

TRANSFER OF HABITUATION SHOWS AN INTERACTION BETWEEN NEURONAL CIRCUITS OF THE GILL WITHDRAWAL REFLEX IN *APLYSIA CALIFORNICA*

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SUMMARY

The gill withdrawal reflex (GWR) and its subsequent habituation can be evoked by tactile stimulation of the siphon or gill when the CNS is either intact or removed. It has been suggested that the neural circuits that mediate the GWR evoked at these two loci are parallel and independent. We provide three lines of evidence which show that these circuits interact and, therefore, comprise a single integrated system. Firstly, siphon and gill stimulation evoked similar excitatory responses in the central gill motor neurones. Secondly, the GWR habituated by repetitive stimulation at one locus was dishabituated by stimulation of the other locus. Thirdly, transfer of habituation occurred. Although the transfer was seen neurally at the level of central gill motor neurones, transfer of habituation also occurred after the CNS was removed. Therefore, the neuronal circuits mediating the reflexes evoked at the siphon and gill interact within both the CNS and PNS. The PNS is largely responsible for mediating this gill behaviour that is based on such interactions, while the CNS provides suppressive and facilitatory plasticity to these responses to enable *Aplysia* to better adapt to a changing environment.

INTRODUCTION

The gill withdrawal reflex (GWR) and its subsequent habituation in *Aplysia* can be evoked by tactile stimulation of the siphon (Pinsker *et al.* 1970), mantle shelf (Carew, Castellucci & Kandel, 1971) and gill (Peretz, 1970). It has been suggested (Kupfermann *et al.* 1971; Kupfermann, Carew & Kandel, 1974) that, upon weak to moderate intensity stimulation, the central nervous system (CNS) mediates the GWR evoked by tactile stimulation of the siphon and mantle, while a peripheral nervous system (PNS) in the gill mediates the reflex evoked by gill stimulation. Moreover, it was said that the circuits were parallel and independent of each other. Thus, activity in one circuit would not affect the response mediated by the other. On the other hand, Peretz & Howieson (1973) found that the CNS exerted suppressive control over the PNS in the mediation of gill behaviours evoked by gill stimulation. In addition, Lukowiak & Peretz (1975) presented further evidence that the CNS and PNS interact and form an integrated system which mediates gill reflex behaviours evoked by tactile stimulation of the gill or siphon.

In an attempt to clarify the situation, we have designed a series of experiments to test whether the neural circuits which mediate the GWR evoked by siphon stimulation are parallel and independent of the neural circuits that mediate the GWR evoked by gill stimulation; or whether they form an integrated system when weak (1 g) tactile stimuli are employed. Basically, we tested if habituation evoked by repeated tactile stimulation at one site transfers its effect to the reflex evoked at the other site. Transfer of habituation is one of the nine parametric characteristics of habituation as outlined by Thompson & Spencer (1966). Interestingly, this is the one parametric characteristic that has not been demonstrated in the *Aplysia* siphon, mantle, gill and abdominal ganglion model preparation (see Carew *et al.* 1971; Kupfermann *et al.* 1971, 1974). If, for example, habituation of the GWR evoked by repeated tactile stimulation of the siphon brought about a reduction in the amplitude of the GWR, and increased the rate of habituation evoked by gill stimulation, this would show a transfer of habituation and an interaction between the neural circuits.

We report here that transfer of habituation occurs in this preparation between the siphon and gill stimulation sites. Thus, the neural circuits mediating the GWR are not parallel and independent, but interact with each other and form an integrated system which normally mediates gill reflex behaviour.

METHODS

Aplysia californica (between 150 and 400 g) were obtained from Pacific Biomarine Laboratories (Venice, Ca.). They were maintained in an aerated 300 gallon aquarium in artificial sea water (Instant Ocean) at 15–16 °C and pH 7.9. The animals were fed weekly, and at least 2 days expired between feeding and the use of an animal (Lukowiak, 1980).

The preparation (Fig. 1) consisted of the siphon, mantle, gill and the abdominal ganglion (the CNS). The siphon (Sn), ctenidial (Ct) and branchial (Br) nerves, by which the ganglion innervates the siphon, mantle and gill were left intact; all other nerves and connectives were severed. The preparation was pinned out in a Sylgard (Dow Corning) coated 500 ml chamber filled with artificial sea water maintained at 15 °C. The siphon was immobilized by attaching one end of a surgical thread to its distal margin and securing the other end with moderate tension to the edge of the recording chamber. This was done to ensure that each tactile stimulus was delivered to the same sensory field. Gill contraction was measured with a force-transducer (Grass FT 03C) connected by fine surgical thread to a single gill pinnule (see Peretz & Lukowiak, 1975). The thread was hung with a slight bow to prevent the exertion of tonic or stimulus-induced stretch on the gill pinnule (see Lukowiak & Peretz, 1977). There was no evidence of gill damage due to this procedure. The output of the transducer was recorded on a polygraph from which the measurements were made.

This method of gill withdrawal measurement has been shown to be equally effective to the photocell technique (Lukowiak & Peretz, 1977). It is preferred because the use of a photocell necessitates removal of the mantle. All preparations tested exhibited the large spontaneous gill respiratory movements (SGMs) which have been attributed to the activity of the Interneuron II network (Kupfermann *et al.* 1974). In all preparations the amplitude of the gill withdrawal reflex evoked by a 1 g stimulus was at least

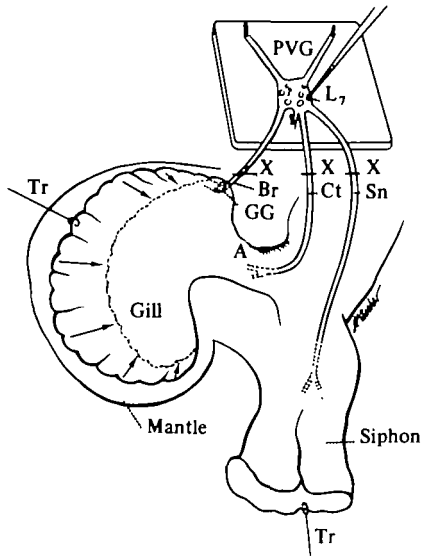


Fig. 1. The abdominal ganglion (parieto-visceral ganglion, PVG) innervates the gill via the branchial (Br), ctenidial (Ct) and a small branch of the siphon nerve (Sn, not shown). The siphon is innervated via Sn. The gill ganglion (GG) is located on Br and may play an important role in the mediation of the interaction between neural circuits mediating the gill withdrawal reflex (GWR). Punctate tactile stimuli were delivered to the siphon or the gill by a mechanical tapper. The GWR evoked by the tactile stimulus applied to the gill or the siphon involved the whole gill whether or not the PVG was present. The arrows indicate the direction of movement of the gill pinnules in response to the tactile stimuli and the dotted line indicates the extent of the contraction. Surgical thread (Tr) was attached to a single gill pinnule and led to a force transducer to record movements. Thread was also attached to the siphon, to immobilize it, so that the same receptive field could be activated each time. The PVG was pinned out on a clear Sylgard platform and trans illuminated which aided the impalement of identified motor neurones such as L_7 . Lines labelled X indicate where the Br, Ct and Sn are cut.

