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LEARNING BY THE *APLYSIA* MODEL SYSTEM: LACK OF CORRELATION BETWEEN GILL AND GILL MOTOR NEURONE RESPONSES

BY ELAINE COLEBROOK AND KEN LUKOWIAK

Department of Medical Physiology, The University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1

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SUMMARY

A semi-intact preparation of *Aplysia californica* was used to monitor simultaneously behavioural and motor neurone responses during classical conditioning of the gill withdrawal reflex. Gill motor neurone responses and gill withdrawal responses were both capable of enhancement in response to the conditioned stimulus after associative training. The neuronal and behavioural responses did not, however, correlate. In 32% of the conditioned (paired) preparations and 27% of the control (unpaired) preparations, the neuronal response was facilitated whereas the gill withdrawal response did not change, or decreased. In addition, amongst those preparations that showed behavioural enhancement, the acquisition of learning of gill withdrawal followed a different pattern from that displayed by the central neurones. This suggests that facilitation of the central sensory–motor neurone synapses is not primarily responsible for conditioning of the gill withdrawal reflex. The gill withdrawal response elicited by direct depolarization of the central motor neurones decreased following the unpaired (control) presentations of the conditioned and unconditioned stimuli, and remained unchanged following paired presentations, suggesting that there is a site of neuronal plasticity in the gill.

INTRODUCTION

The easily accessible 'simple' nervous systems of invertebrates have proved valuable for exploring the cellular basis of learning. Research involving these model systems has provided the means to localize learning-induced changes to individual neurones. Many invertebrates are capable of associative learning; these include the locust (Hoyle, 1979) and the fruit fly (Quinn, Harris & Benzer, 1974), as well as the molluscs, *Pleurobranchaea* (Mpitsos & Collins, 1975), *Limax* (Gelperin, 1975), *Hermissenda* (Crow & Alkon, 1978) and *Aplysia* (Lukowiak & Sahley, 1981; Carew, Walters & Kandel, 1981; Walters & Byrne, 1983; Cook & Carew, 1986).

One of the most detailed hypotheses for the cellular and molecular basis of learning has emerged from research on the opisthobranch mollusc *Aplysia californica* (see Hawkins, 1984). The defensive withdrawal reflex of the mantle organs (the gill and

Key words: *Aplysia*, learning, gill, semi-intact preparation.

siphon) of *Aplysia* habituates if weak stimuli are repeatedly presented to the siphon (Pinsker, Kupfermann, Castellucci & Kandel, 1970). This same reflex becomes sensitized following one or several shocks to the neck or tail (Pinsker *et al.* 1970; Pinsker, Hening, Carew & Kandel, 1973; Frost, Castellucci, Hawkins & Kandel, 1985). Both the siphon withdrawal reflex (SWR) and gill withdrawal reflex (GWR) can be classically conditioned by paired presentations of a conditioned stimulus (CS) (a tap to the siphon) and an unconditioned stimulus (UCS) (tail shock). The enhanced response resulting from paired presentation of the CS and UCS is larger and lasts longer than the sensitized response caused by unpaired or UCS-only training (Carew *et al.* 1981; Carew, Hawkins & Kandel, 1983).

A significant advantage of studying this defensive withdrawal reflex is that its neural circuitry has been characterized in some detail (Kupfermann, Carew & Kandel, 1974). The siphon and gill are each innervated by the central and peripheral nervous systems and the central innervation is derived entirely from neurones in the abdominal ganglion. The GWR and SWR are closely interconnected as they share the same mechanoreceptors and interneurones (Hawkins, Castellucci & Kandel, 1981). A few central motor neurones are known to innervate the gill and, of these, L7 and LDG1 together are believed to contribute to about 75% of the GWR (Kupfermann *et al.* 1974). The majority of the CNS control over the gill withdrawal component of the defensive withdrawal reflex can therefore be monitored by recording from two easily identifiable motor neurones in the abdominal ganglion.

The response of the gill motor neurone L7 to a tap to the siphon, or an action potential elicited in a siphon sensory neurone, increases after a strong dishabituating stimulus is presented to the connective nerves (Castellucci, Pinsker, Kupfermann & Kandel, 1970). Similar synaptic facilitation can also be demonstrated by isolated neurones in culture (Rayport & Schacher, 1986). The central synaptic connections between the siphon sensory neurones and the gill motor neurones can therefore be made more effective by activation of a second sensitizing pathway, a phenomenon known as heterosynaptic facilitation (Kandel & Tauc, 1965). The siphon sensory neurones have been proposed as the neuronal site of GWR and SWR sensitization. The synaptic facilitation is thought to be mediated by an increase in the amount of neurotransmitter that is released from the sensory neurone terminals, and a model of the ionic and molecular events which may underlie this synaptic facilitation has been proposed (Klein & Kandel, 1980; Kandel & Schwartz, 1982; Gingrich & Byrne, 1985; Byrne, 1985, 1987).

Utilizing the isolated central nervous system, Hawkins, Abrams, Carew & Kandel (1983) reported a cellular analogue of differential associative conditioning. Intracellular depolarization of a sensory neurone served as the CS and shocks to the tail or pedal nerves (which innervate the tail) as the UCS. The classically conditioned sensory neurones were presented with five trials of the CS followed 500 ms later by the UCS. An action potential in the paired sensory neurone evoked a larger EPSP in the siphon motor neurone, or in L7, after training. A correlation between associative conditioning of the siphon withdrawal duration (Carew *et al.* 1983) and associative conditioning of the motor neurone EPSP was suggested. A detailed molecular model

for the underlying processes of the behavioural conditioning of the defensive withdrawal reflex has been proposed. This process has been referred to as 'activity-dependent amplification of presynaptic facilitation' and is considered to be an amplification of the events that have been proposed to underlie sensitization (see Kandel *et al.* 1983; Hawkins, 1984; Byrne, 1985, 1987).

