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ON THE TRANSMITTER FUNCTION OF 5-HYDROXYTRYPTAMINE AT EXCITATORY AND INHIBITORY MONOSYNAPTIC JUNCTIONS

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SUMMARY

1. Two symmetrical giant neurones located in the cerebral ganglion of *Aplysia californica* contain 4–6 p-mole 5-hydroxytryptamine (5-HT) and are able to synthesize it (Weinreich, McCaman, McCaman & Vaughn, 1973; Eisenstadt, Goldman, Kandel, Koike, Koester & Schwartz, 1973). Stimulation of each of these neurones evokes excitatory and inhibitory potentials in various cells of the ipsilateral buccal ganglion. In nine buccal neurones it evokes excitatory potentials, in other three, 'classical' inhibitory potentials and in one neurone an 'atypical' inhibitory potential.

2. The connexion between the giant cerebral neurone and the cells receiving either an excitatory or a 'classical' inhibitory input from it are monosynaptic. TEA injection into the cerebral giant neurone, which prolongs the presynaptic spike, causes a gradual increase of both the excitatory and the inhibitory potentials. On the other hand, high Ca^{2+} media, which block polysynaptic pathways, do not suppress these synaptic potentials.

3. The iontophoretic application of 5-HT to the buccal neurones receiving excitatory input from the giant cerebral neurones evokes depolarizations showing the pharmacological properties of both *A*- and *A'*-responses to 5-HT (see preceding paper). Antagonists which block only the *A*-receptors (curare, 7-methyltryptamine, LSD 25) block partially the synaptic depolarizing potentials. Bufotenine, which blocks both the *A*- and *A'*-receptors, completely blocks the excitatory potentials. Thus, the post-synaptic membrane of these buccal neurones appears to be endowed with both *A*- and *A'*-receptors to 5-HT.

4. The 'classical' inhibitory potentials elicited in three buccal neurones are hyperpolarizations which reverse at -80 mV and are due to an increase in K^+-conductance. The iontophoretic application of 5-HT to these post-synaptic neurones evokes hyperpolarizing *B*-responses which are also

generated by an increase in K^+-conductance. Antagonists which block the *B*-responses (bufotenine, methoxygramine) also block the inhibitory potentials.

5. The 'atypical' inhibitory potential evoked in one buccal neurone consists in an hyperpolarization which increases in amplitude with cell hyperpolarization. Iontophoretic application of 5-HT to this buccal cell evokes an hyperpolarizing β-response which also increases in amplitude with cell polarization and results from a *decrease* in both Na^+- and K^+-conductances. The monosynaptic character of the 'atypical' inhibitory potential is not yet fully proven.

6. It can be concluded that the excitatory and inhibitory synaptic effects evoked in the buccal neurones by the stimulation of the 5-HT-containing-giant cerebral neurones are very likely mediated by 5-HT.

INTRODUCTION

In spite of a great deal of circumstancial evidence, the transmitter function of 5-hydroxytryptamine (5-HT) has not yet been convincingly demonstrated. Having been discovered in non-neural tissues (see Erspamer, 1966), its detection in vertebrate C.N.S. (Twarog & Page, 1953; Amin, Crawford & Gaddum, 1954) in a particular regional distribution (Bogdansky, Weissbach & Udenfriend, 1956) immediately suggested the possibility that this indoleamine was a chemical transmitter (Brodie & Shore, 1957). The idea was reinforced by the finding of 5-HT in nerve endings isolated from the brain (Michaelson & Whittaker, 1963; Zieher & De Robertis, 1963), and the tracing of 5-HT containing pathways in the vertebrate C.N.S. (see Fuxe, Hökfelt & Ungerstedt, 1970).

The discovery of 5-HT in the C.N.S. of invertebrates also raised the possibility of its transmitter role there (Welsh, 1957) and subsequent work has suggested that such a role might be more convincingly demonstrated for gastropod central synapses than elsewhere. Thus, as would be expected of a synaptic transmitter, 5-HT was shown to alter the membrane conductance of neurones (see Gerschenfeld & Paupardin-Tritsch, 1974); furthermore, it has been shown for snail neurones that substances which blocked the depolarizing action of 5-HT also blocked some evoked excitatory synaptic potentials (Gerschenfeld & Stefani, 1968).

Recently, a more refined and precise way of looking for tryptaminergic synapses in molluscs has been reported. Cottrell & Osborne (1970) have used the Falck–Hillarp technique to identify two symmetrically located neurones in *Helix* metacerebral ganglia, which, as confirmed by bio-assay and microchemical methods (Cottrell & Osborne, 1970; Osborne, 1972), contain 5-HT. These giant cells, as has been shown by Cottrell (1970, 1971),

provide excitatory inputs to three identified neurones located in the ipsilateral buccal ganglion. Cottrell & Macon (1974) have recently shown that the connexions are probably monosynaptic and that the excitatory potentials are affected by pharmacological agents in a way which suggests that they may be generated by 5-HT.

In parallel studies, Weinreich, McCaman, McCaman & Vaughn (1973) have demonstrated in *Aplysia californica* the existence of a homologous pair of giant neurones in its cerebral ganglia containing 4–6 p-mole 5-HT in their somata and capable of synthesizing 5-HT from 5-hydroxytryptophan. These cells can also synthesize 5-HT from tryptophan (Eisenstadt *et al.* 1973).

The present work is concerned with the connexions made by these *Aplysia* cells. Each of them has been found to make monosynaptic connexions with at least thirteen neurones of the ipsilateral buccal ganglion. In nine the input was excitatory, in four, inhibitory. Evidence has been obtained that the transmitter mediating these excitatory and inhibitory synaptic actions is very likely to be 5-HT and that of the six different 5-HT receptors described in the preceding paper, four (A, A', B, β) are involved in the generation of the synaptic potentials.

Preliminary accounts of some of the results have been already given (Paupardin-Tritsch & Gerschenfeld, 1973; Gerschenfeld & Paupardin-Tritsch, 1973).

METHODS

The general procedures in this work were the same as those described in the preceding paper (Gerschenfeld & Paupardin-Tritsch, 1974).

The preparation consisted of the cerebral and both buccal ganglia of *Aplysia californica* which were removed together with the intact cerebro-buccal connectives. This ganglionic ring (Fig. 1) was fixed to the bottom of a suitable chamber and continuously bathed with circulating artificial sea water. The connective tissue sheaths of the ganglia were removed by dissection. Two double barrelled micropipettes were used, one to impale one of the giant cerebral neurones and the other, one of the ipsilateral buccal neurones. The pipettes were filled with 0·6 M-K_2SO_4 or 3 M-KCl. In some cases (see below) one of the barrels impaling the giant cerebral neurone was filled with a 0·5 M solution of tetraethylammonium (TEA) chloride or bromide. TEA ions were injected into the neurone by passing suitable currents either between the barrels (Eccles, Eccles & Ito, 1964), or between the TEA pipette and the earth electrode. In some experiments, a third external pipette filled with a saturated solution of 5-HT creatinine sulphate solution was used to apply iontophoretically 5-HT to the buccal cells.

RESULTS

At their resting potential (of about -60 mV), the 5-HT containing giant cerebral neurones of *Aplysia* are generally silent. When required, they were stimulated to give a train of action potentials by passing square

pulses of outward current across the cell. This will be referred to as a presynaptic stimulation (Fig. 2 *et seq.*). A systematic screening of the neurones of the ipsilateral buccal ganglion revealed that such stimulation excited at least nine cells and inhibited at least four other neurones. All these thirteen neurones were located on the caudal face of the buccal ganglion. The two largest of these cells have been described by Gardner (1971) as BR1 (or BL1) and BR2 (or BL2); the approximate location of the others, which have not been previously studied, is shown in Fig. 1 (squares). After dissection, it was difficult to identify the buccal cells individually, from preparation to preparation, and no distinction between them will be made in this paper.