35% of the estimated SGM amplitude. These preparations, therefore, met the minimal response criterion as outlined by Carew *et al.* (1979).

Tactile stimuli were delivered to the siphon and gill by a 'Tapper', which was a plastic coated wire, 1 mm diameter, connected to a solenoid (see Peretz & Lukowiak, 1975 for complete description). The duration and amplitude of the voltage impressed across the solenoid, and the distance between the tapper and the stimulation site (1 mm) determined the force applied. The stimulus intensity normally used was 1000 mg. The force exerted by the tactile stimulator was calibrated against known weights with the force transducer. All experiments reported here used the 'Tapper' to evoke the GWR. It needs to be noted that the stimulator used by Byrne, Castellucci & Kandel (1974) did not evoke a gill withdrawal reflex after removal of the CNS, whereas the 'Tapper' did. It is not understood why one type of stimulus can activate the PNS and not the other. Stimulation sites were restricted to the distal portions of the siphon and gill. Any locus within these regions was suitable. For example, the pinnule coupled to the transducer was used as a stimulus target in several experiments, while other pinnules were used in the remaining experiments. This variable had no effect on the results. It was necessary, however, to keep the stimulation sites constant throughout the course of a single experiment.

Neurone identification was consistent with that of Koester & Kandel (1977) and was based on several criteria. For neurone L₇, the criteria included: soma location, size, synaptic activity observed during SGMs, the appearance of one-for-one activity recorded from a gill pinnule by a suction electrode with an intracellularly recorded action potential (AP), and the type of gill movement elicited by depolarization of the neurone.

Micropipettes filled with 3 M-KCl and having a resistance of 5–50 MΩ were used. A bridge circuit in the electrometer (Getting M-5) allowed simultaneous recording and stimulation.

Preparations were allowed to rest for at least 1 h following surgery before any experiments were conducted in order to minimize the possible effects of the dissection. A 3 h rest interval was interposed between habituation sessions to allow for the complete recovery from the effects of the habituation (Peretz & Howieson, 1973; Lukowiak, 1979). Interposition of a 3 h rest between sessions prevents the occurrence of long term habituation. Carew & Kandel (1973) demonstrated incomplete recovery with rest periods of 1.5 h duration. Their training schedule also led to the acquisition, and up to one week retention of long term habituation.

RESULTS

(A) *Central v. peripheral mediation of the GWR*

Preparations used in this study met the minimal response criterion as outlined by Carew *et al.* (1979); that is, the amplitude of the evoked reflex must be at least 35% of the amplitude of the spontaneous gill respiratory movements (SGMs). These gill respiratory movements are not to be confused with the smaller more frequent (1/60–90 s vs 1/6 min) pinnule flare movements (PFs). An example of each of the centrally generated spontaneous movements, along with the concomitant synaptic activity in a central siphon motor neurone (LB_{S1}) are shown in Fig. 2. LB_{S1} is one of several motor neurones that receive inhibitory synaptic input from Interneurone II, a central network of respiratory command cells that synapse onto motor neurones to produce the SGM (Byrne & Koester, 1978). Therefore, LB_{S1} provides certain identification of this spontaneous gill behaviour. Note that the peak of the SGM was clipped due to the magnitude of the contraction. However, the curvature of the trace immediately preceding the clipped portion suggests that only a small percentage of the contraction was not recorded.

In the same preparation, the GWR evoked by a 1 g stimulus applied to the siphon and gill are also shown in Fig. 2, both with and without the abdominal ganglion present. Although the GWRs evoked by gill stimulation are also slightly clipped, the response evoked in each case clearly exceeds the minimal response criterion. Notice that in this criterion preparation there was essentially no difference in reflex amplitude before and after removal of the abdominal ganglion. Data similar to these were obtained in all preparations used in this study ($n = 40$ preparations). These data show that in preparations which meet the minimal response criterion, the basic GWR, whether evoked by tactile stimulation of the siphon or gill, is primarily mediated by the PNS. The CNS is involved however in these gill behaviours since neural activity