Simultaneous associative conditioning of the EPSP and the behavioural response has not yet been reported. A causal relationship has not been demonstrated between the changes that are observed in the isolated nervous system and those displayed by the intact animal after paired presentation of stimuli.

A semi-intact preparation provides a means of simultaneously monitoring cellular and behavioural responses during learning. Semi-intact preparations of *Aplysia californica* have already proved capable of associative conditioning (Lukowiak & Sahley, 1981; Lukowiak, 1986). One of these studies, in which the semi-intact preparation was presented with a tap to the siphon as the CS and a series of taps to the gill as the UCS, revealed that the responses of the gill and its motor neurones may not correlate during learning acquisition (Lukowiak, 1986).

To determine the relationship between the behavioural conditioning and the central synaptic facilitation that has been observed in the isolated nervous system, a semi-intact preparation was developed for the present study. The stimulus parameters that have proved successful in previous studies for training the intact animal (Carew *et al.* 1983) and the isolated nervous system (Hawkins *et al.* 1983) were followed closely in an attempt to bridge observations.

The first aim of these experiments was to determine whether the central sensory–motor neurone synapses and the GWR of the semi-intact preparation could be classically conditioned within 10 trials. The second aim was to question whether the activity-dependent facilitation that has been observed at these central synapses is sufficient, or necessary, for classical conditioning of the defensive withdrawal reflex. We also monitored changes in the ability of the central motor neurones to elicit a gill withdrawal before and after conditioning, so that learning-induced changes in the peripheral modulation of the GWR could be identified.

We found that the semi-intact preparation is capable of associative learning. Activity-dependent facilitation at the central sensory–motor neurone synapses is evident in the absence of behavioural conditioning and is therefore not sufficient for conditioning of the GWR. Other changes occur in the periphery as a result of associative conditioning; this is reflected in a relative enhancement of the gill withdrawal elicited by depolarization of the motor neurone in conditioned preparations as compared with controls.

MATERIALS AND METHODS

The preparation

Aplysia californica were obtained from Sea Life Supply or Pacific Biomarine. They weighed 100–200 g and were maintained in a 1200-l seawater-filled tank at 15–16 °C, pH 7·9. Animals were fed once a week with dried red seaweed. Food-

satiated *Aplysia*, which show suppressed gill behaviour (Lukowiak, 1980), were not used for these experiments. All animals were anaesthetized with isotonic (0·33 mol l^{-1}) $MgCl_2$ prior to dissection.

The semi-intact preparation consisted of the siphon, mantle, gill, abdominal ganglion, head ganglia and tail (Fig. 1). The siphon, branchial and ctenidial nerves were left intact as were the pleuroabdominal connectives and the two posterior pedal nerves; all other nerves and connectives were severed. The preparation was pinned, dorsal-side-down, to the clear Sylgard (Dow Corning) coated base of a Lucite chamber. The abdominal and head ganglia were further pinned out on clear Sylgard platforms.

The abdominal ganglion was bathed in hypertonic sucrose/seawater solution (2 mol l^{-1} sucrose diluted 1:1 with artificial sea water) for 15 min prior to removal of the connective sheath (Connor, 1979). This facilitates desheathing by causing a slight shrinkage of the 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 solution was removed and the ganglion washed 4–5 times in artificial sea water. A thread was attached to a single gill pinnule at one end and to a force transducer (Narco myograph F60) at the other, the thread tension being adjusted to avoid stretching the pinnule. The gill was not damaged by this procedure and the muscle did not fatigue (spontaneous movements remained constant throughout the training period). The output of the transducer was displayed on analogue (Tektronix 5113) and digital (Nicolet 2090-3) storage oscilloscopes. The amplitude of the gill withdrawal was used as a measurement of response magnitude, and these measurements were taken from the digital oscilloscope. The tension transducer method has previously been shown to be as accurate as other methods commonly used for measuring gill response magnitude (Lukowiak & Peretz, 1977). After dissection, 45 min was allowed before the neurones were impaled. The preparation was bathed in artificial sea water (Instant Ocean, Aquarium Systems) at 15–16 °C throughout the experiment.

The motor neurones

The abdominal ganglion was transilluminated to aid neurone impalement. Single-barrelled micropipettes (filled with 3 mol l^{-1} KCl) of resistance 10–20 MΩ were used. A Getting M-5 or Dagan 8700 cell explorer electrometer containing a bridge circuit allowed simultaneous recording and current injection through the electrode. One or both of the major gill motor neurones (L7 and/or LDG1) were impaled and monitored in each preparation. Identification of these neurones was based on the correlation of their activity with gill behaviour, as well as their position in the ganglion and their pattern of activity (Koester & Kandel, 1977). EPSP amplitudes in response to the CS were measured using the digital oscilloscope. Neurones were hyperpolarized by about 10 mV throughout the conditioning sessions to reduce spontaneous activity and to enable measurements of the evoked EPSP amplitude. In

