

CLASSICAL CONDITIONING ALTERS THE EFFICACY OF IDENTIFIED GILL MOTOR NEURONES IN PRODUCING GILL WITHDRAWAL MOVEMENTS IN *APLYSIA*

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Summary

In a semi-intact preparation of *Aplysia californica* Cooper, classical conditioning training leads to changes in the synaptic strength at the sensory–motor neurone synapse. However, these changes are neither necessary nor sufficient to bring about the observed behavioural changes of the gill withdrawal reflex. We therefore tested whether the ability of a gill motor neurone to elicit a gill withdrawal response was altered following classical conditioning training of the reflex. We found that following classical conditioning training, the ability of a gill motor neurone to elicit a gill withdrawal response was significantly potentiated. In addition, in control preparations which did not receive classical conditioning training, the ability of a gill motor neurone to elicit a gill response was decreased. Thus, associative learning of this reflex appears to involve alteration in neuronal activity at loci distal to the sensory–motor neurone synapse.

Introduction

In an attempt to ascertain the underlying neuronal basis of learning and memory in man, many investigators have turned to the relatively simple nervous systems of invertebrates. From these invertebrate model systems valuable information has been obtained about the neuronal basis of learning (Carew & Sahley, 1986; Mpitsos & Lukowiak, 1986; Byrne, 1987; Farley & Alkon, 1987). By utilizing these systems, it has been possible to localize learning-induced changes in individual identifiable neurones.

One of the most extensively studied of these model systems is the marine mollusc *Aplysia californica*. Investigators have made use of the well-described neuronal circuitry that mediates the defensive gill withdrawal reflex (GWR) (Kupfermann *et al.* 1974; Leonard & Lukowiak, 1987; Leonard *et al.* 1988). The GWR undergoes non-associative learning (habituation, dishabituation and sensitization) (Pinsker *et al.* 1970, 1973); the well-described changes in neuronal activity which accompany these behavioural changes may in part mediate these adaptive behaviours (Kandel & Schwartz, 1982). Thus, it is not too surprising that this

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reflex has been used in studies of associative learning. Initial experiments utilized the intact animal (Carew *et al.* 1981, 1983); paired presentations of a conditional stimulus (CS) (tactile stimulus to the siphon or mantle area) and an unconditional stimulus (UCS) (tail-shock) led to conditioning of both the siphon and gill withdrawal reflexes. The enhanced response to the CS resulting from pairing of the CS–UCS was larger and persisted longer than the sensitized response elicited by unpaired presentations of the stimuli or the presentation of the CS alone. It is not clear whether this is a true example of classical conditioning or of α -conditioning but there is little doubt that it is associative learning. This associative learning paradigm was then applied to the isolated ganglia preparation to obtain a neural correlate of associative learning. Paired presentation of the CS (an action potential, AP, in an abdominal ganglion sensory neurone) with the UCS (electrical stimulation of the pedal nerve which innervates the tail) resulted in an alteration of the synaptic efficacy between the siphon sensory neurones and the follower motor neurone (either a siphon motor neurone or L₇, a gill motor neurone) (Hawkins *et al.* 1983). Again, paired presentation of the CS–UCS led to a larger and more persistent increase in the EPSP amplitude than the sensitized response elicited by unpaired presentation of the stimuli. A specific mechanism, activity-dependent neuromodulation, was proposed to account for these and similar changes seen in tail sensory neurones (Walters & Byrne, 1983). Similar changes in synaptic efficacy have now been demonstrated in tissue culture preparations, consisting of sensory and motor neurones, with the application of exogenous serotonin as the UCS (Rayport & Schacher, 1986). The correlation between the changes in synaptic efficacy observed in the isolated ganglia and in the dissociated cell cultures fits well with the observed behavioural changes. However, these changes are only correlative: it has not yet been demonstrated that these changes in synaptic efficacy are either sufficient or necessary for the behavioural changes.