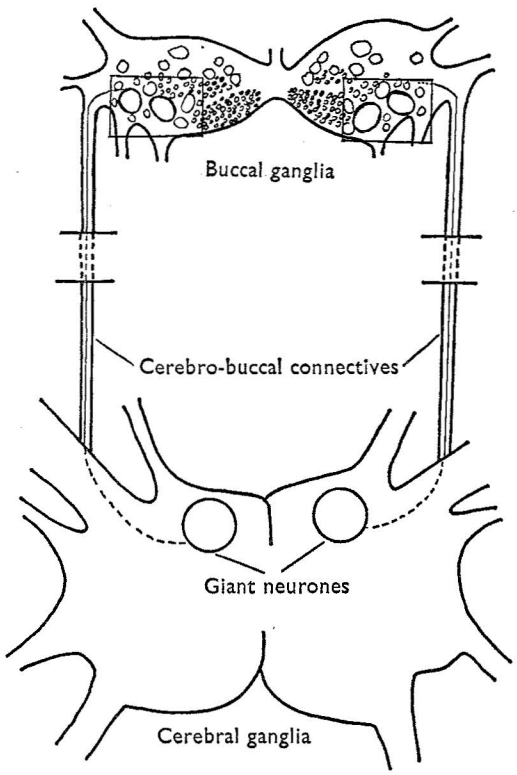

Fig. 1. Schematic drawing of the preparation (see description in the text).

Excitatory connexions between the cerebral giant cell and the buccal neurones

The response of each of the nine buccal neurones to presynaptic stimulation generally consists of a slow wave of depolarization. The amplitude of this response varies from cell to cell as can be seen from Fig. 2 which

illustrates the responses of three different cells to the same train of spikes of the same presynaptic neurone. This variability probably results from the location of the excitatory synapses. Occasionally, discrete excitatory synaptic potentials result from each spike of the giant cerebral neurone; some examples will be given later.

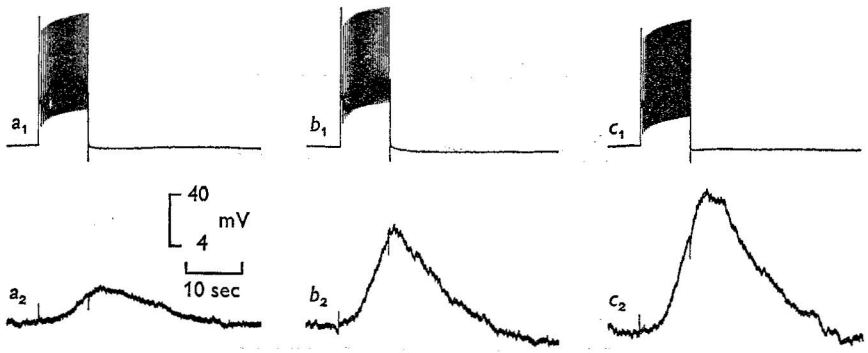

Fig. 2. Excitatory synaptic responses of three different buccal neurones to the stimulation of the same ipsilateral giant cerebral neurone. Recordings in a_1, b_1, c_1 are similar train of spikes evoked in the same giant cerebral neurone; a_2, b_2, c_2 recordings from three different buccal neurones, all of them previously hyperpolarized by inward current to -55 mV. Notice the different amplitudes of the excitatory potentials (see text).

The analysis of the synaptic responses of the buccal cells was hindered by the marked fall in their input resistance with hyperpolarization ('anomalous rectification'). This effect is illustrated in Fig. 3 which shows that hyperpolarization reduces both the responses to presynaptic stimulation and to current pulses.

Monosynaptic character of the excitatory connexions. Since unfortunately, no anatomical evidence is available, the first question to be decided about the excitatory potentials recorded from the buccal neurones was whether they were produced directly by transmitter release from the giant cerebral neurone or if there were other neurones interposed in the pathway. Two different tests were made to answer this question.

The first was that used by Kehoe (1972*c*) to demonstrate the monosynaptic character of the connexions of a cholinergic neurone in the pleural ganglion of *Aplysia*. It is based on the well established fact that TEA ions injected into excitable cells block the K^+-conductance increase associated with the action potential (see Hille, 1970), thus prolonging its duration. As demonstrated by Katz & Miledi (1967) and by Kusano, Livengood & Werman (1967), the prolongation of the depolarization of the nerve terminal (either by a spike or a depolarizing pulse) results in an increase of the amount of transmitter released by the presynaptic endings and thus

in an increased amplitude and duration of the presynaptic potential. Thus if there were a monosynaptic connexion between the giant cerebral neurone and the buccal neurones, it would be expected that the injection of TEA into the cerebral cell, after a sufficient time to allow its intra-

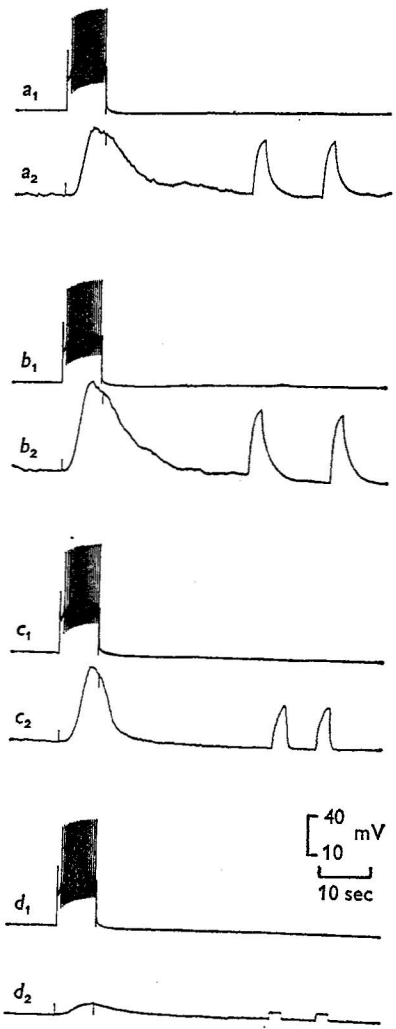

Fig. 3. The stimulation of the same giant cerebral neurone in a_1, b_1, c_1, d_1 evokes excitatory potentials in a buccal cell (a_2, b_2, c_2, d_2). At the end of each buccal cell recording, two square current pulses are passed across the membrane to measure the buccal neurone input resistance. The buccal neurone is hyperpolarized by inward current from -50 mV (a_1), successively to -60 (b_2), -70 (c_2) and -80 mV (d_2). Notice the parallel behaviour of both the synaptic and the electrotonic pulses, slightly increased in b_2 and markedly decreased in c_2 and d_2 (see text).

cellular diffusion, would prolong the presynaptic spike and thus produce a gradual and continuous augmentation of the post-synaptic response. Fig. 4 shows that this is exactly what happened. The discharge of a train of 19 spikes in a giant cerebral neurone evoked a slow depolarization of a buccal neurone in which no discrete excitatory potentials could be recognized (Fig. $4a_1$–a_2). After injecting TEA ions for 45 min into the giant cerebral cell, the same presynaptic stimulation caused both a remarkable increase in the amplitude of the slow depolarization and the

