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AN ELECTROPHYSIOLOGICAL STUDY OF 5-HYDROXYTRYPTAMINE RECEPTORS OF NEURONES IN THE MOLLUSCAN NERVOUS SYSTEM

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SUMMARY

1. 5-Hydroxytryptamine (5-HT) has been iontophoretically applied to the membrane of central neurones of *Cryptomphallus aspersa*; CILDA neurones (cells with inhibition of long duration) (Gerschenfeld & Tauc, 1964) are the only cells sensitive to 5-HT. The responses to 5-HT is always a depolarization. The CILDA cells studied were also depolarized by ACh.

2. From experiments in which pulses of 5-HT and ACh were applied from a double-barrelled micropipette to the CILDA cell soma, it has been calculated that 5-HT and ACh receptors were located at different distances from the injecting micropipette tip. It has also been calculated from the diffusion equation that in the same CILDA cell a 5-HT concentration of $8{\cdot}2 \times 10^{-9}$ M and a ACh concentration of $1{\cdot}3 \times 10^{-8}$ M caused a similar peak depolarization.

3. CILDA neurones show 'anomalous' rectification. 5-HT increases the membrane conductance of CILDA.

4. 5-HT receptors of CILDA neurone are desensitized by repeated application of 5-HT. The desensitization lasts for *ca.* 40 sec.

5. 5-HT receptors are blocked by lysergic acid diethylamide and its derivatives. Morphine chlorhydrate blocks them non-competitively.

6. Some inhibitors of monoamine oxidase (trancylpromine, isocarboxazide, iproniazide and nialamide) have been tested. They do not prolong the action of 5-HT, but block the 5-HT receptors.

7. No crossed desensitization between 5-HT and ACh has been observed. Atropine blocks both ACh-receptors and 5-HT receptors, 5-HT receptors appear to be blocked to a greater extent.

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8. The data presented support the assumption of a excitatory transmitter role of 5-HT to CILDA neurones, but further evidence is necessary to confirm this hypothesis.

INTRODUCTION

Since the demonstration of 5-HT in vertebrate brain by Twarog & Page (1953) and Amin, Crawford & Gaddum (1954) an impressive amount of work has been done (see Esparmer, 1961) in an attempt to elucidate its physiological role in the nervous system. On the basis of its distribution in specific areas of the brain (Bogdansky, Weissbach & Udenfriend, 1956) a transmitter role of 5-HT in the parasympathetic system was postulated by Brodie & Shore (1957). This assumption has recently found support in cell fractionation methods, which have demonstrated that 5-HT is principally located in isolated nerve endings in mammalian brains (Michaelson & Whittaker, 1963; Zieher & De Robertis, 1963; Maynert, Levi & De Lorenzo, 1964).

In a number of investigations, 5-HT has been applied iontophoretically to neurones in various parts of the vertebrate central nervous system. No effect was observed in the spinal cord (Curtis, Phillis & Watkins, 1961) or in the brain stem (Curtis & Koizumi, 1961). However, application of 5-HT to neurones of the lateral geniculate body, blocks their orthodromic responses (Curtis & Davis, 1962).

In a survey of the 5-HT content of invertebrates Welsh & Moorehead (1960) found an especially high concentration in ganglia of Annelids and Molluscs. The present work constitutes an attempt to find physiological support for the postulated transmitter role of 5-HT. It has been found that certain neurones in the central ganglia of a mollusc are endowed with 5-HT receptors and the properties of these receptors have been analysed by electrophysiological methods. The results obtained support the idea that 5-HT is a transmitter. A preliminary account of some of the results has been published (Gerschenfeld & Stefani, 1965).

METHODS

Specimens of the Argentinian land-snail *Cryptomphallus aspersa* were maintained at room temperature in a cage with a wet grass floor. Unanaesthetized animals were dissected and the perioesophageal ganglionic ring with the associated peripheral nerves were isolated and pinned on to the wax-bottom of a lucite chamber of 5 ml. capacity, adapted for continuous circulation of a physiological solution. This solution (Chiarandini, 1964) contained 110 mM-NaCl; 4·9 mM-KCl; 18·3 mM-Na_2HCO_3; 3. 5 mM-$MgSO_4$ and 6·4 mM-Ca-gluconate, previously equilibrated to pH 7·5 by gassing with a 95 % O_2 + 5 % CO_2 mixture.

The abdomino-visceral ganglionic mass comprising both pleural and both pallial ganglia and the visceral ganglion was carefully dissected in order to remove the connective tissue sheaths, thus allowing the observation under a stereoscopic microscope of naked neuronal perikarya of 70–120 μ diameter. Snail neurones are monopolar and their axo-axonal synaptic

contacts are located in the neuropile beyond the axon-hillock (Gerschenfeld, 1963). Orthodromic electrical stimulation of the neurones was done through both pallial, the anal and the visceral nerves and both cerebro-abdominal connectives placed on suitable pairs of Ag–AgCl electrodes.

The cells were impaled with two independent micro-electrodes of 0·5–1 μ tip diameter or a double-barrelled micro-electrode of 1–1·5 μ tip diameter. The resistances were of 15–30 MΩ and they were pulled with a Fonbrune microforge. One of the micro-electrodes was filled with a 3 M solution of KCl and was used for recording; the other micro-electrode, filled with a 0·6 M solution of Na_2SO_4, was used for injecting inward or outward current with respect to an earthy-electrode placed at the bottom of the chamber in contact with the saline solution through an agar-Ringer bridge. Double-barrelled micro-electrodes presenting coupling resistances higher than 100 kΩ were discarded. The membrane resistance was measured by passing square pulses of inward or outward current between the Na_2SO_4 filled micro-electrode and the ground lead.

