, 1959) and assuming 1:1 electron to ion transfer, the maximum dosage which could be ejected with these currents would range between 5 and 150 $\times 10^{-14}$ gram equivalents per second of electrophoresis. Each of the drugs listed above was tested on at least 8 neurons sensitive to that amine (ACh, NE, and 5-HT) to which the drug was pharmacologically related. In two experiments an aqueous solution of lyophilized reserpine phosphate was ejected by the application of pressure to avoid precipitation of the alkaloid by Cl^- ions.

The analysis was confined to the layer of the olfactory bulb containing mitral cells. These cells may be identified by the antidromic spike discharge occurring within 2 milliseconds after electrical stimulation of their centripetal processes in the ipsilateral LOT (Green *et al.*, 1962). Other spontaneously active cells not exhibiting the antidromic spike response were included in the study only if the shape of the field potential evoked by LOT stimulation indicated that the tip of the micropipette was within the mitral cell layer (Green *et al.*, 1962; Phillips *et al.*, 1963). Unless otherwise specified all drugs were administered electrophoretically.

RESULTS. Data reported are based upon 258 spontaneously active neurons whose discharge rate was decreased by electrophoresis of ACh, NE or 5-HT. All of these cells were also tested with various drugs known to act peripherally as synergists or antagonists of one or the other of the three amines. Fifty-five additional spontaneously active neurons which responded only to the pharmacological agents but not to ACh, NE or 5-HT are also included.

The absolute dose of the drug that reaches neuronal receptors cannot be accurately determined with the electrophoretic technique. Aside from uncertainty as to the amount of ions ejected by any given current, the effects of diffusional and enzymatic barriers through which the wave of ions must pass also escape rigorous evaluation. Therefore, the results have been expressed in terms of the qualitative response, *i.e.*, increase or decrease of discharge rate during or after treatment with a particular drug and the modification of the response to ACh, NE or 5-HT.

Acetylcholine. As previously reported (von Baumgarten *et al.*, 1963), a small proportion of olfactory neurons in decerebrated rabbits respond to ACh by increasing their discharge rate. Most of the responsive neurons decrease their rate of discharge when tested with ACh, but these responses were seldom dramatic or intense. In these neurons, electrophoretic administration of physostigmine—which also slowed many neurons whether or not they were sensitive to ACh—prolonged, but usually did not potentiate, the intensity of the ACh response. Moreover, physostigmine never caused an ACh insensitive unit to become ACh sensitive. Dihydro-β-erythroidine, atropine, hexamethonium, chlorisondamine, and DMPP slowed the discharge rates of units which were either slowed or unaffected by ACh. All these drugs, with the exception of DMPP, blocked

the ACh response. Often this blockade lasted only 0.5 minute. Atropine stopped neuronal firing for 1 to 3 minutes; consequently its effects upon the responsiveness to ACh could be tested only during the early stages of recovery from atropine. Dihydro-β-erythroidine was tested on 33 units, in which it blocked responses to ACh but failed to interfere with responses to either 5-HT or NE. The blockade of ACh responses was not due to tachyphylaxis to ACh, as consecutive subsequent ACh administrations at constant intervals of at least 30 seconds did produce responses after termination of blocker electrophoresis.

Norepinephrine. In decerebrated rabbits, NE exclusively reduced the spontaneous discharge rate of responsive units. Decreased rates of discharge were also observed after the electrophoresis of tyramine and metaraminol, but neither of these drugs affected subsequent responses to NE nor caused tachyphylaxis. Dibenamine and phentolamine, two blockers of peripheral sympathetic

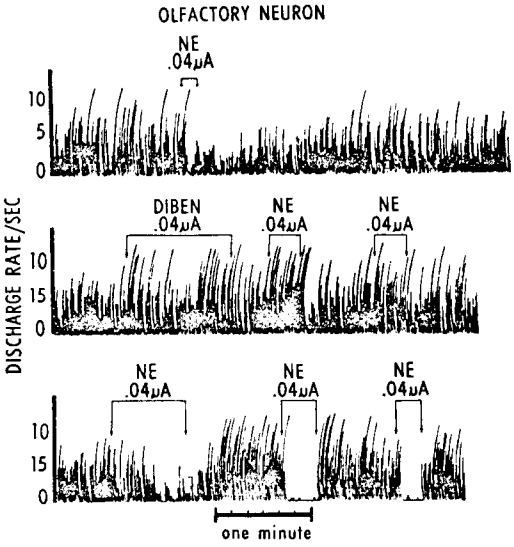

FIG. 1. Continuous polygraph records of discharge rate of one olfactory neuron responding to brief NE electrophoresis with prolonged reduction in rate of firing.