In vitro semi-intact preparations, which provide a means of simultaneously monitoring cellular and behavioural responses during learning, are capable of undergoing associative learning (Lukowiak & Sahley, 1981; Lukowiak, 1986). Furthermore, Lukowiak (1986) showed that changes in synaptic efficacy at the sensory–motor neurone synapse did occur during the course of learning but that these changes only occurred over the first 10 trials, after which there appeared to be no further increase in synaptic efficacy as judged by the number of action potentials evoked in the motor neurone by the CS, even though the GWR continued to increase in size. However, this preparation did not approximate either the intact animal used in the study of Carew *et al.* (1983) or the isolated ganglia preparations of Hawkins *et al.* (1983), and the results from these two groups cannot therefore be directly compared.

Colebrook (1986) and Colebrook & Lukowiak (1988), however, attempted to mimic, as far as possible, the whole-animal preparation of Carew *et al.* (1983). This preparation was also similar to the Hawkins *et al.* (1983) isolated ganglia preparation, the only substantial difference being that the siphon, mantle and gill

were left intact. In this preparation, therefore, changes in neuronal activity could be measured simultaneously with changes in gill reflex behaviour. It was found (Colebrook, 1986; Colebrook & Lukowiak, 1988) that the changes in synaptic efficacy between the siphon sensory and gill motor neurones were neither necessary nor sufficient for changes in gill behaviour. Thus changes must be occurring at other sites within the nervous system.

We therefore tested the hypothesis that classical conditioning training would lead to changes in the ability of identified gill motor neurones to elicit a gill withdrawal. We report here that following classical conditioning training, the ability of a gill motor neurone to elicit a gill withdrawal is potentiated. However, when the stimuli are presented in an explicitly unpaired manner, the ability of the motor neurone to elicit a gill response is decreased. These data indicate that important changes in the central nervous system which underlie learning take place distal to the sensory-motor neurone synapse. The results presented here have appeared in abstract form (Lukowiak & Colebrook, 1987).

Materials and methods

Preparation

Aplysia californica obtained from Sea Life Supply weighing between 100 and 200 g and maintained in a 1200-l aquarium (Instant Ocean) at 15–16°C and pH 7.9 were used. Animals were fed weekly with dried red seaweed. Food-satiated *Aplysia*, which show suppressed gill behaviour (Lukowiak, 1980; Leonard & Lukowiak, 1986), were not used for these experiments. The semi-intact preparation consisted of the siphon, mantle, gill and abdominal ganglion. This preparation has been referred to as the 'Lukowiak' preparation to differentiate it from the 'Colebrook' preparation which also included the head ganglia and tail (see Colebrook & Lukowiak, 1988, fig. 1). The siphon, branchial and ctenidial nerves which innervate the mantle organs were left intact. All other nerves and connectives were severed. The preparation was pinned dorsal side down to a clear Sylgard (Dow Corning) coated base of a Lucite chamber. The abdominal ganglion was further pinned out on a clear Sylgard platform.

The ganglion was bathed in a hypertonic sucrose/seawater solution [2 mol l^{-1} sucrose diluted 1:1 with artificial sea water (ASW)] for 15 min prior to removal of the connective sheath. This facilitates desheathing of the ganglion by causing a slight shrinkage of cells away from the sheath. The flap of sheath covering the left dorsal surface of the ganglion was removed with fine scissors. Following desheathing, the sucrose was removed and the ganglion washed 4–5 times in ASW. Gill movements were monitored by attaching a 5.0 gauge suture to a single gill pinnule and to a tension transducer (Narco F60). The tension was adjusted so as not to stretch the pinnule. The gill was not damaged by this procedure and the amplitude of the spontaneous gill movements remained relatively constant throughout the experiment. The output of the transducer was displayed on analogue (Tektronics 5113) and digital (Nicolet 2090-3) storage oscilloscopes, as

well as on a Gould Polygraph. Following dissection, a rest of at least 1 h was allowed before neurones were impaled.

Central gill motor neurones

The abdominal ganglion was transilluminated to aid neurone impalement. Single-barrel micropipettes (filled with 3 mol l^{-1} KCl) with a resistance of 10–20 M Ω were used. A Dagan 2700 cell explorer electrometer containing a 'bridge-circuit' allowed simultaneous recording and current injection through the electrode. Identification of the motor neurones, L₇, LDG₁ and LDG₂, was made both by correlation of the synaptic activity with spontaneous gill movements and more importantly by the type of gill movements they caused when depolarized. The gill movements have been adequately described by Kupfermann *et al.* (1974) and Leonard & Lukowiak (1987).

