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THE SYNAPTIC JUNCTIONS OF LE AND RF CLUSTER SENSORY NEURONES OF *APLYSIA CALIFORNICA* ARE DIFFERENTIALLY MODULATED BY SEROTONIN

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Summary

The monosynaptic component of withdrawal reflexes in *Aplysia californica*, from sensory neurones to motor neurones, is a critical site of the synaptic modulation occurring during short-term and long-term behavioural changes. There are four clusters of sensory neurones (LE, rLE, RE, RF) innervating the receptive field for the gill and siphon withdrawal reflex. The receptive fields of these cells are located on the siphon, the mantle, the branchial cavity and the gill itself. In most studies, the synapses made by the sensory neurones of the LE cluster of the abdominal ganglion or the VC cluster of the pleural ganglion have been investigated and shown to be facilitated by the neuromodulator serotonin (5-hydroxytryptamine, 5-HT). In this report, we have examined the effect of 5-HT on the synaptic junctions of the RF cluster neurones. The duration of action potentials in these cells, unlike those of the other clusters, is barely affected by serotonin.

We found that while the LE synapses are facilitated by 5-HT (10 μmol l^{-1}), the RF synapses are not. In fact, the RF–L14 connections are actually depressed by 5-HT; this effect is not due to shunting in the postsynaptic neurone. The RF–L7 connections are also depressed by 5-HT, although the effect is smaller. The RF–L14 connections are blocked by the non-NMDA receptor agonist CNQX (100 μmol l^{-1}), suggesting that the transmitter and the postsynaptic receptors involved are similar to those present on the LE or VC cluster cells. The absence of serotonin-induced facilitation of the RF cluster cells may provide the animal with a means of reducing the nonspecific effects of aversive sensitization and therefore of allowing a greater specificity and more flexibility in plastic behavioural changes.

Key words: serotonin, 5-hydroxytryptamine, *Aplysia californica*, modulation, synaptic junction.

Introduction

The gill and siphon withdrawal reflex and the tail withdrawal reflex are two excellent systems for investigating the cellular and molecular mechanisms underlying short-term and long-term behavioural modifications (Hawkins et al., 1993; Lechner and Byrne, 1998). The neuronal networks causally related to these reflexes include two main components, a monosynaptic one, the sensory-to-motor neurone synaptic junctions, and a polysynaptic one made up of various excitatory and inhibitory interneuronal synapses (Frost et al., 1988; Trudeau and Castellucci, 1992; White et al., 1993). There are many sites that can be modified when the reflex is facilitated or depressed (Fischer and Carew, 1995; Trudeau and Castellucci, 1993a; Xu et al., 1995). The importance of a specific site and the mechanism involved may depend on the particular protocol being used (Mackey et al., 1987; Byrne and Kandel, 1996). It is readily apparent that a large amount of complexity can be achieved even at the level of a simple withdrawal reflex and in a relatively simple neuronal network. This paper documents another degree of complexity resulting from the fact that the sensory neurones of the RF cluster differ from the sensory neurones in the other clusters.

It is known that the monosynaptic component of withdrawal reflexes in *Aplysia californica* is a critical site of synaptic modulation occurring during short-term and long-term behavioural changes (Byrne and Kandel, 1996; Castellucci et al., 1978). In these studies, the connections made by the LE sensory neurone cluster of the abdominal ganglion or the VC sensory cluster of the pleural ganglion were investigated. It was repeatedly observed that these synaptic junctions are facilitated by the neuromodulator serotonin (5-hydroxytryptamine, 5-HT) (Ghirardi et al., 1995; Wu et al., 1995). Four sensory neurone clusters have been described in the abdominal ganglion; the LE, rLE, RE and RF clusters (Byrne, 1980; Dubuc and Castellucci, 1991).