After administration of Dibenamine response to NE was blocked. During early stages of recovery from Dibenamine, rate of discharge is slightly faster than before Dibenamine. Effect of NE appears to be intensified, but note that NE effect now does not extend beyond period of administration (compare with top row response). In this and subsequent illustrations taken from individual experiments, the animal preparation was the unanesthetized midcollicularly decerebrated rabbit, and μA refers to the electrophoretic current in microamps.

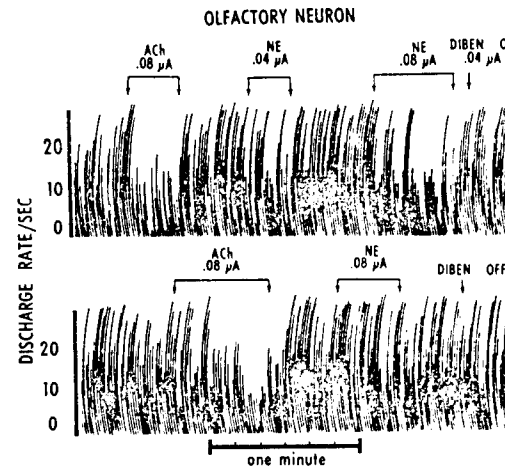

FIG. 2. Polygraph record of discharge rate of one olfactory neuron responding to both ACh and NE with reduction in rate of firing.

Tests with both amines repeated at 1- to 2-minute intervals during 15 minutes of Dibenamine (0.08 microamp) electrophoresis between upper and lower records. NE response was blocked by Dibenamine, but ACh response was not.

α receptors, suppressed responsiveness to NE when administered for 0.5 to 15 minutes. Occasionally, blockade of responses lasted longer than 30 minutes. For some cells, the electrophoretic administration of Dibenamine caused a biphasic effect: the NE response was enhanced either prior to the onset of NE blockade or during the period of recovery from Dibenamine electrophoresis (fig. 1). Dibenamine administration, particularly in NE sensitive units requiring prolonged Dibenamine treatment to produce blockade, caused a sustained moderate increase in discharge rate for the duration of the NE blockade. In no case did Dibenamine affect ACh or 5-HT responses (fig. 2). DCI, a peripheral β receptor blocker, decreased the rates of spontaneous discharge of NE sensitive cells, but did not interfere with their responsiveness to NE. Dibenzyline, guanethidine, and bretylium reduced spontaneous activity of neurons regardless of their sensitivity to NE. However, with pipettes filled with any one of these three drugs, electrophoresis was particularly difficult to control and no meaningful data concerning the effects of these drugs on NE responses were obtained.

Serotonin. All 5-HT sensitive neurons reacted to the amine by slowing their discharge rate. Although LSD-25 and BOL-148 specifically block the response of smooth muscle to 5-HT (Gaddum,

1953; Gaddum and Hameed, 1954), in 21 olfactory neurons from 4 rabbits, these drugs never blocked 5-HT responses without also blocking the response to NE (figs. 3 and 4). Despite our attempts to avoid leakage of LSD-25 and BOL-148 from the pipette, the number of NE-sensitive neurons encountered per microelectrode penetration was much less when the pipette contained LSD (168 NE-sensitive cells in 98 penetrations without LSD in the pipette *versus* 8 NE-sensitive cells in 16 penetrations with LSD). Application of LSD-25 and BOL-148 occasionally slowed the spontaneous rate of unit discharge, but equally acidified saline had no slowing effect.