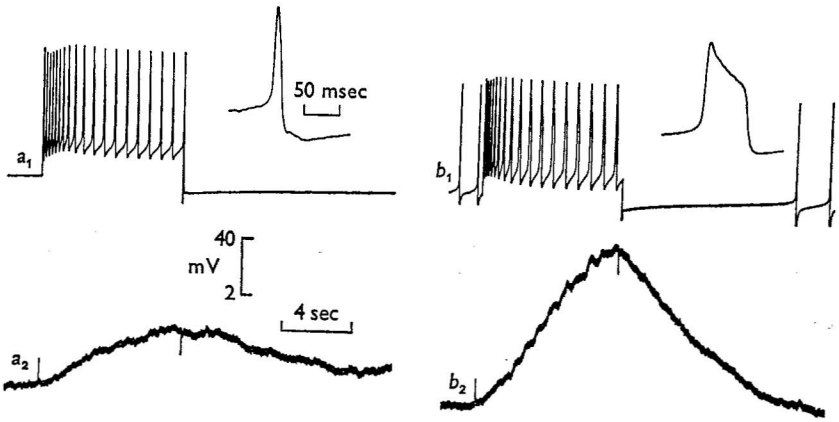

Fig. 4. Intracellular injection of TEA in the cerebral giant neurone. a_1, a_2, a train of spikes of the giant cerebral neurone evoked a low voltage depolarization in a buccal neurone previously hyperpolarized by inward current to -60 mV. The inset in a_1 corresponds to one of the presynaptic cell spikes recorded at high speed. b_1, b_2, after injecting TEA into the giant cerebral neurone for 45 min, the same number of presynaptic spikes causes now both a large depolarization of the buccal cell (b_2) and a discharge of discrete excitatory potentials superimposed on the depolarizing wave. The marked prolongation of the duration of the action potentials of the presynaptic cell is illustrated in the inset of a_2.

appearance of discrete excitatory synaptic potentials superimposed on the slow component (Fig. $4b_1$–b_2). The effects of presynaptic TEA injection were even more striking in records from a buccal neurone where discrete excitatory potentials were observed following the discharge of each spike in the giant cerebral neurone (Fig. $5a_1$–a_2). The intracellular injection of TEA into the cerebral cell for 30–40 min caused a progressive increase in both the amplitude and the duration of each one of the discrete excitatory synaptic potentials (Fig. $5b_1$–b_2).

The other procedure used to test the nature of the connexions between the giant cerebral neurone and the buccal neurones was to increase the extracellular Ca^{2+} concentration. The effect of this would be to (1) increase

transmitter release, (2) increase the threshold of interneurones. With very high Ca^{2+} concentrations the second effect is dominant and results in blockade of polysynaptic excitatory potentials (Stuart, 1970). Fig. 6 illustrates one of the experiments using this technique. In Fig. 6a_1–a_2,

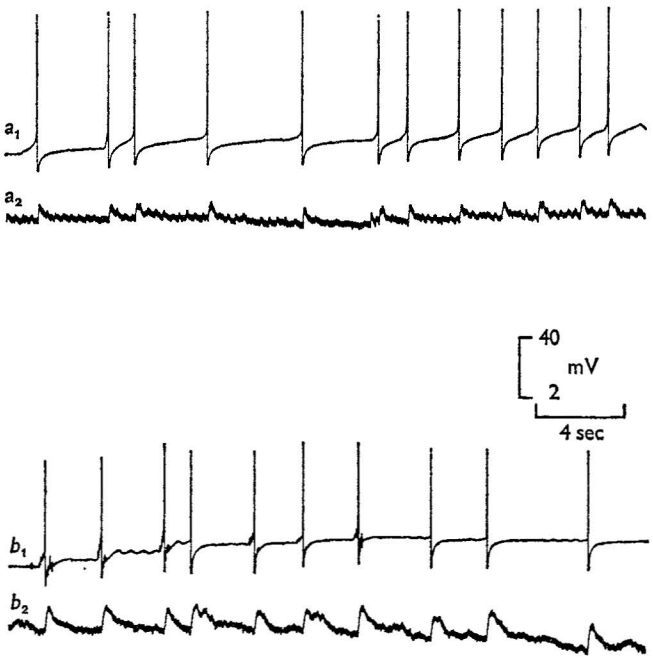

Fig. 5. Another example of the effects of an intracellular injection of TEA into a giant cerebral neurone. In a_1, a_2, the giant cerebral neurone is discharged at low frequency by passing a steady outward current across the membrane. Each spike evokes a discrete excitatory synaptic potential in a buccal neurone artificially hyperpolarized to -55 mV. (In this particular case, the synapses between the two types of cells are probably located near the soma of the buccal neurone).

In b_1, b_2, after injecting TEA into the presynaptic cell for 40 min, the discrete synaptic potentials evoked by each presynaptic spike are much increased in amplitude.

presynaptic stimulation evoked in a buccal neurone, already showing 'spontaneous' inhibitory potentials, a 'biphasic' effect consisting of an early discharge of a series of inhibitory potentials, summating to give an hyperpolarizing wave, followed by a late depolarization. The increase in extracellular Ca^{2+} from 10 to 60 mM caused a striking effect on the buccal cell response: both the 'spontaneous' and evoked inhibitory potentials disappeared whereas the depolarization became fully visible. This showed

that the inhibitory potentials blocked by high Ca^{2+} were polysynaptic whereas the depolarization was monosynaptic. It can be concluded that the excitatory connexions between the giant cerebral neurones and the nine buccal neurones are monosynaptic.

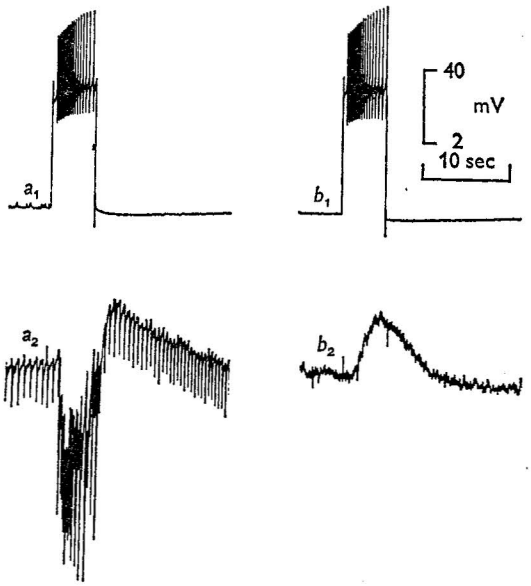

Fig. 6. Lack of effect of a high Ca^{2+} external medium on the excitatory potentials. a_1, a_2, in a normal medium containing 10 mM-Ca^{2+} a train of seventeen action potentials in a giant cerebral neurone evokes a complex synaptic activity in a buccal neurone previously hyperpolarized by inward current to -50 mV. This complex synaptic response consists of the discharge or large inhibitory potentials summating in a slow hyperpolarizing wave which is followed by a late hyperpolarization. Notice that the buccal neurone also shows 'spontaneous' hyperpolarizing synaptic potentials. b_1, b_2, after increasing the external Ca^{2+} concentration to 60 mM, the same presynaptic stimulation only evokes a depolarization of the buccal cell. Both the 'spontaneous' and the evoked inhibitory potentials become blocked, probably because of the increase by high Ca^{2+} medium of the firing threshold of the interneurones.

5-HT receptors and excitatory potentials. Iontophoretic applications of 5-HT to the buccal neurones which receive excitatory inputs from the giant cerebral cell demonstrated that all these neurones were depolarized and excited by 5-HT (Fig. 7 *et seq.*). As will be shown immediately below, the majority of these buccal cells appear to have a combination of both A and A'-receptors on their membranes. The ionic mechanism and the pharmacological sensitivity of the A- and A'-responses recorded in the

buccal cells were exactly the same as those described in the preceding paper (Gerschenfeld & Paupardin-Tritsch, 1974).

