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SYNAPTIC CONNEXIONS OF TWO SYMMETRICALLY PLACED GIANT SEROTONIN-CONTAINING NEURONES

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SUMMARY

1. Each giant serotonin cell in *Helix pomatia* makes synaptic connexions with three non-amine-containing neurones: the anterior, middle and posterior buccal cells.

2. Individual e.p.s.p.s, of 500–600 msec duration, were observed in both left and right middle cells following each evoked giant serotonin cell action potential. They were facilitated with repetitive stimulation of the giant serotonin cells and summed to give rise to an action potential. The membrane resistance of the middle cells was reduced when the giant serotonin cells were stimulated to fire rapidly. Evidence is presented which suggests that the link between each giant serotonin cell and each middle cell is monosynaptic.

3. Iontophoretically applied serotonin produced a depolarizing potential change in the middle cell perikaryon; the response rapidly desensitized on repetitive application.

4. Morphine abolished reversibly the middle cell serotonin potential and antagonized transmission from the giant serotonin cells to the middle cells. Lowering the Na concentration of the medium reversibly diminished the size of the serotonin potential and the giant serotonin cell elicited e.p.s.p.s in the middle cells.

5. Reserpine, which depletes serotonin in the giant serotonin cell, impaired transmission from these cells to the middle cells.

6. The results suggest that serotonin is the synaptic transmitter released from the giant serotonin cells on to the middle cells and that this system is a suitable model for further analysis of the neuronal role of serotonin.

INTRODUCTION

It has been suggested that serotonin (5-hydroxytryptamine) is a synaptic transmitter substance (Welsh, 1957; Brodie & Shore, 1957; see Gerschenfeld, 1973). Because many of the neurones of gastropod molluscs are accessible, large and identifiable, they are more easily studied with existing techniques than neurones of vertebrate species; consequently they allow a more detailed analysis of the cellular role of serotonin. Iontophoretic studies have demonstrated serotonin receptors on specific regions of certain neurones of the snail, *Cryptomphallus aspersa* (Gerschenfeld & Stefani, 1968). Gerschenfeld (1971) has distinguished three different types of neuronal receptors in terms of their pharmacological properties and induced ionic permeability changes. Type A receptors result in depolarization and excitation whereas Types B and C result in hyperpolarization and inhibition. The effects of stimulating selected nerve inputs have been compared with the excitatory action of serotonin and the data obtained are consistent with a transmitter role for serotonin (Gerschenfeld & Stefani, 1968). However, presynaptic serotonin-containing neurones have not been located in this system, preventing a more complete analysis of the possible role of serotonin as a transmitter substance.

Recently it was shown that there is a giant serotonin-containing neurone in each cerebral ganglion of the snail *Helix pomatia*, and also in other closely related species (Cottrell & Osborne, 1970; Osborne & Cottrell, 1971). The serotonin within these cells (giant serotonin cells) can be detected histochemically, by bio-assay, and by microchromatography (Osborne & Cottrell, 1972).

The experiments reported in this paper were made on the two giant serotonin cells, and on other giant neurones found to be innervated by them. The results provide further evidence for a transmitter role for serotonin, and describe certain properties of the serotonin receptors and the presumed serotonergic synapses. Part of this work has already been published in note or abstract form (Cottrell, 1970*a*, *b*).

METHODS

Preparation. Specimens of *Helix pomatia* were supplied by Gerrard & Haig Ltd., East Preston, Sussex. The snails were kept at 7–12° C until required for use, when they were hydrated and fed lettuce. The circum-oesophageal ganglionic complex, together with the buccal ganglia and connective nerves, was dissected from active animals and pinned to a plastic sheet fixed at the bottom of a small bath (vol. 0·7 ml.).

The preparation was illuminated with a simple light guide made from a tapered glass rod fixed to a microscope lamp. Usually, the neurones could be seen and identified readily with a stereomicroscope (× 20 to × 40). Each giant serotonin cell is easily found because of its position close to the inner lip nerve on the ventral

surface of the cerebral ganglion. In all iontophoretic studies of the buccal ganglion cells, the cerebro-buccal connective and the circum-oesophageal ganglionic complex remained intact.

Most of the connective tissue layer above the neurones to be impaled was removed by careful dissection with fine-tipped forceps. In a few experiments, the inner thin, but tough, connective tissue layer was softened by applying one or two specks of Pronase (BDH, specific activity 45,000 PUK units/g) to the saline just above the cells to be impaled, and leaving the enzyme in contact with the tissue for 5 min. No differences in responses to drugs or to giant serotonin cell stimulation were observed in preparations treated with Pronase compared with untreated preparations. All iontophoretic studies were made on cells untreated with Pronase. All the experiments were made at room temperature (18–25° C).

Electrodes and electrical measurements. Double-barrelled, or two single, glass micro-electrodes were used to impale the neurones to study the synaptic connexions. The current passing barrel was usually filled with 0·6 M-Na_2SO_4, or in some cases with 3 M-KCl. The recording barrel was filled with 3 M-KCl. Micro-electrode resistances varied from 7 to 30 MΩ. Input leads from the electrodes were fed through cathode followers to a dual beam Tektronix 502A oscilloscope. Permanent recordings of electrical activity were made of unamplified signals from the cathode followers on a Devices multichannel recorder, or of the amplified signal by connecting the Y plate output terminals of the oscilloscope to a Brush 220 series two-channel ink recorder.

Iontophoretic studies were made on cells penetrated by a single micro-electrode filled with 4 M-K acetate which was used both for recording and passing current by means of an M4 Electrometer (WPI Instruments, Inc.) connected to a dual-beam Tektronix 565 oscilloscope and a DC voltage source. Permanent recordings of the amplified signal were photographed from a Tektronix Type 564B dual-beam storage oscilloscope or taken from a Brush 220 two-channel ink recorder. Iontophoresis was accomplished by passing positive square pulses from a Grass 548 pulse generator through a 50 MΩ resistor to the serotonin-containing iontophoretic glass micro-electrode. A constant backing current to prevent diffusional outflow of serotonin was applied from a 9 V battery and potentiometer in series with the iontophoretic micro-electrode. The iontophoretic micro-electrodes were filled with 0·15 M serotonin creatinine sulphate (pH 3·5), or 1 M acetylcholine chloride, by means of the glass-fibre technique; resistances varied between 20 and 40 MΩ. The iontophoretic current was monitored as the potential drop across a 1 MΩ resistor in series with the iontophoretic micro-electrode and displayed on the dual-beam oscilloscope.

Physiological solutions. The composition of normal snail medium (Meng, 1960) was (mM): NaCl 60, KCl 5·8, $CaCl_2$ 10, $MgCl_2$ 16, and $NaHCO_3$ 13; pH 7·8. Solutions with lowered Na concentration had the composition (mM): KCl 5·8, $CaCl_2$ 10, $MgCl_2$ 16, $NaHCO_3$ 12 with Tris HCl added to maintain the Cl concentration at 117 mM and the pH at 7·8. In some experiments, NaCl was replaced with 120 mM sucrose or 60 mM-LiCl.

In certain studies on synaptic transmission, physiological solution with the following composition was used (mM): NaCl 80, KCl 4, $CaCl_2$ 7, $MgCl_2$ 5, Tris HCl buffer 5 to give a final pH of 7·8 (Chamberlain & Kerkut, 1969). Similar results were obtained in both normal physiological solutions. To study the effect of high Ca concentrations on the e.p.s.p.s, the final concentration of $CaCl_2$ was increased to 60 mM without compensation to maintain the original osmotic pressure of the solution. The preparations were washed in a continuous flow of solution.

Drugs. Drugs were applied to the preparation by addition of known amounts to the flow of physiological solution, by addition to the bath when the flow was discontinued, or by iontophoresis from a drug-filled micropipette. The drugs used in

this study were: serotonin creatinine sulphate (BDH and Nutritional Biochemicals), tryptamine HCl (Calbiochem), D-lysergic acid diethylamide tartrate (LSD-25), methysergide (UML-491) (Sandoz), bromolysergic acid diethylamide tartrate (BOL-148) (DHEW, U.S. Public Health Service, NIMH), reserpine phosphate (CIBA), acetylcholine chloride (ACh) (Calbiochem), tubocurarine chloride (Burroughs Wellcome), neostigmine bromide (Hoffman–La Roche), and tetraethylammonium bromide (TEA) (BDH).

Dye injection. Experiments with Procion Yellow were made following the procedure outlined by Stretton & Kravitz (1968). Because of the presence of an 'anti-Dusting' agent in the Procion Yellow available, samples of the dye were washed several times with diethyl ether before use.

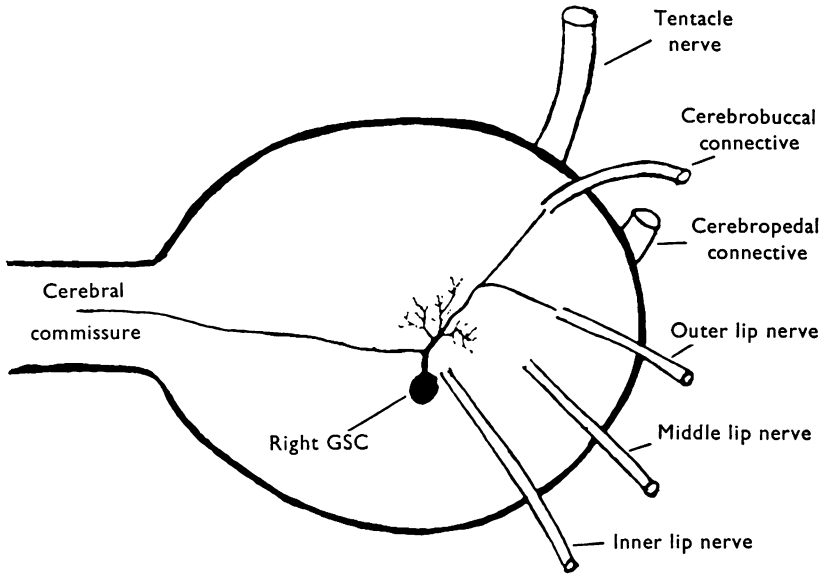

Fig. 1. Diagram of the antero-ventral surface of the right cerebral ganglion showing the perikaryon and main processes of the right giant serotonin cell (GSC) within the ganglion.