Reserpine. Neurons originally sensitive to ACh, NE and 5-HT were continuously studied for periods of 90 to 220 minutes after the intravenous injection of 1 mg/kg of reserpine to deplete brain stores of NE, 5-HT and dopamine (Brodie *et al.*, 1961). Electrophoretic administration of each of the three amines was then repeated at intervals of 5 to 30 minutes. In seven rabbits the unit responses to the electrophoresis of each of the three amines remained unaltered after the i.v. injection of reserpine (fig. 5). Because reserpine alkaloids precipitate in ionic media, local electrophoretic administration of this drug was not possible. However, in two experiments reserpine was ejected from the pipette by pressure (see METHODS). This direct application of reserpine produced transient slowing of unit discharges, after which the rate of discharge gradually increased above control rates. Responses to ACh, NE and 5-HT remained unchanged.

In another series of experiments 25 animals were chronically treated with reserpine prior to surgery by various dosage schedules, but none of these rabbits survived the electrolytic decerebration.

α-Methyl-m-tyrosine (α-MMT). In rabbits, α-MMT selectively depletes brain NE (Hess *et al.*, 1961; Porter *et al.*, 1961; Costa *et al.*, 1962). Seven days after treatment with α-MMT, its decarboxylated metabolites are undetectable in brain although NE depletion still endures for several days (Gessa *et al.*, 1962). The time course of NE depletion by α-MMT excluded the possibility of using the same animal as its own control. In experiments carried out 7, 9 and 11 days after the intravenous injection of 100 mg/kg of α-MMT, neurons responsive to ACh, 5-HT and NE were readily encountered. In a rabbit 9 days after α-MMT, neurons initially unresponsive to tyramine became responsive to it for about 30 minutes after a 35-second electrophoresis of NE (fig. 6), a dose which in normal animals did not affect responses to subsequent electrophoresis of tyramine. In none of the 25 neurons tested with ACh, NE or 5-HT in 3 α-MMT treated animals was there any qualitative difference in their response from that of the untreated animals.

DISCUSSION. The purpose of this study was to investigate the effects of various drugs on the responses of olfactory bulb neurons to 3 endogenous brain amines: ACh, NE and 5-HT. These amines and their synergists and antagonists were administered electrophoretically in the immediate vicinity of individual nerve cells. The data ob-

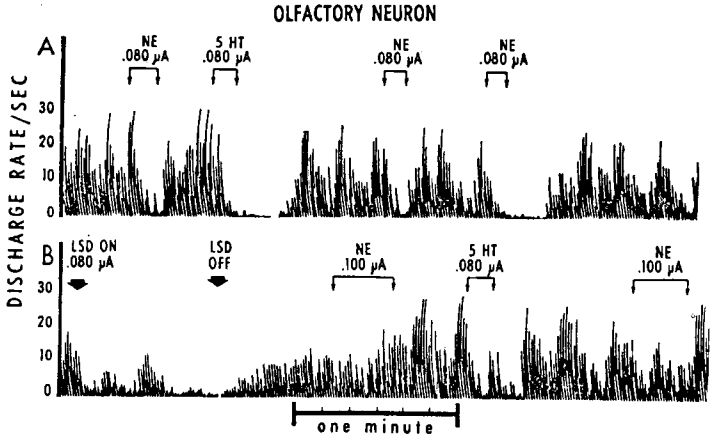

FIG. 3. Continuous polygraph record of discharge rate of one olfactory neuron responding to both NE and 5-HT with reduction in rate of firing.
NE response was blocked after LSD (0.10 microamp) electrophoresis, while 5-HT response was not.