In Fig. $7a_2$, a_3, responses both to presynaptic stimulation and to the iontophoretic application of 5-HT were recorded in a buccal neurone. The preparation was perfused with a 5×10^{-4} M solution of (+)-tubocurarine (TC) which partially blocked both responses (Fig. $7b_2$, b_3). No further

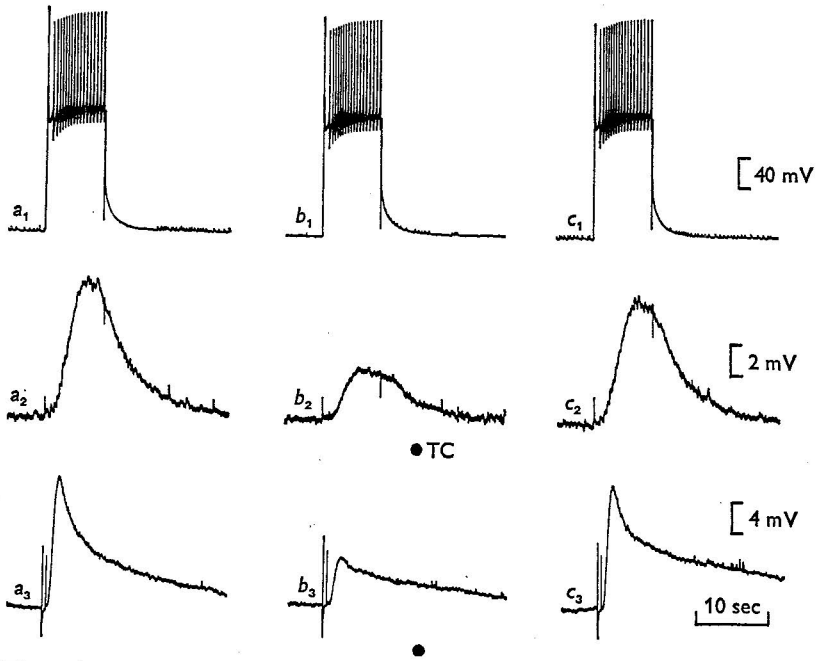

Fig. 7. Effects of curare on the 5-HT response and the excitatory synaptic response of buccal neurones. a_1, a_2, stimulation of a giant cerebral neurone evokes a depolarizing response in a buccal neurone hyperpolarized to -70 mV; a_3, the iontophoretic application of 5-HT to the same buccal neurone also produces a depolarization. b_1, b_2, b_3, bath application of a 5×10^{-4} M solution of TC causes a partial blockade of both the iontophoretic and the synaptic responses. c_1, c_2, c_3, the responses recover their previous amplitudes after removal of the antagonist (see text).

blockade was achieved by increasing the TC concentration. As was shown in the preceding paper, TC blocks the A-receptors without affecting the A'-receptors. The parallel effects of TC on the iontophoretic and the synaptically evoked responses suggests that the latter also involved A'- as well as A-receptors.

Fig. 8 demonstrates that the action of TC on the depolarizations of the buccal neurones could not be explained by the existence of a cholinergic component. A bath application of hexamethonium at a 10^{-4} M concentra-

tion, which has been shown to block all excitatory cholinergic responses in molluscan ganglia (Tauc & Gerschenfeld, 1961; Kehoe, 1972*b*) did not at all affect the responses of the buccal neurones to presynaptic stimulation (Fig. $8b_1, b_2$).

As was described in the preceding paper, 7-methyltryptamine (7-MT) is a 5-HT antagonist which also blocks the *A*-receptors specifically. Fig. 9 illustrates the similarity of actions of 7-MT on a response to presynaptic stimulation and a response to 5-HT iontophoretically applied to a buccal neurone. In both cases (Fig. $9b_2, c_2$), the blockade was not complete. As in the case of the partial block by TC, this suggests the presence of a composite population of 5-HT receptors in the synaptic membrane. It may be recalled that neither TC nor 7-MT generally affect the input resistance of the buccal cells and, as is evident in Figs. 9 and 10, these antagonists did not affect the firing of the giant cerebral neurone.

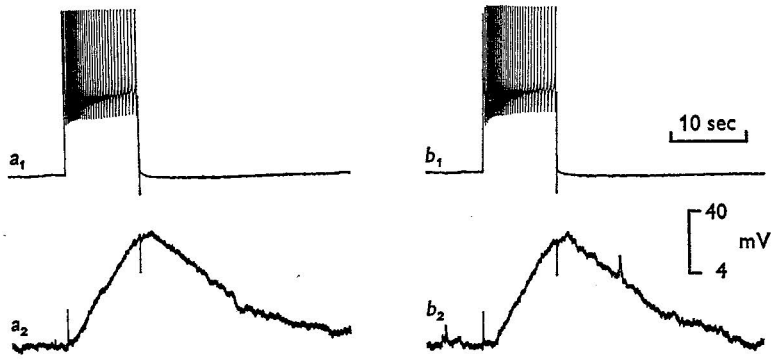

Fig. 8. a_1, a_2, presynaptic stimulation evokes, in a buccal neurone whose membrane potential has been driven to -60 mV, an excitatory potential. b_1, b_2, perfusion of a 10^{-4} M solution of hexamethonium bromide does not affect the synaptic response.

The idea that both *A*- and *A'*-receptors are involved in the generation of buccal neurone synaptic potentials was strongly supported by the effect of bufotenine which, as was shown in the preceding paper, blocks both the *A*- and *A'*-responses to 5-HT. In the experiment of Fig. 10, the buccal neurone responses to presynaptic stimulation were augmented by the previous injection of TEA and consisted of a series of a discrete excitatory potentials superimposed on a slow depolarizing wave (Fig. $10a_1, a_2$). Iontophoretic applications of 5-HT to the same neurone caused a long-lasting depolarization (Fig. $10a_3$). Perfusion of the preparation with a 10^{-6} M solution of bufotenine completely blocked both the slow depolarization and the discrete potentials (Fig. $10b_2$) and at 10^{-5} M completely

blocked the iontophoretic response (Fig. $10b_3$). The recovery of both responses after removing the antagonist was complete (Fig. $10b_3$, c_3). Bufotenine did not affect the firing of the presynaptic neurone. Moreover, it has been shown that this antagonist was totally ineffective when assayed on six other cholinergic and non-cholinergic synapses of the pleural ganglion of *Aplysia* (J. S. Kehoe, personal communication).

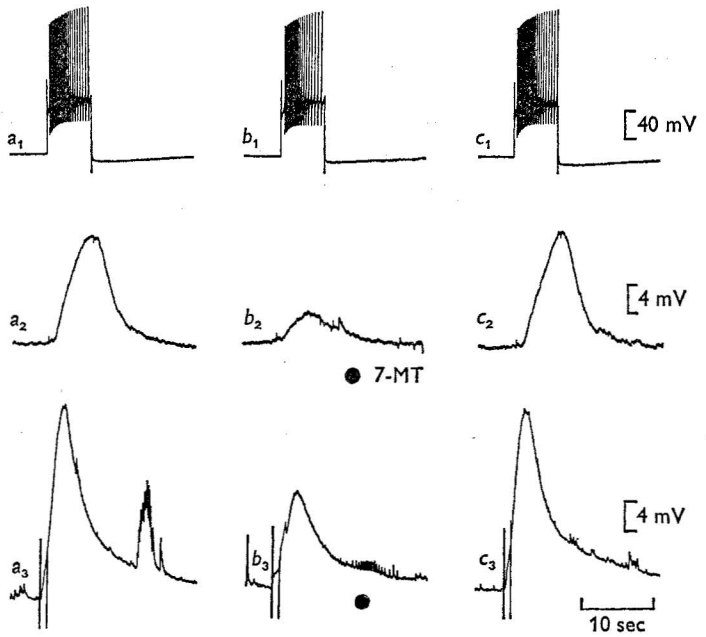

Fig. 9. Effect of 7-methyltryptamine on the 5-HT and excitatory synaptic responses. a_1, a_2, presynaptic stimulation elicits an excitatory potential in a buccal neurone artificially hyperpolarized by inward current to -63 mV. In a_3, 5-HT application on another buccal neurone also receiving an excitatory synaptic input from the giant cerebral neurone evokes a depolarization. On the falling phase, a burst of spontaneous synaptic activity can be observed. b_1, b_2, b_3, both the synaptic and the 5-HT responses are partially blocked by the bath application of 5×10^{-5} M solution of 7-MT. c_1, c_2, recovery of both responses after removal of the blocking agent.