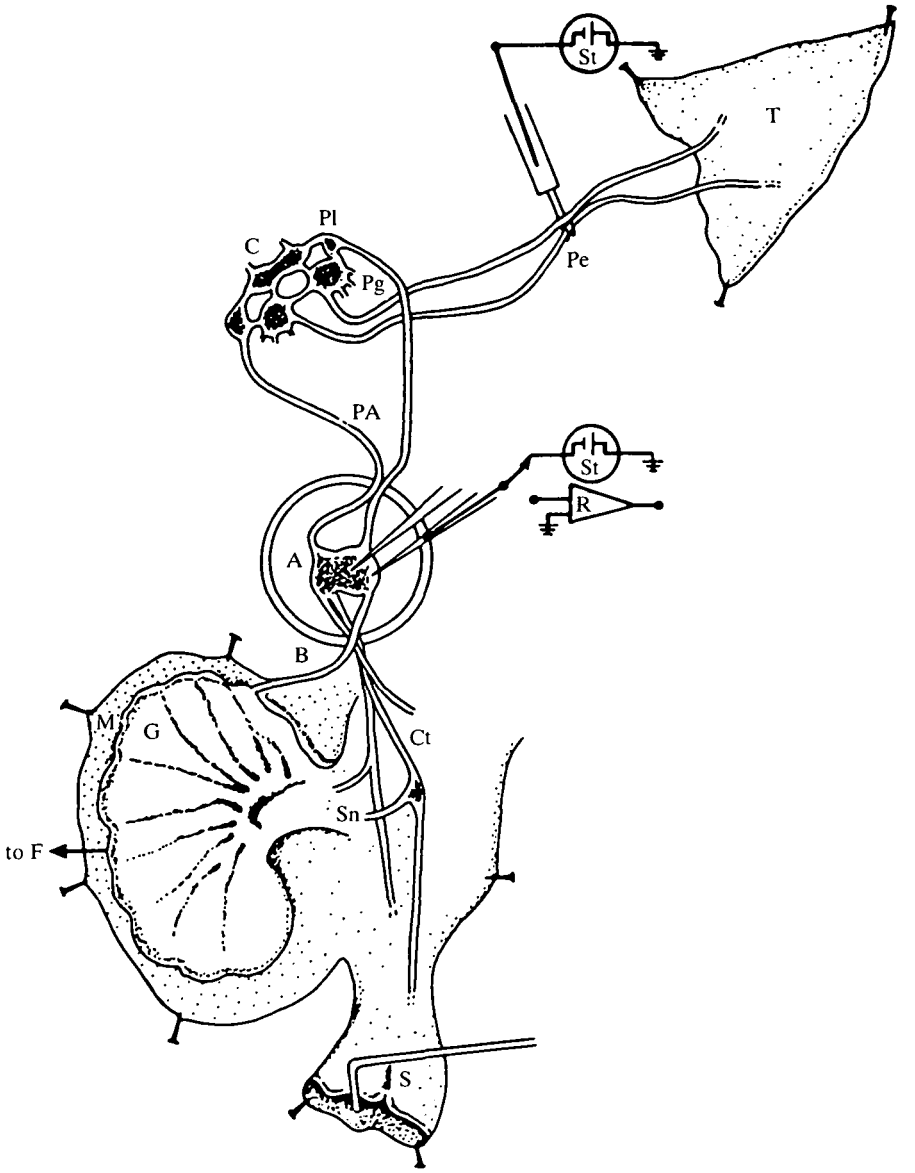

Fig. 1. The semi-intact preparation. The preparation consisted of the siphon (S), gill (G), tail (T), mantle (M), abdominal ganglion (A), pleural ganglia (Pl) and cerebral ganglia (C). The gill and siphon were innervated by branchial (B), ctenidial (Ct) and siphon nerves (Sn) from the abdominal ganglia. The head ganglia were left connected to the abdominal ganglia *via* the pleuroabdominal connective nerves (PA). A suture was secured to a single gill pinnule and led to a tension transducer (F) for the measurement of gill contractions. Tactile stimuli were delivered to the siphon with a mechanical tapper. The abdominal ganglion was isolated in a chamber sealed with Vaseline, the nerves passed underneath the chamber *via* a small Vaseline-filled notch. Intracellular recordings were made from motor neurones in the abdominal ganglion. St, stimulating electrodes; R, recording electrodes; Pe, posterior pedal nerves; Pg, pedal ganglia.

many cases, however, the cells could not be prevented from spiking in response to the CS. On these occasions, the number of action potentials, rather than the EPSP amplitude, was recorded. In the experimental group ($N = 22$) EPSP amplitude was used as a measure for 12 of the preparations, and number of action potentials for the remaining 10. In the control group ($N = 11$) the EPSP amplitude was measured for six of the preparations and the number of action potentials was counted for the other five. Changes in the EPSP amplitude or in the number of action potentials were considered to be equivalent measures of a change in the response of the motor neurone to the CS. These parameters were also considered sufficient and adequate measures of the neurone's response to the simple conditioned stimulus.

The conditioning stimuli

The conditioned stimulus consisted of a 'tapper' stimulus (see Peretz & Lukowiak, 1975) of 600 mg intensity and 50 ms duration applied to the pinned-out siphon. This stimulus was just sufficient to cause an EPSP in the gill motor neurone(s) and usually a small withdrawal of the gill. The same patch of siphon skin was used as the stimulation site throughout the training period and tests. The CS used in these experiments was similar to that used previously to condition the intact animal (Carew *et al.* 1981, 1983).

The unconditioned stimulus was a train of shocks to the pedal nerves [similar to that used by Hawkins *et al.* (1983) as the UCS for conditioning of the isolated nervous system]. These were delivered *via* bipolar silver hook electrodes. The nerves were hooked over the electrodes and were lifted out of the water, for stimulation, with the use of an x–y–z manipulator. A 3 s, 10 Hz train of 3 ms pulses was sufficient to cause a brisk burst of action potentials in the motor neurone(s) and a large gill withdrawal.

The protocols

For the classical conditioning (CC) protocol (Fig. 2A) the UCS was specifically paired to the CS and delivered 500 ms after the CS. The intertrial interval was 5 min and each animal received 10 training trials. Control animals (Fig. 2B) received specifically unpaired presentations of the CS and UCS; the UCS was presented 2·5 min after the CS for the 10 trials. Conditioning in *Aplysia* has been found to have a steep interstimulus interval function and no significant learning is expected to occur at CS–UCS intervals greater than 2 s (Kandel *et al.* 1983). A CS–UCS interval of 2·5 min has previously been used as a control for conditioning intact animals (Carew *et al.* 1983) and isolated nervous systems (Hawkins *et al.* 1983). The GWR amplitude and neurone EPSP amplitude (or number of action potentials) in response to the CS were simultaneously monitored throughout the training trials, and also at CS-only test trials 5 min prior to training, T(0), and 30 min after training, T(30). The 30 min post-training test was chosen to comply with a previous report on conditioning of *Aplysia* (Carew *et al.* 1983).