Conditioning stimuli

The conditional stimulus (CS) consisted of a 'tapper' stimulus (Peretz & Lukowiak, 1975) of 600 mg intensity and 40 ms duration applied to the siphon. The CS normally evoked an AP in the motor neurone and sometimes a small gill movement. This is the same CS that was used in the Lukowiak (1986) and Colebrook & Lukowiak (1988) studies. The unconditional stimulus (UCS) was a stronger train of tactile stimuli delivered by a second tapper to the gill. The UCS evoked a large gill withdrawal reflex and many APs in the gill motor neurone.

Experimental protocol

For the classical conditioning training (CC) protocol, the UCS was specifically paired to the CS and delivered 500 ms after it. The intertrial interval was 2 min and each animal received at least 10 training trials. In some preparations, 10 more conditioning trials were delivered 1 h after the first series. Control preparations received specifically unpaired presentations of the CS and UCS; the UCS was presented 1 min after the CS.

Motor neurone depolarization

The ability of an identified gill motor neurone to elicit a gill withdrawal response was tested at an intertrial interval of 20 min. The motor neurone was depolarized for 2 s and this elicited 10–25 APs in it, causing a gill withdrawal. Two control depolarizations were given and the experiment was only continued if the amplitudes of the contractions were within 10% of each other and if the same number of APs was elicited in each trial. Following the second control depolarization, the preparations received either 10 paired presentations of the CS–UCS (CC group) or the explicitly unpaired presentation of the CS–UCS (control group). Two minutes after the last tactile stimuli had been presented, the gill motor neurone was again depolarized. Data were only used if approximately the same number of APs was evoked (a difference of 1–2 action potentials was acceptable). Some preparations received explicitly unpaired stimuli after the

second control depolarization and the motor neurone was then tested. These same preparations were then given a 1-h rest, after which they received paired presentations of the CS–UCS before the motor neurone was depolarized again.

Statistical procedure

Differences between the ability of a gill motor neurone to elicit a gill withdrawal response following classical conditioning training and that following specifically unpaired presentations of the CS–UCS were determined using a paired *t*-test. Two sets of data were assumed to be different at $P < 0.05$.

Results

Twenty-three animals were used: 10 received CC training; seven received explicitly unpaired CS–UCS; three received explicitly unpaired stimuli, then received CC training; and three received CC training (20 trials instead of 10). Fig. 1 shows data taken from individual preparations which received explicitly unpaired stimuli (the control group). In these preparations the ability of a gill motor neurone to elicit a gill withdrawal response was depressed following the presentation of the 10 explicitly unpaired stimuli. There did not appear to be any differential effect on the specific type of motor neurone. When the data from all the preparations tested in this manner are grouped together (Fig. 2), it is apparent that the presentation of unpaired stimuli leads to a significant decrease (approximately 40%) in the ability of the gill motor neurone to elicit a gill withdrawal response ($P < 0.01$).

The situation was quite different in the CC group. In these preparations, the ability of a gill motor neurone to elicit a gill withdrawal response was greatly facilitated. Representative data obtained from individual preparations are shown (Figs 3, 4, 5) and the group data are presented in Fig. 6. This paired presentation of the stimuli augmented the ability of LDG₂ to elicit a gill withdrawal response. Similar data were obtained with motor neurones L₇ and LDG₁.

In some preparations, the elicited withdrawal following 10 paired stimuli was not greatly increased; in these preparations ($N = 3$) a 1-h rest was interposed, then the preparation received 10 more CC training trials (Fig. 4). CC training between T₁ and T₂ facilitated the gill response by about 40%; however, following 10 more CC trials, the response was facilitated by approximately 320%. Moreover, the increased ability of LDG₁ to produce a gill withdrawal response persisted for at least 1 h after CC training (T₄). Finally, after another 1-h rest (T₅) the response was similar to the control (T₁).

We also determined if, in the same preparation, we could see the effects of giving unpaired and paired presentations of stimuli (Fig. 5). Following two control trials, the preparation received 10 unpaired presentations of the stimuli. When LDG₁ was then depolarized (T₃), it produced a smaller response. The preparation was given a 1-h rest and then it received 10 paired presentations of the CS–UCS.