Partial blockade of the synaptic responses was also obtained by applying LSD 25 and tryptamine which were shown, in the preceding paper, to block the *A*-receptors. In summary, the post-synaptic membrane of the buccal neurones receiving an excitatory input from the giant cerebral cell is endowed with a composite population of *A*- and *A'*-receptors to 5-HT.

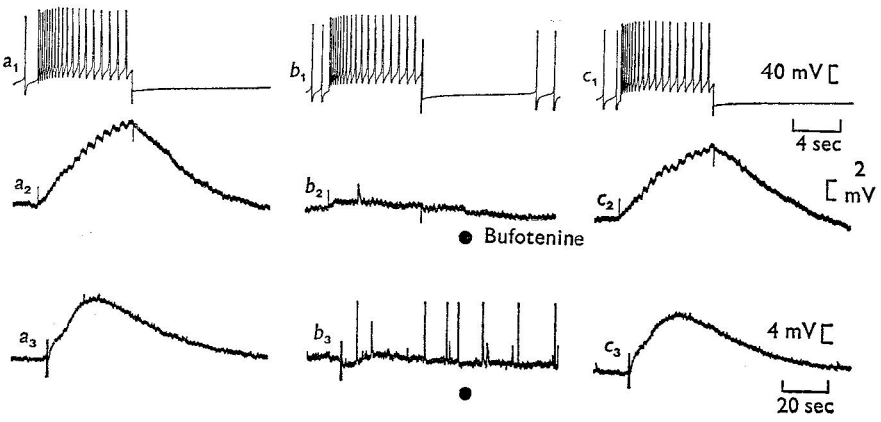

Fig. 10. Blockade of synaptic and 5-HT responses by bufotenine. a_1, a_2, the discharge of a train of spikes in a giant cerebral neurone evokes in a buccal neurone a slow depolarizing wave and the discharge of discrete excitatory potentials superimposed on the depolarization. TEA was injected into the presynaptic cell 1 hr before the recording of this response; a_3, the iontophoretic application of 5-HT to the same buccal neurone also evokes a depolarization. The membrane potential of the buccal neurone between stimulations was held at -60 mV.

b_1, b_2, b_3, bath application of bufotenine 10^{-6} M blocks completely both components of the synaptic response. In b_3, a 10^{-5} M solution of bufotenine was necessary to block completely the 5-HT response; the excitatory potentials observed are probably due to an indirect activation of an interneurone and do not seem to be affected by bufotenine. c_1, c_2, c_3, both types of responses recover their previous amplitudes after prolonged washing with normal sea water.

Inhibitory connexions between the giant cerebral neurone and the buccal neurones

The giant cerebral neurone establishes inhibitory contacts with a group of 4 neurones located on the caudal face of the ipsilateral buccal ganglion, at the periphery of the area occupied by the neurones which receive excitatory input from the giant cerebral neurone. Three buccal neurones show similar inhibitory potentials, but the fourth shows a different response which will be called 'atypical' inhibitory potential (see below).

In none of these cells, did presynaptic stimulation give rise to discrete inhibitory potentials, probably because the inhibitory synapses were located rather far from the neuronal somata. Very intense firing of the presynaptic neurone was always necessary to obtain the slow hyperpolarizing wave, which generally did not reach amplitudes larger than 2–3 mV (Fig. 11 *et seq.*). The declining phase of these inhibitory potentials was also rather slow.

Monosynaptic character of the inhibitory potentials. As in the case of the

excitatory input from the giant cerebral neurone, no anatomical information was available about the inhibitory connexions. The same two methods, intracellular TEA injections and high extracellular Ca^{2+}, were used to investigate whether the inhibitory potentials were due to the activation of a mono- or a polysynaptic pathway.

In Fig. 11 a_1, a_2, presynaptic stimulation resulted in an hyperpolarizing potential of a buccal neurone. Intracellular injection of TEA into the

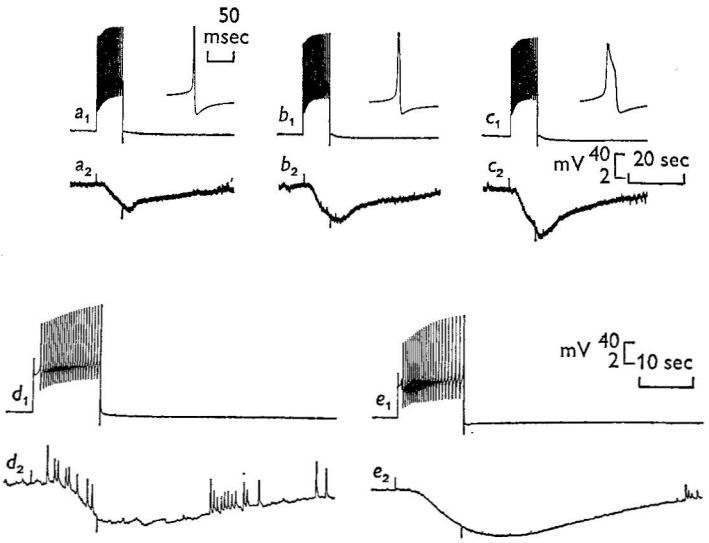

Fig. 11. Monosynaptic character of the inhibitory potentials. a_1, a_2, repetitive stimulation of the giant cerebral neurone evokes a low amplitude inhibitory potential in a buccal neurone previously hyperpolarized by inward current to -50 mV. The inset in a_1 shows a single spike of the giant cerebral neurone recorded at high speed. b_1, b_2, the same pair of neurones is recorded after a 30 min injection of TEA into the presynaptic cell. Concomitantly to the prolongation of the presynaptic neurone spike (inset in b_1), the same train of spikes produces now a synaptic inhibitory potential of increased amplitude. c_1, c_2, the same experiment after one hour of TEA injection into the giant cerebral neurone. The presynaptic spike has been further prolonged in duration (inset in c_1), and there is a further increase in amplitude of the inhibitory potential (see text).

d_1, d_2, another experiment in which presynaptic stimulation also evokes in a buccal neurone (membrane potential: -60 mV) an inhibitory synaptic potential. Other fast synaptic potentials are also recorded in a_2, and probably are caused by the 'spontaneous' firing of some interneurones.

e_1, e_2, the same experiment as in d_1, d_2. The external Ca^{2+} concentration has been raised from 10 to 50 mM. The inhibitory synaptic potential evoked by presynaptic stimulation remains unaltered whereas the 'spontaneous' synaptic potentials, probably involving polysynaptic pathways, become blocked.

giant cerebral neurone for 30 and 60 min respectively resulted in a gradual increase in duration of the presynaptic cell spike (Fig. $11b_1$ and c_1 respectively). Concomitantly, the inhibitory responses of the buccal neurones inhibitory potentials increased in both amplitude and duration (Fig. $11b_2, c_2$). These findings may be interpreted as indicating that the connexion between the giant cerebral neurone and the buccal neurone is monosynaptic. The results of the experiments in which the extracellular Ca^{2+} concentration was increased were also in agreement with such an interpretation. In Fig. $11d_1, d_2$, presynaptic stimulation elicited an inhibitory post-synaptic potential in a buccal neurone which also received other synaptic inputs. A fivefold increase of the extracellular Ca^{2+} concentration (from 10 to 50 mM) resulted in the disappearance of large part of the 'spontaneous' synaptic activity of the buccal neurone, whereas the inhibitory potential evoked by the presynaptic stimulation increased in amplitude (Fig. $11e_1, e_2$).