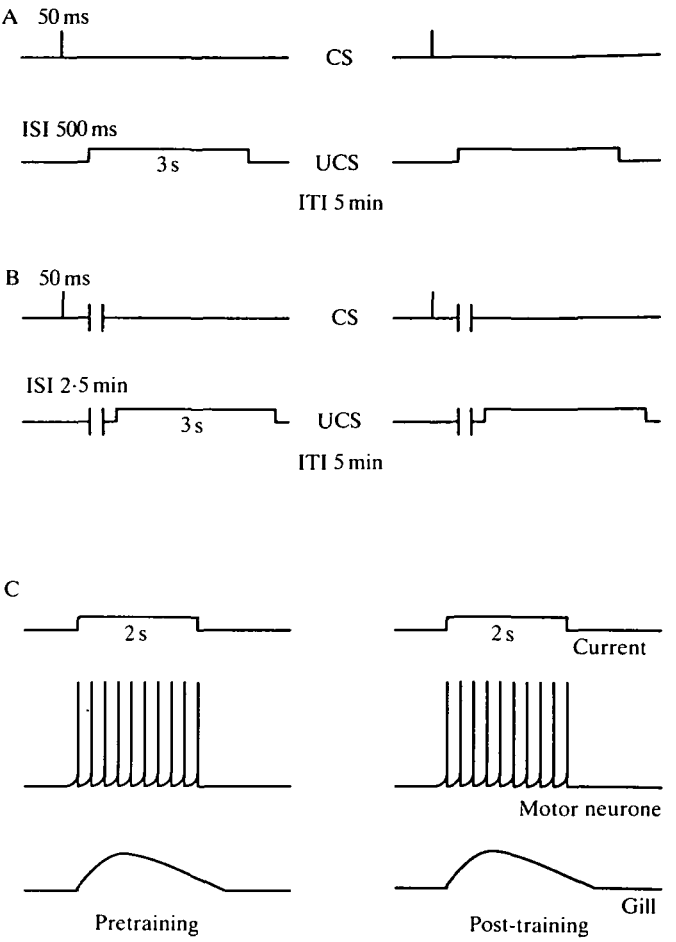

Fig. 2. The stimulation protocols. (A) The classically conditioned animals received 10 paired presentations of the conditioned stimulus (CS) (siphon tap) followed 500 ms later by the unconditioned stimulus (UCS) (pedal nerve stimulation). The intertrial interval (ITI) was 5 min. ISI, interstimulus interval. (B) Control animals received 10 specifically unpaired presentations of the CS followed 2·5 min later by the UCS. The ITI was again 5 min. The response to the CS was tested before training [T(0)] and 30 min after training [T(30)] in both groups. (C) Prior to paired or unpaired training (pretraining) some of the motor neurones were depolarized for 2 s and a burst of action potentials was evoked. The same number of action potentials was elicited after training (post-training). The resulting gill withdrawal responses were measured.

Depolarization of the motor neurones

The ability of the gill motor neurone(s) to move the gill was monitored before and after training in some of the paired and unpaired animals. Twenty minutes before T(0) the motor neurone was depolarized for 2 s; a steady train of 10–20 action potentials was induced in this way and the amplitude of the elicited gill withdrawal

was recorded. An identical test was performed 5 min after T(30) (Fig. 2C). The number and frequency of action potentials were the same in both tests.

Statistical procedures

Differences between the paired- and the unpaired-induced changes in CS-elicited GWRs and cell responses were tested with the Mann–Whitney *U*-test. The chi-squared test was used to analyse the distributions of outcomes following paired or unpaired training. Estimates for correlation were determined by the Pearson's Product Moment Correlation Coefficient. The paired *t*-test was utilized for all other statistical testing. Two populations of data were assumed to be different if $P < 0{\cdot}05$.

RESULTS

Twenty-two semi-intact preparations were presented with paired stimuli (the conditioned group) and 11 preparations received specifically unpaired stimuli (the control group).

To determine whether this *semi-intact* preparation was capable of associative conditioning, the responses (of both the gills and motor neurones) of the paired group were compared with those of the control group (Fig. 3). In the control group, both the motor neurone response and the GWR elicited by the CS showed a very slight facilitation (or sensitization) 30 min after the tenth trial; these enhancements were not statistically significant.

In the CC group, however, the CS elicited a greatly enhanced motor neurone response and a facilitated behavioural response 30 min after the tenth paired trial. A

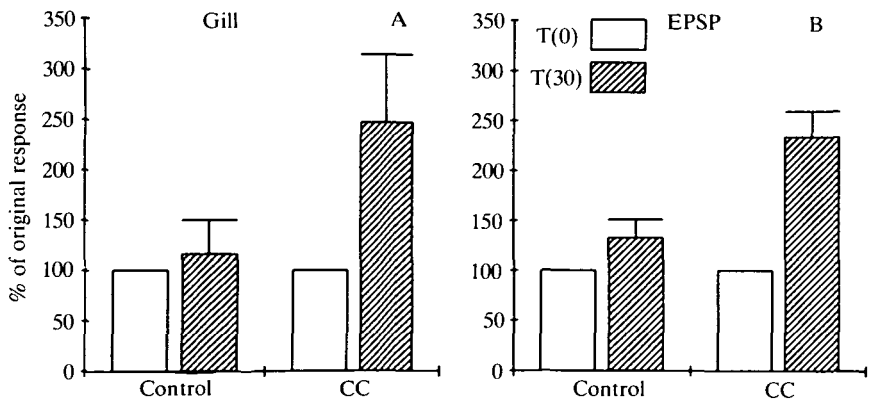

Fig. 3. The effect of associative training on gill motor neurone responses and gill withdrawal reflex amplitudes. Both motor neurone (EPSP) and gill withdrawal responses to the conditioned stimulus were normalized to the response before training [T(0)]. The mean behavioural response (A) was slightly facilitated after unpaired training (to 116 %) and greatly increased after paired training (to 248 %). The mean EPSP response (B) was slightly enhanced after unpaired training (to 133 %) and significantly facilitated (to 234 %) after paired training. The means ± S.E.M. of all animals are plotted. CC, classical conditioning. $N = 11, 22$.

significantly greater change in response amplitude between T(0) and T(30) was found in the CS-elicited response of the motor neurones in the conditioned group when compared with that of the control group ($P < 0{\cdot}05$). In addition, a within-group comparison showed that the neuronal responses to the CS of the conditioned group exhibited significant enhancement of EPSP amplitudes, or number of action potentials, compared with their own T(0) scores ($P < 0{\cdot}005$).