5-HT was generally applied to the neurones by iontophoresis (Nastuk, 1953; del Castillo & Katz, 1955) under microscopic control. The injecting micro-electrodes were also pulled with the Fonbrune microforge and had resistances of 35–60 MΩ. 5-HT was injected as a cation from a 0·15 M aqueous solution of 5-HT creatinine-sulphate freshly prepared, at a pH of 3·2. When double-barrelled micro-electrodes were used for injection, the second barrel was filled with a 1 M aqueous solution of acetylcholine chloride (ACh). Braking currents were used to avoid drug leakage from micro-electrodes. Possible electrical artifacts were controlled by reversing the polarity of the injecting current.

If 5-HT had no effect on a neurone, one of two procedures was used to check that the micropipette was close to a cell: (*a*) when a single pipette was used, it was introduced into the cell and after confirming its intracellular position, by passing a square pulse of current, it was pulled out again and the injection repeated; (*b*) when a double-barrelled micropipette was used, ACh was applied from the second barrel: a response to ACh confirmed the localization of the micro-electrodes. When an injection was made through a double-barrelled micropipette braking currents were always applied to the other barrel, to prevent leakage of the non-injected drug. The injecting current was monitored with a RCA micro-ammeter.

In some experiments drugs such as blocking agents or enzyme inhibitors were added to the fluid perfusing the preparation. Such solutions were applied in volumes of at least 20 times the content of the chamber. In the case of drug applications longer than a few minutes the bath was continuously renewed during the perfusion.

Dose–response curves were prepared by plotting the effect of the injections against the logarithm of the injecting current in nA. The effect of iontophoretic injections was expressed as the percentage of a control maximal effect (Figs. 5–7). Before the experiments were made the injecting micropipettes were tested for a linear relation between current and voltage (see example in Fig. 7*B*). Those with a non-linear relation were discarded.

The experiments were performed at temperatures between 20° and 24° C. Although the ganglia remained in good condition for 6–8 hr the experiments were never prolonged beyond the 4th hr. The present work was performed on 118 preparations. From a total number of 366 cells explored, seventy-three neurones with 5-HT receptors on their membrane were found. Each of the experiments described in the Results was confirmed in at least six to eight cells.

RESULTS

Characterization of 5-HT receptors. A survey of iontophoretic microinjections of 5-HT on to the somatic membrane of *Cryptomphallus* neurones showed that only few cells in each ganglion were responsive to 5-HT. The neurones having 5-HT receptors were also characterized by receiving a

complex synaptic input which gives rise to excitatory post-synaptic potentials (EPSP) and to a peculiar long lasting wave of hyperpolarization that Tauc (1959, 1960) first described by the name of 'inhibition of long duration'. These cells have been named CILDA (cells with inhibition of long duration, Gerschenfeld & Tauc, 1964). Although all the cells responsive to 5-HT were of the CILDA type, five neurones of this type did not respond to 5-HT injections.

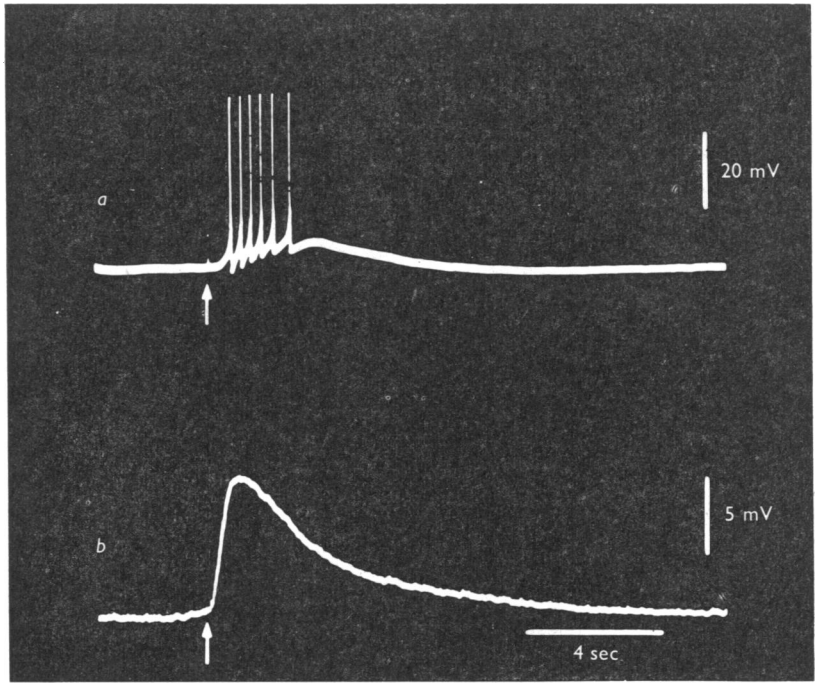

Fig. 1. Effects of iontophoretic applications of 5-HT (arrows) on to a CILDA neuron. *a*, at the resting potential (−47 mV); *b*, after artificially hyperpolarizing the cell to −80 mV to avoid the spike firing. Current intensity: 5×10^{-8} A; duration 500 msec.

5-HT always depolarized and excited CILDA neurones; in no case were hyperpolarizing or inhibitory actions observed. Figure 1*a* shows a typical response to 5-HT of one of these neurones at the resting membrane potential of −47 mV. The response consisted of a depolarization accompanied by a train of spikes. When the cell was artificially hyperpolarized to −80 mV, the response to a similar injection consisted of a depolarization (5-HT potential) (Fig. 1*b*) with a rapid rising phase followed by a relatively slow return to the initial potential level.