As in the case of the excitatory connexions, these results are strongly in favour of the monosynaptic nature of the inhibitory connexions between the giant cerebral neurone and three of the buccal cells.

5-HT receptors and inhibitory synaptic potentials. When 5-HT was applied to two of the three buccal cells shown above to receive a monosynaptic input from the giant cerebral neurone they were hyperpolarized and inhibited.

The other buccal neurone of the group of three responded to 5-HT with a triphasic response composed by an early fast depolarization followed by both a slow hyperpolarization and a late slow depolarization. The pharmacological analysis of this composite response showed that it was due to the activation of *A*, *B* and *A'*-receptors respectively. Nevertheless, the response to presynaptic stimulation was always monophasic and similar to the inhibitory potentials recorded in the other two neurones.

The inhibitory responses of the buccal cells to iontophoretically applied 5-HT were of the *B*-type which, as was shown in the preceding paper, are associated with an increase in K^+-conductance. A strict parallelism was observed in the effects of 5-HT antagonists on both the inhibitory potential and the *B*-responses to 5-HT. Fig. $12a_1, a_2$ is from an experiment where a buccal neurone was inhibited by presynaptic stimulation. 5-HT iontophoretically applied to the same neurone also evoked a *B*-hyperpolarization (Fig. $12a_3$). The bath application of a 10^{-5} M solution of bufotenine blocked both the inhibitory synaptic potential and the *B*-response to 5-HT (Fig. $12b_2, b_3$). The blocking effect of bufotenine was fully reversible (Fig. $12c_2, c_3$).

A similar blocking effect was produced by 5-methoxygramine (5-MG) which, as previously demonstrated (Gerschenfeld & Paupardin-Tritsch,

1974), is a highly specific blocking agent of the *B*-responses. Thus, in Fig. 13b_2, the perfusion of the preparation by a 10^{-5} M solution of 5-MG almost completely blocked the synaptic response. The iontophoretic response was in great part abolished (b_3). Further increase in the concentration of the antagonist completely blocked it (not shown). These blocking actions were reversible (Fig. 13c_2–c_3).

Neither the inhibitory synaptic potentials evoked by presynaptic stimulation nor the *B*-responses of the buccal cells were affected either by TC or 7-MT, but both were blocked by LSD 25 and tryptamine.

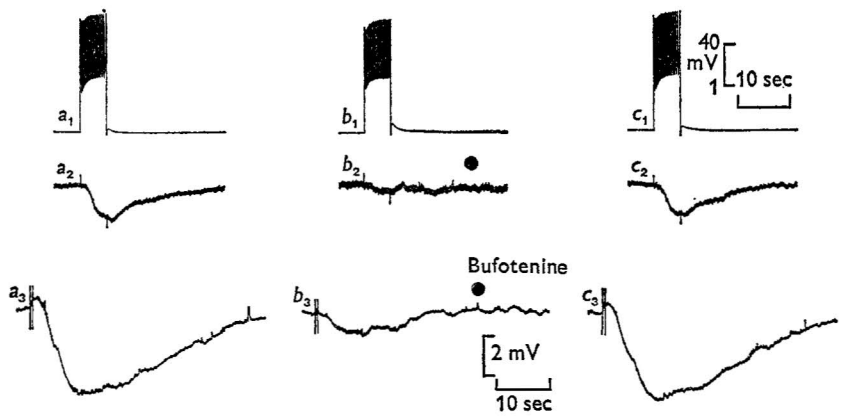

Fig. 12. Blocking effect of bufotenine. a_1, a_2, intense firing of a giant cerebral neurone evokes an inhibitory potential in a buccal cell hyperpolarized by inward current to -60 mV; a_3, the iontophoretic application of 5-HT to the same buccal neurone evokes an hyperpolarizing *B*-response. Notice the difference in calibration in the recordings of the synaptic and the iontophoretic responses.

b_1, b_2, b_3, bath application of a 10^{-5} M solution of bufotenine blocks completely both the synaptic potential and the *B*-response to 5-HT. c_1, c_2, c_3, after prolonged washing of the preparation with sea water the responses recover their original amplitudes.

Ionic mechanism of the inhibitory synaptic potentials. It was of great interest to see if the synaptic inhibitory potentials resulted from the same alteration of the ionic membrane conductance as those caused by 5-HT acting on *B*-receptors.

Although in general, presumably because the synapses were located far from the somata, it was not possible to reverse the inhibitory synaptic potentials by hyperpolarizing the buccal neurones by inward current, in two experiments the reversal potentials of the inhibitory synaptic potentials were measured.

In Fig. 14*b–e*, a buccal neurone receiving an inhibitory input from the giant cerebral neurone was hyperpolarized from −50 to 90 mV. The inhibitory potential reached a null value at about −80 mV, and beyond this level, in spite of the 'anomalous rectification', a reversal of the response could be observed; this value of the reversal level coincided with the known value of the K^+ equilibrium potential, E_K, in *Aplysia* neurones (Kehoe, 1972*a*; Russell & Brown, 1972). When the K^+ content of the

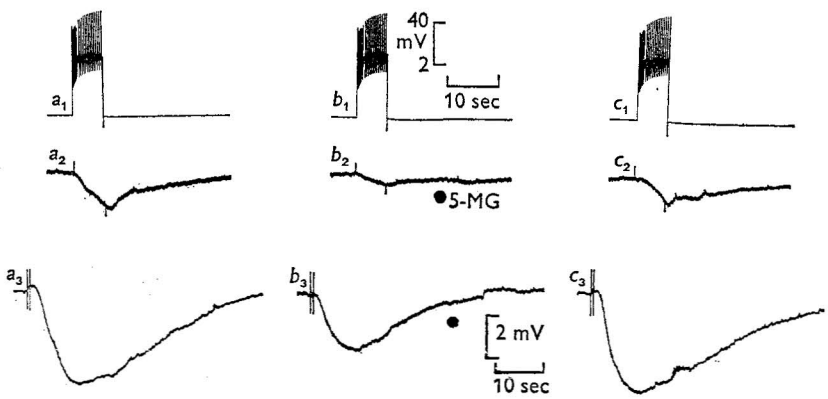

Fig. 13. Effect of 5-methoxygramine. a_1, a_2, stimulation of a giant cerebral neurone evokes an inhibitory potential in a buccal neurone whose membrane potential was previously driven to −60 mV; a_3, a iontophoretic application of 5-HT evokes an inhibitory *B*-response.

b_1, b_2, b_3, bath application of a solution of 5-MG blocks completely the synaptic response and a significant part of the iontophoretic response. The latter could be blocked by increasing of the 5-MG concentration (not shown).

c_1, c_2, c_3, both responses recover their amplitude after removing the antagonist.

environment was increased from 10 to 20 mM, a shift in the reversal potential from −80 to −64 mV was observed (Fig. 14*f–i*). This shift is almost identical to the displacement of E_K predicted by the Nernst relation for such a change in K^+ external concentration (17 mV at 20° C). This result indicates that the inhibitory potential generated by presynaptic stimulation in three buccal neurones is due to a selective increase of the membrane K^+-conductance.