The receptive fields of these cells are located on the siphon, the mantle, the branchial cavity and the gill itself (Dubuc and Castellucci, 1991), which is the receptive field of the withdrawal reflex. The properties of these sensory neurones are similar except that the action potentials of the RF neurones are not as sensitive to known modulators of the defensive reflex (Dubuc and Castellucci, 1991). Since the synaptic output of the sensory neurones is to the various motor neurones of the gill and siphon as well as to some motor neurones involved in inking (L14), we wanted to determine whether the RF synaptic junctions were modulated like the other sensory neurones involved in the aversive reflex. Previous studies have shown that pairing sensory neurone tetanization with connective stimulation produces much larger synaptic facilitation than that obtained by tetanization alone (Dubuc, 1990). This was true for all neurones of the LE, rLE and RE clusters, the exceptions being the neurones from the RF cluster. In the latter case, tetanization alone produces a larger effect than that due to pairing with connective stimulation (Dubuc, 1990). Because the connectives are known to contain serotoninergic axons (Longley and Longley, 1986; Hawkins, 1989; Mackey et al., 1989), we thought that this depressing effect could be attributed in part to serotonin. We therefore examined the effects of 5-HT on the RF neurone synapses and compared the results with those for the LE neurone synapses.

Materials and methods

Preparation

Aplysia californica (mass 100–300 g) were purchased from Marine Specimen Unlimited (Pacific Palisades, CA, USA) or *Aplysia* Resource Facilities (Miami, FL, USA). They were maintained in a large (900 l) tank at 15 °C. All experiments were performed at room temperature (22 °C) on isolated abdominal ganglia. Before dissection, animals were anaesthetized with an injection of an isotonic $MgCl_2$ solution (385 mmol l^{-1}) corresponding to approximately one-third of their volume. Dissection of the abdominal ganglion was performed in an extracellular medium consisting of equal volumes of 385 mmol l^{-1} $MgCl_2$ and artificial sea water (ASW). The ganglia were pinned to the bottom of a Sylgard-coated chamber (2 ml volume) and the sheath surrounding them was cut using scissors and removed using fine forceps. All preparations were rested under constant superfusion with ASW for at least 2 h before the start of an experiment.

Electrophysiology

Sharp microelectrodes were pulled from omega-dot borosilicate glass (WPI, Sarasota, FL, USA) and filled with 2 mol l^{-1} potassium acetate. Their resistances were between 10 and 20 MΩ. Experiments were performed in current-clamp mode, and the voltage signals were recorded through Axoclamp 2B amplifiers (Axon Instruments). Computer