RESULTS

Processes and electrical properties of the giant serotonin cells

The cell body and processes of the giant serotonin cell within the right cerebral ganglion were visualized by injecting Procion Yellow (Fig. 1). The pattern of the processes from the left giant cell was very similar to, but the mirror image of, the right one. Each giant cell sends processes through both buccal ganglia since stimulation of both groups of buccal nerves gives rise to an antidromic spike in each giant cell (cf. Kandel & Tauc, 1966). In Fig. 2, for example, the lateral buccal nerves of the left buccal ganglion were stimulated and intracellular records made from both giant serotonin cells simultaneously. Hyperpolarization of each giant

serotonin cell by about 15 mV blocked the spike invading the soma, and further hyperpolarization diminished and then abolished the axon spike without the appearance of an excitatory post-synaptic potential (e.p.s.p.).

The electrical properties of both giant serotonin cells were very similar. The membrane potential of the cells was usually in the range of -50 to -60 mV. Occasionally, small spontaneous e.p.s.p.s were observed, but in

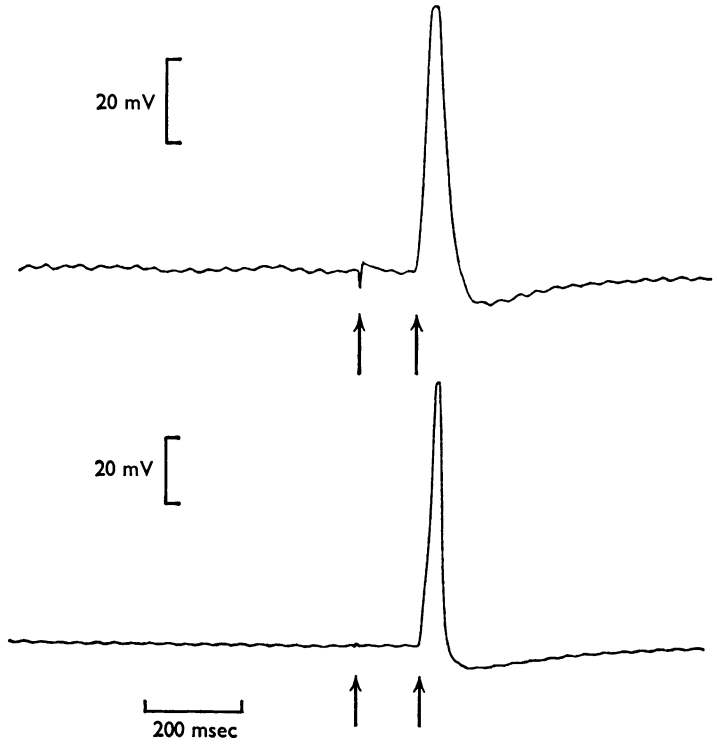

Fig. 2. Antidromic action potentials recorded in the left (above) and right giant serotonin cells following stimulation of the left buccal nerves. Ipsilateral latency was 60·0 msec and the contralateral latency was 68·8 msec. Arrows indicate stimulus artifacts and onset of action potentials (the peaks of the spikes were lost because the pens had reached their maximum upwards excursion).

most cases action potentials were not seen after the initial penetration, unless the cell was stimulated when 70–80 mV action potentials were observed. No connexion could be detected between right and left giant cells (cf. Kandel & Tauc, 1966).

Buccal cell responses to giant serotonin cell stimulation

Because of the presence of giant serotonin cell processes in the cerebrobuccal connectives, the buccal ganglia were explored for cells receiving inputs from these cells. In these experiments, a double-barrelled electrode was inserted into one of the giant serotonin cells and another double-barrelled electrode, or two single electrodes, used to impale different

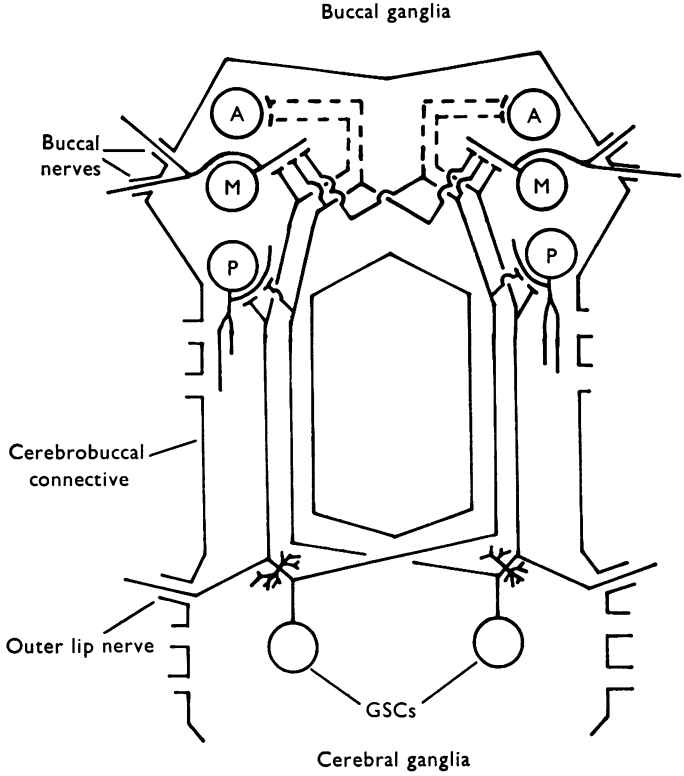

Fig. 3. Diagram of the cerebral and buccal ganglia showing the giant serotonin cells and their main processes and the anterior (A), middle (M) and posterior (P) giant buccal cells. The presumed synaptic connexions on to the buccal cells are also shown. The large processes of the M and P cells were traced with Procion Yellow. The processes of the giant serotonin cells (GSCs) passing into the buccal nerves have been omitted for simplicity.

neurones in the buccal ganglia. Three giant neurones in each buccal ganglion responded to stimulation of the giant serotonin cells. Their positions are indicated in Fig. 3. For convenience they will be referred to as the anterior, middle and posterior cells.

The resting potential of both middle cells was usually between -45 and

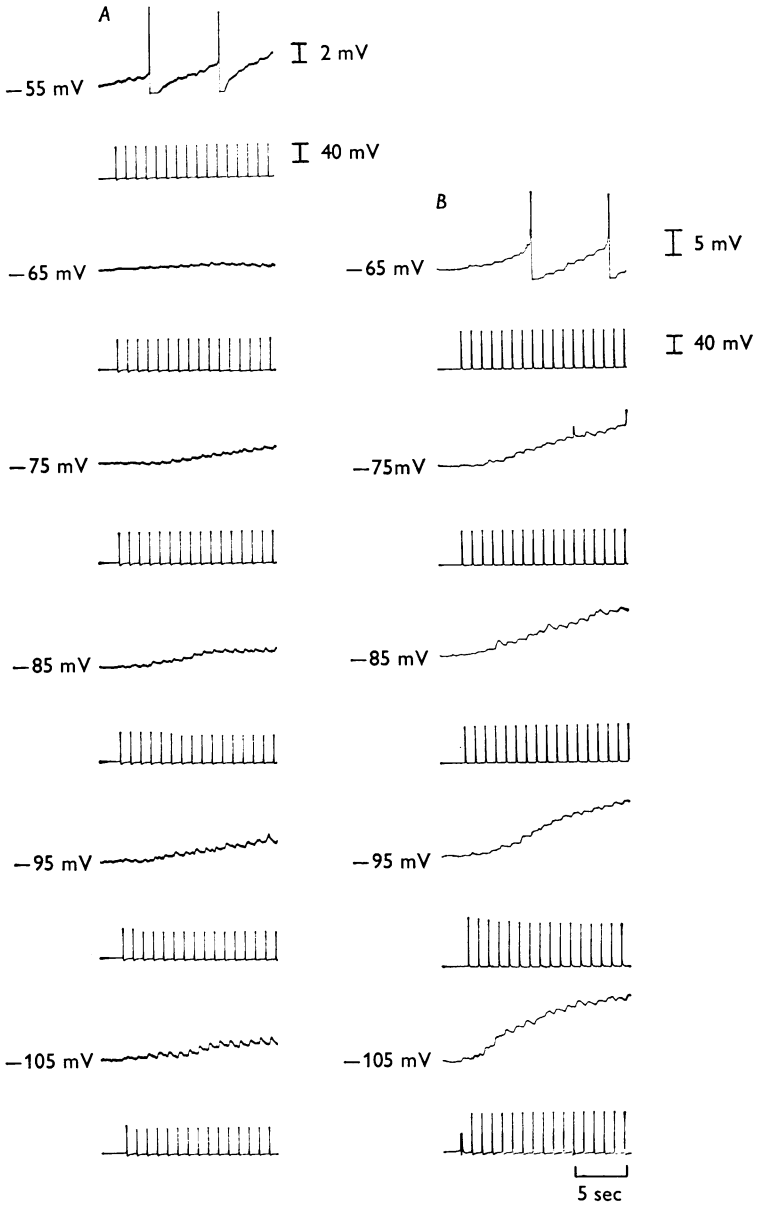

Fig. 4. Effect of hyperpolarization on the left middle cell responses to stimulation of the left giant serotonin cell. The middle cell was penetrated with two separate electrodes, the recording electrode was filled with KCl and the current passing electrode with Na_2SO_4. *A*, preparation immersed in normal medium. *B*, the same preparation immersed in KCl-free medium (the middle cell hyperpolarized by 5 mV in this medium). Note the different calibrations in *A* and *B*, and the steeper relationship between the increase in the size of the e.p.s.p.s and the level of hyperpolarization in *B* (see text).