Sensitivity and localization of the receptors. Figure 2*A* shows an experiment in which were compared the depolarizations evoked by the injection

of ACh and 5-HT from a double-barrelled micropipette on to a CILDA neurone artificially hyperpolarized to -60 mV. The injection of ACh (current, 3×10^{-7}A for 10 msec) caused a depolarization of 3 mV (Fig. 2*A*, *a*). The injection of 5-HT (2×10^{-6}A for 10 msec) produced a depolarization of 4 mV (Fig. 2*A*, *b*).

Approximate calculations of the probable maximum concentrations of 5-HT and ACh at the receptors can be made from a suitable solution of the diffusion equation (Carslaw & Jaeger, 1947). del Castillo & Katz (1955) assumed that when a substance is applied instantaneously from a point source in a homogeneous medium, the peak concentration at a point at distance r from the source is given by the equation

$$C = \frac{Q}{8\,(\pi DT)^{1.5}} \times \exp\left(-\frac{r^2}{4DT}\right),$$

where C is the peak concentration, Q is the amount of substance applied, D the diffusion constant of the substance and T the time to the peak of the response.

In the case of iontophoretic injections r represents the distance between the tip of the injecting pipette and the receptors and according to del Castillo & Katz (1955) it may be calculated from the equation $r^2 = 6DT$. In the case of the injections of Fig. 2*A*, $T_{5\text{-HT}} = 2370$ msec and $T_{\text{ACh}} = 300$ msec. Since an exact value of the diffusion constant of 5-HT was not available, a value of $6{\cdot}5 \times 10^{-6}$ cm^2/sec corresponding to the diffusion constant of 5-hydroxytryptophan was used (Longsworth, 1953). The values of r thus obtained were:

$$r_{5\text{-HT}} = 96\,\mu, \quad r_{\text{ACh}} = 38\,\mu.$$

Since 5-HT and ACh were applied from the same point, the receptors to the drugs were at different points on the cell.

The values of $Q_{5\text{-HT}}$ and Q_{ACh} were calculated from the charge transferred assuming a transport number for ACh of 0·3 and for 5-HT of 0·5. This last figure was assumed since the pipettes were filled with a solution of a sulphate-creatinine complex of 5-HT. The calculated values of Q were

$$Q_{5\text{-HT}} = 10^{-13}\text{M}, \quad Q_{\text{ACh}} = 10^{-14}\text{M}.$$

Then, $$C_{5\text{-HT}} \propto 8{\cdot}2 \times 10^{-9}\text{M}, \quad C_{\text{ACh}} \propto 1{\cdot}3 \times 10^{-8}\text{M}.$$

The peak maximum concentration of 5-HT producing a depolarization of 4 mV was $8{\cdot}2 \times 10^{-9}$ M. A depolarization of 3 mV was caused by peak ACh concentration of $1{\cdot}3 \times 10^{-8}$ M at the receptor level.

Effect of 5-HT on the membrane resistance of CILDA neurones. The effect of 5-HT on CILDA neurone resistance was analysed by using two independent intracellular micropipettes and passing square pulses of currents through the membrane (see Methods).

Figure 2*C*,*a* illustrates the changes in amplitude of the electrotonic potentials with constant increments of polarizing current in a CILDA cell (resting potential = −50 mV). The filled circles curve of Fig. 2*B* is the current–voltage relation of the membrane of the same CILDA cell, showing that it presents an 'anomalous' rectification.

In Fig. 2*C*, *b* analogous increments of current were applied to the same CILDA cell after perfusing the preparation for 10 sec with a 5×10^{-4} M

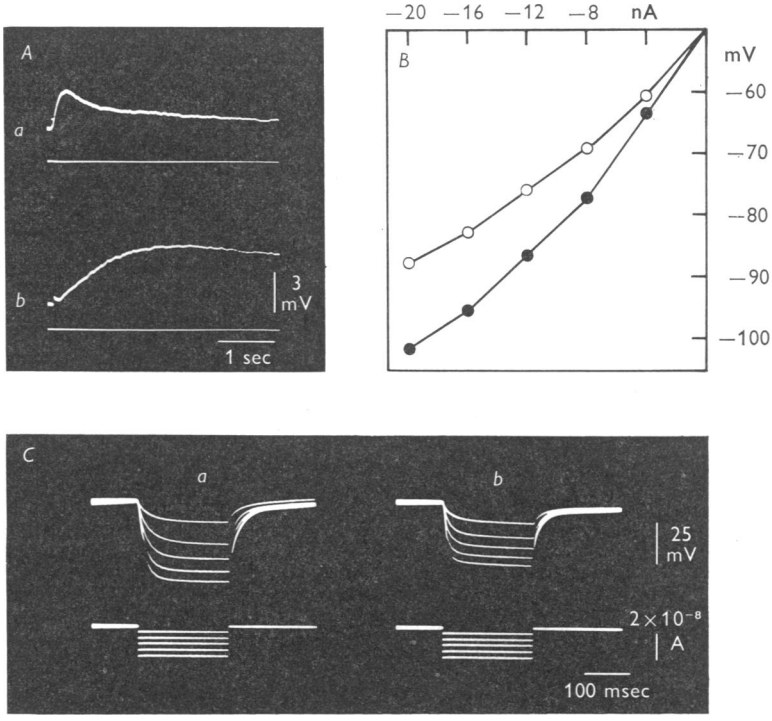

Fig. 2. *A*. Iontophoretic application of ACh and 5-HT from a double-barrelled micropipette to the same CILDA neurone. *a*, ACh (current of 3×10^{-7} A for 10 msec); *b*, 5-HT (current of 2×10^{-6} A for 10 msec).