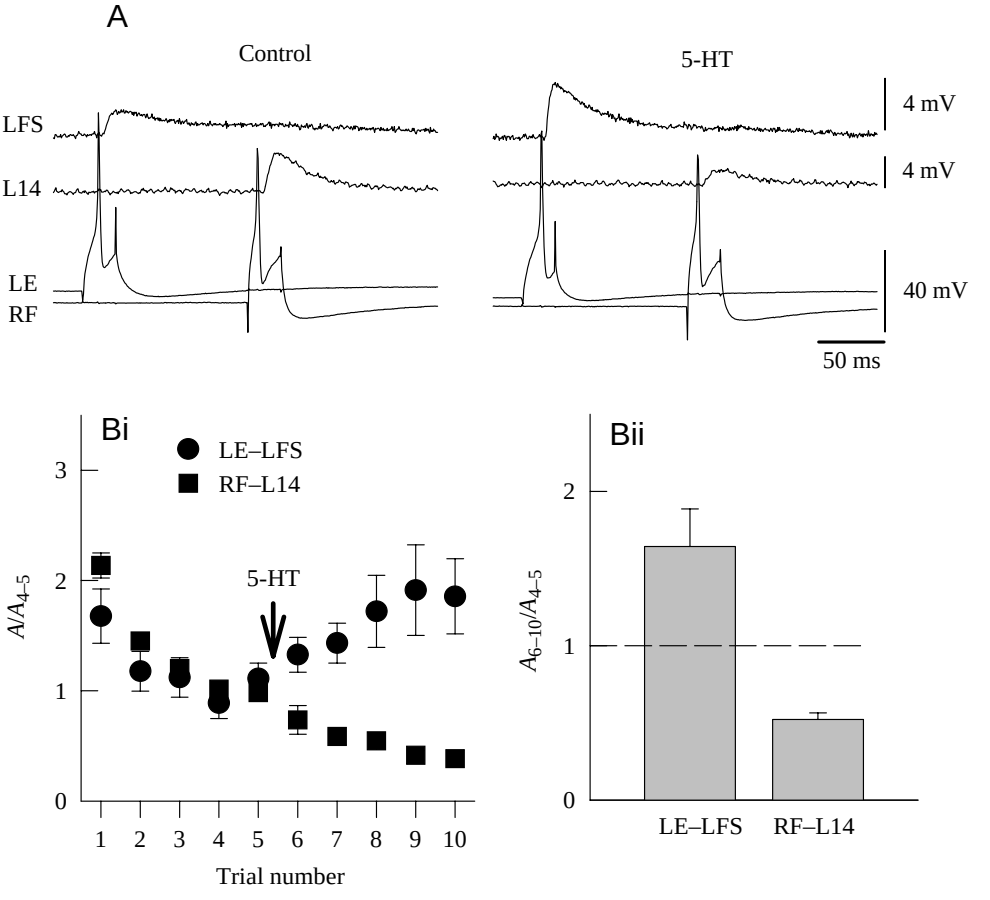

Fig. 1. Effects of serotonin (5-HT) on the RF–L14 and LE–LFS synaptic junctions. (A) Simultaneous recordings were obtained from two pairs of neurones, LE to LFS and RF to L14. While the LE–LFS connection is facilitated by 5-HT, the RF–L14 connection is not. The traces illustrated are the fifth (control) and the eighth (5-HT) trials. (B) A summary of the results from six separate experiments (four preparations). The EPSP amplitudes (A) in this graph and the following ones were normalized to the mean amplitudes of trials 4 and 5 ($A_{4–5}$). The interstimulus interval was 30 s; 5-HT (10 µmol l^{-1}) was added after trial 5 (at the arrow). (Bi) The time course of the effect of 5-HT. Values are means ± S.E.M. (Bii) The mean effect of 5-HT on LE–LFS and RF–L14 connections in the same experiments. The mean EPSP amplitude in the presence of 5-HT (trials 6–10) ($A_{6–10}$) was compared with the mean amplitude of the respective last two control or reference measurements (trials 4 and 5) ($A_{4–5}$).

acquisition and analysis of the data were performed using PClamp. A modified version of CLAMPEX (Dr M. V. Storozhuk) was also used to measure evoked excitatory postsynaptic potential (EPSP) amplitudes. Data are presented as means ± S.E.M. Student's *t*-test was used for statistical comparisons; all probabilities reported are two-tailed.

Drugs and solutions

Artificial sea water (ASW) was composed of (in mmol l^{-1}): NaCl, 460; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 55; and Hepes buffer, 10 (pH 7.6). The composition of the 2:1 ASW, which contained twice the normal concentration of Mg^{2+} and 1.25 times the normal concentration of $CaCl_2$ was (in mmol l^{-1}): NaCl, 368; KCl, 8; $CaCl_2$, 13.8; $MgCl_2$, 110; and Hepes buffer, 10 (pH 7.6) (Trudeau and Castellucci, 1992). Experiments with 5-HT (and corresponding controls) summarized in this report were performed in the modified ASW (2:1 ASW) to minimize possible indirect effects of 5-HT on the connections under study. Similar results, however, were observed in ASW containing normal concentrations of Mg^{2+} and Ca^{2+}.

Results

In pilot experiments, we rarely observed short (<5–7 ms) and constant-latency EPSPs in ink motor neurones (L14 neurones; Carew and Kandel, 1977a) when the presynaptic neurone was an LE cell; longer-latency (10–15 ms) EPSPs were usually observed. The latencies of these EPSPs were highly variable from trial to trial and they were abolished in bathing solution containing a high concentration of divalent cations (2:1 ASW; Trudeau and Castellucci, 1992), suggesting that these synaptic inputs are indirect. It was important to compare the action of 5-HT on RF and LE synapses in the same preparation to minimize the variation between animals, and we therefore used synaptic junctions with good convergence (more than 50 % of the sampled neurones had a connection), the RF–L14 and LE–LFS neuronal pairs.

Differential effects of 5-HT on the RF–L14 and LE–LFS synaptic junctions

In our experiments, the presynaptic neurones were stimulated intracellularly every 30 s and, after the fifth trial, a small volume of 5-HT solution was added to the chamber to obtain a final 5-HT concentration of 10 μmol l^{-1}. The results of the first series of experiments are summarized in Fig. 1. We found that the LE–LFS connections were facilitated in the presence of 5-HT, but there was no facilitation at the RF–L14 connections. On average, the amplitude of the EPSPs of LE neurones for trials 6–10 was increased by 64±24 % (P<0.05, N=6) compared with the control trials (mean of trials 4 and 5). In contrast, the amplitude of the EPSPs of the RF sensory neurones was decreased by 47.8±4.3 % (P<0.01, N=6).