In summary, it may be concluded that the inhibitory synaptic potentials evoked by the stimulation of the giant cerebral neurone in the three buccal cells are due to the activation of receptors similar to the *B*-receptors to 5-HT and that, like the *B*-responses, they result from an increase in K^+-conductance.

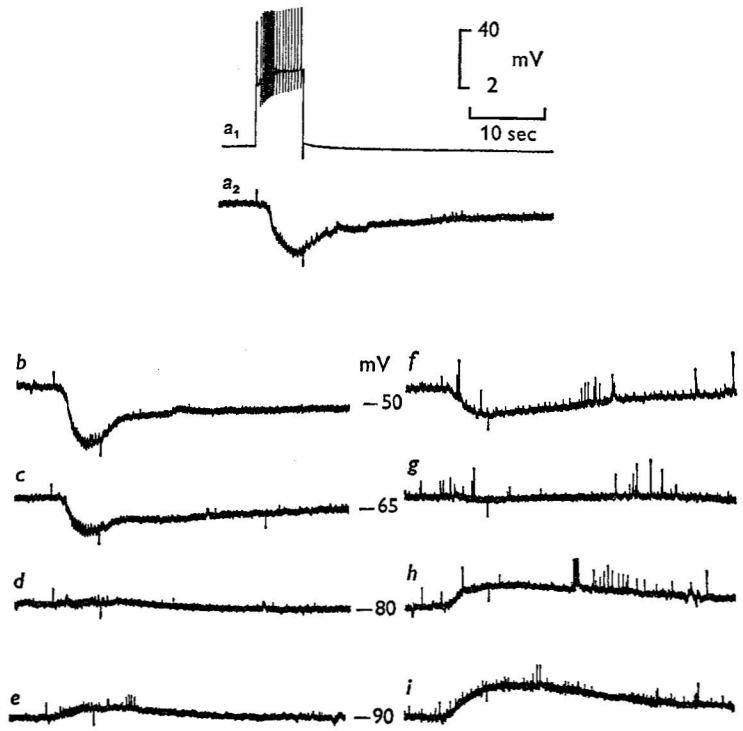

Fig. 14. Ionic basis of the inhibitory potentials. a_1, a_2, control inhibitory potential evoked in a buccal neurone, at −50 mV, by presynaptic stimulation. In *b* to *i* only the recordings from the buccal cells are shown.

b–e, in a normal external medium where the K^+ concentration is 10 mM, the inhibitory synaptic potential is recorded at different membrane potentials: −50 (*b*), −65 (*c*), −80 (*d*) and −90 mV (*e*). Notice in *d* that the hyperpolarization reverses at −80 mV.

f–i, the K^+ external concentration is increased to 20 mM, and the inhibitory potential recorded at the same membrane potential levels as before. Notice the shift of the reversal potential to about −65 mV (*g*) (see text).

'Atypical' inhibitory potentials evoked by stimulation of the giant cerebral neurone

In a few experiments on a small buccal neurone which could not be visually differentiated from the cluster of three neurones which respond with a K^+-dependent inhibitory potential to presynaptic stimulation, another type of hyperpolarizing synaptic response was observed. This differed in many respects from the K^+-dependent inhibitory response described above and will be called an 'atypical' inhibitory potential.

At the resting potential, the 'atypical' inhibitory response did not look

different from the K-dependent inhibitory potentials. However, in contrast to the behaviour of the latter, the 'atypical' inhibitory potentials, instead of decreasing in amplitude and reversing when the neurone was hyperpolarized, gradually grew in amplitude (Fig. 15a_2–d_2). This behaviour evidently resembled that of the β-responses to 5-HT, described in the

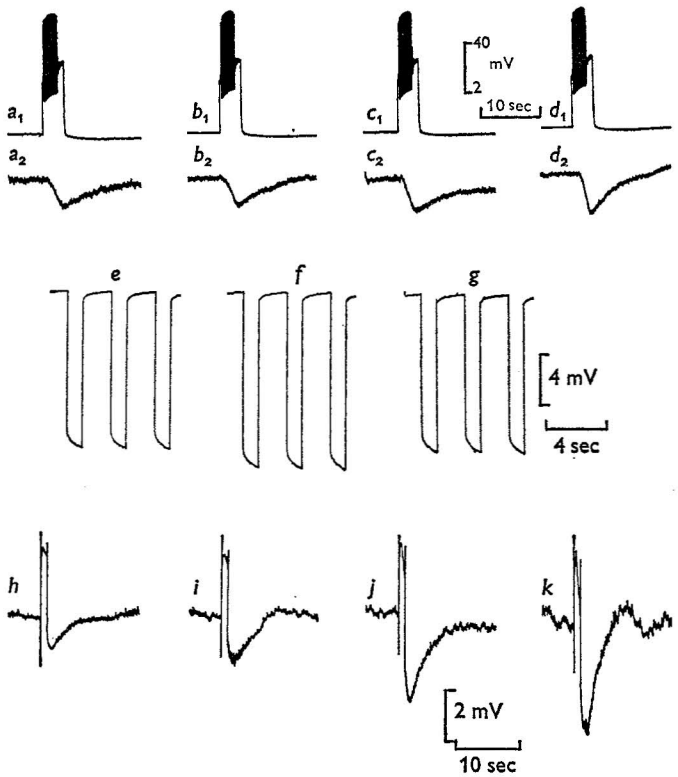

Fig. 15. Atypical inhibitory potentials. a_1, a_2, presynaptic stimulation evokes an inhibitory potential in a buccal neurone hyperpolarized by inward current to -50 mV. b_2–d_2, when the buccal cell membrane potential is successively driven to -60 (b_2), -70 (c_2) and -80 mV(d_2), the inhibitory potential, instead of decreasing, increases in amplitude. e–g, square pulses of current are passed across the membrane of the buccal neurone to measure the input resistance. In e, control electrotonic potentials. In f, bath application of 10^{-5} M-5-HT causes an increase in the amplitude of the electrotonic potentials. In g, the effect of 5-HT disappears after removal of the amine. h–k, iontophoretic application of 5-HT on a buccal neurone responding to presynaptic stimulation by an 'atypical' potential. The hyperpolarizing response grows in amplitude when the cell membrane potential is successively driven from -50 mV (h) to -60 (i), -70 (j) and -80 mV (k) (see text).

preceding paper. As it may be recalled, these hyperpolarizing responses to 5-HT are caused by a decrease in both Na^+- and K^+-conductances. The likelihood that both the β-responses to 5-HT and the 'atypical' inhibitory potential were generated by the same mechanism was increased by the analysis of the effects of 5-HT on the cells showing the 'atypical' inhibitory potential. When the input resistance of these neurones was monitored by passing square current pulses (Fig. 15*e*) and 5-HT was applied to the neurone (Fig. 15*f*) it could be seen that the input resistance increased, the effect being totally reversible (Fig. 15*g*). Moreover, the iontophoretic application of 5-HT to these neurones also resulted in an hyperpolarization which increased in amplitude when the buccal neurone was hyperpolarized by inward current (Fig. 15*h–k*). This was then a β-response to 5-HT, and from Fig. 15*h–k*, the reversal potential was calculated to be close to -38 mV; thus this response was due to a decrease in both Na^+- and K^+-conductances (see preceding paper).