– 55 mV. Occasionally, middle cells exhibited large e.p.s.p.s. When either of the giant serotonin cells was stimulated, each cell action potential caused a small e.p.s.p., of 500–600 msec duration, in each middle cell (Fig. 4). E.p.s.p.s in response to giant serotonin cell stimulation have been

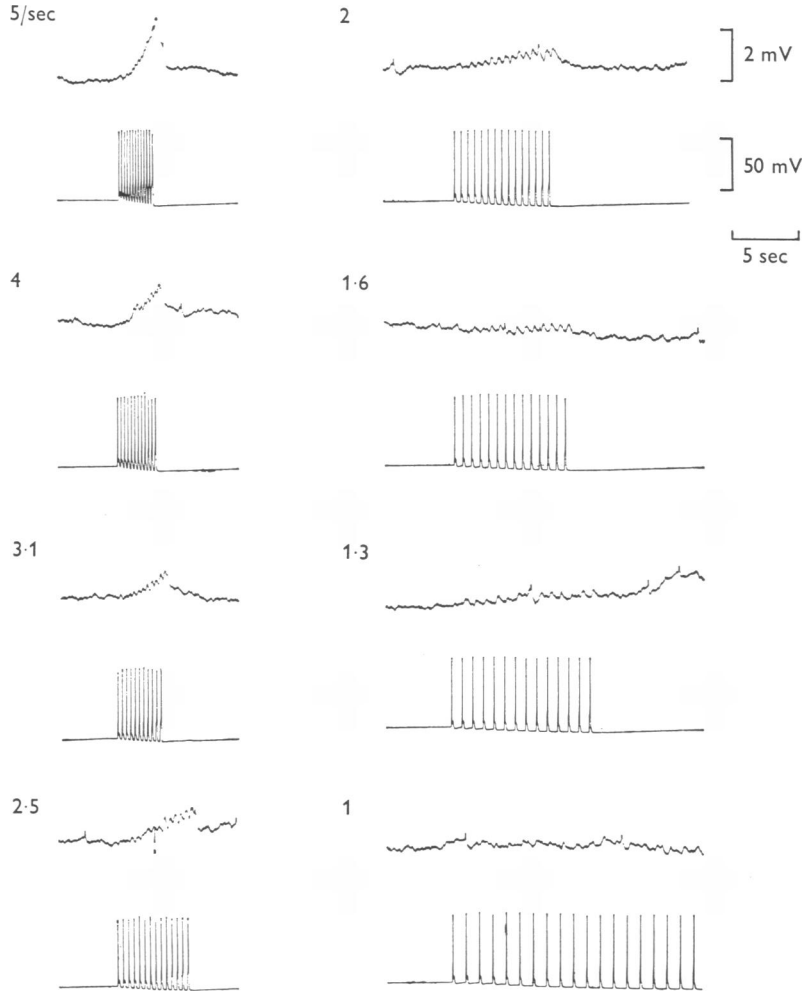

Fig. 5. Effect of frequency of giant serotonin cell action potentials on e.p.s.p. summation and facilitation in the middle cell. The middle cell was impaled with a KCl/Na_2SO_4 double-barrelled electrode. Top trace in each pair is the middle cell membrane potential and the lower trace the giant serotonin cell membrane potential. The middle cell resting potential was – 60 mV, but was artificially hyperpolarized to – 100 mV during the experiment; the giant serotonin cell resting membrane potential was – 50 mV. Summation of e.p.s.p.s occurred at frequencies of 2 Hz or above, whereas facilitation occurred at lower frequencies.

recorded in more than eighty middle cells. On repetitive stimulation of the giant serotonin cell the middle cell responses summed and gave rise to an action potential. The responses could be increased by artificially hyperpolarizing the middle cell. This effect was more marked when the bathing solution was replaced with KCl-free solution (Fig. 4*B*), in agreement with the observation that the M cells exhibit anomalous rectification (J. B. Macon, unpublished observations) and that K-free physiological solution diminishes anomalous rectification in *Anisodoris* G neurones (Marmor, 1971). Summation of e.p.s.p.s occurred only at GSC spike frequencies of 2 Hz or above, whereas facilitation in the size of the e.p.s.p.s could be observed at lower frequencies, down to about 0·5 Hz (Fig. 5). Rapid firing of the giant serotonin cells caused a reduction in the membrane resistance of the middle cells. This effect was more pronounced when the preparation was immersed in KCl-free solution (Fig. 6), presumably due to the reduction in anomalous rectification.

The responses of the posterior cells were very similar to those of the middle cells, but usually the e.p.s.p.s recorded were smaller and could not be seen without artificially hyperpolarizing the neurone. Posterior cells rarely showed e.p.s.p.s without giant serotonin cell stimulation.

Responses were only seen in the anterior cell during continued stimulation and repetitive firing of one of the giant cells. For example, during a burst of fifty giant serotonin cell action potentials at a frequency of 3 Hz, the anterior cell gradually depolarized and, approximately half-way through the burst, commenced firing action potentials which continued several seconds after stimulation of the giant serotonin cell was ceased. Individual e.p.s.p.s were not seen in response to giant cell stimulation even when the anterior cell was artificially hyperpolarized to -120 mV, but at the resting membrane potential characteristic small fluctuations, or oscillations, of 1–5 mV in amplitude and 500–800 msec duration were often observed during giant serotonin cell stimulation, and in the 15 sec period following stimulation.

Connexions between the different identified neurones

In spite of an extensive series of experiments, no connexions were found among the identified buccal cells. Efforts were also made to determine the pathways taken by the processes of the giant serotonin cells to the buccal ganglia by experiments in which one or the other of the cerebrobuccal connectives was cut and the response of the buccal cells compared with those observed before nerve section. It was found that the contralateral connexions are made by the branch of each giant serotonin cell which crosses to the contralateral cerebrobuccal connective, and that ipsilateral connexion is made by the shortest route, along the ipsilateral connective

(Fig. 3). However, in some experiments, very small e.p.s.p.s could still be observed in the ipsilateral middle cell when the giant cell was stimulated after completely sectioning the ipsilateral connective; they disappeared after cutting the contralateral connective. Thus each middle cell receives two inputs from the ipsilateral giant serotonin cell; one link is direct, the other is made by a circular route (Fig. 3).

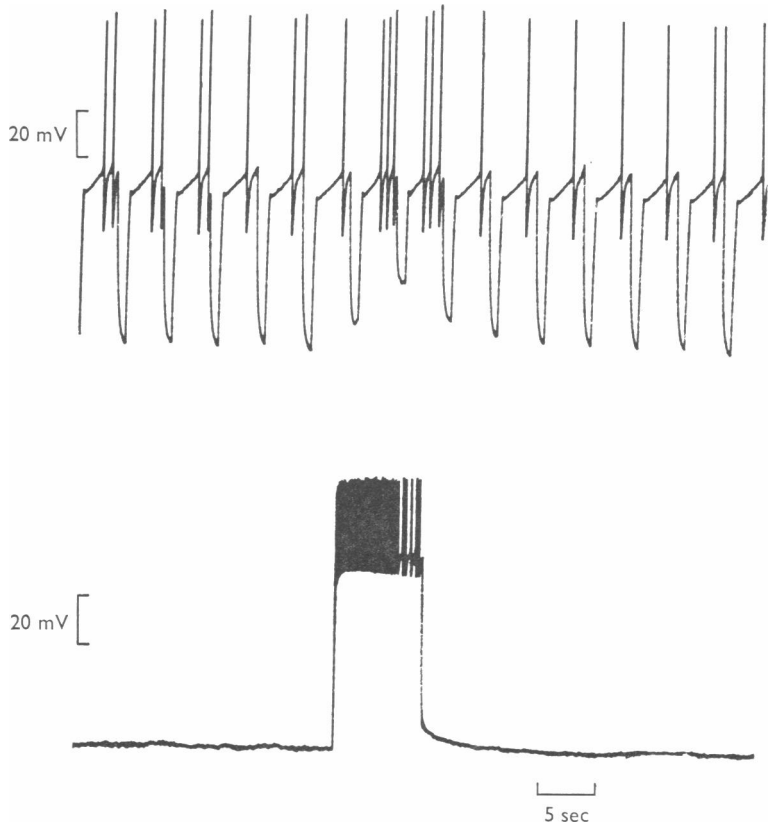

Fig. 6. Effect of rapid firing of the giant serotonin cell (lower trace) on the membrane resistance of the middle cell. The resting membrane potential of the middle cell, impaled with two separate KCl electrodes, was −50 mV and that for the giant serotonin cell was −55 mV. Membrane resistance was monitored by passing 1 sec hyperpolarizing pulses into the cell. Action potentials were seen in the middle cell at resting membrane potential. The giant serotonin cell was artificially hyperpolarized to −80 mV and then rapidly depolarized to −30 mV to make it fire rapidly. The firing rate of the middle cell increased and there was a marked reduction in the amplitude of the potential changes caused by the hypolarizing pulses, showing that there was a drop in membrane resistance. The recordings were made in KCl-free medium (Chamberlain & Kerkut, 1969).