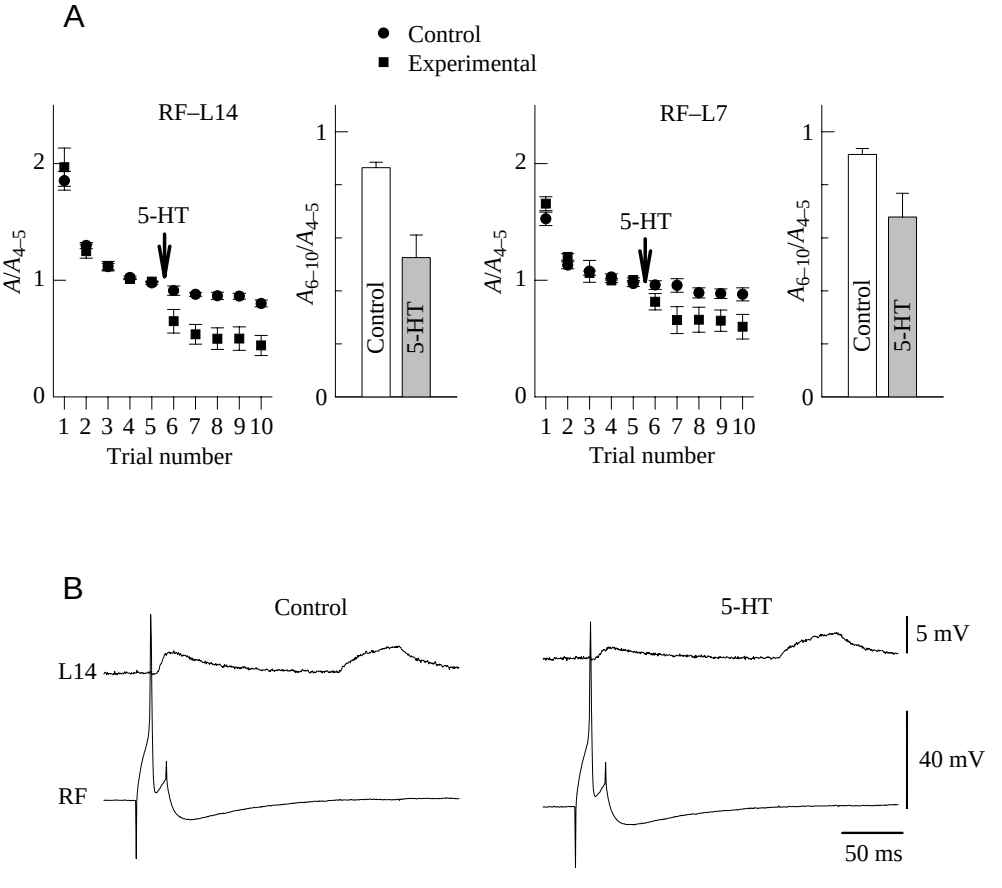

Fig. 2. The effects of serotonin (5-HT) on RF–L14 and RF–L7 connections. (A) Summary of the inhibitory effect on RF–L14 and RF–L7 connections. The EPSPs in two postsynaptic neurones were recorded simultaneously in each experiment. The interstimulus interval was 30 s; 5-HT (10 μmol l^{-1}) was added after trial 5 in the experimental group (at the arrow). The respective bar graphs represent the normalized mean EPSP amplitude (A) of trials 6–10 in the control (N=10) and experimental (N=5) groups compared with values in trials 4–5 ($A_{4–5}$). The values for the experimental groups were significantly different from their respective control groups (L14, P<0.001; L7, P<0.01). Values are means ± S.E.M. (B) The effect of 5-HT on RF–L14 connections is not due to a decreased input resistance in the postsynaptic neurone. Responses to RF stimulation in control or reference conditions (trial 5) and in the presence of 5-HT (trial 8) are shown. The input resistance in L14 was measured by an intracellular current pulse (50 ms) at the end of each sampling period.