Although the mean GWR amplitude of the conditioned group in response to the CS had increased, the change in GWR amplitude between T(0) and T(30) was not significant when compared with the unpaired group. Also, the behavioural responses of the paired group were not significantly enhanced when compared with their own pretest scores. The lack of significant behavioural change following classical conditioning appears to be due to the large variability in the T(30) GWR amplitude values; the mean percentage increase in the gill response amplitude is greater than that shown by the motor neurone responses.

In addition, only those preparations which demonstrated increased behavioural responses at T(30) ($N = 11$) were compared with the controls. The neuronal responses of these conditioned preparations were significantly greater than those of the control preparations ($P < 0{\cdot}01$). There was also a significantly greater effect of paired training on the gill responses when the two groups were compared ($P < 0{\cdot}01$), indicating that behavioural 'learning' had occurred in a subset of the preparations that were exposed to paired stimuli.

If facilitation at the central sensory–motor synapses is responsible for the behavioural learning, any increase, or learning, that occurs in the input to the motor neurone as a result of the conditioning procedure should also be apparent in the gill's response to the CS. When the response to each preparation was examined, four different outcomes at T(30) were identified (Table 1). Both the classically conditioned group and the control group included preparations that demonstrated a facilitation of both the motor neurone response and the GWR at T(30) and

Table 1. *Summary of the responses of the gills and motor neurones to the conditioned stimulus at T(30)*

	CC group ($N = 22$)	Control group ($N = 11$)
Both	45·5 % (10)	27·3 % (3)
Cell only	31·8 % (7)	27·3 % (3)
Gill only	4·5 % (1)	9·1 % (1)
Neither	18·2 % (4)	36·3 % (4)

Both: increases in both the cell and gill responses to the conditioned stimulus (CS) at T(30).

Cell only: facilitation of the motor neurone response but no concomitant increase in the gill withdrawal reflex (GWR) to the CS at T(30).

Gill only: an enhancement of the GWR but no facilitation of the motor neurone response at T(30).

Neither: neither an increased cell response nor an increased gill response at T(30).

See text for an explanation of T(30).

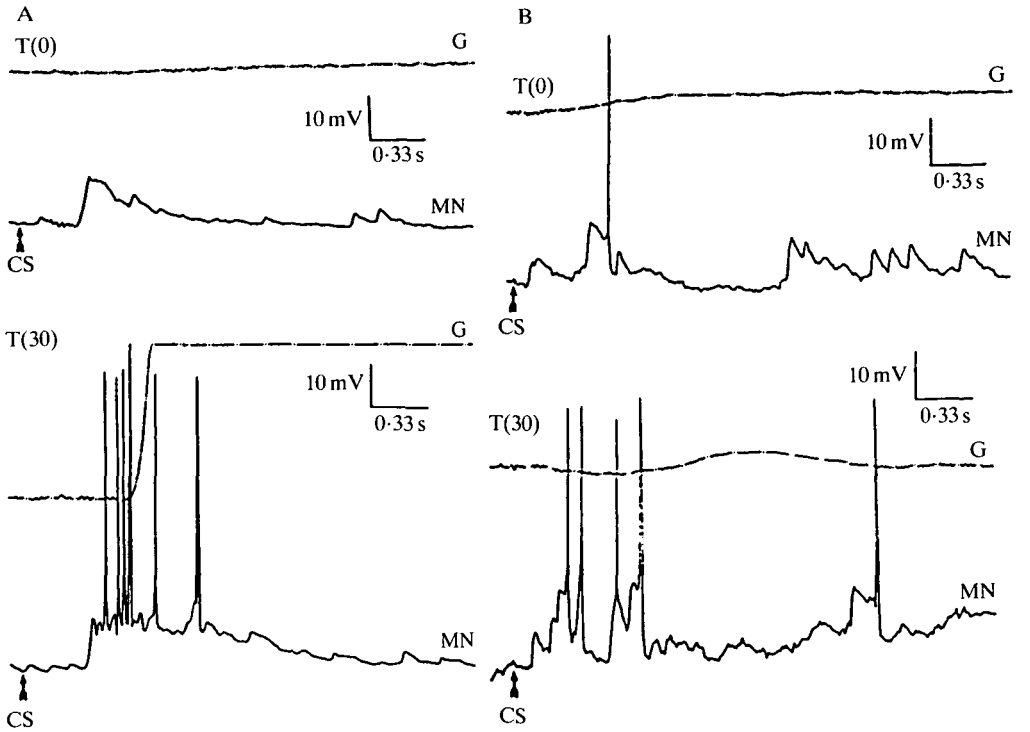

Fig. 4. Examples of motor neurone responses and gill withdrawals evoked by the conditioned stimulus (CS) before [T(0)] and 30 min after [T(30)] training. (A) A preparation from the *both* response group. After paired training both the gill (G) and the motor neurone (MN) response to the CS were facilitated. (B) A preparation from the *cell only* response group. After paired training the cell response to the CS was facilitated, the gill withdrawal response was unchanged.

preparations that showed no evidence of either gill or motor neurone enhancement at T(30). However, 31·8% of the CC group and 27·3% of the control group preparations responded to the CS with an enhanced motor neurone response and either no change or a decrement in the GWR. One preparation in each group demonstrated facilitation of the GWR with no concomitant increase in the response of the motor neurone. The two distributions, that following paired training and that following unpaired training, were significantly different from each other ($P < 0{\cdot}01$). The type of outcome following training was not related to whether the motor neurones reached threshold. Of the 10 conditioned preparations which showed both gill withdrawal and motor neurone enhancement, the change in EPSP amplitude had been used as a measure of neurone response in five of them, and the number of action potentials had been used in the other five. The other 'types of outcome' groups were equally evenly distributed.