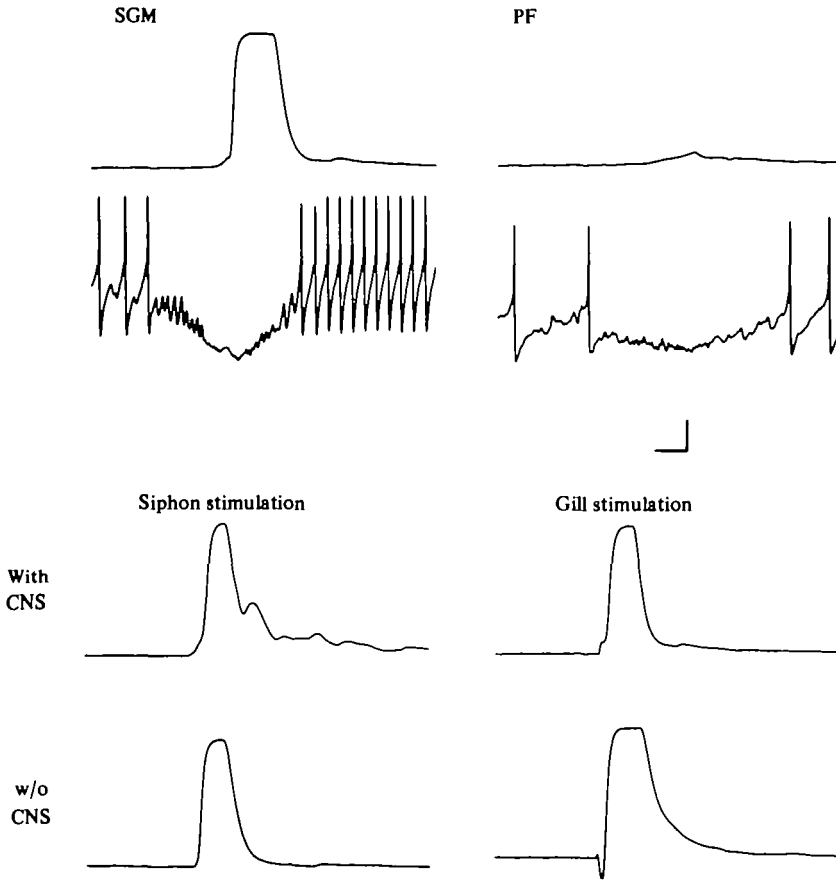


Fig. 2. Comparison between the amplitude of spontaneous gill respiratory movements and the evoked gill withdrawal reflexes. The large spontaneous gill movement (SGM; upper trace) is due to the activity of the Interneurone II network. A reflection of the activity of this network is seen in siphon motor neurone LB_{81} (lower trace). Gill motor neurone L_7 receives similar synaptic input during an SGM. The SGM was so large that the amplifier was blocked. The pinnule flare (PF) is a spontaneous gill movement of a lower amplitude than the SGM; but it occurs at a higher frequency (1/60–90 s *v.* 1/6–9 min). Associated with the PF is a weaker inhibition of LB_{81} (lower trace) and L_7 . In the same preparation the GWRs evoked by tactile stimulation (1 g) of the siphon and gill with and without the abdominal ganglion (CNS) are shown. All of the GWRs had amplitudes estimated to be in excess of 35% of the SGM and thus met the minimal response criterion. In addition, there was no decrease in the reflex amplitude following removal of the CNS with either siphon or gill stimulation. The small artifacts preceding the GWRs evoked by gill stimulation result from the proximity of the stimulation site to the recording site. Scale: 30 mV; 1 s.

is evoked in central gill motor neurones by both gill and siphon stimulation (Figs. 3 A, 5 B, 6). These data are in agreement with previously reported findings of Peretz & Howieson (1973) and Lukowiak (1979), but they do not agree with the recent findings of Carew *et al.* (1979).

(B) Transfer of habituation

To examine whether there is a transfer of habituation between siphon and gill, a frequency of stimulation had to first be determined which did not lead to habituation

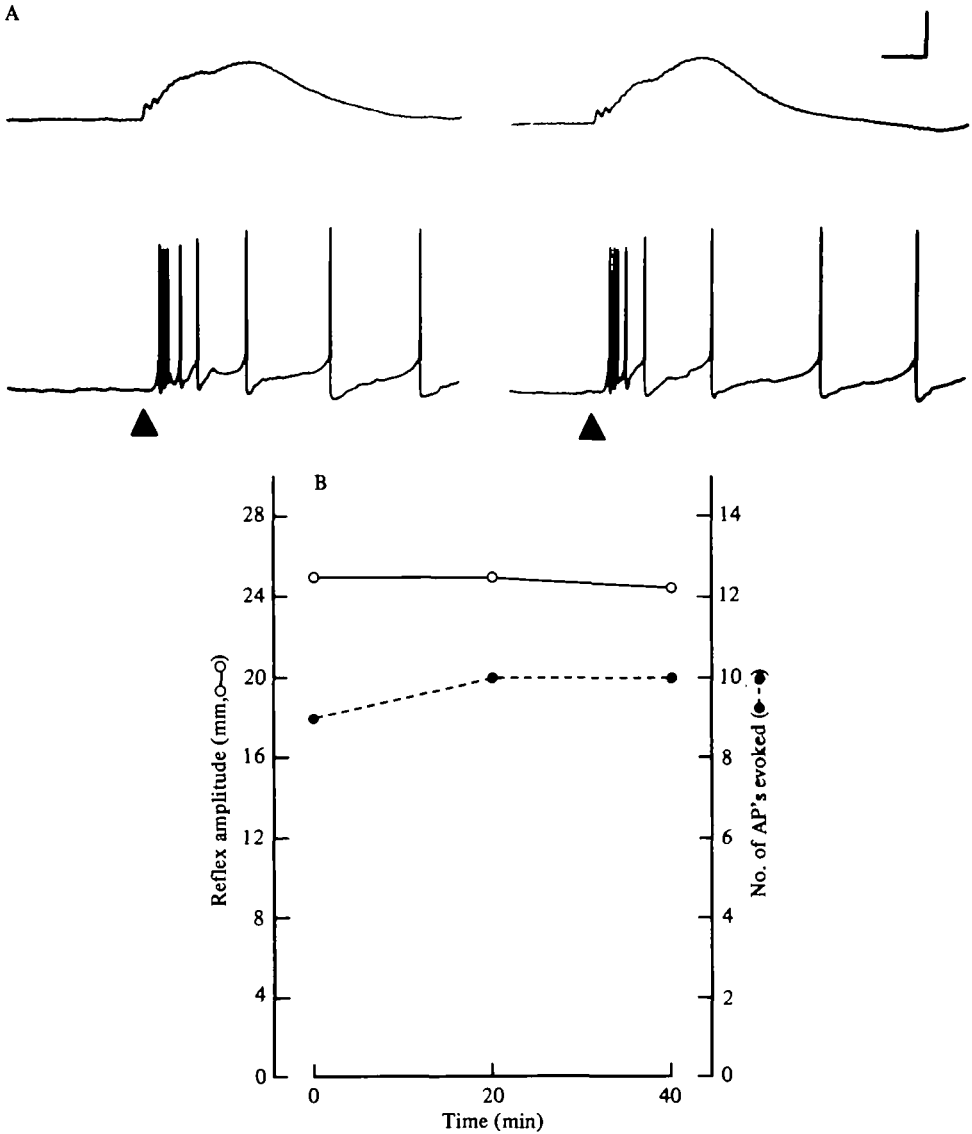


Fig. 3. An interstimulus interval of 20 m does not produce habituation. (A) A 1 g stimulus was presented to the gill (arrow) with an ISI of 20 min which did not result in habituation of the GWR amplitude nor in the number of APs evoked in L_7 . (B) A 1 g stimulus was presented to the siphon at an ISI of 20 m. Again, this frequency did not lead to habituation or facilitation of the GWR (O—O) or significant changes in the number of APs evoked in L_7 (● - - - ●). Scale: 20 mV; 400 ms.

or sensitization of the GWR. As shown Fig. 3A), with an interstimulus interval (ISI) of 20 min there was no significant change in either the reflex amplitude or the number of APs evoked in gill motor neurone L_7 by gill stimulation. Thus, we used a 20 min ISI to test for the transfer of habituation. When the siphon was stimulated with an ISI of 20 m (Fig. 3B, 3 trials) there was also no significant change in the