B. Current–voltage relation of a CILDA neurone bathed in physiological solution (●) and after perfusing the entire preparation with solution containing 5×10^{-4} M 5-HT creatinine sulphate (○). Each point is the mean value of eight measurements (S.E. range from 0·13 to 0·42 mV).

C. In *a*, same CILDA neurone as in *B* bathed in physiological solution (resting potential −50 mV). The electrotonic potentials were evoked passing square pulses with constant increments in polarizing current through the membrane. Each current step is 4×10^{-9} A. In *b*, the same pulses were repeated 10 sec after the beginning of the perfusion of the whole preparation with a 5×10^{-4} M solution of 5-HT creatinine sulphate. The drug depolarized the cell by 9 mV, but the membrane potential was adjusted to −50 mV just before the record. The recording and the polarization were performed using two independent micropipettes.

solution of 5-HT creatinine sulphate complex. The drug depolarized the cell by 9 mV, but just before recording the membrane potential was adjusted to − 50 mV by passing a steady current. 5-HT caused a diminution of *ca.* 25 % in the amplitude of the electrotonic potentials. The open circles curve of Fig. 2*B* corresponds to the current–voltage relation under 5-HT.

Desensitization of 5-HT receptors. Successive applications of 5-HT to a CILDA neurone give rise to diminishing responses and the response to a steady application of 5-HT is not maintained. At the resting potential level, successive applications of 5-HT result in a decrease in the frequency of the spikes, or they may fail to arise at all. The desensitization is, however, best studied in cells which are kept artificially hyperpolarized, thus avoiding the discharge.

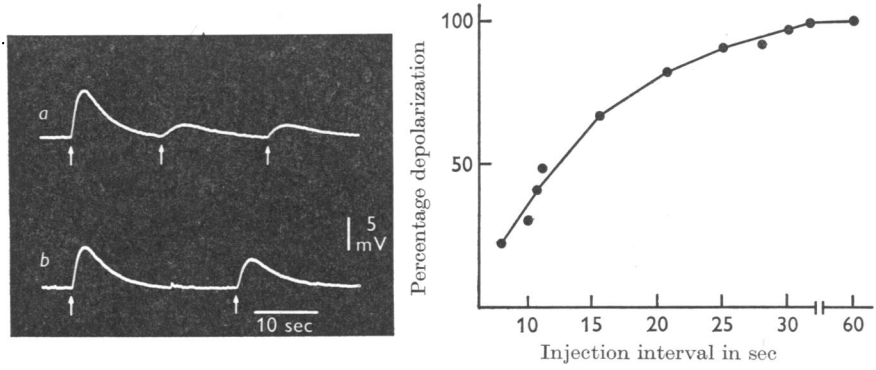

Fig. 3. Desensitization of 5-HT receptors. CILDA neurone artificially hyperpolarized to − 90 mV. In *a* and *b*, the repetition of the iontophoretic application of 5-HT (arrows) at different delays after the first application diminishes the amplitude of the successive 5-HT potentials. Delivering current $0{\cdot}6 \times 10^{-9}$ A, duration: 1 sec.

At right, desensitization curve, Abscissa: intervals between two successive identical injections. Ordinate: effect of the second injection expressed as percentage of the depolarization provoked by the first injection.

In the experiment of Fig. 3, the membrane potential was displaced from the resting value of − 45 to −90 mV; the records show that repetition of 5-HT injections at short intervals gives rise to a diminution of the amplitudes of the successive 5-HT potentials. In the curve of Fig. 3 the abscissae are the intervals separating two successive and identical 5-HT 'pulses' and the ordinates are the amplitudes of the 5-HT potentials evoked by the second injection expressed as a percentage of the amplitude of the 5-HT potential caused by the first. It can be seen that the second injection produces a response to equal that of the initial injection only after an interval of *ca.* 40 sec.

The records of Fig. 4 are from a CILDA cell in which pairs of 5-HT

micro-injections were separated by a constant interval of 20 sec. The intensity of the first (conditioning) injection was varied, while the intensity of the second (testing) injection was maintained constant. The diminution of the conditioning injection produces an increase of the 5-HT potential in response to the testing injection (Fig. 4*b*). On the other hand, the latter becomes diminished when the injecting current of the conditioning injection is increased (Fig. 4*c*). The curve of Fig. 4 relates the amplitude of the 5-HT potentials provoked by both the conditioning injection (I) and the testing injection (II). The stepwise increase of the amplitude of the first 5-HT potential is linearly related to the decrease of the amplitude of the second one.

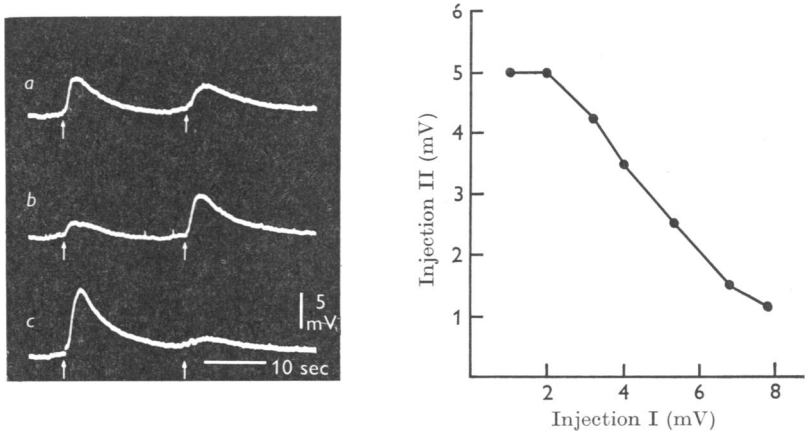

Fig. 4. CILDA neurone artificially hyperpolarized to −85 mV. In *a*, *b* and *c* two successive 5-HT applications (arrows) are applied with a constant interval of 20 sec. The delivering current of the second (testing) application in all the records was $6{\cdot}2 \times 10^{-8}$ A. First application: *a*, $6{\cdot}2 \times 10^{-8}$ A; *b*, 5×10^{-9} A; *c*, 10^{-7} A. The duration of all delivery current was 400 msec.