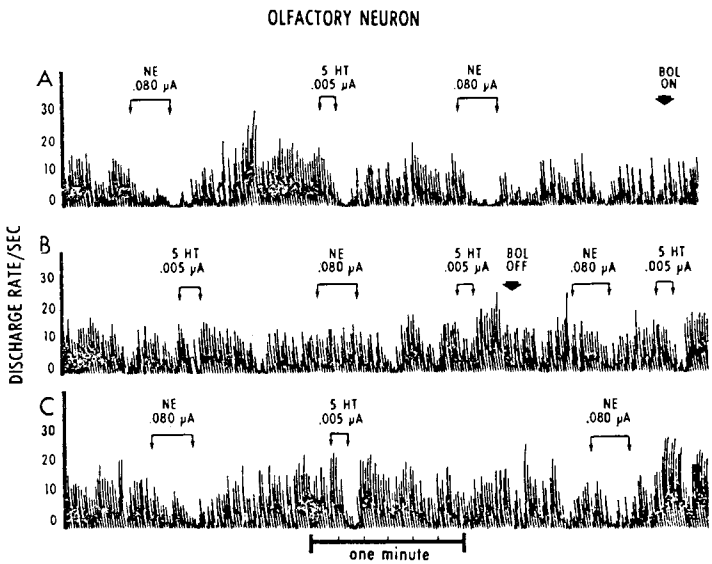

FIG. 4. Polygraph record of discharge rate of one olfactory neuron responding to both NE and 5-HT. Each record (A, B, C) is separated by 3 minutes. Both NE and 5-HT responses were reduced by BOL-148 (0.04 microamp) administered between heavy arrows. Note the increase of the overall discharge rate of the unit after BOL-148.

tained show that some of the drugs tested selectively block the action of the amines at this central site. Furthermore, our results in reserpine-treated rabbits indicate that the ACh, NE and 5-HT effects do not require the presence of intact neuronal stores of NE, 5-HT and dopamine.

Electrophoretic administration of ACh has been shown to increase the discharge rate of certain spinal (Curtis and Eccles, 1958a,b), medullary (Anderson and Curtis, 1964) and cortical neurons (Krnjević and Phillis, 1963a,b) in anesthetized animals. It also increases activity of caudate nucleus neurons in unanesthetized cats (Bloom *et al.*, 1964). In contrast, the activity of olfactory bulb neurons in decerebrated rabbits is, with few exceptions, reduced by ACh (von Baumgarten *et al.*, 1963). The cholinesterase inhibitor physostigmine potentiated olfactory neuron responses to ACh less efficiently than described in medulla (Salmoiraghi and Steiner, 1963) and in visual cortex of cats (Spehlmann, 1963), but more actively than in somatosensory cortex (Krnjević and Phillis, 1963a,b). The slowing of spontaneous activity caused by electrophoresis of physostigmine would be compatible with the view that ACh might be an important physiological transmitter in an inhibitory pathway for mitral cells. It is not clear, however, why ACh blockers should also slow spontaneous neuronal activity. It is probable that a number of pharmacological actions are elicited by a given drug, for example, atropine is known to have local anesthetic properties (Curtis and Phillis, 1960; Krnjević and Phillis, 1963a) in addition to antimuscarinic activity. Thus local anesthetic actions may also be involved in the suppression of activity caused by the electrophoretic administration of atropine and some of the other cholinolytic drugs. In any event, our data at present do not permit us to make a distinction between nicotinic and muscarinic ACh receptors nor do they *per se* exclude the presence of a cholinergic olfactory pathway.

Electrophoretic administration of NE has been shown to increase the discharge rate of units in the brain stem of decerebrated cats (Bradley and Wolstencroft, 1962), the olfactory bulb of anesthetized rabbits (von Baumgarten *et al.*, 1963), and the hypothalamus of anesthetized cats (Bloom *et al.*, 1963a). 5-HT in large doses also increased activity of cortical neurons in anesthetized cats, rabbits and monkeys (Krnjević and Phillis, 1963c). In small doses, both NE and 5-HT slowed the discharge rate of neurons in the lateral geniculate nucleus (Curtis and Davis, 1962) and cortex (Krnjević and Phillis, 1963c). In the olfactory bulb of unanesthetized rabbits, NE and 5-HT invariably decreased the spontaneous