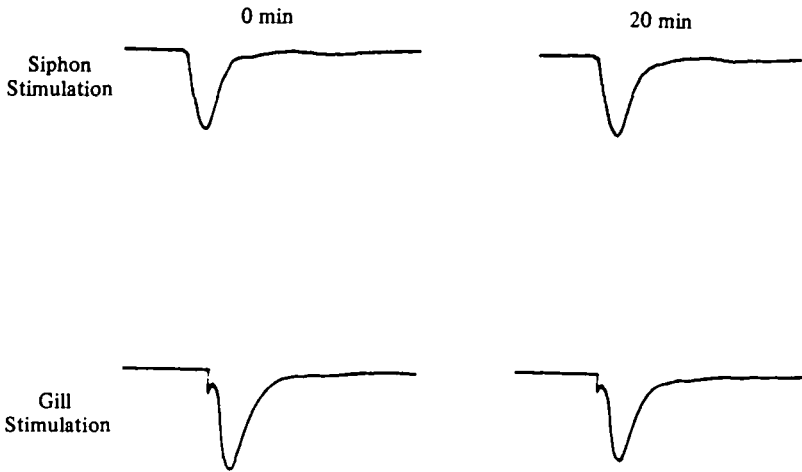


Fig. 4. An interstimulus interval of 20 min does not produce habituation following removal of the abdominal ganglion. Following removal of the CNS an ISI of 20 m did not lead to habituation or facilitation of the GWR evoked by siphon or gill stimulation (1 g).

GWR amplitude, nor in the number of APs evoked in L_7 . As shown in Fig. 4, an ISI of 20 min also did not produce habituation or sensitization following removal of the abdominal ganglion.

The experimental paradigm employed to determine whether transfer of habituation occurs is illustrated in Fig. 5A. The tactile stimulus is presented to one of the loci with an ISI of 20 m. The first stimulus (at time 0) serves as the control, while the second (at 20 m) serves as the test stimulus. Fifteen minutes after the control stimulus, a series of 10 stimuli were presented to the other locus with an ISI of 30 s. This ISI produces short-term habituation of the GWR. The test stimulus was delivered 30 s after the completion of the habituation run and 20 m after the control. If transfer of habituation occurs, the GWR evoked by the test stimulus should be significantly smaller than that produced by the control stimulus. If no transfer occurs, there should be little or no difference in reflex amplitude.

In the experiment shown (Fig. 5A), the control and test stimuli were presented to the gill (○—○) while the habituation stimuli (●—●) were presented to the siphon. As a result of the interposed habituation run, the GWR evoked by the test stimulus was markedly reduced from that evoked by the control presentation (3 vs 30 mm). In addition, the number of APs evoked in L_7 by the test stimulus was also reduced compared to control (■—■; 3 v. 11 APs).

The raw data from this experiment are shown in Fig. 5B. The transfer of habituation was seen behaviourally as a reduction in GWR amplitude and neurally as a reduction in the number of APs evoked in L_7 . It should be noted that gill stimulation evoked activity in L_7 much as does siphon stimulation.

When the stimulating sites were reversed, similar data were obtained (Fig. 6). The control and test stimuli were presented to the siphon, and stimuli were presented to the gill at an ISI of 30 s to produce habituation. As can be readily seen, the amplitude of the GWR evoked by the test stimulus was significantly smaller than that evoked

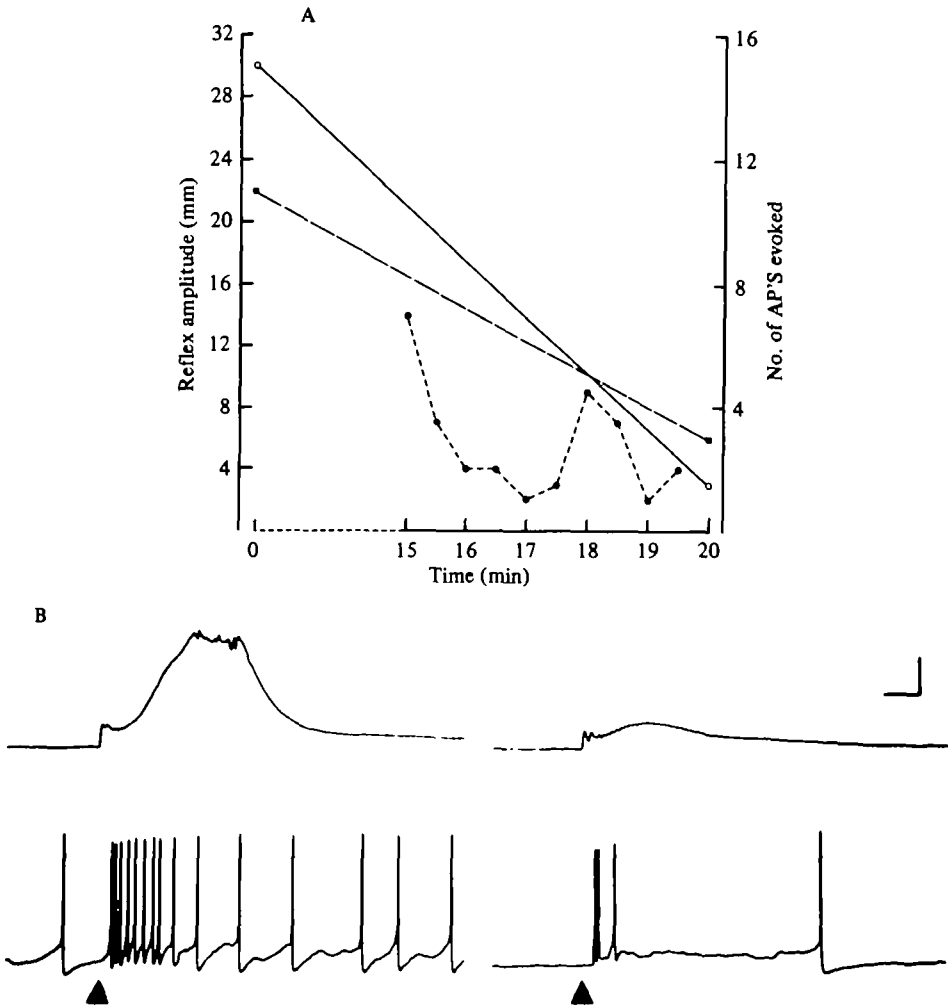


Fig. 5. Transfer of habituation. (A) The gill was stimulated (1 g) at an ISI of 20 m (○—○). However, at the 15 m mark 10 stimuli (ISI 30 s) were presented to the siphon (1 g; ●---●). This resulted in habituation of the GWR. This habituation of the GWR evoked by siphon stimulation led to a reduction in the amplitude of the GWR evoked by the gill stimulus. In addition to affecting the amplitude of the GWR evoked by the stimulus presented to the gill, the interposition of the siphon stimuli also brought about a reduction in the number of APs evoked in L_7 by the gill stimulus (■—■). Compare these data to those presented in Fig. 3A. (B) The raw data plotted in A. The interposition of the stimuli to the siphon which produce habituation lead to a reduction in the amplitude of the GWR and the number of APs evoked in L_7 by the gill stimulus (arrows). Scale: 25 mV; 400 ms.

by the control stimulus. Again, notice that the number of APs evoked in L_7 by the test stimulus was also significantly reduced compared to control.