The curve at right represents the relation between the amplitudes of the 5-HT potentials obtained with the two applications.

Effect of antagonists of 5-HT on the CILDA neurone 5-HT receptors. In the guinea-pig ileum Gaddum & Picarelli (1957) described two major types of 5-HT receptors distinguishable by different blocking agents: *D* receptors of visceral smooth muscles blocked by lysergic acid diethylamide (LSD 25) and its derivatives and *M* receptors localized in nervous components and blocked by morphine but not by LSD 25. Figure 5*A* shows an experiment with the bromo-derivative of LSD 25 (BOL 148). The upper record (*a*) shows the response to a control 5-HT micro-injection on to a CILDA neurone artificially hyperpolarized to −85 mV. The middle record (*b*) shows the effect on the 5-HT potential of a 10^{-3} M solution of BOL 148 applied to the whole preparation for a few minutes; in the lower

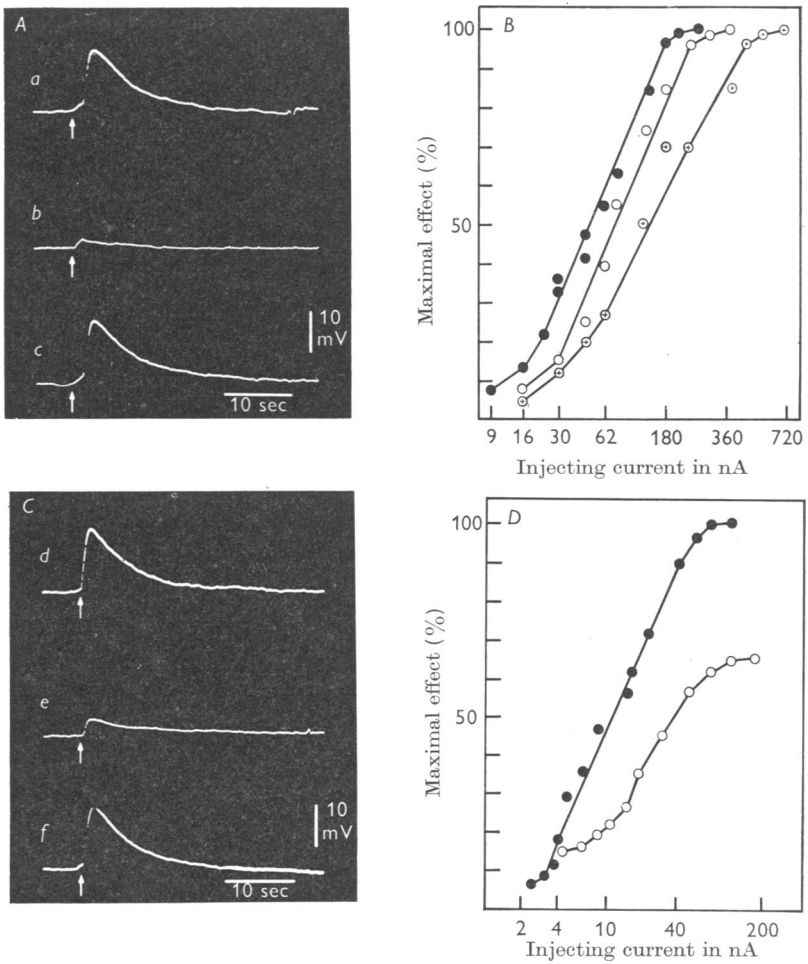

Fig. 5. CILDA neurone artificially hyperpolarized to −85 mV. *a*, control 5-HT potential produced by a delivering current pulse of 5×10^{-8} A and 1 sec duration. *b*, effect of the same pulse (arrows) 2 min after the application by perfusion to the whole preparation of a 10^{-3} M solution of bromolysergic acid (BOL 148). *c*, the initial amplitude of 5-HT potential is restored by a short washing.

B, dose–response curves obtained by plotting the logarithm of the delivering current in nA (abscissa) against the response (ordinate) expressed as percentage of the maximal effect obtained in the control curve (●). Middle and right curves obtained in the presence of $2{\cdot}5 \times 10^{-8}$ M (○) and $2{\cdot}5 \times 10^{-7}$ M (⊕) of bromolysergic acid. Notice the similar slope and maximal effect of the three curves.

C, CILDA neurone hyperpolarized to −85 mV. *d*, control 5-HT potential produced by a delivering pulse of 5×10^{-8} A and 1 sec duration. *e*, presence of a 10^{-3} M solution of morphine chlorhydrate in the bath block the effect of 5-HT application (arrows). *f*, after the removal of morphine the effect of the application is restored.

D, The dose–response curve obtained in the presence of a 3×10^{-5} M solution in the bath (○) presents a different slope and does not attain the maximal effect of the control curve (●). The difference in the values of the abscissa in *B* and *D* mainly depends on a difference in the distance of the injecting micro-electrodes.

record (*c*), the 5-HT potential recovered its original amplitude after the preparation was washed.