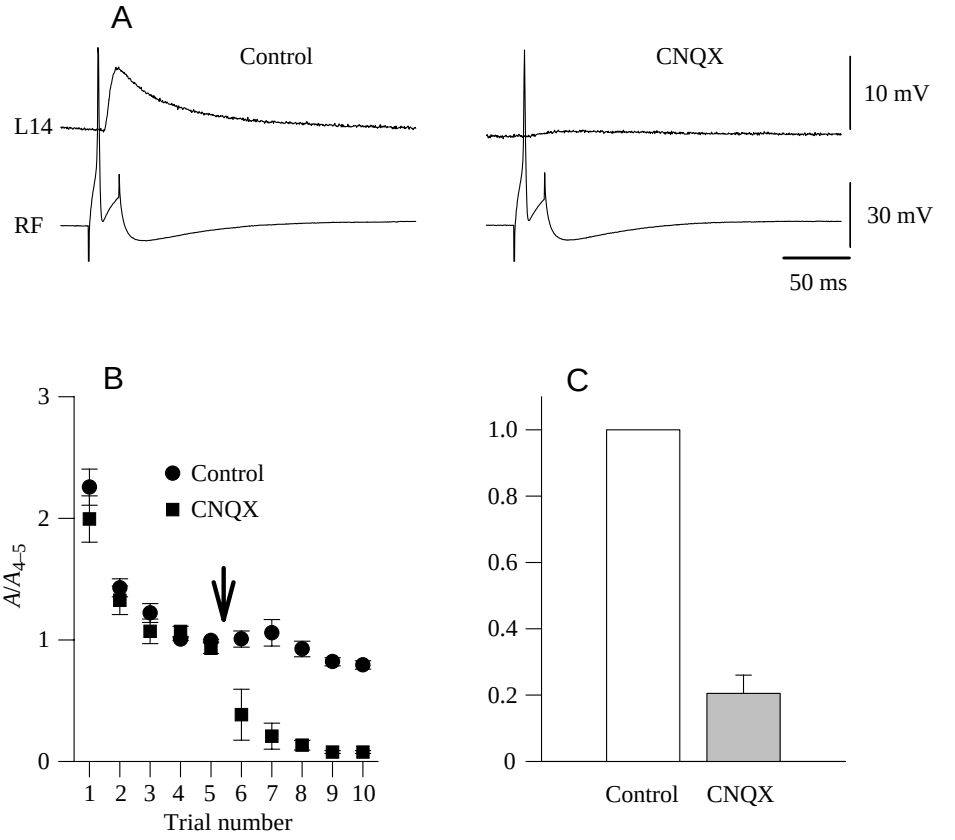

Fig. 3. CNQX blocks RF–L14 connections. (A) An example of the effect of CNQX (100 μmol l^{-1}) on an RF–L14 EPSP. Responses of trial 5 (control) and trial 8 (in the presence of CNQX) are shown. (B) Time course of the effect of CNQX: comparison of four control and three experimental preparations when CNQX was added after trial 5 (at the arrow). Values are means ± S.E.M. (C) Summary of the effect of CNQX. When normalized to the two trials preceding the application of CNQX, the values for the experimental group were 20.5±5.5 % of the corresponding values for the control group. The difference between the two groups was statistically significant (control, N=4; experimental, N=4; P<0.001). In one additional experiment, CNQX was added earlier than trial 5.

The effect of 5-HT on RF connections is inhibitory

To distinguish between an inhibitory effect or a simple depression of the EPSPs of the RF neurones, we performed a series of parallel control experiments in which no 5-HT was added. To test the generality of the observation, we also examined another RF follower neurone, the gill motor neurone L7. The results of this series of experiments are summarized in Fig. 2A. We found that the synaptic depression was normal in the control experiments (N=10) and that in presence of 5-HT there was no facilitation but an inhibition in addition to the synaptic depression. On average, the amplitude of the RF–L14 EPSPs (trials 6–10) was 52.6±8.5 % (N=5) compared with the control or reference trials (mean of trials 4 and 5). The mean amplitude of the RF–L7 EPSPs was 67.9±9 % (N=5). The corresponding control values obtained in the absence of 5-HT were 86.4±2.1 % for the RF–L14 connections and 91±5.4 % for the RF–L7 connections. Thus, both RF–L14 and RF–L7 connections were inhibited by 5-HT, although the effect tended to be less pronounced in L7. Compared with their respective control groups, the EPSP amplitudes in the two experimental groups were decreased by 39.1 % in L14 (P<0.001) and by 25.4 % in L7 (P<0.01). It is unlikely that the effect of 5-HT is due to shunting of the postsynaptic cell. As illustrated in Fig. 2B, no change in input membrane resistance was observed in neurone L14 (N=4).