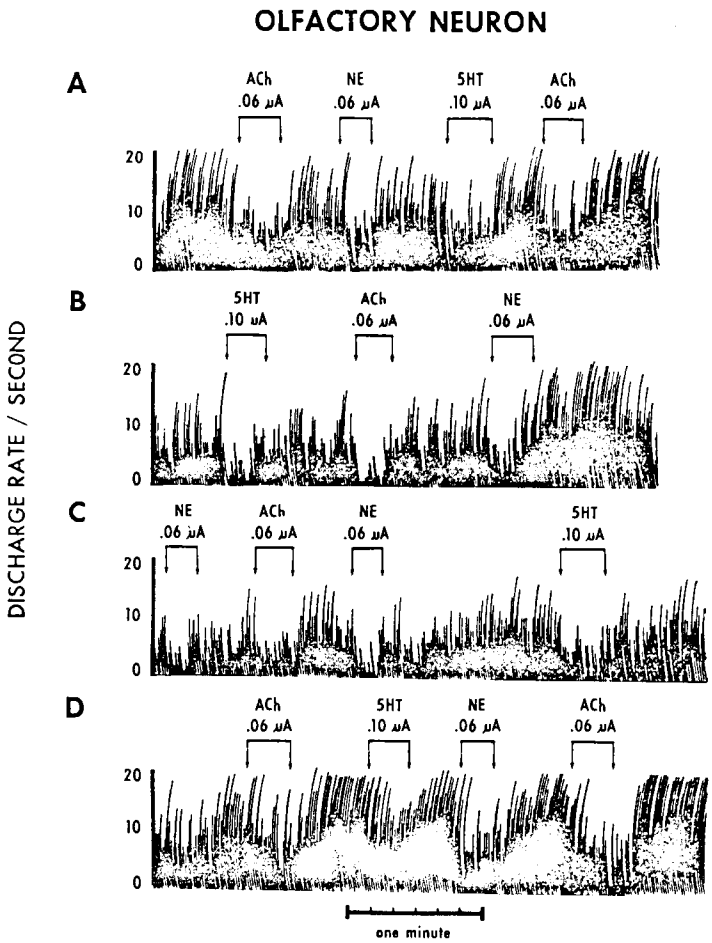

FIG. 5. Polygraph record of the discharge rate of one olfactory neuron responding to ACh, NE and 5-HT with reduction in firing rate.

Following record A, reserpine (1 mg/kg) was administered intravenously. B, C and D show typical persistence of responsiveness to each of 3 amines at 60, 120 and 210 minutes, respectively, after the reserpine injection. Note the increase of the overall discharge rate in record D.

discharge of responsive units and similar effects were produced by tyramine and metaraminol, which are known to release NE from peripheral axon terminals (Hertting *et al.*, 1961; Weiner *et al.*, 1962).

A distinct site of action for NE in olfactory neurons is emphasized by the selective effect of Dibenamine in antagonizing the decreased firing rate elicited by NE but not that elicited by ACh or 5-HT. It is noteworthy that increased unit activity was occasionally seen after electrophoresis of Dibenamine (Bloom *et al.*, 1964) and following depletion of brain catechols by local injection of reserpine. Thus, the results obtained using drugs that either deplete the stores of NE and 5-HT or block NE activity are compatible with the view that these endogenous amines possess an inhibitory role in the function of olfactory bulb.

The evidence that NE has inhibitory effects when released from brain stores is still meager. However, this view is supported by the action of tyramine in α-MMT treated rabbits; in fact, in this experiment tyramine reduced the unit activity of olfactory neurons only after a 35-second electrophoresis of NE (fig. 6). On the other hand, NE, ACh and 5-HT continued to cause decreased discharge rates in reserpine and α-MMT treated rabbits. The latter results cannot be used as proof that ACh or 5-HT does not act by releasing NE, since neither reserpine nor α-MMT affects brain