Transfer of habituation was analyzed quantitatively to determine the magnitude of this learning behaviour, and its variation from animal to animal. It became immediately apparent that the 'behavioural state' (level of arousal) of the animal was the crucial factor in this analysis. Animals exhibiting behavioural suppression were not examined, as they failed to meet the minimum response criterion described above (see

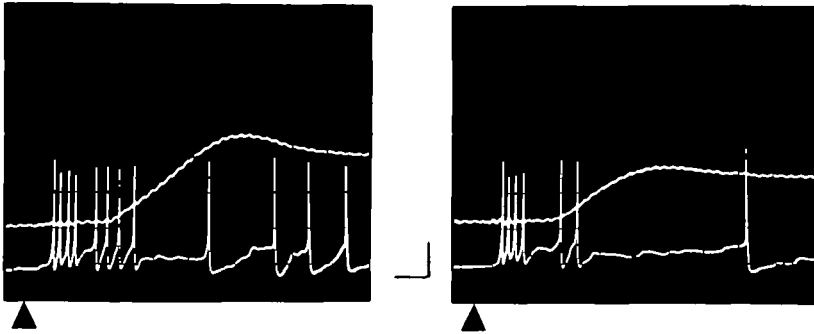


Fig. 6. Transfer of habituation in the other direction. The siphon was stimulated with an ISI of 20 ms and 10 stimuli were interposed to the gill (ISI 30 s) which resulted in habituation of the GWR. As a result of this habituation there was a reduction in the amplitude of the GWR and the number of APs evoked in L_7 by the siphon stimulus. Scale: 20 mV; 200 ms.

Lukowiak, 1980). However, animals displaying a facilitated behavioural state yielded markedly different results from those displaying 'normal' gill behaviour. The distinction between normal and facilitated behavioural state was made on the basis of the extent of habituation, and type of contractions evoked by tactile stimulation. In normal preparations, there was greater than 50% reduction in response amplitude over the course of a habituation session (Fig. 5A). Behaviourally facilitated preparations, on the other hand, did not exhibit response decrement with repeated stimulation. In these cases, the GWR amplitude either remained the same or increased with repeated tactile stimulation. In addition, the reflex contractions were usually much stronger and of longer duration than those observed in the normal preparations. The facilitated state was never observed after removal of the abdominal ganglion (CNS).

A normal behavioural 'state' was observed in 75% of the experiments performed with the CNS intact. The grouped data for these experiments are presented in Fig. 7A. The reduction in reflex amplitude due to the interposition of habituation stimuli at the opposite site was at least 50% (open bars) regardless of whether the control and test stimuli were delivered to the siphon (Siphon Stim) or gill (Gill Stim). Preparations in which the CNS was removed always displayed a normal behavioural state. The grouped data from these animals are also seen in Fig. 7A (striped bars). The GWR was reduced by 30% when the control and test stimuli were delivered to the gill, and 68% when the control and test stimuli were delivered to the siphon. Therefore, transfer of habituation was clearly evident, both before and after removal of the CNS.

As an internal control, the recovery of response amplitude was examined in five experiments. After a 3 h rest period, the response amplitude recovered to a mean of 109% of the original control amplitude (range 90–150% of control). The data from the facilitated state preparations are shown in Fig. 7B. In these experiments, a facilitation of the GWR was observed, rather than a transfer of habituation. The increase in reflex amplitude (open bars) was at least 50% over that of control regardless of which site was used for control-test stimulation and habituation stimulation.

The data (Fig. 7) show that habituation stimuli applied to the gill had a greater effect on the GWR evoked by siphon stimulation than vice versa. However, there is

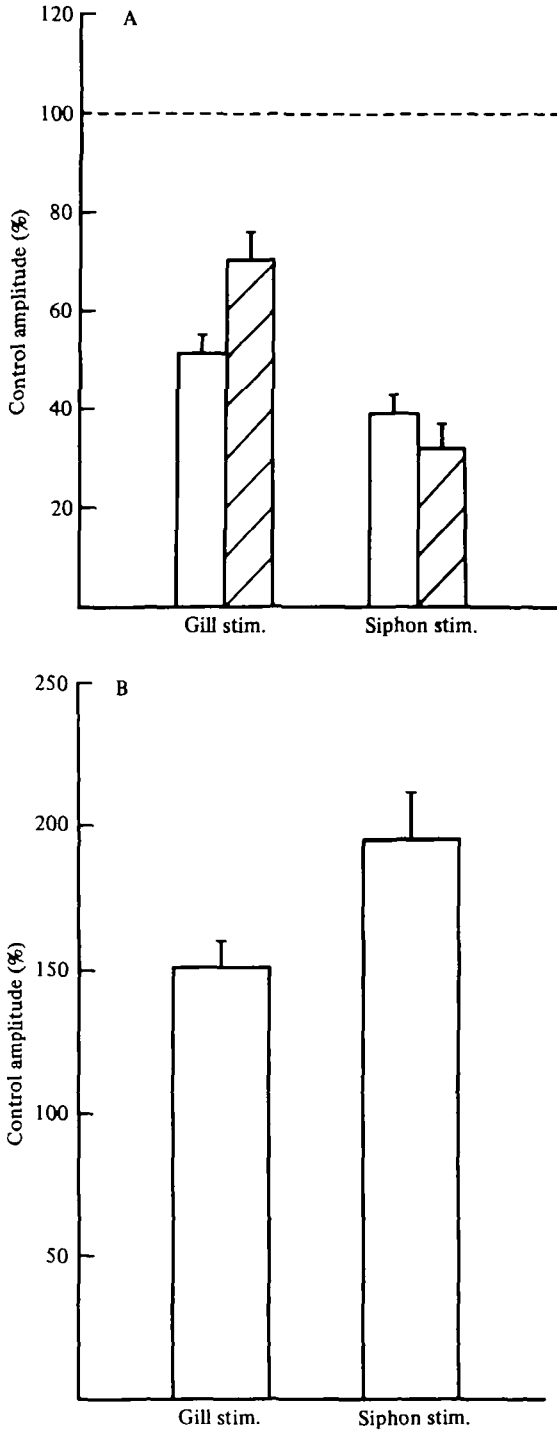


Fig. 7. Quantitative measure of habituation transfer. With the CNS intact, the occurrence of transfer of habituation depended on whether the preparation displayed normal state behaviour (7A) or facilitated state behaviour (7B). With the CNS removed, normal state behaviour and transfer of habituation were always observed (A).