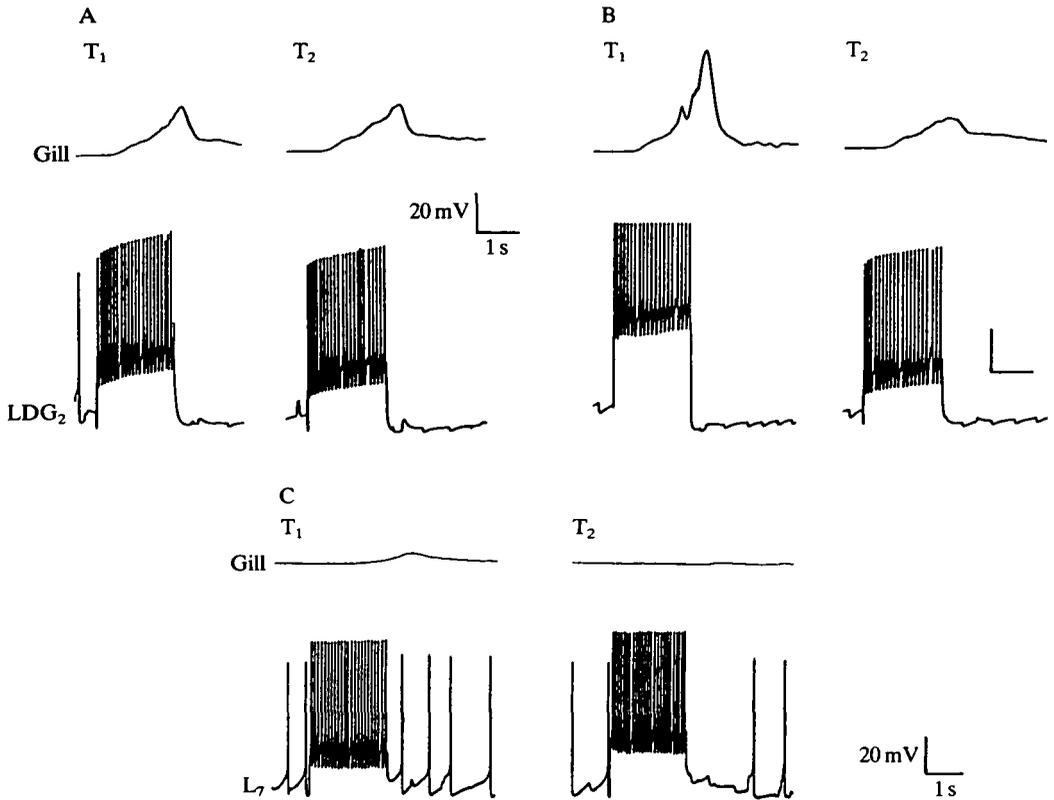


Fig. 1. Unpaired CS and UCS leads to a decrease in the ability of a gill motor neurone to elicit a gill withdrawal response. (A) Gill motor neurone LDG₂ was depolarized to produce 22 APs for 2 s at an interstimulus interval of 20 min; only the second control stimulus (T₁) is shown. Following T₁, the preparation received 10 explicitly unpaired CS-UCS presentations. Following the tenth trial LDG₂ was again depolarized to produce 22 APs. (B) In another preparation, LDG₂ was again tested as in A. In this preparation 20 APs were elicited on T₁ and 21 on T₂. Even though one additional AP was elicited by the depolarizing current on T₂, a much smaller gill withdrawal response was elicited following the unpaired CS-UCS stimuli. (C) Gill motor neurone L₇ in a third preparation was tested as above. Again following the unpaired stimuli, the ability of L₇ to elicit a gill response was reduced. In this preparation 23 APs were elicited on T₁ and 25 on T₂.

When LDG₁ was then depolarized again (T₄), it produced a facilitated response. Data similar to these were obtained in the other two preparations tested in this manner.

From the combined data from the 10 experiments in which the preparations received 10 paired CS-UCS trials (Fig. 6), it is apparent that classical conditioning training had a significant potentiating effect on the ability of the gill motor neurone to elicit a gill withdrawal response ($P < 0.01$).