It is not entirely clear if the connexion between the giant cerebral neurone and the buccal neurone which shows the 'atypical' inhibitory potential is monosynaptic. In favour of such an idea is the evidence from a small number of experiments where the Ca^{2+} content of the external medium was increased five times without causing a significant reduction in amplitude of the response. However, more information is required.

No blocking agents have yet been found which are specific for the β-receptors and thus, only negative evidence could be obtained from a comparison of the pharmacological properties of these with the receptors responsible for the 'atypical' inhibitory potentials. The most interesting of these negative findings was the observation that bufotenine which blocks the *B*-inhibitory responses to 5-HT did not affect either the atypical inhibitory potentials nor the β-responses to 5-HT.

DISCUSSION

Fig. 16 summarizes our main conclusions. The present results demonstrate that the firing of each of the giant cerebral neurones evokes in different ipsilateral buccal neurones excitatory and inhibitory synaptic potentials. The transmitter which mediates these synaptic actions is very probably 5-HT. Such an interpretation is supported by the following evidence:

(1) the giant cerebral neurones synthesize and contain fair amounts of 5-HT (Weinreich *et al.* 1973; Eisenstadt *et al.* 1973);

(2) the excitatory and inhibitory pathways from the giant cerebral cells to the buccal neurones are direct;

(3) the properties of the extrasynaptic 5-HT receptors and the receptors to the transmitter are identical.

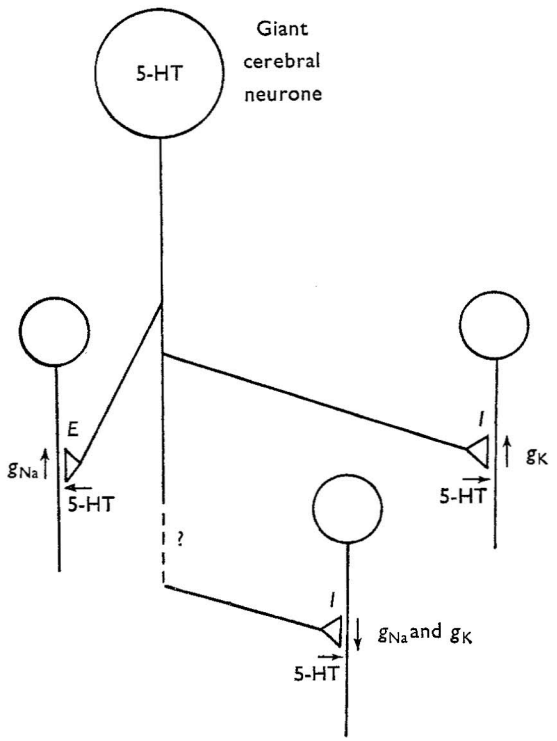

Fig. 16. Schematic drawing summarizing the main conclusions of this work. A giant cerebral neurone, which synthesizes and contains 5-HT, releases this amine through different endings which establish synaptic connexions with different buccal neurones. 5-HT thus released from some of the giant cerebral neurone terminals will activate *A*- and *A'*-receptors, exciting some buccal neurones by causing an increase in their Na^+ conductance. At the same time, 5-HT liberated from other endings will activate *B*-receptors in other neurones, inhibiting them by causing an increase in K^+ conductance. It is also possible that the liberation of 5-HT through other giant cerebral neurone terminals will activate β-receptors in other neurones, inhibiting them by causing a decrease in both Na^+ and K^+ conductances.

The evidence presented here also strongly suggests that of the six different kinds of 5-HT receptors described in the preceding paper, at least four intervene in the generation of the synaptic potentials. It therefore follows that 5-HT released from a single neurone is then able to mediate opposite synaptic actions on different buccal cells: excitation associated with a Na^+-conductance increase and inhibition resulting from an increase in K^+-conductance. The fact that the same transmitter substance can mediate opposite synaptic actions is well known since the pioneer work of Loewi (1933) and Dale (1937) on acetylcholine, which

was shown in vertebrates to be the transmitter of both vagal inhibition and of neuromuscular excitation. Moreover, such a dual action of the same transmitter released from a single neurone was shown to be an important organizational feature of central nervous systems of Molluscs, as has been already demonstrated in *Aplysia*, where acetylcholine released from cholinergic neurones may excite or inhibit post-synaptic neurones by increasing their membrane conductances to either Na^+ or Cl^- or K^+ (Kandel, Frazier, Waziri & Coggeshall, 1971; Kehoe, 1972*c*; see Gerschenfeld, 1973). Recent reports also suggest that a single dopamine-containing neurone in *Planorbis* may excite or inhibit different postsynaptic neurones (Berry & Cottrell, 1973).

The presence of more than one receptor to the same transmitter on the membrane of a single cell is also not an uncommon finding in molluscan synapses (Kehoe, 1967; Wachtel & Kandel, 1967; Gardner & Kandel, 1972). For 5-HT, it is possible that the presence on the same synaptic membranes of A- and A'-receptors may provide a way to achieve, by changing the permeability to the same ionic species, both a rapid and a slow excitatory action.

If it is allowed that 5-HT mediates synaptic transmission both for excitation and for a K^+-dependent inhibition of buccal cells, some ancillary questions arise which deserve consideration:

(*a*) *The problem of the atypical inhibitory synaptic potential.* It has been shown above that besides the 'classical' K^+-dependent inhibitory potentials, presynaptic stimulation elicited in buccal neurones a second type of inhibitory synaptic potential, the 'atypical' inhibitory potential. The evidence for the monosynaptic character of the 'atypical' inhibitory potentials cannot yet be considered so compelling as that obtained for the other synaptic events. However, the parallel behaviour of the 'atypical' inhibitory responses and the β-responses to 5-HT recorded from the same cell when the cell is hyperpolarized, together with the negative pharmacological evidence, are consistent with the idea that the 'atypical' inhibitory responses are generated by the action of 5-HT on a receptor similar to the β-receptor.

(*b*) *The problem of 5-HT inactivation.* From the work on vertebrate brain, it is well known that the enzyme involved in the degradation of 5-HT is monoamine-oxidase (see Blaschko & Levine, 1966). It has been reported that although a monoamine-oxidase exists in molluscan nervous tissue, it is not able to metabolize a tryptamine or 5-HT substrate (Cardot, 1964). Another inactivation pathway which has been proposed for several transmitters is a re-uptake mechanism. An uptake of 5-HT has been demonstrated in brain synaptosomes (see Snyder, Shaskan & Kuhar, 1973) and Taxi & Gautron (1969) were able to show by auto-

radiography that radioactive 5-HT is also taken up by the terminals of cardiac nerves in *Aplysia* heart auricles. Since there is now good evidence that cardiac motoneurones contain and synthesize 5-HT (Eisenstadt *et al.* 1973; Mayeri, Koester, Kupfermann, Liebeswar & Kandel, 1974), this could be taken to indicate that 5-HT is inactivated by uptake. In the central nervous system of *Aplysia*, an autoradiographic exploration of this uptake system has been shown to be impracticable because of an intense non-specific uptake of labelled 5-HT by the connective tissue sheaths (Ascher, Glowinski, Tauc & Taxi, 1968). However, recent experiments (D. Paupardin-Tritsch & H. M. Gerschenfeld, unpublished) have demonstrated that drugs which are known to block 5-HT uptake, such as chlorimipramine (Carlsson, Corrodi, Fuxe & Hökfelt, 1969; Alpers & Himwich, 1969), cause an increase in both the amplitude and the duration of the excitatory potentials, without affecting the firing of the giant cerebral neurone or the responses of the buccal cells to 5-HT. These findings suggest that the uptake may play a physiological role in the inactivation of 5-HT when released at the synapses analysed in the present paper.

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