Figure 5*B* shows a series of dose–response curves from the same cell. The dose is expressed as the logarithm, of the injecting current, and the response is expressed as a percentage of the maximal effect obtained in the control series of injections (left curve, filled circles). The middle curve (open circles) has been drawn with the values obtained from a series of injections in the presence of a $2{\cdot}5 \times 10^{-8}$ M solution of BOL 148, and the right curve (crossed circles) from another series of micro-injections in the presence of a $2{\cdot}5 \times 10^{-7}$ M solution of BOL 148. The three S-shaped curves are of similar slope and show that a maximal effect was obtained in the presence of the antagonist. Similar experiments with LSD 25 show that this drug behaves like its bromo-derivative. On the other hand, neither LSD 25 nor its bromo-derivative alter the CILDA neurone membrane conductance, even in the more concentrated solutions.

Figure 5*C* shows how a 5-HT potential in a hyperpolarized CILDA neurone (*d*) is blocked by a 10^{-3} M solution of morphine chlorhydrate (*e*). This effect is reversible since the 5-HT potential returns to its initial value (*f*) after 10 min of washing. However, in this case, the curves of Fig 5*D* show that the right S-shaped curve (open circles) obtained in the presence of a 3×10^{-5} M solution of morphine chlorhydrate, presents a different slope than the control curve (filled circles) and only attains 65 % of the maximal effect.

Other antagonists of 5-HT have been tested. Tryptamine, cyproheptadine hydrochloride and dibenamine block irreversibly the receptors. The blockade of 5-HT receptors by atropine sulphate will be analysed below.

Effect of some inhibitors of monoamine oxidase on 5-HT receptors. The action of some well known inhibitors of monoamine oxidase, the inactivating enzyme of 5-HT, was studied on CILDA neurones. The substances analysed were: tranylcypromine (SKF-trans 385), iproniazid, nialamide and isocarboxazide (Marplan). The action of all these drugs is similar and Fig. 6 illustrates an example of the action of isocarboxazide. Figure 6*a* shows a control 5-HT potential from a CILDA neurone artificially hyperpolarized to -90 mV. In Fig. 6*b* and *c* the whole preparation was bathed in a 4×10^{-4} M solution of isocarboxazide and it may be seen that the amplitude of the 5-HT potential has diminished. The effect may be observed within 1 min of the application of the drug. The removal of isocarboxazide restores (Fig. 6*d*) the initial amplitude of the 5-HT potential. It may be noticed that the background 'spontaneous' excitatory post-synaptic potentials in the record of Fig. 6*a* appear to increase in frequency during perfusion with isocarboxazide, and diminish in amplitude after the removal of the drug.

The curves of Fig. 6 are two dose–response plots obtained from two series of 5-HT injections on to the same CILDA neuron as in the experiment of Fig. 6*a–d*. Both the control curve (filled circles) and the curve obtained in the presence of a 4×10^{-4} M solution of isocarboxazide (open circles) have similar slopes and maxima. None of the inhibitors of monoamine oxidase assayed was found to affect the membrane resistance.

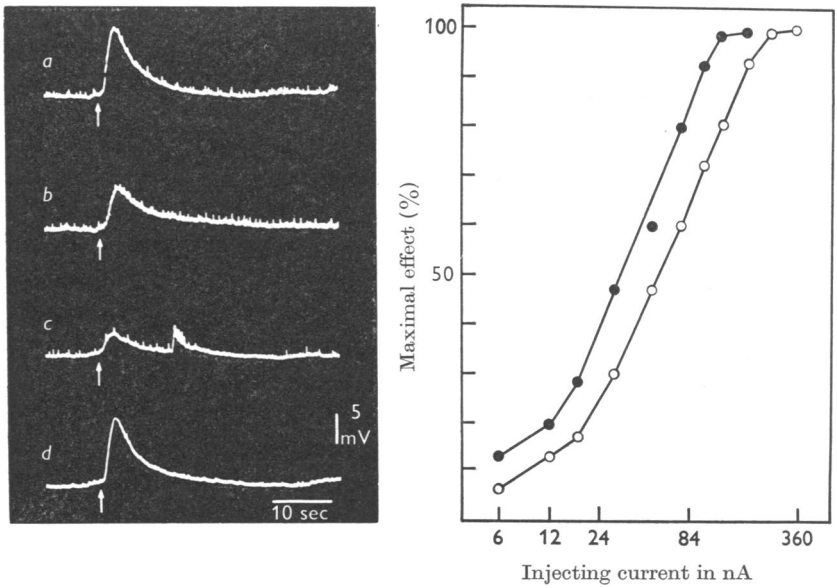

Fig. 6. CILDA neurone artificially hyperpolarized to −90 mV. *a*, control depolarization produced by 5-HT application (2×10^{-9} A and 1 sec duration). *b* and *c*, diminution of effect of the same 5-HT dose (arrows) 5 and 8 min after the perfusion of the preparation with a 4×10^{-4} M solution of isocarboxazide. *d*, the initial effect of 5H-T is restored by washing of the preparation. Notice that the synaptic noise present in *a* increases in frequency in *b*, diminishes in *c* and almost disappears in *d*.

At right, dose–response curves. Both the control curve (●) and that obtained in the presence of a 4×10^{-4} M solution of isocarboxazide (○) present similar slopes and attain the same maximum.

Differences between cholinergic and tryptaminergic receptors present on the same membrane. As was shown above, CILDA neurones may also have cholinergic receptors on their membrane and the location and the sensitivities of the receptors to 5-HT and to ACh have been compared.