The T(0) and T(30) responses of a preparation in which both the GWR and sensory–motor neurone synapses became facilitated after CC training are shown in Fig. 4A. Before training the CS elicited no GWR, and the motor neurone responded

with a small EPSP. After training, however, there was a large GWR in response to the CS and the motor neurone responded with several action potentials. The depolarization elicited in the motor neurone by the CS was also longer in duration after training than before training. Fig. 4B shows the T(0) and T(30) responses of a preparation whose motor neurone response was facilitated at T(30), even though its GWR did not increase significantly. The CS elicited a small EPSP and one action potential before CC training, and this response increased to four action potentials after training. The gill responded to the CS with a slight withdrawal before training, and this response remained the same after training.

No correlation existed between the neuronal response and the GWR of each individual preparation to the CS following training ($P > 0{\cdot}05$). The observation that the response of the motor neurone, as measured in the soma, does not correlate with the GWR amplitude indicates that changes observed in the motor neurones of an isolated nervous system are not necessarily representative of changes in the behaviour.

In those preparations ($N = 6$) in which both LDG1 and L7 were monitored simultaneously, the two cells responded similarly at T(30): if the input to one cell increased, the input to the other cell also increased. This suggests that the facilitation that occurs at the central sensory–gill motor neurone synapses occurs at all of these synapses simultaneously. Four of these six preparations demonstrated both increased neurone and increased gill responses at T(30), indicating that slight hyperpolarization of both motor neurones did not interfere with conditioning of the GWR.

Analysis of the mean learning curves of the 10 animals that underwent enhanced gill and enhanced motor neurone responses after associative conditioning (Fig. 5) revealed that the mean neuronal response was gradually facilitated during training. The behavioural response, however, did not demonstrate facilitation during training, in fact a slight decrement in response amplitude is evident. The response of the gill greatly increased between the last trial and the test 30 min later. The motor neurones and the gill therefore show different patterns of acquisition during classical conditioning.

Training-induced changes in the elicited gill withdrawal

Our next aim was to determine if CC training affected the ability of a gill motor neurone to elicit a gill withdrawal. This would indicate whether associative learning in this system involves changes at sites distal to the sensory–motor neurone synapse. Seventeen motor neurones of the CC group and seven cells of the control preparations were depolarized to produce a set number of action potentials before and after the CS–UCS presentations (see Fig. 2C). The amplitude of gill withdrawal measured after training was calculated as a percentage of the amplitude of withdrawal generated before training. Three types of outcome were observed (Table 2). A large proportion of the control group (86%) showed a decrease in the amplitude of the elicited withdrawal (Fig. 6). In only 14% (one preparation) of the motor neurones in

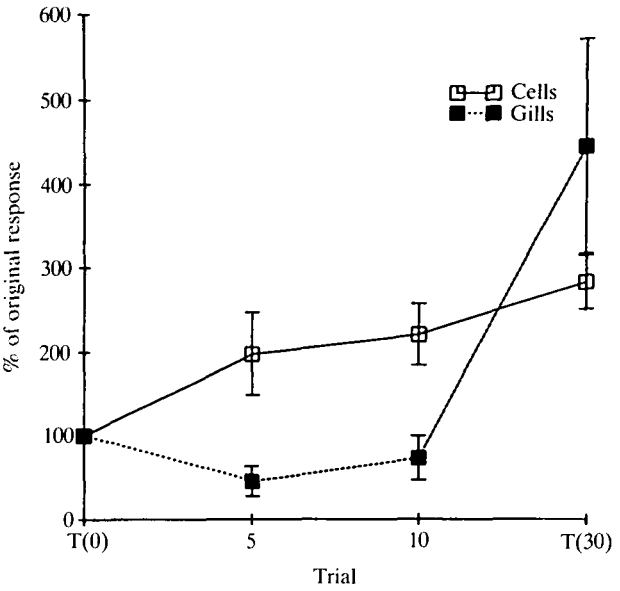

Fig. 5. The acquisition of learning by the motor neurones and gills. All amplitudes were normalized to T(0). Mean responses of those preparations from the classical conditioning group which demonstrated both gill and cell learning ($N = 10$) are plotted. The motor neurone response amplitude (□) increased gradually over the course of associative conditioning. The gill withdrawal reflex amplitude (■) did not demonstrate facilitation until after the last training trial. Means ± S.E.M. are plotted.

the control group was a larger withdrawal elicited after training than before training. In the CC group, however, 47 % showed a decrease in their elicited gill withdrawal after associative conditioning, 24 % did not change and 29 % displayed an increased gill withdrawal amplitude (Fig. 7).

The control group showed a mean decrease of 50 % in the gill withdrawal elicited by the motor neurone. This was a significant decrement ($P < 0{\cdot}05$). The paired group demonstrated only a slight mean decrease of 7 % ($P > 0{\cdot}05$) (Fig. 8). Unpaired training, therefore, caused a decrement in the ability of the motor neurone to elicit a gill withdrawal. Such a decrement was not evident after paired training (classical conditioning).