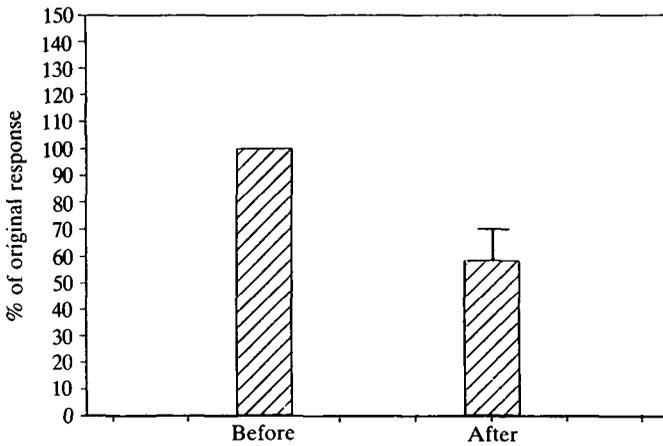


Fig. 2. Grouped data ($N=10$) showing the effect of the presentation of the CS and UCS in an explicitly unpaired paradigm on the ability of a gill motor neurone to elicit a gill withdrawal. On average, the ability of the motor neurone to elicit a gill response was decreased by 40% following 10 control trials. This is a significant decrease ($P < 0.01$). Vertical bar shows 1 S.E.M.

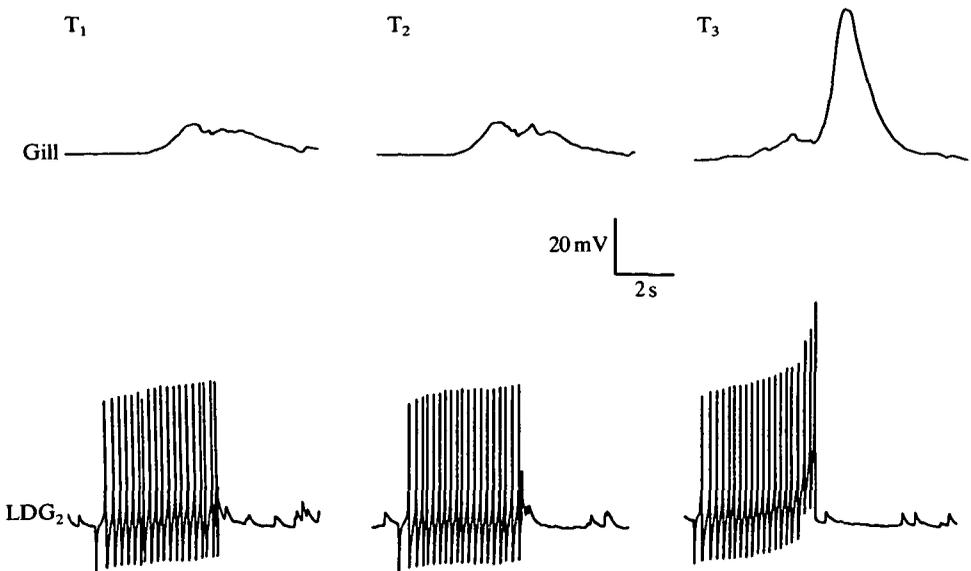


Fig. 3. Paired presentation of the CS and UCS augments the ability of a gill motor neurone to elicit a gill withdrawal response. The two 20-min control depolarizations are shown (T_1 and T_2). Twenty APs were elicited on T_1 and 19 APs on T_2 and the gill contractions were of similar amplitude. Following T_2 the preparation received 10 paired presentations of the CS–UCS which led to classical conditioning of the GWR. When LDG_2 was again depolarized to produce 20 APs (T_3) a much larger gill withdrawal response was seen.