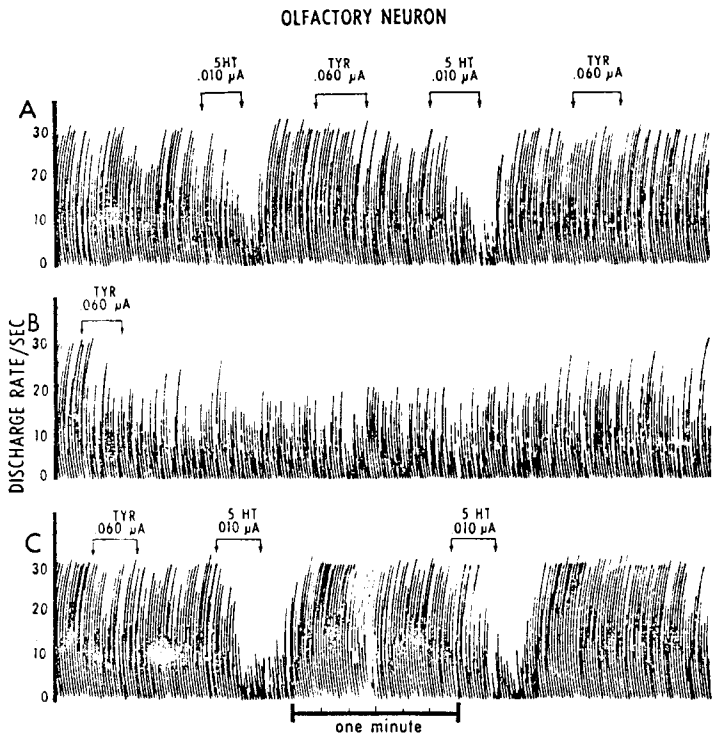

FIG. 6. Polygraph record of discharge rate of one olfactory neuron 9 days after 100 mg/kg α-MMT (IV). The unit initially was unresponsive to tyramine (TYR). Between A and B, NE (0.06 microamp) was administered electrophoretically for 35 seconds. Subsequently (record B) this neuron demonstrated prolonged decrease in discharge rate after brief electrophoretic administration of TYR. Record shows diminished TYR response 30 minutes after B, at which time 5-HT response was unaffected (see text).

NE biosynthesis. The rate of formation, especially after depletion of the NE stores (Brodie *et al.*, 1959), may still be sufficient to maintain a NE tissue level compatible with indirect effects of ACh and 5-HT.

One further point to be considered is that the introduction of the pipette may disrupt cellular membranes interposing artificial drug-binding sites between neurons and pipette which could prevent or modify the extent of the qualitative responses observed. Inactivation by Dibenamine of nonreactive binding sites may explain the increased effectiveness of NE just before or after the termination of Dibenamine electrophoresis (fig. 1).

The data presented here, regarding the responsiveness of olfactory neurons to 3 endogenous brain amines, are directly applicable in the testing of another of the criteria for the identification of neurohumoral agents (Paton, 1958; Florey, 1960; Curtis, 1963). Thus the suspected brain substance must interact with pharmacological synergists and antagonists in a manner identical with that of the transmitter at the particular synapse under investigation. For example, an adrenergic synapse should be blocked or impaired by drugs which block or deplete the stores of NE. With this thought in mind, many of the units considered in this qualitative study were also simultaneously tested for a possible alteration in the physiological response to specific pathways of synaptic impingement arising from the lateral olfactory tract. The alterations of particular physiological mechanisms induced by these drugs in the olfactory bulb will be reported elsewhere.

SUMMARY

The reduction of spontaneous discharge rate of olfactory neurons by electrophoretic administrations of ACh, NE or 5-HT to individual nerve cells has been investigated in unanesthetized rabbits. Electrophoretic administration of dihydro-β-erythroidine, atropine, hexamethonium and chlorisondamine to ACh-sensitive units,

blocked their response to ACh. Physostigmine prolonged the duration of the ACh response. Dihydro-β-erythroidine specifically blocked the effects of ACh, but not of NE or 5-HT administration. Both the ACh blocking agents and physostigmine temporarily suppressed discharge rate regardless of ACh responsiveness.

Electrophoretic administration of NE and 5-HT always resulted in slowing of the unit's spontaneous discharge. Similar responses were seen after administration of tyramine and metaraminol. Dibenamine and phentolamine specifically blocked NE responses and did not interfere with the response of 5-HT. LSD-25 and BOL-148 did interfere with responses to 5-HT, but never without prior or concurrent blockade of responses to NE. The spontaneous discharge rate was at times increased after the unit's response to NE was blocked by Dibenamine.

The ability of ACh, NE or 5-HT to slow spontaneous discharge rate was qualitatively unchanged 4 hours after local administration or intravenous injections of reserpine or 7 to 11 days after intravenous injection of α-MMT, suggesting that the amines are not dependent for their effect upon intact brain stores of NE, 5-HT or dopamine. In α-MMT treated rabbits, the effect of tyramine was not apparent until after electrophoresis of NE.

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