Table 2. *Summary of changes in the gill withdrawal elicited by depolarization of the motor neurone*

	CC group ($N = 17$)	Control group ($N = 7$)
Decreased elicited withdrawal	47·1 % (8)	85·7 % (6)
No change in elicited withdrawal	23·5 % (4)	0·0 % (0)
Increased elicited withdrawal	29·4 % (5)	14·3 % (1)

DISCUSSION

The results obtained from conditioning of these semi-intact preparations demonstrate that synaptic facilitation occurs between the central siphon sensory neurones

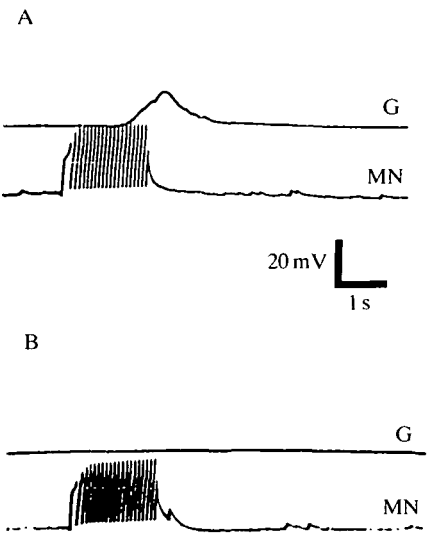

Fig. 6. An example of a decrease in L7's elicited gill withdrawal. (A) The gill withdrawal reflex (G) elicited by depolarization of L7 (MN) before 10 unpaired trials. (B) The same gill and motor neurone after 10 unpaired trials and 35 min rest. The elicited withdrawal has decreased to zero.

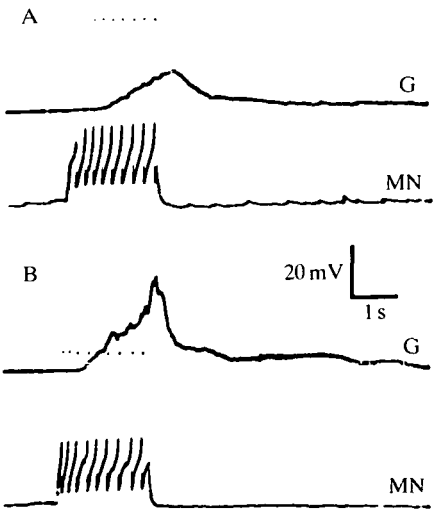

Fig. 7. An example of an increase in L7's elicited gill withdrawal. (A) The gill withdrawal reflex (G) elicited by depolarization of L7 (MN) before 10 paired trials. (B) The same gill and motor neurone after 10 paired trials and 35 min rest. The elicited gill withdrawal was enhanced after associative conditioning.

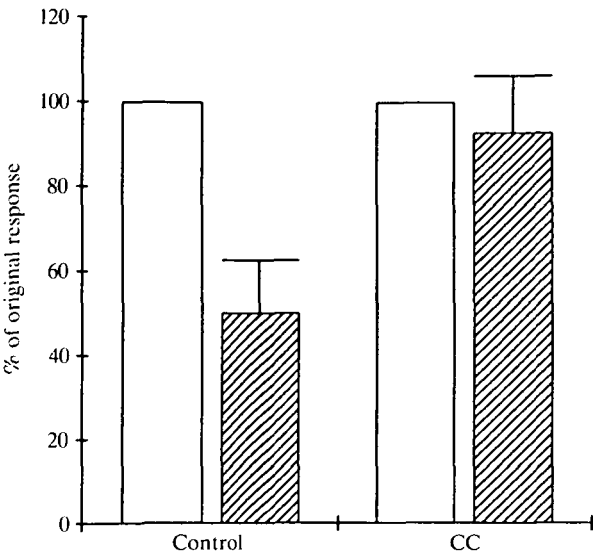

Fig. 8. Mean amplitudes of the gill withdrawals elicited by direct depolarization of the gill motor neurones. The withdrawal elicited after 10 trials (cross-hatched bars) was calculated as a percentage of that elicited before training (open bars). After paired training (CC) no change was observed in the elicited withdrawal. Unpaired training (control) resulted in a significant decrement of the original elicited withdrawal. Means ± S.E.M. are plotted. $N = 7$, 17.

and gill motor neurones as a result of associative training. This result is in agreement with previous observations made with the isolated nervous system (Hawkins *et al.* 1983); similar stimulus parameters were used in the present study. These results are also complementary to a recent report (Lukowiak, 1986) in which the gill and motor neurones of a semi-intact preparation demonstrated facilitation after 40 paired trials. The facilitated mean GWR observed after 10 paired trials in the present study was not statistically significant, indicating that the behavioural learning does not occur within 10 trials in all animals, although it has been demonstrated to occur within 40 trials. When only those animals that demonstrated enhanced responses at T(30) were compared with controls, however, significant behavioural learning was observed. It seems that a sub-group of these semi-intact preparations is capable of demonstrating classical conditioning of the gill withdrawal reflex after only 10 paired trials.

A previous report (Carew *et al.* 1981) demonstrated that associative conditioning of the GWR develops more slowly than conditioning of the SWR. The same siphon mechanoreceptors make synaptic connections with both the gill and the siphon motor neurones (Castellucci *et al.* 1970). It is feasible, however, that conditioning of the GWR involves a larger contribution from the periphery than conditioning of the siphon component. Our preparation could be used in the future to determine the contribution of central activity-dependent synaptic facilitation to conditioning of the SWR by simultaneous monitoring of the siphon motor neurones and the siphon withdrawal amplitude. The semi-intact preparation is therefore a suitable model

system for investigating classical conditioning of the gill and siphon withdrawal reflexes and the underlying neuronal mechanisms.

The gill withdrawal reflex is very complex and is made up of many components (Kupfermann *et al.* 1974; J. L. Leonard, J. Edstrom & K. Lukowiak, in preparation). In the present study the GWRs were not broken down into their separate components. The semi-intact preparation provides a useful system with which changes in the complexity of the gill withdrawal reflex could be observed whilst recording the activity of the central sensory and motor neurones.