Connexions between the giant serotonin cells and middle cells

For the purpose of investigating the role of serotonin in the giant neurones, subsequent experiments were made on the connexions of the giant serotonin cells on to the middle cells, because the results already described suggested that these connexions may be monosynaptic.

Experiments on the giant serotonin cells that elicited e.p.s.p.s were made over a period of $2\frac{1}{2}$ yr. During the latter half of February, and the months of March and April, responses in the middle cells were smaller than at other times of the year; frequently individual e.p.s.p.s could not be recorded at this time. In the course of more than eighty experiments, completed at other times of the year, it was observed that a one giant serotonin cell action potential to one e.p.s.p. relationship always applied, even after the giant serotonin cell has been stimulated to fire hundreds of action potentials. There was, however, a relatively long delay between the giant cell action potential and e.p.s.p. recorded in the middle cell. Since e.p.s.p.s were very small, rarely exceeding 0·5 mV and had a relatively slow rate of rise, it was difficult to measure their latency accurately. Nevertheless, the delay was constant within the limits of error of measurement, and it was shorter for the ipsilateral connexions. Analysis of variance of three separate experiments in which it was possible to measure the latency of both ipsi- and contralateral connexions showed that there is a difference ($P < 0{\cdot}001$) between the ipsilateral ($110{\cdot}8 \pm 2{\cdot}2$ msec S.D.) and the contralateral ($120 \pm 2{\cdot}2$ msec S.D.) connexions (cf. latency for antidromic spikes in Fig. 2).

One test for a monosynaptic connexion is to inject TEA into the presynaptic neurone and to monitor the size of the e.p.s.p. in the postsynaptic neurone. This technique (cf. Kehoe, 1972) depends on the injected TEA passing to the nerve terminals and increasing transmitter release. TEA increases the duration of the action potential (by an action on K permeability) and hence amount of transmitter released (Katz & Miledi, 1967; Kusano, Livengood & Werman, 1967). If the connexion is direct, the e.p.s.p.s would be expected to increase in size. The result of one TEA experiment is shown in Fig. 7. The right giant serotonin cell was penetrated with a double-barrelled electrode at the onset, one barrel was filled with 10% TEA and the other with Na_2SO_4 solution. Current (approx. 4 nA) was passed between the barrels for 1 hr, the positive source being connected to the TEA electrode, and then the electrode removed. The ipsilateral M cell was impaled, and the giant serotonin cell reimpaled, 6 hr later. E.p.s.p.s in the middle cell were recorded in response to giant serotonin cell stimulation at this time and also at intervals for a further 4 hr. When the e.p.s.p.s were first recorded (i.e. 6 hr after TEA injection),

they already appeared slightly larger than normally encountered, and untypically, a relatively large e.p.s.p. was observed in response to the initial giant serotonin cell spike of a burst. Recordings made 10 hr after injection showed that the amplitude of the e.p.s.p.s approximately doubled

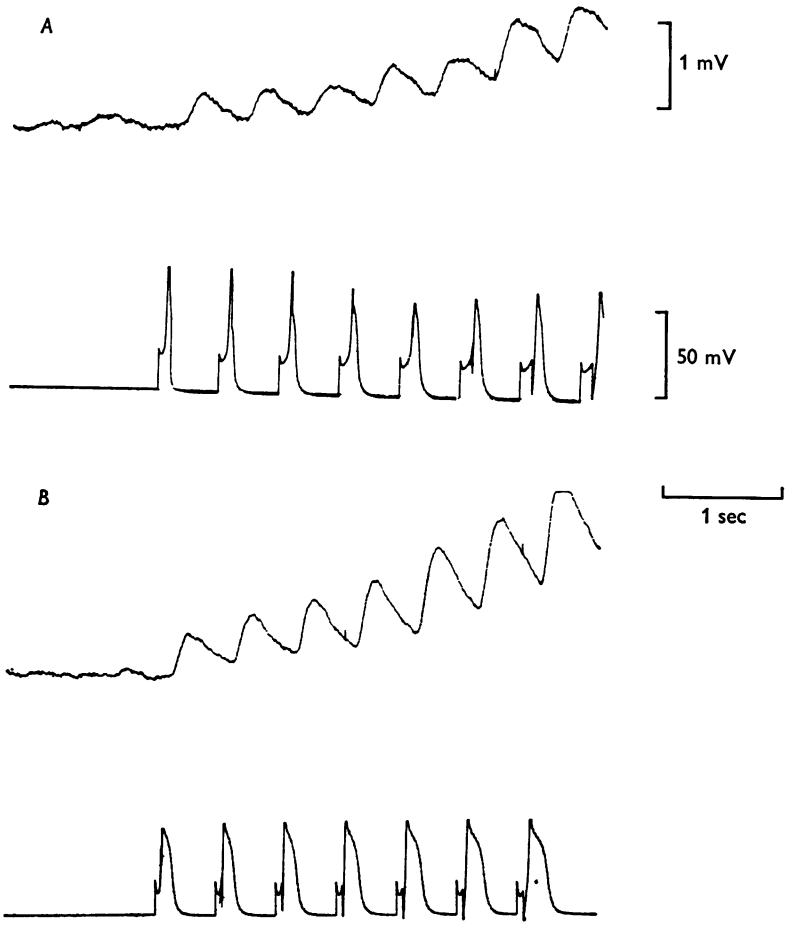

Fig. 7. Effect of TEA injection into the giant serotonin cell on the e.p.s.p.s of the right middle cell. The giant serotonin cell resting membrane potential was − 50 mV and the middle cell resting membrane potential was − 55 mV. During periods of recording, the middle cell (upper trace in each pair) was hyperpolarized by 40 mV. *A*, 6 hr after TEA injection. *B*, 10 hr after TEA injection (see text).

in size compared with those observed 6 hr after injection. The same result was obtained in a second experiment of the same type. When the experiment was repeated, but with Na injected (5 nA for 1 hr), the largest e.p.s.p.s recorded were 0·6 mV in amplitude. In other preparations, the

effect of TEA was tested by comparing the size of the e.p.s.p.s recorded 12 hr after TEA injection (4–8 nA current for 1 hr) with those recorded after giant serotonin cell injection of Na (4–11 nA current for 1 hr). The e.p.s.p.s recorded from the TEA injected preparations were generally larger (maximum amplitude 0·7–1 mV) than those seen in the control preparations (maximum amplitude 0·6–0·7 mV).

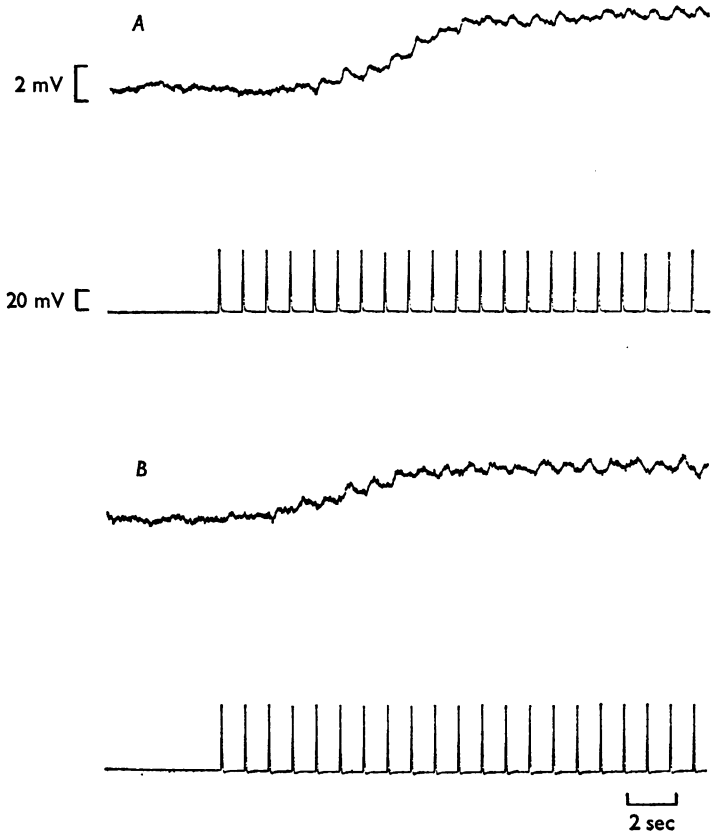

Fig. 8. Absence of effect of high Ca medium on the middle cell e.p.s.p.s. The middle cell was impaled with two separate KCl electrodes and was hyperpolarized to −95 mV during periods of recording. The resting membrane potential of the middle cell was −55 mV and that of the giant serotonin cell −50 mV. *A*, responses in normal medium (Chamberlain & Kerkut, 1969). *B*, responses after exposure to 60 mM-Ca medium for 30 min.

A further test for a direct monosynaptic connexion was made by exposing preparations to physiological solution containing a high concentration of Ca. In *Aplysia*, Austin, Yai & Sato (1967) have shown that a high Ca concentration greatly increases spike threshold. If the connexion from each giant serotonin cell to each middle cell is not monosynaptic, the

occurrence of an e.p.s.p. in the middle cells will depend on whether the interneurones fire. When the threshold of the interneurones is increased, it is less likely that an e.p.s.p. will be seen in response to a stimulus applied to the giant cell and the one giant cell spike to one e.p.s.p. relationship would be expected to be lost. If, on the other hand, the connexion is monosynaptic, high Ca solution would not be expected to block the e.p.s.p.s. In these experiments, the concentration of $CaCl_2$ was increased to 60 mM without osmotic compensation. Spike threshold was greatly increased but e.p.s.p.s were still seen following spiking in either giant serotonin cell (Fig. 8) and the relationship of one giant serotonin cell action potential to one e.p.s.p. was maintained.