Figure 7*B* shows that the voltage–current relation is linear for each of the barrels of the double-barrelled micro-electrode used to inject ACh and 5-HT in the experiments of Fig. 7*A*. The upper record of Fig. 7*A* shows a typical desensitization of 5-HT receptors. The middle record (*b*) shows that the desensitization of ACh receptors by a series of four successive ACh injections does not affect the response to an injection of 5-HT. In the

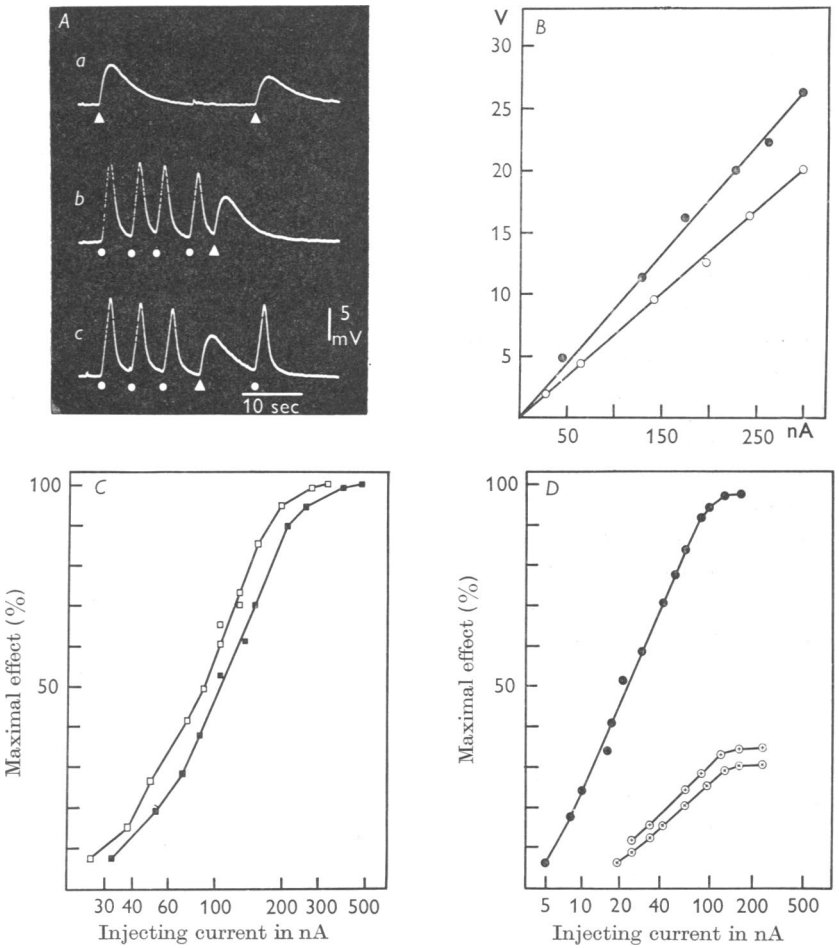

Fig. 7. *A*, Absence of crossed desensitization between 5-HT and ACh in the same CILDA neurone hyperpolarized to -90 mV. All the iontophoretic applications of ACh (○) were done with an intensity of $1{\cdot}4 \times 10^{-9}$ A and 1 sec duration. 5-HT was always applied using a current of 6×10^{-9} A for 1 sec. *a*, 5-HT desensitization. *b*, 5-HT potential is not affected after the ACh-receptor desensitization by four successive ACh injections. *d*, 5-HT potential interposed between the third and the last ACh injections, is not affected by ACh receptor desensitization and does not influence the last ACh potential.

B, linear voltage–current relation of the micro-electrodes utilized for injecting ACh (○) and 5-HT (●).

C, Dose–response curves of ACh. Both the control curves (□) and that obtained when a $7{\cdot}2 \times 10^{-6}$ M solution of atropine sulphate is present in the bath (■) attain the maximum effect.

D, The dose–response curves of 5-HT obtained in the presence of $7{\cdot}2 \times 10^{-6}$ M and $1{\cdot}6 \times 10^{-5}$ M solutions of atropine sulphate (⊕⊗) have a different slope from the control curve and do not attain the same maximum.

lower record (*c*) a 5-HT injection has been interposed between the third and the last of a series of four ACh injections. The amplitude of the 5-HT potential has not been changed by the previous ACh receptor desensitization and at the same time its presence has not affected the last ACh injection which after the delay introduced by the 5-HT potential has almost recovered its original amplitude. These experiments show the absence of crossed desensitization between 5-HT and ACh.

Atropine sulphate blocks the cholinergic response of molluscan neurones (Tauc & Gerschenfeld, 1961, 1962) and may also block the 5-HT response of CILDA neurones.

In Fig. 7*C* the left curve (open squares) corresponds to a control dose–response curve obtained from a series of ACh injections on to the same CILDA neurone membrane of Fig. 7*A*. The right curve (filled squares) was obtained in the presence in the medium of $7{\cdot}2 \times 10^{-6}$ M atropine sulphate. The two curves have similar slopes and maxima.

Figure 7*D* corresponds to the action of atropine sulphate on the 5-HT receptors of the same CILDA cell. The left curve (filled circles) is the control and the two other curves (crossed circles) were obtained in the presence of two different solutions of atropine sulphate: $7{\cdot}2 \times 10^{-6}$ M and $1{\cdot}4 \times 10^{-5}$ M respectively. The slope of these two curves is different from that of the control and they only attain 37 % of the maximal effect.

DISCUSSION

A survey of the responses of neurones of *Cryptomphallus aspersa* to 5-HT applied by iontophoretic injection shows that only a limited number of cells have 5-HT receptors on their membranes. This observation is at variance with previous results obtained in *Aplysia* and *Helix* by perfusing the whole ganglia which provoked more generalized responses (Gerschenfeld & Tauc, 1961). The difference is possibly due to the excitation by 5-HT of excitatory interneurones giving rise to a secondary activation of many neurones.