(A) These preparations displayed normal state behaviour. In each experiment, the amplitude

no doubt that the interaction is bi-directional and can occur in the absence of the abdominal ganglion.

(C) *Dishabituation*

A further demonstration of the interaction between pathways was obtained by showing that activity in one pathway could dishabituate the reflex evoked by repeated stimulation of the other pathway. As can be seen (Fig. 8A) in data obtained from a preparation in which the siphon was repetitively stimulated (1 g, 1/30 s), the interposition of a 2 g gill stimulus between trials 20 and 21 dishabituated the reflex. Notice that although gill motor neurone LDG₂ received intense excitation as a result of the dishabitulatory stimulus, the activity evoked in it by the next siphon stimulus was not different from that on trial 20.

Another example of dishabituation is seen in Fig. 8B, along with the simultaneous activity in L₇. The habituation was evoked by repetitive siphon stimulation, while a gill pinch was used as a dishabitulatory stimulus. Although trial 11 produced behavioural dishabituation, the activity evoked in L₇ was not different than that evoked in Trial 10. These data are thus similar to those reported by Jacklet & Rine (1977). Note that the gill pinch itself did evoke intense excitation in L₇. Although not shown here, tactile stimulation of the siphon can dishabituate the reflex habituated by repeated tactile stimulation of the gill. If the neural circuits mediating these gill reflex behaviours were parallel and independent, dishabituation could not be obtained by the interposition of activity in the other circuit. The dishabituation therefore demonstrates an interaction between the neural circuits.

DISCUSSION

The GWR in *Aplysia* can be evoked by tactile stimulation of the siphon or gill. The neural circuits which mediate the GWR evoked by stimulation of either locus could be parallel to and independent of each other, or they could interact and form an integrated system. Kupfermann *et al.* (1971, 1974) reported that the neural circuits which mediated the GWR evoked by weak to moderate intensity siphon and gill

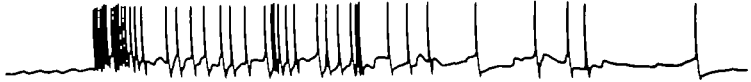
of the control reflex was normalized to 100% (dotted line) while the amplitude in each experiment of the reflex evoked following the interposition of habituating stimuli to the other site was expressed as a percentage of the control amplitude. Thus, the height of each bar is the mean amplitude (\pm S.E.M.) of the test amplitude in relation to the control amplitude. Gill Stim refers to the situation where the control and test stimuli were presented to the gill and siphon stimuli were interposed while Siphon Stim refers to the situation where the control and test stimuli were delivered to the siphon. The striped bars in each case are the data obtained after removal of the CNS. With Gill Stim the test stimulus was reduced to 51% of that of control when the CNS was ($n = 29$) present and to 71% of the control amplitude after the CNS was removed. With Siphon Stim the test stimulus was reduced to 39% of control with the CNS present and 32% after CNS removal ($n = 13$).

(B) These preparations (CNS intact) displayed facilitated state behaviour. In these experiments, the interposition of habituating stimuli to the other site resulted in facilitation of the GWR rather than in a reduction. The control amplitudes were again normalized (100%) and the amplitudes evoked by the test stimulus were expressed as a percentage of the control value (open bar). When the control and test stimuli were presented to the gill (Gill Stim), the mean test amplitude was 152% of that of control ($n = 7$) while with Siphon Stim ($n = 4$) the mean test amplitude was 195% of control.

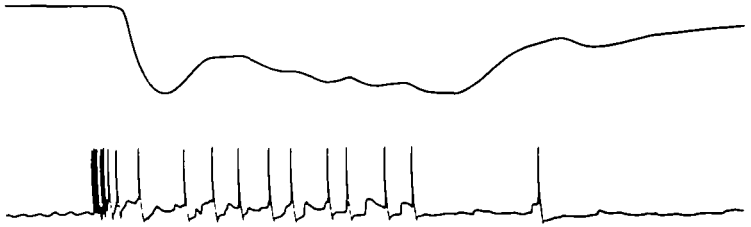
8A



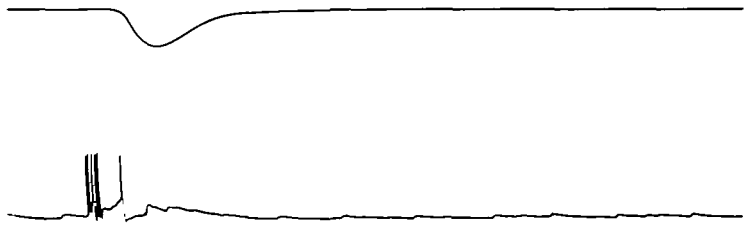
Trial 1
(1 g siphon stim.)



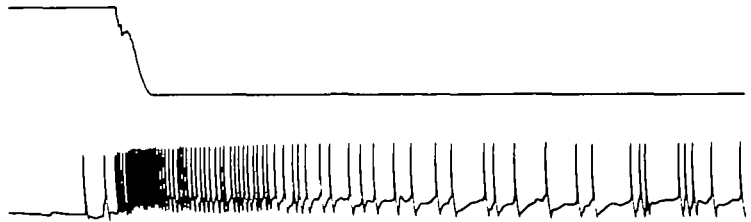
Trial 10



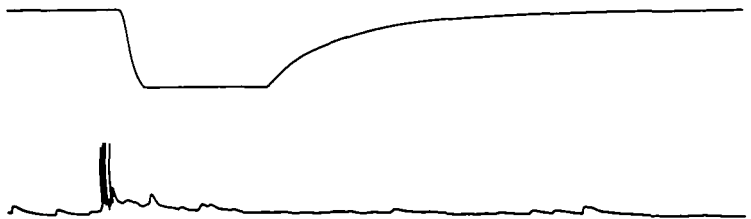
Trial 20



Novel stimulus
(2 g gill stim.)