Iontophoretic application of serotonin on to the middle cells

Response and sensitivity to serotonin

Serotonin applied locally to the soma membrane produced a depolarizing potential change. It was essential to place the iontophoretic pipette as close as possible to the middle cell membrane in order to obtain a maximal response. The thin inner connective tissue layer still covering the buccal cells was penetrated with the iontophoretic electrode, which was then manipulated as close as possible to the middle cell membrane without impaling the cell. It was not possible to determine visually the position of the electrode tip relative to the middle cell axon hillock, except occasionally in cells possessing an orange pigment in the region of the hillock. In these cells, the greatest sensitivity to serotonin iontophoresis was in the region of the orange pigment suggesting that the receptors are located along the axon or on axon hillock of the middle cell.

It soon became apparent that the serotonin receptors are rapidly desensitized (see below), consequently in all the iontophoresis experiments it was necessary to adjust carefully the backing current to avoid leakage and receptor desensitization. In Fig. 9*A*, a current pulse of 20 nA for 500 msec produced a potential change of 4–5 mV with a duration of 7–10 sec. In this and most subsequent records of the serotonin potential, the middle cells were artificially hyperpolarized by 10–20 mV to prevent action potential firing. Both middle cells responded in a similar way to iontophoresed serotonin. A depolarizing response of 2–3 mV and duration 4–5 sec was observed in some left and right middle cells with an iontophoretic current pulse of 20–40 nA for 25–50 msec (Fig. 9*B*, *C*). Some middle cells had a sensitivity to serotonin as high as 5 mV/nC although sensitivity varied greatly among different middle cells, probably partly due to the variability in location of the electrode tip in relation to the axon hillock (cf. Ascher, 1972).

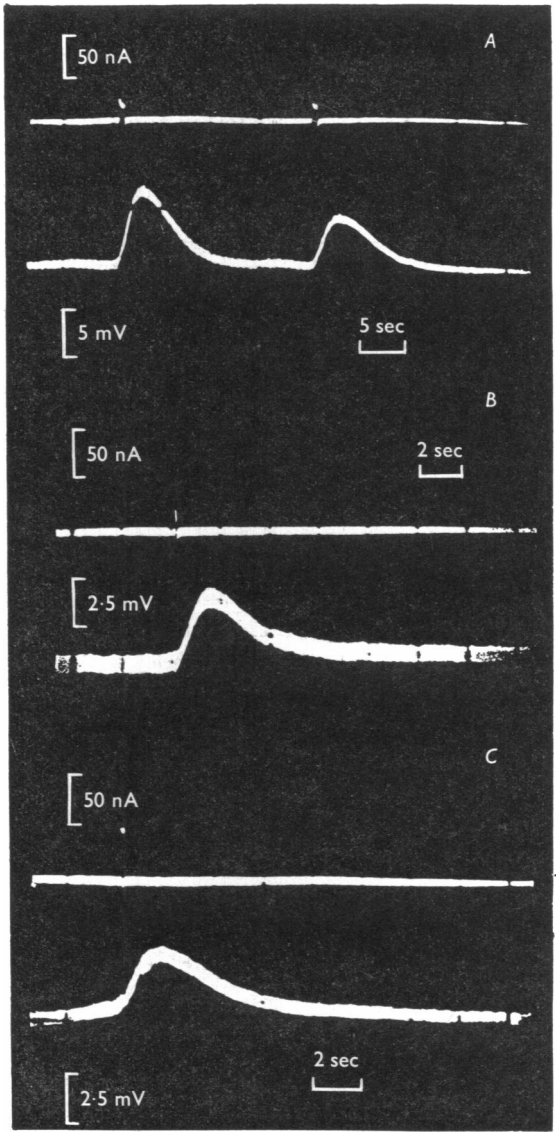

Fig. 9. Iontophoretic application of serotonin. In each record the top trace shows the iontophoretic current and the lower trace the middle cell potential. The middle cells were artificially hyperpolarized by 20 mV in *A* and *B* and by 15 mV in *C*. *A*, response of the right middle cell to two iontophoretic pulses (20 nA for 50 msec). The resting membrane potential was −45 mV. *B*, responses of another right middle cell (resting membrane potential −45 mV) to an iontophoretic current pulse of 10 nA for 50 msec. *C*, responses of the left middle cell to a pulse of 40 nA for 25 msec.

Serotonin receptor desensitization

When two iontophoretic pulses were applied within a 25 sec interval, the second serotonin potential was reduced in amplitude, probably due to desensitization of the receptors. The shorter the pulse interval the more marked was the desensitization (Fig. 10*A*). Repetitive application at 10 sec pulse intervals led to pronounced desensitization (Fig. 10*B*). When

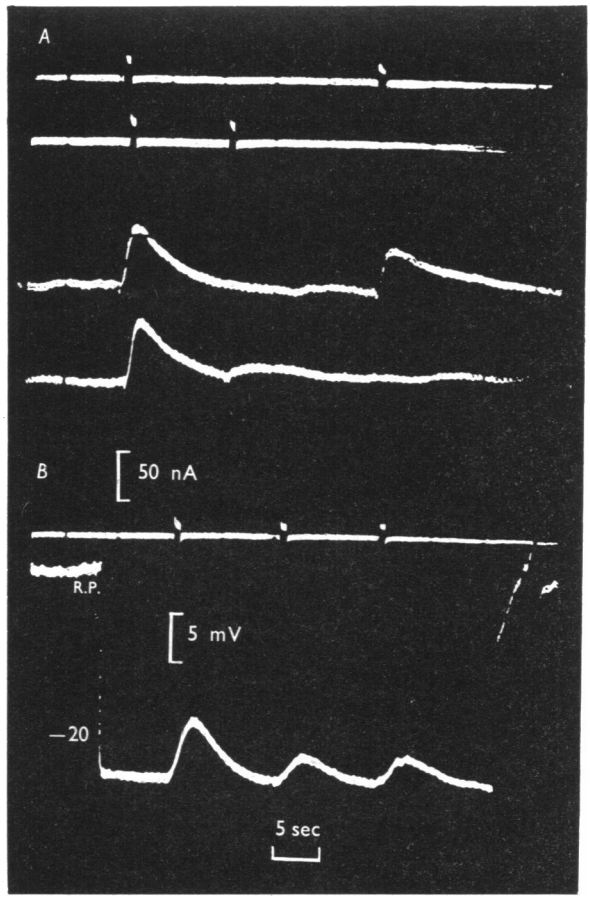

Fig. 10. Desensitization of the middle cell serotonin receptors. *A*, upper two traces iontophoretic current, lower traces membrane potential. The left middle cell was artificially hyperpolarized by 15 mV to −70 mV. There was only slight reduction of the second serotonin potential when the pulse interval is 25 sec, but marked reduction of the potential occurs when the pulse interval was 10 sec. The current pulses were 30 nA for 500 msec. *B*, upper trace current pulses and lower trace right middle cell potential. The cell was artificially hyperpolarized by 20 mV to −65 mV. The current pulses were 20 nA for 500 msec.

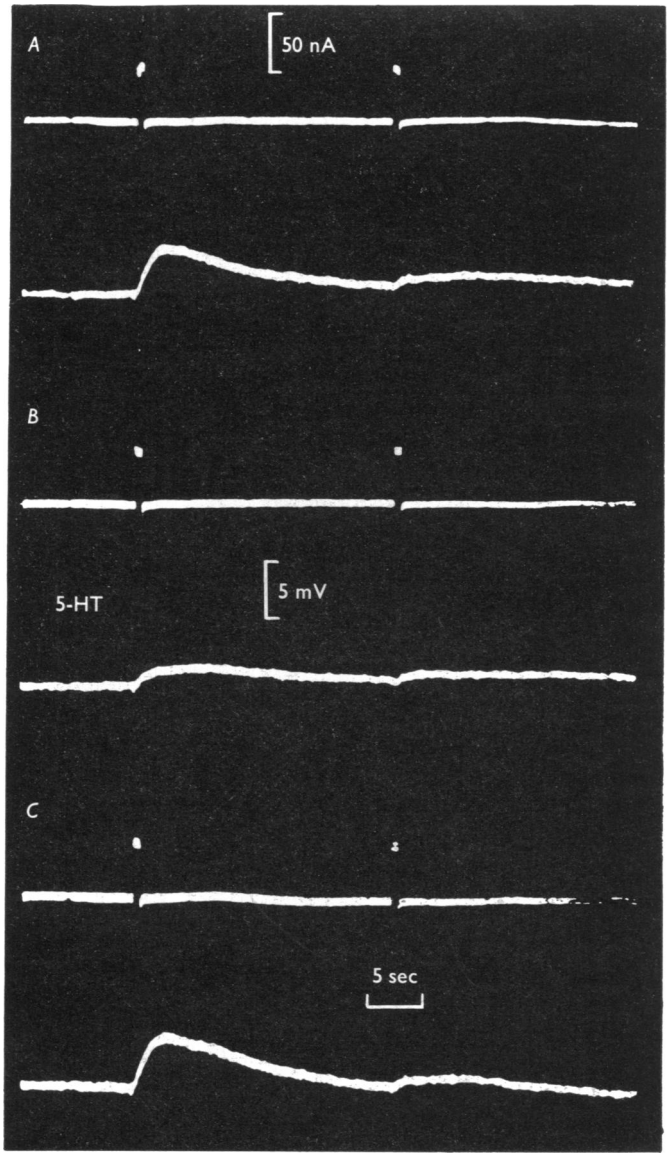

Fig. 11. Effect of exposing the preparation to a low concentration of serotonin on the middle cell serotonin potential. The resting membrane potential was −50 mV and the cell was artificially hyperpolarized to −70 mV. The iontophoretic pulse was 50 nA for 500 msec. *A*, responses in normal medium. *B*, responses after 3 min in the presence of 5×10^{-8} M serotonin. There was no depolarization of the middle cell membrane by this concentration of serotonin in the medium. *C*, serotonin potential after washing the preparation for 5 min with normal medium.

the preparation was exposed for 3 min to a low concentration of serotonin (5×10^{-8} M), the serotonin potential was greatly reduced in amplitude (Fig. 11), which caused only a slight depolarization of the middle cell, with little alteration of firing rate. This effect was reversed by washing the preparation for 5 min. Addition of a higher dose of serotonin (5×10^{-5} M), leading to a marked depolarization of the middle cell (5–10 mV) and an increased firing rate, abolished the serotonin potential. This effect was not reversible on washing the preparation for 30 min.