In nervous ganglia of *Cryptomphallus aspersa* 5-HT receptors have been found exclusively in the membrane of the so-called CILDA neurones and never in the other cell types described (Gerschenfeld & Tauc, 1961, 1964). However, in five neurones of a total of seventy-eight CILDA neurones studied, the iontophoretic application of 5-HT produced no response. In some of these neurones the injection of ACh was effective indicating that the insensitivity to 5-HT was not due to anatomical barriers hindering the access of the drug to the receptor.

CILDA neurones were always depolarized by iontophoretic injections of 5-HT and inhibitory effects of the drug were never observed. ACh injec-

tions also evoked depolarization in these neurones. Calculations based on the application of the diffusion equation for a point source in an homogeneous medium (del Castillo & Katz, 1955) demonstrated that when 5-HT and ACh are injected from the same point with a double-barrelled micropipette, the distance from the 5-HT micro-electrode to the receptors is longer than the distance between the ACh source and the receptors. Since there is good evidence that ACh receptors are present on the entire somatic membrane of monopolar molluscan neurones (Tauc & Bruner, 1963), it may be assumed that 5-HT receptors are located in the region of the soma close to the axon hillock or beyond it. The sensitivity of 5-HT receptors of CILDA neurones appears to be compatible with sensitivity expected from a transmitter receptor responding with a depolarization of 4 mV to a calculated 5-HT concentration of $8{\cdot}2 \times 10^{-9}$ M. The ACh receptors of CILDA neurones responded by a depolarization of 3 mV to a calculated ACh concentration of $1{\cdot}3 \times 10^{-8}$ M. It may be remembered that in molluscan nervous system ACh is probably the transmitter of excitation to the so-called D neurones and of inhibition to the conventionally named H neurones (Tauc & Gerschenfeld, 1961, 1962).

A fundamental requirement that a synaptic transmitter must fulfil is the property of altering the membrane resistance of the supposed postsynaptic cell (del Castillo & Katz, 1954; see Eccles, 1964). Therefore, if 5-HT is expected to be a transmitter to CILDA neurones, it should be capable of changing the membrane resistance. The membrane of CILDA neurones presents 'anomalous' rectification, previously analysed in *Helix* giant cells by Tauc & Kandel (1964). The experiments presented here show that 5-HT increases the CILDA membrane conductance by about 40%. Previous results (Gerschenfeld & Stefani, 1965) have shown that 5-HT does not affect other types of neurones found in *Cryptomphallus* ganglia.

5-HT receptors of CILDA neurones show desensitization phenomena. This property of receptors has been described by Katz & Thesleff (1957) for ACh receptors of the frog skeletal muscle. Desensitization also occurs with other receptors of molluscan neurones, i.e. those sensitive to ACh (Tauc & Bruner, 1963) and to glutamate (Gerschenfeld & Lasansky, 1964). Takeuchi & Takeuchi (1964) have also recently analysed desensitization of glutamate receptors of crustacean muscle.

Many different substances block the action of 5-HT on CILDA neurones. A comparison of the dose–response curves for 5-HT in the presence of BOL 148 and of morphine suggests that the blockade by BOL 148 is different from that produced by morphine, and that the latter appears to block the 5-HT receptors non-competitively. A competitive blockade of 5-HT receptors of rat uterus by LSD 25 was first shown by Gaddum (1953). Recently Marchbanks, Rosemblat & O'Brien (1964) have shown

that the binding of 5-HT to subcellular particles of nerve endings was also inhibited by LSD 25.

Atropine also blocks 5-HT receptors of CILDA neurons. Comparing this effect with the blockade of the ACh receptors of the same cells by atropine, it may be said that atropine blocks 5-HT receptors to a greater extent. The calculated difference in location of 5-HT and ACh receptors, the failure of crossed desensitization between ACh and 5-HT and the differences in atropine action all suggest that 5-HT receptors are not the same as the ACh receptors.

A series of inhibitors of mono-amino oxidase, the enzyme inactivating 5-HT (Sjoerdsma, Smith, Stevenson & Udenfriend, 1955), such as trancylpromine, isocarboxazide, nialamide and iproniazide, not only fail to produce the expected prolongation of 5-HT effects (i.e. such as produced by anticholinesterase agents in the case of ACh) but moreover block 5-HT receptors of CILDA neurones. The absence of a prolongation of the antagonistic effect is not so surprising in view of the demonstration in subcellular fractions of vertebrate brain (Rodriguez de Lores Arnaiz & De Robertis, 1962) that monoamine oxidase is located in mitochondria and that it is not related to the cell membrane.

5-HT has been recently found in the nervous system of different species of snails, such as *Helix pomatia* (Cardot & Ripplinger, 1963), *Helix aspersa* (Kerkut & Cottrell, 1963) and *Cryptomphallus aspersa* (L. M. Zieher & H. M. Gerschenfeld, unpublished results). In the last species it is present in a labile store, which may be depleted by reserpine. There is also good evidence for the existence in snail ganglia of the enzymes responsible for the synthesis (Cardot, 1963; Kerkut & Cottrell, 1963) and inactivation (Cardot, 1964) of 5-HT.

It has been observed that BOL 148 may block the excitatory postsynaptic potentials of some CILDA cells in Aplysia (Gerschenfeld & Tauc, 1964) and *Cryptomphallus aspersa* (H. M. Gerschenfeld & E. Stefani, unpublished results). In the case of *Aplysia*, BOL 148 has also a strong action on the nerve conduction (H. M. Gerschenfeld, P. Ascher & L. Tauc, unpublished).

The available data therefore make 5-HT a suitable candidate as a synaptic transmitter. However, further work is necessary to establish that 5-HT is an excitatory synaptic transmitter to molluscan CILDA neurons.

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