Trial 21



8B

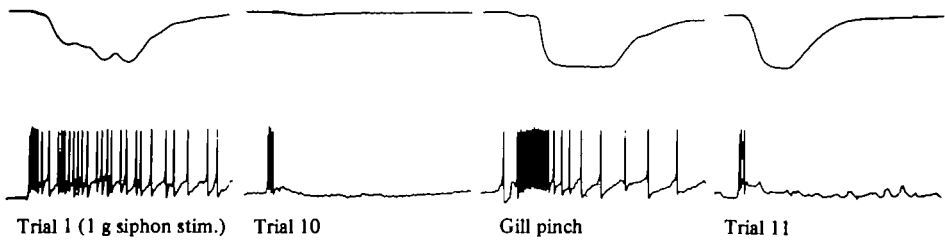


Fig. 8. Dishabituation of the GWR. (A) Repetitive tactile stimuli (1 g) were presented to the siphon (1/30 s), resulting in habituation of the GWR and a concomitant decrement in the number of APs evoked in LDG₂. Shown are trials 1 and 20. Between trials 20 and 21, a 2 g tactile stimulus was presented to the gill (gill stim). This dishabitatory stimulus evoked high frequency activity in LDG₂, and led to dishabituation of the GWR (trial 21). There was no difference in evoked LDG₂ activity in trial 20 and 21. (B) The GWR was again habituated by repetitive tactile stimulation of the siphon (1 g, 1/30 s) and a concomitant decrease in the number of APs evoked in L₇ was observed. Following the interposition of a gill pinch between trials 10 and 11, the amplitude of the GWR was dishabituated but there was not any concomitant increase in evoked activity in L₇ on trial 11. Notice again that the gill stimulus evoked high frequency activity in L₇.

stimulation were parallel and independent. Their conclusion was based on both neurophysiological and behavioural evidence. They found that only a very intense shearing stimulus applied to the gill could evoke activity in central gill motor neurones; and even then the input was minimal. On the other hand, weak punctate stimuli to the siphon were quite effective in evoking excitatory input to the same gill motor neurones. Punctate stimuli applied to the gill were not effective in evoking activity in gill motor neurones. They further reported that there was not transfer of habituation between the siphon and gill. Stimuli applied to one site had no effect on the response evoked by stimulation of the other site.

In contrast to the earlier reports of Kupfermann *et al.* (1971, 1974), our data leads us to favour the second possibility, that of an interaction between the neural circuits. Three lines of evidence indicate that the circuits interact and form an integrated system. Firstly, the central gill motor neurone pool received similar excitatory input whether the 1 g stimulus produced by the 'Tapper' was applied to the gill or siphon. For example, the activity evoked in L₇ by the 1 g gill stimulus (Fig. 3A, 5B) is quite similar to that evoked by a 1 g siphon stimulus (Fig. 6). We have found that a 1 g gill stimulus evokes short latency excitatory input to central gill motor neurones L₇, L₉, LDG₁, LDG₂, as well as central siphon motor neurones (Goldberg & Lukowiak, 1980). However, the excitatory input evoked in these central motor neurones by gill stimulation is of a slightly smaller magnitude than that evoked by siphon stimulation. It needs to be emphasized that this is not the first report that a weak stimulus applied to the gill evoked activity in a central gill motor neurone. Peretz & Howieson (1973) showed that a water drop falling 2 cm (approx 600 mg force) onto the gill evoked an excitatory post synaptic potential (EPSP) in L₇. The EPSP amplitude decremented with repeated presentation of this stimulus; as did the evoked GWR. Peretz & Lukowiak (1975) showed that in young *Aplysia*, a stimulus as weak as 200 mg could evoke an EPSP or AP in L₇ when applied to the gill. Lukowiak & Peretz (1977) also

showed that a 1 g stimulus applied to the gill evoked activity in L_7 , although they did not stress that this demonstrated an interaction. Thus, there is ample evidence in the literature to show that gill stimulation (weak to moderate intensity) can evoke activity in central gill motor neurones, similar to siphon stimulation.

The second line of evidence in support of the interaction between neural circuits is that of dishabituation. Dishabituation has been viewed as a superimposed facilitatory process on a decremental process, and not simply the removal of the habituating or suppressive process (Groves & Thompson, 1970). That is, the stimulus used to dishabituate the particular response must evoke activity in the circuit in such a way as to produce facilitation in the response pathway. For example, Castellucci & Kandel (1976) have shown that dishabituation of the GWR by the interposition of electrical stimulation of the pleural-abdominal connectives is brought about by a process of pre-synaptic facilitation of the central sensory neurone input to the central gill motor neurones. In other words, those two particular systems interact. Here (Fig. 8) we have shown that a tactile stimulus applied to gill can dishabituate the reflex evoked by siphon stimulation. Thus, activity in one circuit affected the response mediated by the other circuit, demonstrating an interaction between circuits.

It has been our experience that the dishabituation of the GWR produced by the interposition of a gill or siphon pinch was not as evident at the neural level as it was at the behavioural level (Fig. 8). That is, there was not much difference in the number of APs evoked in LDG_2 or L_7 following the dishabituating stimulus as the trial preceding it; even though there was a large increase in the amplitude of the GWR. Similar data have been reported previously by Jacklet & Rine (1977), and dishabituation was attributed to facilitation at the neuromuscular junction. The facilitation was due presumably to the high frequency activity evoked in the central gill motor neurones by the dishabituating stimulus as was shown here in Fig. 8. In addition, at least part of the dishabituation must be due to activity in the PNS (see below).

The third line of evidence was that of transfer of habituation. The data clearly show that habituation of the GWR by stimulation of one pathway affected the response evoked by stimulation of the other. When stimuli were presented at an ISI of 20 min. the responses were virtually identical (Fig. 3, 4), yet in most preparations when an habituation series was imposed between the control and test stimuli, the test response was significantly reduced (Fig. 7A). In those preparations where the response was not reduced, it was greatly facilitated. Again, if the neural circuits which mediate the GWR evoked by siphon or gill stimulation were parallel and independent, we would not expect to obtain these results. But, if the circuits do interact and form an integrated system, then such results should be expected.