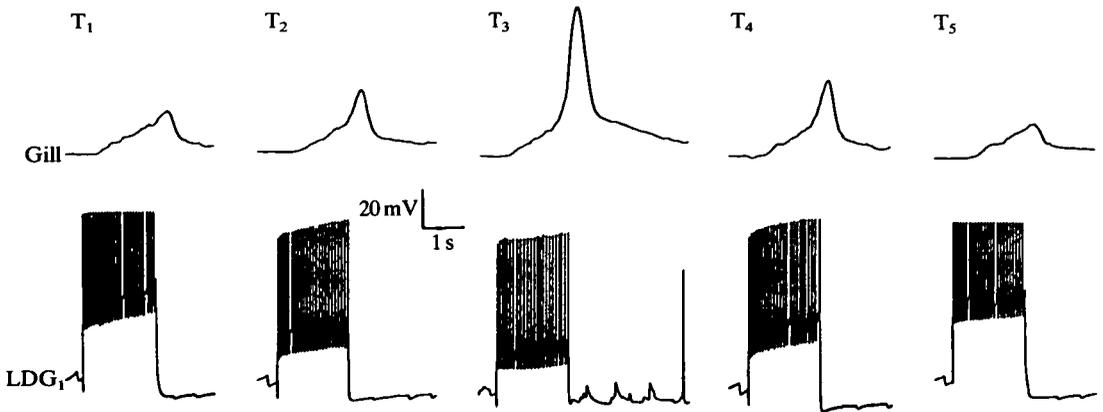


Fig. 4. Increased classical conditioning training leads to an increased ability of a gill motor neurone to elicit a gill withdrawal response. The second control is shown (T₁) in which LDG₁ was depolarized to produce 25 APs for 2 s. The preparation then received 10 paired CS–UCS trials and when the neurone was again depolarized to produce 25 APs (T₂) there was a slight (40%) increase in amplitude. The preparation was given a 1-h rest and then it received 10 more classical conditioning trials. Following this second training interval, when the motor neurone was depolarized (T₃) to produce 23 APs, a much larger response was seen. The preparation was then rested for another hour and, when the neurone was depolarized (T₄) to produce 24 APs, a somewhat reduced response compared with T₃ but larger than the control (T₁) was produced. Finally, after a further 1-h rest (T₅) depolarization of the neurone to produce 24 APs elicited a gill withdrawal response similar to the control (T₁).

Discussion

The results presented here show that classical conditioning training in *Aplysia* enhances the ability of gill motor neurones to elicit a gill withdrawal response. In addition, we have also found that the ability of the gill motor neurones to elicit a gill movement is suppressed by the presentation of the explicitly unpaired stimulus. Therefore, alterations in neuronal efficacy occur at multiple sites within the nervous system of *Aplysia* to mediate adaptive behaviour. Such changes include, but are not limited to, alterations in the sensory input to the motor neurones; necessary and important changes occur distal to the sensory–motor neurone synapse.

Earlier investigations which delved into the neuronal mechanisms that underlie associative learning in *Aplysia* have, for the most part, concentrated on changes in synaptic efficacy that occur at the sensory–motor synapse. Those studies by Kandel's group are important and seminal (e.g. Kandel *et al.* 1983); for along with the results obtained by Alkon's group using *Hermissenda* (see Farley & Alkon, 1987), they have shown that it is possible to look at changes in neuronal efficacy, and their underlying biochemical and molecular mechanisms, as a means of beginning to explain associative learning.