The combined data showing the behavioural responses and the motor neurone responses after training (Fig. 3) appear very similar to those presented by Hawkins *et al.* (1983). If these graphs were to be considered alone the conclusion might be drawn that changes at the sensory–motor synapses reflect changes in the behavioural response and *vice versa*. However, the finding that some of the preparations demonstrated synaptic facilitation without showing a concomitant GWR enhancement (Table 1) indicates that activity-dependent amplification of presynaptic facilitation at the central sensory–motor neurone synapses (Kandel *et al.* 1983) is not sufficient to mediate the behavioural learning. The control group also included some preparations in which only the response of the motor neurone increased, which suggests that presynaptic facilitation of the central sensory–motor neurone synapses (Castellucci & Kandel, 1976) is not sufficient to mediate sensitization of the gill withdrawal response. These results suggest that at least one other site of facilitation is involved in mediating conditioning and sensitization of the gill withdrawal response. Such sites may be located both centrally and peripherally, but at least one change occurs distal to the motor neurone cell body.

The additional sites of facilitation may also be capable of mediating associative learning, and sensitization, of the reflex independently of central synaptic facilitation. This is suggested by the observation that one preparation in the CC group and one preparation in the control group demonstrated enhanced GWRs in the absence of any central synaptic facilitation. Further experiments are required to determine whether central presynaptic facilitation is necessary for associative and non-associative learning of the GWR. LDG1 and L7 together contribute about 75% of the gill withdrawal response (Kupfermann *et al.* 1974) and were observed to respond similarly when the responses from both cells were recorded. It is therefore unlikely, though not impossible, that other central sensory–motor synapses mediated the GWR facilitation in those preparations that demonstrated only enhanced gill responses after training.

The associative conditioning protocol was not 100% successful (Table 1). This has also been observed in associative training studies using other preparations, such as *Limax* (Chang & Gelperin, 1980). It would be of interest to compare the success rates for conditioning of the isolated *Aplysia* nervous system and the intact animal. The current study would predict a higher success rate for conditioning of the isolated nervous system.

The mean GWR increased substantially between the last CC training trial and the test at T(30) (Fig. 5). Some form of facilitation therefore occurs during the 30 min

rest after the last paired trial. The change that mediated this facilitation was not seen in the gill motor neurones, whose response showed only a slight increase after the 30 min rest. The mechanism that mediates the enhanced behavioural response must, therefore, occur peripheral to the motor neurone cell body.

The mean neuronal response of the facilitated preparations was 195% on trial 5 (Fig. 5). This response is similar to that observed by Hawkins *et al.* (1983), in which an EPSP amplitude of 170% of the pretraining response was recorded after five paired trials. This suggests that the siphon tap and intracellular depolarization of the siphon sensory neurones are equally effective as a stimulus for conditioning of the sensory–motor synapses.

The motor neurones were slightly hyperpolarized by current injection during the training. Despite this, many of the cells responded to the CS with one or more action potentials, and all fired a brisk train of action potentials in response to the UCS. In addition, it was found that the GWR was equally likely to become conditioned in those preparations in which both LDG1 and L7 were hyperpolarized ($N = 6$) as it was in those in which just one of the cells was hyperpolarized. It seems, therefore, that slight hyperpolarization of the motor neurone did not interfere with conditioning of the GWR.

When the motor neurones were depolarized by current injection at T(30) the majority of the preparations in the control group responded with a smaller GWR after training than they did before training (Table 2). Less than half of the CC group demonstrated such a decreased elicited withdrawal. The fact that the animals in the control group underwent a significant decrease in their motor neurone-elicited gill withdrawals suggests that habituation of the neuromuscular junction occurred over the course of the unpaired training, supporting the conclusions of Jacklet & Rine (1977) and Lukowiak (1977) that habituation can occur in the peripheral nervous system.

It seems likely that some form of facilitation, peripheral to the motor neurone soma (similar to that described by Jacklet & Rine, 1977), is counteracting the habituation in the CC group. If facilitation did not occur in the periphery due to the paired presentations of the CS and UCS, habituation of the elicited gill withdrawal would be expected to be equally evident in the control and in the CC group. The elicited withdrawal did not habituate in more than half of the CC preparations however, and 29% of them were facilitated.

The additional or alternative sites of facilitation that are involved in mediating the learned withdrawal response have not been characterized or localized in this study. As the changes seem to occur at a site distal to the motor neurone cell bodies, the peripheral nervous system is a likely candidate. Synapses within the peripheral nervous system may become facilitated by activity-dependent amplification of presynaptic facilitation, as described by Kandel *et al.* (1983) for the central sensory–motor neurone synapses, or alternative mechanisms may be involved.

One or more central neurones having a direct effect in the periphery may be additional or alternative mediators of the conditioned response. The gill motor neurone L9, for example, can potentiate L7's ability to elicit a gill withdrawal

(Lukowiak, 1979*a*) and can also prevent, or reverse, habituation of the GWR (Lukowiak, 1979*b*). L9's modulation of the GWR is mediated peripherally, making it a possible candidate for mediating non-associative and/or associative learning of the GWR either by a direct effect on the gill muscle itself or by modulation of the terminals of central or peripheral motor neurones. A recent report in which voltage-sensitive dyes were used to monitor the activity of cells within the abdominal ganglion before and after sensitization has revealed that more central neurones are active after exposure to sensitizing stimuli than before (London, Cohen & Zecevic, 1986). Central modulators of peripheral activity, such as L9, may be included amongst these cells.

Many loci and mechanisms are likely to be involved in conditioning of the *Aplysia* GWR. No one locus or mechanism may be necessary or sufficient, and it is likely that higher centres and local changes combine to produce behavioural modification. This report emphasizes that any study undertaken in the hope of revealing the contributions of various systems and mechanisms to a learned behaviour should include as much of the nervous system, and indeed the animal itself, as possible. The method of examining learning in the isolated nervous system is a valuable means for determining cellular and subcellular methods of modification. The contribution of these isolated changes to learning in the intact organism must, however, be established before a molecular model for behavioural learning can be accepted.

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