Effects of serotonin blocking drugs

A number of substances have been shown to be effective antagonists of serotonin (Gaddum & Picarelli, 1957; Gerschenfeld, 1973). These include LSD-25 and related compounds, morphine, tryptamine and tubocurarine. In this part of the study, efforts were concentrated on finding a selective blocking agent, with minimal side effects, which could be used to investigate the e.p.s.p.s mediated by giant serotonin cells.

LSD-25 at $1{\cdot}5 \times 10^{-4}$ M produced a 5–10 mV depolarization of the middle cell and a prolonged burst of action potentials. After 15 min, the sensitivity to applied serotonin was markedly reduced but was restored after washing for 10 min. Lower concentrations of LSD-25 (10^{-5} M) produced a less marked reduction in the serotonin potential. The related compounds BOL-148 and UML-491 ($2{\cdot}5 \times 10^{-4}$ M) produced less depolarization but a similar antagonism of iontophoresed serotonin developed after about 15 min.

Morphine, in a slightly lower concentration (2×10^{-5} M), also reversibly blocked the serotonin potential (Fig. 12). Unlike LSD-25, it did not depolarize the cells even in a concentration of 10^{-4} M.

Tryptamine (10^{-6} M) and curare ($1{\cdot}5 \times 10^{-4}$ M) also reversibly blocked the serotonin potential without causing depolarization. As with the depolarizing, Type A, serotonin receptors characterized by Gerschenfeld (1971), the serotonin response was unaffected by neostigmine bromide at a concentration (6×10^{-6} M) adequate to enhance the amplitude and prolong the duration of an acetylcholine potential recorded in other neurones (the posterior cells).

Ionic mechanism of the serotonin potential

A characteristic of the depolarizing, Type A, serotonin receptor is its dependence on Na (Gerschenfeld, 1973). When the preparation was immersed in physiological solution containing 12 mM-Na with the remainder of the NaCl replaced by addition of Tris-HCl (pH 7·8), the serotonin potential was abolished within 5 min (Fig. 13). Washing with normal physiological solution for 10 min restored the serotonin potential to its

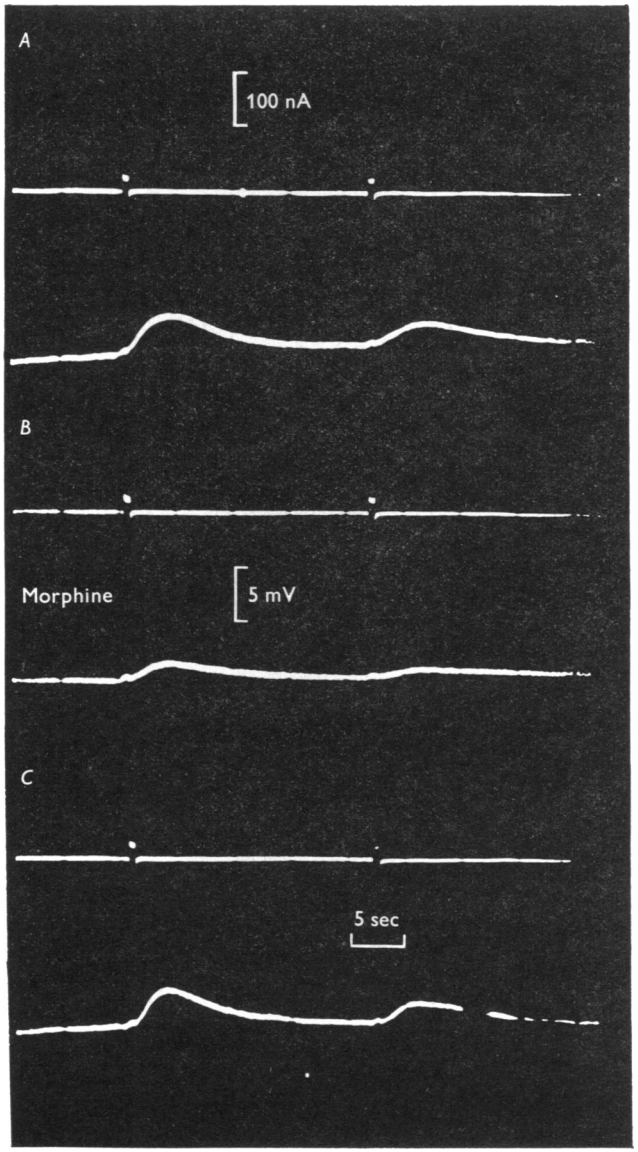

Fig. 12. Effect of 2×10^{-5} M morphine sulphate on the middle cell serotonin potential. Upper trace in each record is the current pulse and the lower trace the middle cell potential. The iontophoretic current pulse was 30 nA for 500 msec. The resting membrane potential was -65 mV. Responses: in normal medium (*A*), after exposure to morphine sulphate for 12 min (*B*), and 10 min after washing the preparation in normal medium (*C*). The morphine sulphate did not depolarize the cell.

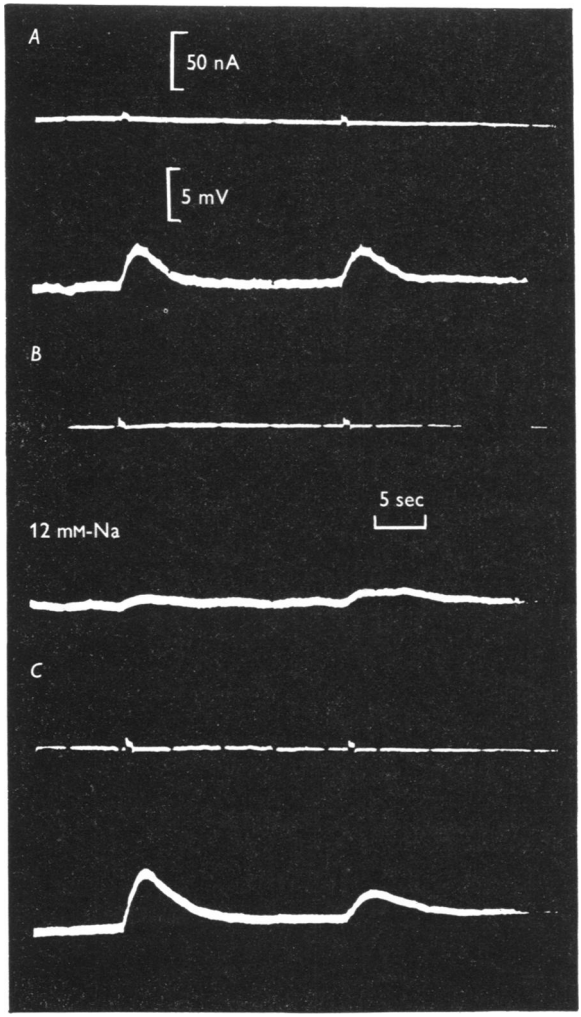

Fig. 13. Effect of low Na medium on the middle cell serotonin potential. Upper trace in each record is the iontophoretic current pulse (10 nA for 500 msec), and the lower trace is the right middle cell membrane potential. The middle cell was artificially hyperpolarized by 10 mV to −55 mV. *A*, serotonin potential in normal medium containing 71 mM-Na. *B*, responses after 5 min exposure to 12 mM-Na medium (NaCl replaced by Tris-HCl). The middle cell hyperpolarized by 4 mV due to the reduction of external Na, but the membrane potential was artificially adjusted to −55 mV. *C*, serotonin potentials after washing for 10 min in normal medium. The middle cell potential returned to its original value.

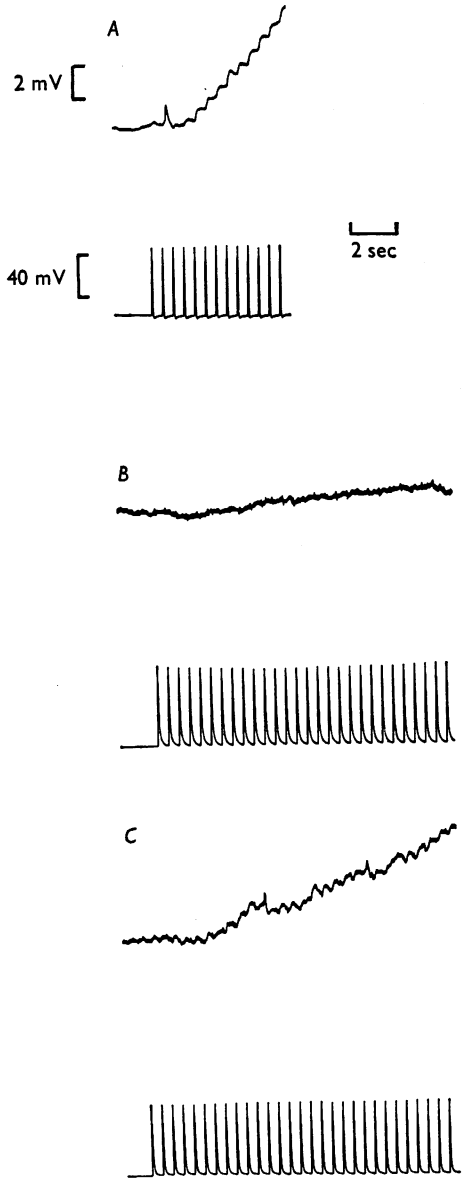

Fig. 14. The effect of $1{\cdot}3 \times 10^{-4}$ M morphine sulphate on the e.p.s.p.s of the middle cell resulting from giant serotonin cell stimulation. The upper trace in each record is the middle cell potential and the lower trace the giant serotonin cell potential. The middle cell, impaled with a KCl/Na_2SO_4 double-barrelled electrode, had a resting membrane potential of -60 mV; the giant serotonin cell resting membrane potential was -60 mV. During periods of recording, the middle cell was hyperpolarized to -110 mV. Responses: before drug application (*A*) and (*B*) after 30 min exposure to morphine sulphate. *C*, responses after 3 hr washing in normal medium.