Based on the work of Carew *et al.* (1983) in the intact animal and that of

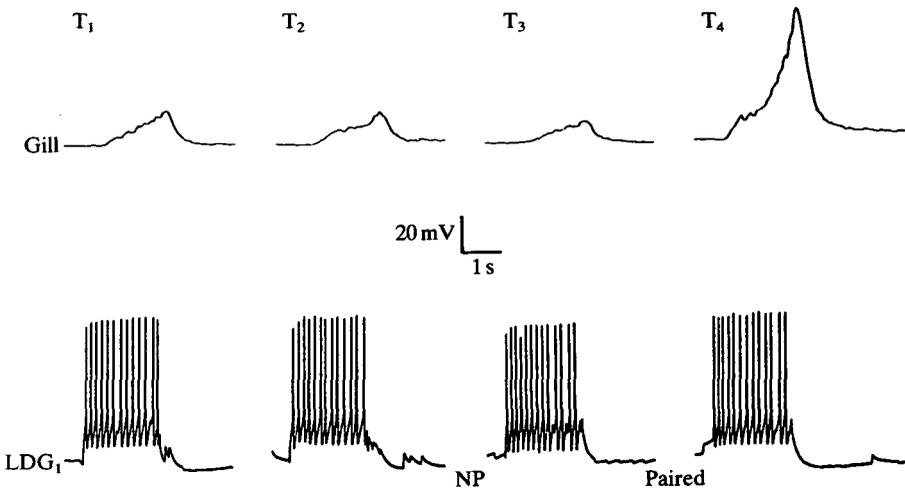


Fig. 5. The effect of unpaired and paired stimuli on the ability of a gill motor neurone to elicit a gill withdrawal movement. In two control depolarizations (T_1 and T_2), LDG_1 was depolarized to produce 13 APs; the amplitudes of the elicited gill withdrawal responses were similar. Following T_2 , the preparation received the explicitly unpaired presentations (NP) of the CS and UCS. Depolarization (T_3) of the neurone to produce 13 APs resulted in a smaller gill withdrawal response. Following a 1-h rest, the preparation received 10 paired presentations of the CS-UCS (Paired). Now when LDG_1 was depolarized to produce 13 APs, it elicited a much larger gill withdrawal response.

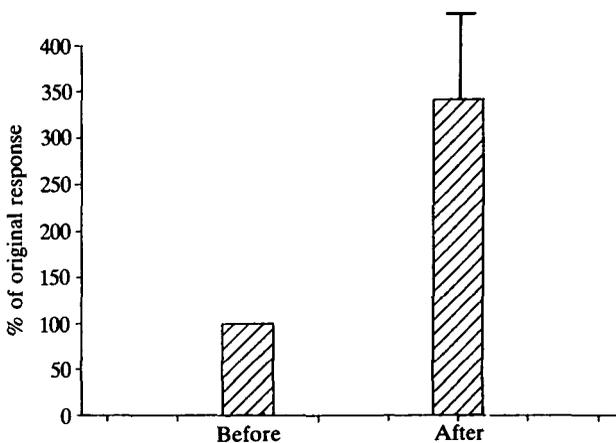


Fig. 6. Grouped data ($N = 10$) showing the effect of classical conditioning training on the ability of a gill motor neurone to elicit a gill withdrawal response. 100% was the amplitude of the second 20-min control depolarization. There was a significant increase ($P < 0.01$) in the gill withdrawal response. Vertical bar shows 1 s.e.m.

Hawkins *et al.* (1983) in an isolated ganglia preparation, it seems obvious that the changes observed in synaptic efficacy at the sensory–motor neurone synapse, which are so similar to the observed behavioural changes in the intact animal, are responsible in large measure for the mediation of associative learning. However, that conclusion is based only on correlational inferences. Simultaneous measurements of alterations in synaptic efficacy and behaviour of the siphon and gill responses were not made.

Lukowiak (1986), using a semi-intact preparation, found that there were changes in synaptic efficacy at the sensory–motor neurone synapse which occurred early on during the course of associative learning, but that there were no further increases in synaptic efficacy (as judged by the number of APs evoked in the motor neurone beyond trial 10–15), even though the GWR amplitude continued to increase with training. Thus, sites in the nervous system other than the sensory–motor neurone synapse must be involved.

Colebrook & Lukowiak (1988), using a preparation similar to that of the Hawkins *et al.* (1983) study, but with the siphon, mantle and gill left intact, found that changes in synaptic efficacy at the sensory–motor neurone synapse were neither necessary nor sufficient to account for the alterations in gill reflex behaviour. Changes in synaptic efficacy, like those shown in the Hawkins *et al.* (1983) study, certainly did occur, but these changes by themselves could not explain the learned behaviour.

Since changes in synaptic efficacy at the sensory–motor neurone synapse are not by themselves sufficient to explain the increased GWR brought about by classical conditioning training, changes must be occurring at other loci in the nervous system. One such locus could be the motor neurone itself or its peripheral terminations in the gill. This was the hypothesis tested in the present series of experiments. It is already known that the peripheral terminations of the central gill motor neurones are sites of adaptive change. Lukowiak & Peretz (1977) showed that repeated activation of L_7 and LDG_1 led to alterations in their ability to cause a gill movement and that there were differences in the peripheral terminations of these neurones, both with respect to each other and with respect to the nerve by which they innervated the gill.