The RF–L14 connections are blocked by CNQX

Previous studies have suggested that the transmitter of the LE and VC connections is an excitatory amino acid. It has also been shown that these synaptic connections were blocked by antagonists of non-NMDA receptors such as CNQX or DNQX (Trudeau and Castellucci, 1993b; Dale and Kandel, 1993). The unusual effect of 5-HT at the RF connections led us to examine whether these synaptic junctions were differently affected by antagonists such as CNQX. We found that the RF–L14 synaptic connections were not unusual in this regard: they were effectively blocked by CNQX (100 μmol l^{-1}). One example of such an experiment is illustrated in Fig. 3A. In these experiments, CNQX was added after the fifth trial (N=3) or after the third trial (N=1). The time course of the effect is shown in Fig. 3B (control, N=4; experimental, N=3). A summary of the results of the four experiments is shown in Fig. 3C; when normalized to the two trials preceding the application of CNQX, the values for the experimental group were 20.5±5.5 % of the corresponding values for the control group. The difference between the two groups was statistically significant (P<0.001). These results are similar to those obtained for the LE connections by Trudeau and Castellucci (1993).

Discussion

Differential properties of RF sensory neurones

Previous studies have shown that inking and gill withdrawal behaviour in *A. californica* are rather different from each other in several aspects. Inking is a high-threshold behaviour with a

steep input/output relationship approaching an all-or-none response; gill withdrawal is a low-threshold behaviour with a graded input/output relationship, and it is graded as a function of the stimulus intensity (Carew and Kandel, 1977a; Shapiro et al., 1979). These differences are at least partly due to the high threshold and electrical coupling of L14 neurones (Carew and Kandel, 1977a–c). The absence of inking sensitization to strong repeated neck stimuli (Carew and Kandel, 1977a) may indicate other differences between the inking and withdrawal circuits. In this regard, the RF neurones may contribute to these differences. In the present study, we observed a differential effect of 5-HT on the EPSPs evoked by the LE and the RF sensory neurones: while LE–LFS connections were facilitated by 5-HT, the RF–L14 connections were not (Figs 1, 2). Although our results may seem to be unusual for the sensory neurones of the gill and the siphon reflex, they are not unique. It has been shown previously (Rosen et al., 1989), that 5-HT inhibits some sensory neurone connections to neurones in the cerebral ganglion. Our experiments extend this observation for the RF cluster of the abdominal ganglion. We think that modulation of RF connections by 5-HT is likely to occur in simple learning protocols such as sensitization or classical conditioning. This view is supported first by the fact that many serotoninergic processes are found in the neuropile of the abdominal ganglion and, second, that serotoninergic neurones send their processes to the abdominal ganglion and that they are activated by tail shocks (Glanzman et al., 1989; Hawkins, 1989; Longley and Longley, 1986; Mackey et al., 1989) or connective stimulation.

Mechanism of the inhibitory effect of 5-HT on RF connections

It is unlikely that shunting contributes to the inhibitory effect of 5-HT on RF connections. For example, the 5-HT effect is not accompanied by any change in input resistance in cell L14 (Fig. 2B). However, we cannot exclude the possibility that local shunting occurs in the neuropile region. Since the mechanoreceptor properties and the receptive field types of the RF neurones do not differ from those of the other cluster cells (Dubuc and Castellucci, 1991), we wanted to determine whether any differences other than the modulation by 5-HT existed. One obvious test was to verify whether the RF connections were blocked by the same AMPA/kainate receptor antagonist, CNQX, that is effective at the LE or VC connections (Trudeau and Castellucci, 1993; Dale and Kandel, 1993). We found that the RF connections are blocked by CNQX (Fig. 3), suggesting that, in this respect, RF–L14 connections are similar to the connections made by the sensory neurones from the other clusters. A similar conclusion (Gapon and Kupfermann, 1996) was drawn for the sensory connections in the cerebral ganglion, which are also inhibited by 5-HT (Rosen et al., 1989). It has been shown that, in the presence of 5-HT, there is an increase in cyclic AMP levels in the sensory neurones (Bernier et al., 1982). Thus, there is a possibility that a different type of 5-HT receptor is present on the RF neurones which could be associated with a decrease in cyclic AMP level; such a receptor has recently been cloned from the central nervous system of *A. californica* (Angers et al., 1998). It is not yet known whether it is present on the RF neurones.

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