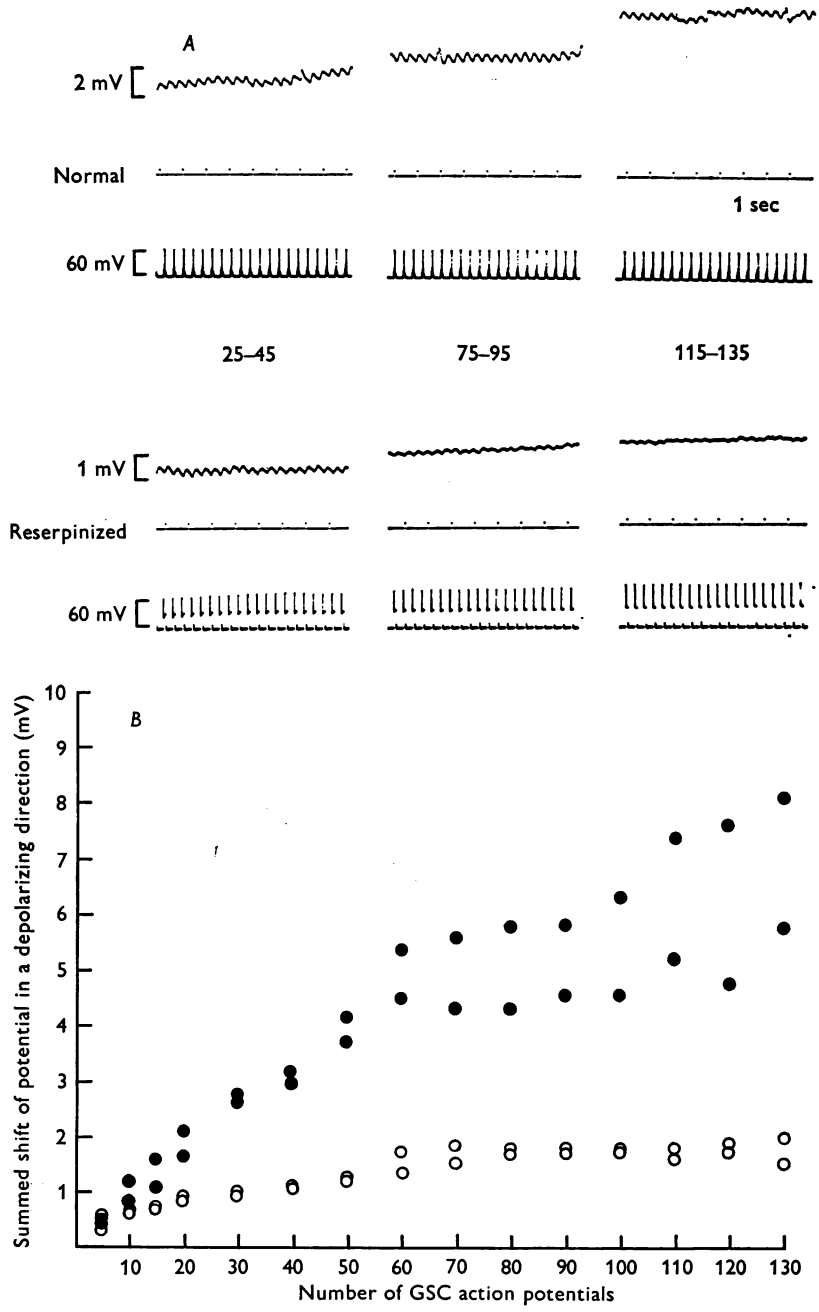

Fig. 15. For legend see facing page.

original amplitude. Identical results were obtained when the NaCl was replaced by sucrose or LiCl. It is interesting to note that the amplitude of the middle cell action potentials (60–65 mV) in 12 mM-Na was the same as in normal medium.

Effects of different agents on the e.p.s.p.s

Serotonin antagonists

The clearest results were obtained with morphine sulphate. At a concentration of 5×10^{-5} M and above, it reduced the size of the e.p.s.p.s (Fig. 14). The effect could be partially reversed by washing for 2 or 3 hr. Other experiments showed that morphine sulphate at $1{\cdot}3 \times 10^{-4}$ M did not block action potential conduction along the giant serotonin cell axons nor reduce the membrane resistance of the middle cells.

Neither LSD-25 nor UML-491 produced a consistent measurable reduction in size of the e.p.s.p.s in low concentrations (10^{-6}–3×10^{-5} M). Higher concentrations produced complex effects. LSD-25, at 3×10^{-5} M to 3×10^{-4} M, depolarized and stimulated the middle cells to fire action potentials. The e.p.s.p.s were reduced in size, or even abolished, in 3×10^{-4} M-LSD-25, but it could not be determined to what extent blockade of serotonin receptors accounted for the reduction in size of the e.p.s.p.s. UML-491 in concentrations of 3×10^{-5} to $1{\cdot}5 \times 10^{-4}$ M produced only a small drop in membrane resistance with very little, if any, increase in the firing rate of the middle cells. Exposure to $1{\cdot}5 \times 10^{-4}$ M-UML-491 abolished the initial e.p.s.p.s produced by a train of giant serotonin cell spikes. This

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Fig. 15. Reserpine and transmission from the giant serotonin cells (GSCs) to the middle cells. *A*, results of two parallel experiments are shown. In each case, the right giant serotonin cell was stimulated at 3 Hz for about 1 min (lower trace in each experiment). The resting membrane potential of the giant serotonin cells: upper −50 mV, lower −55 mV. The middle cell was artificially hyperpolarized by 40 mV to a level of about −95 mV in each experiment. The e.p.s.p.s recorded in response to giant serotonin cell spikes 25–45, 75–95 and 115–135 are shown. The results in the upper records were made from a normal, and those below from a reserpinized, preparation (see text). In the normal preparation e.p.s.p.s were observed in response to each giant serotonin cell spike and summation of the e.p.s.p.s resulted in considerable depolarization. E.p.s.p.s were also observed in the reserpinized preparation, but with repetitive stimulation the size of the e.p.s.p.s rapidly diminished and there was much less depolarization of the cell. *B*, results of four separate experiments expressed graphically from two control (●) and two reserpine injected (○) animals. In each experiment, the middle cell was impaled with a KCl/Na_2SO_4 double-barrel electrode and hyperpolarized to a level of about −95 mV and the shift of membrane potential in a depolarizing direction recorded during a burst of 130 action potentials, at a frequency of 3 Hz, in one of the giant serotonin cells.

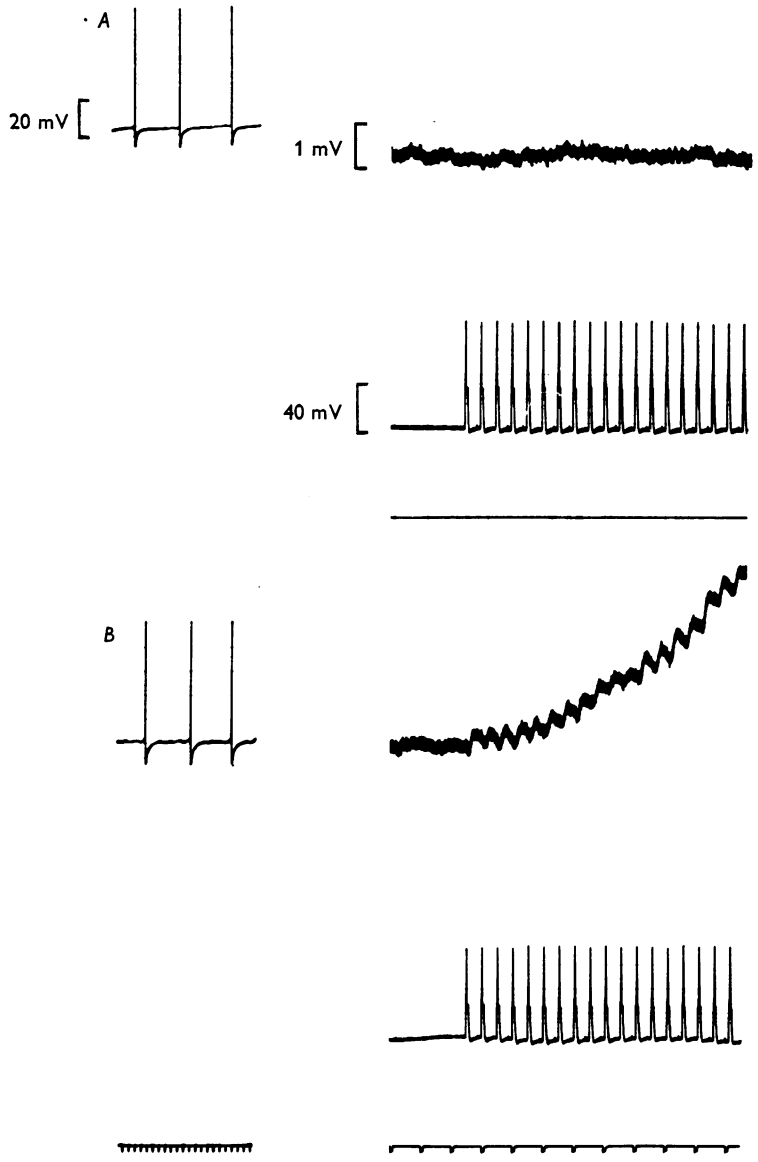

Fig. 16. Effect of low Na medium (12 mM-NaCl and 68 mM-LiCl) on the middle cell e.p.s.p.s. The middle cell was impaled with two separate KCl electrodes and hyperpolarized by 40 mV to −95 mV during periods of recording. Upper trace in each pair is the middle cell potential; lower trace is the giant serotonin cell potential. *A*, absence of middle response to giant cell stimulation after exposure to low Na medium for 30 min. *B*, e.p.s.p.s recorded after 12 min exposure to normal medium. Note that the height of the middle cell action potential (shown on the left) was the same in low Na medium as in the normal medium.