However, there appears to be a discrepancy between those data and the data shown here. In the earlier study, it was shown that, in at least one case, the ability of L_7 to elicit a gill withdrawal response was facilitated following the presentation of 10 tactile stimuli to the siphon (1 g, interstimulus interval 45 s), which led to habituation of the GWR. In the present study we showed that the ability of L_7 to produce a gill response decreased when the stimuli were unpaired. This discrepancy may be due to the different types of stimulus used. In the Lukowiak & Peretz (1977) study, an interval of only 8 min occurred between the L_7 depolarizations, whereas in the present study there was a 22-min interval. Finally, in the present study, unpaired stimuli were given in an explicitly unpaired manner; whereas, in the 1977 study, only single punctate stimuli were given. It was sometimes our experience that the strong UCS stimuli, although producing an initial period of

sensitization (see Lukowiak, 1986), often led to prolonged suppression of the reflex at later times. This is similar to some of the recent studies, which show that in many cases there is profound suppression of the reflex following sensitization training (Rankin & Carew, 1987; Marcus *et al.* 1987). In any case, the present results show that even in the same preparations, presentation of unpaired stimuli leads to a suppression of the ability of the motor neurone to elicit a response, whereas presentation of paired stimuli leads to an increase.

Lukowiak (1979a) also showed that induced activity in a modulatory motor neurone, L₉, could selectively lead to a potentiation of the ability of L₇ to elicit a gill withdrawal response and the ability of the reflex to undergo habituation (Lukowiak, 1979b). During L₉ activation, the reflex did not habituate; thus stimuli that might induce L₉ (i.e. classical conditioning training) to become more active would have a facilitatory effect, both on the ability of L₇ to elicit a gill contraction and on the ability of a CS to elicit a gill movement. The mechanism by which L₉ potentiates the ability of L₇ to elicit a gill response is not known, although dopamine release in the gill has been implicated (Ruben & Lukowiak, 1983). In addition to the effect produced by dopamine perfusion of the gill, two other endogenous neurotransmitter substances affect gill behaviour: SCP_B and FMRFamide. The perfusion of SCP_B through the gill brings about suppression of the GWR and increases the rate of gill reflex habituation (Cawthorpe *et al.* 1985) and FMRFamide perfusion leads to facilitation of the reflex and prevents its habituation (Cawthorpe *et al.* 1988). We suggest here that classical conditioning training could bring about its effect on motor neurone efficacy by activating such peripheral systems.

At present we do not know much about the interactions between the central (CNS) and peripheral (PNS) nervous systems in the mediation of gill reflex behaviour, other than that interactions do occur (Lukowiak & Peretz, 1977). It is possible that, in addition to making direct synaptic connections to gill muscle, the central gill motor neurones (e.g. L₇) make synaptic connections to peripheral motor neurones. For example, Kurokawa & Kuwasawa (1985) showed that L₇ makes synaptic connections with gill ganglion (a part of the PNS) neurones in a related species of *Aplysia*. Moreover, Colebrook & Lukowiak (1987) have shown that motor neurones in the gill ganglion receive synaptic inputs from neurones in the abdominal ganglion as well as receiving synaptic input from stimulation of the UCS pathway (pedal nerve stimulation) in the Colebrook preparation. In addition to the motor neurones in the gill ganglion, there are motor neurones in the gill itself, some of which are in close apposition to FMRFamide-containing neurones (Weiss *et al.* 1984). It is possible that CC training increases the synaptic efficacy between the central motor neurone and the peripheral motor neurones, thus bringing more of these PNS motor neurones into play. This could be accomplished by the release in the gill of facilitating substances such as dopamine (Ruben & Lukowiak, 1983) or FMRFamide (Weiss *et al.* 1984; Cawthorpe *et al.* 1988). It is also possible that there are alterations in excitation-contraction coupling in gill muscle. Thus changes could be occurring simultaneously at different sites within

the periphery. Obviously, many more experiments need to be performed to unravel these possibilities.

The mediation of associative learning in even a 'simple' model system is relatively complicated; it involves changes in neuronal activity at multiple sites within the nervous system. In the mediation of associative learning in *Aplysia*, it is probable that no single site is absolutely essential, and no one site paramount, but rather that neuronal changes underlying this form of learning are distributed throughout the nervous system, including the interface between the CNS and PNS. This statement is not meant to suggest that a detailed analysis of the mechanisms that underlie the changes in synaptic efficacy at the sensory-motor neurone synapse are not important but, rather, that important changes are occurring throughout the integrated system. Thus, to obtain an understanding of how the nervous system mediates associative learning, account must be taken of the multiple sites in the nervous system which mediate the learning.

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