As previously mentioned, Kupfermann *et al.* (1971, 1974) failed to find transfer of habituation between siphon and gill stimulation sites, and, therefore, concluded that the pathways mediating the GWR evoked at either locus were parallel and independent. A possible explanation as to why they failed to observe the transfer and we did may be attributed to the stimuli used. In their earlier studies, two different stimuli were used – one for each site. The stimulus presented to the siphon was produced by a jet of sea water (800 ms duration and between 3–6 g) while a shearing stimulus (unknown duration and intensity) was applied to the gill. A more controlled measure of habituation transfer would have been obtained if the stimulus location was

the only variable in the experiment. In our experiments, the siphon and gill were presented with identical stimuli (40 ms duration, 1 g). It is also possible that the stimulus produced by the 'Tapper', when applied to the gill, may better activate the neural circuit mediating the GWR than the brush strokes.

Transfer of habituation was the one parametric characteristic of habituation, as outlined by Thompson & Spencer (1966), that previously was not exhibited by this preparation. In addition to Kupfermann *et al.* (1971, 1974), Carew *et al.* (1971) also did not observe transfer or generalization of habituation between the siphon and mantle shelf sites used to evoke the GWR. Transfer was not seen either behaviourally or neuronally. This was consistent with the finding that each stimulation site was subserved by a particular set of central mechano-receptor neurones (Byrne *et al.* 1974), which then synapse onto the central gill motor neurones. We, however, found that transfer of habituation was manifested at both the behavioural and neural level (Fig. 5-7). Since the transfer does not occur at the level of the central gill motor neurones, as indicated by the Carew *et al.* (1971) study; the transfer possibly occurs 'upstream' at the level of the sensory neurones, or at the level of interneurones which presynaptically gate the sensory input onto the gill motor neurones (Lukowiak & Peretz, 1980). Experiments are presently underway using an isolated siphon preparation, in which the PNS between siphon and gill has been removed (see Kupfermann *et al.* 1971, 1974; Lukowiak, 1977) in an attempt to distinguish between these two possible explanations.

The PNS and gill behaviour

Recently, Carew *et al.* (1979) concluded that the CNS mediated 90-95% of the GWR evoked by siphon stimulation if two important conditions were satisfied: (1) the servo-controlled probe (see Byrne *et al.* 1974) is used to produce the tactile stimulus; (2) the reflex amplitude evoked must be at least 35% of the amplitude of the SGMs. In the experiments reported here, we tested whether a conclusion similar to that reached by Carew *et al.* (1979) could be drawn when the 'Tapper' was used. We employed the same minimal response criterion (Fig. 2) but we found no difference in the reflex amplitude before and after the abdominal ganglion was removed (Fig. 2). Therefore, the PNS in the gill, and between the siphon and gill, appears to be responsible for mediating the GWR evoked by the 'Tapper'. It remains to be determined why the stimulus produced by the 'Tapper' is capable of evoking the GWR in the absence of the CNS while that produced by the servo-controlled probe is incapable of evoking it, particularly since both stimulators evoke similar activity in central gill motor neurones.

The data further show that the PNS, in addition to mediating the GWR, its habituation, and its dishabituation (see Peretz, Jacklet & Lukowiak, 1976), can also mediate the transfer of habituation. Thus, the neural circuits which make up the PNS between the siphon and gill which mediate the GWR evoked by siphon stimulation, interact with the PNS in the gill which mediates the GWR evoked by gill stimulation. How and where this interaction occurs is not known, but the collection of neurones on the branchial nerve in the gill periphery, known as the gill ganglion (Fig. 1; Peretz & Moller, 1974; Peretz & Estes, 1974) may play an important and necessary role. This remains to be determined.

Unlike the gill-reflex behaviour discussed above, spontaneous gill behaviour changes markedly upon removal of the CNS. Rhythmic SGMs no longer appear, as they are mediated by a central pattern generator located within the abdominal ganglion (Byrne & Koester, 1978). However, spontaneous non-rhythmic gill contractions do occasionally occur in the absence of the abdominal ganglion (unpublished observations). Therefore, it is increasingly apparent that the PNS is a complex nervous system that cannot be disregarded in an analysis of gill behaviour.

CNS and gill behaviour

As indicated above, removal of the abdominal ganglion (i.e. CNS) did not affect the amplitude of the GWR, its habituation or the transfer of habituation. The question that must therefore be raised is what is the role of the CNS in the mediation of gill reflex behaviour? It appears that the CNS's major role is to exert suppressive and facilitatory control over the reflex which is basically mediated by the PNS (Lukowiak, 1977; 1979). Depending on the 'state' of the animal, the stimulation site, and the nature of the stimulus, gill reflex habituation can be prevented or augmented by the activity of control neurones in the CNS. For example, the CNS's suppressive influence of the PNS is greater in satiated animals than in unsatiated ones (Lukowiak, 1980). The suppressive influence exerted by the CNS over the PNS can also be increased by perfusing an endogenous neuropeptide hormone, arginine vasotocin (Lukowiak *et al.* 1980) over the abdominal ganglion (10^{-18} M). On the other hand, it is not well understood why the *Aplysia* exhibits facilitated state behaviour. However, this state can be induced by dopamine perfusion (10^{-7} M) through the gill (Ruben & Lukowiak, 1979). In this present study, the facilitated state was expressed in 25 % of the preparations tested. The test for transfer of habituation resulted in an increase in reflex amplitude in these preparations, rather than the decrease observed in the 'normal' preparations (Fig. 7). When the CNS was removed, the facilitation was abolished and transfer of habituation was then observed. Thus, the CNS mediated a facilitatory influence over gill-reflex behaviour. The PNS does not appear capable of mediating a similar facilitatory 'state' by itself. The PNS is less able to mediate the full range of adaptive gill-reflex behaviour. However, in conjunction with the CNS, a wide range of adaptive gill-reflex behaviour in response to various environmental and stimulus conditions is possible.

CONCLUSION

The data presented here further support the hypothesis that the CNS and PNS interact and form an integrated nervous system which mediates adaptive gill reflex behaviour. Only if the systems which mediate the GWR evoked by siphon or gill stimulation interact, could transfer of habituation occur. The PNS by itself is capable of mediating the GWR, its habituation and the transfer of habituation. However, transfer of habituation is seen at the neuronal level in the CNS and the CNS plays an important role in exerting control over the PNS. The CNS control bestows greater plasticity to the gill reflex behaviour. The finding that transfer of habituation occurs in *Aplysia* means that this model system now possesses all the parametric charac-

teristics of habituation. Finally, the data again emphasize the need to take into account the entire integrated system in any analysis of the neural mechanisms of habituation in this preparation.

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