effect became more pronounced with continued exposure (up to 90 min), until no e.p.s.p.s at all were seen during a train of 100 evoked giant serotonin cell action potentials. Further experiments showed, however, that at this concentration UML-491 could reversibly block giant serotonin cell action potential conduction along the cerebrobuccal connectives. A similar effect of BOL-148 has been observed in *Aplysia* (see Gerschenfeld & Stefani, 1966).

Tryptamine and serotonin depolarized and stimulated the middle cells in concentrations of about $2{\cdot}5 \times 10^{-6}$ M. Lower concentrations did not block the e.p.s.p.s.

Reserpine

The serotonin content of the giant serotonin cells is depleted by reserpine (Cottrell & Osborne, 1970). The effect of reserpine was therefore tested on the e.p.s.p.s to establish whether they are influenced by reduced serotonin levels, as would be expected if serotonin is the transmitter substance. Animals were injected with a total of 1 mg of soluble reserpine over a period of 3 days immediately before recording the e.p.s.p.s. The effect of the reserpine injections was tested by monitoring the level of serotonin in giant serotonin cells by fluorescence histochemistry using the method of Corrodi & Jonsson (1967). Reserpine markedly reduced the efficacy of transmission from the giant cells to the middle cells; there was a gradual diminution in the size of the e.p.s.p.s during a period of prolonged stimulation and the summed shift of the potential in a depolarizing direction was greatly reduced compared with the results of control experiments (Fig. 15).

Effect of reducing the external Na concentration

As already noted, the serotonin potential in middle cells is reduced in size when the Na concentration is lowered to 12 mM, although normal action potentials are still recorded. The effect of lowered Na was tested on the e.p.s.p.s. These were reversibly reduced in size and abolished when preparations were exposed to physiological solution in which the NaCl was reduced to 12 mM and replaced by adding either LiCl (Fig. 16) or Tris-HCl.

DISCUSSION

There are two important problems to be discussed. The first is: do the giant serotonin cells make monosynaptic connexions with the middle cells? If this is the case, the other problem is whether serotonin is released from the giant serotonin cell endings to mediate the excitatory response recorded from the middle cells.

Synaptic connexions between the giant serotonin cells and the middle cells

Providing conclusive evidence for a monosynaptic connexion between two gastropod neurones is difficult. At present, there is no method known to us which gives direct unequivocal evidence for a chemical monosynaptic connexion. The available experimental data do strongly suggest, however, that the link between each giant cell and each middle cell is monosynaptic. Electrophysiological results show that each giant serotonin cell sends an axon branch through each buccal ganglion; such branches must pass very close to the middle cells. Within the limits of error of measurement, there is a constant latency between the action potential recorded in each giant serotonin cell soma and the e.p.s.p. in each middle cell. There is a one-to-one relationship between giant serotonin spikes and middle cell e.p.s.p.s and this relationship is maintained in saline containing a very high concentration of Ca, which would be expected to block interneurone connexions because of the increase in spike threshold (Austin *et al.* 1967). Experiments made by M. S. Berry (personal communication) provide clear evidence that saline containing 60 mM-Ca is also of value in blocking one-to-one transmission between strongly electrically coupled giant neurones in *Planorbis*. An action potential in one neurone invariably causes a soma or axon spike in the other. Immersion of the preparation in saline containing 60 mM-Ca causes a loss of this one-to-one coupling of the *Planorbis* neurones, such that frequently a spike in one cell was not followed by a soma or axon spike in the other. TEA injection of the giant serotonin cell enhances the middle cell e.p.s.p.s, presumably by passage to the giant serotonin cell terminals and causing an increase in transmitter released on to the middle cell (see above). The results of these experiments therefore strongly suggest that the connexions are monosynaptic. The experimental results obtained with reserpinized animals, where a progressive diminution of the e.p.s.p.s was observed with repetitive stimulation, are also most readily explained in terms of a monosynaptic connexion between each giant serotonin cell and each middle cell.

Is serotonin the transmitter released on to the middle cells?

It is not possible to demonstrate directly whether serotonin is released at the synapses to cause the observed responses. Instead we must make an appraisal of the probability that serotonin is the responsible transmitter based on various pieces of less direct evidence.

Previous work has shown that serotonin is located in the presynaptic neurones; each giant serotonin cell perikaryon contains about 1 ng serotonin. The perikaryon can take up 5-hydroxytryptophan and can convert it to serotonin (Cottrell & Powell, 1971; Osborne, 1972).

The iontophoresis experiments show that the middle cells possess serotonin receptors. These receptors are rapidly desensitized by serotonin. Similar desensitization has been observed for serotonin receptors of certain *Cryptomphallus aspersa* neurones (Gerschenfeld & Stefani, 1966) and central neurones in the cat (Roberts & Straughan, 1967), for ACh at the frog neuromuscular junction (Katz & Thesleff, 1957), and for glutamate at the crayfish neuromuscular junction (Takeuchi & Takeuchi, 1964). The data suggest that the middle cell possesses Type A receptors according to the classification of Gerschenfeld (1971).

Both the serotonin potential and the e.p.s.p. are dependent upon a large Na electrochemical gradient (Figs. 13, 16). The serotonin potentials of CILDA neurones of *Cryptomphallus aspersa* are also Na-dependent (Gerschenfeld & Stefani, 1968). The serotonin potential ionic mechanism and that for the e.p.s.p.s are clearly more Na-dependent than the middle cell action potential since the latter persists in 12 mM-Na without a reduction in amplitude. This enabled us to observe the effect of reduced Na on the e.p.s.p.s. Na-independent action potentials have been observed in other snail neurones (Gerasimov, 1964; Kerkut & Gardner, 1967; Meves, 1968; Chamberlain & Kerkut, 1969).

Morphine sulphate blocked both the serotonin potential and the e.p.s.p.s but a higher concentration was required to block the e.p.s.p.s ($1{\cdot}3 \times 10^{-4}$ M) than the iontophoresed serotonin (2×10^{-5} M). The difference in concentration required for blockade may be due to the presence of the synapses deep in the neuropile as well as to possible extra barriers to passage of the drug to the receptors located on the post-synaptic membrane, compared with the superficial location of receptors activated by iontophoresed serotonin. It is also possible that very high concentrations of transmitter occur in the confined space of the synaptic gap, during release, and consequently a concomitantly higher concentration of antagonist is required.

One obvious discrepancy between the e.p.s.p.s and the serotonin potential was the effect of exposure to low concentrations of tryptamine (10^{-6} M) and serotonin (5×10^{-8} M) itself. At these concentrations, both agents reversibly antagonized the serotonin potential, but had little if any effect on the e.p.s.p.s. As above, the difference may be explained in terms of the accessibility of the receptors and concentration of transmitter released into the synaptic gap. Another factor may be the active uptake mechanism for serotonin which occurs in fine axonal processes (presumed serotonergic endings) in the neuropile of the ganglia (Pentreath & Cottrell, 1972). Such a process might be expected to reduce the amount of serotonin, and perhaps also tryptamine, available for combination with receptors, particularly in synaptic areas.

In reserpine-treated animals, in which the serotonin content of the giant

serotonin cells was greatly reduced, transmission from these cells to the middle cells was less effective; there was a progressive decline in the e.p.s.p. amplitude on prolonged giant serotonin cell stimulation (Fig. 15). Also, the level of depolarization achieved by the reserpine treated preparations was much less than that of the controls, although the initial summed depolarizing response was very similar. This suggests that action potential propagation to the nerve terminals and the level of sensitivity of the post-synaptic membrane to released transmitter are unchanged by reserpine, but that the drug has reduced the total amount of transmitter available for release from the terminals of the giant serotonin cells.

The size of the giant serotonin cell elicited e.p.s.p. in the middle cells was smaller during late February, March and April. This may be related to a seasonal fluctuation in serotonin; Cardot (1971) has shown that there is a reduction in the serotonin level of the central ganglion of *Helix pomatia* which is particularly apparent in March and April.

Thus, although some discrepancies remain, the present results, together with previous studies, suggest that serotonin within the giant serotonin cell serves a mediator role, transmitting excitatory activity from the giant serotonin cells to the middle cells. However, certain criteria remain to be satisfied in order to establish the point beyond doubt. In particular it is necessary to demonstrate that serotonin is released into the perfusion medium following giant serotonin cell stimulation.

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