HABITUATION AND INHIBITION OF THE CRAYFISH LATERAL GIANT FIBRE ESCAPE RESPONSE

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SUMMARY

1. Decrement of the lateral giant fibre escape response was studied in intact, restrained, crayfish and in those with the ventral nerve cord transected at the thoracic-abdominal level.

2. Taps (delivered at rates of 1 per 5 min to the abdomen) depressed responsiveness to about 50% of its initial value in 10 trials, for both intact and operated animals.

3. With additional stimulation, responsiveness dropped to near zero for both groups. Recovery was negligible 2 h later, but nearly complete after an additional 24 h rest.

4. Protection against response decrement in this situation was obtained by directly activating the cord giant fibres 30 msec prior to the tactile stimulus. The directly-elicited giant fibre spikes which follow the tactile stimulus do not influence the course of response decrement.

5. The results establish the decrement as centrally mediated habituation, and minimize the role of receptor alterations or descending neuronal influences in the behavioural change.

6. A comparison is made between the properties of habituation and the homosynaptic depression of afferent to interneurone synapses that is presumed to be the physiological mechanism of habituation in this situation.

INTRODUCTION

Crayfish escape behaviour includes a rapid abdominal flexion mediated by a large pair of command interneurones (the lateral giant fibres, or LGs), which are triggered by phasic tactile input to the abdomen (Wine & Krasne, 1972). Escape behaviour in general is quite variable (Krasne & Wine, 1975) and, in particular, the lability of the LG response has been documented in physiological preparations of isolated abdomens. The amplitude of the compound EPSP, elicited by shocks to a segmental root and recorded from the LG, shows a profound decrement at stimulus frequencies in excess of 1 per min. Recovery occurs rapidly and seems to be largely complete within 15 min (Krasne, 1969). Physiological analysis of this short-term decrement indicates that the
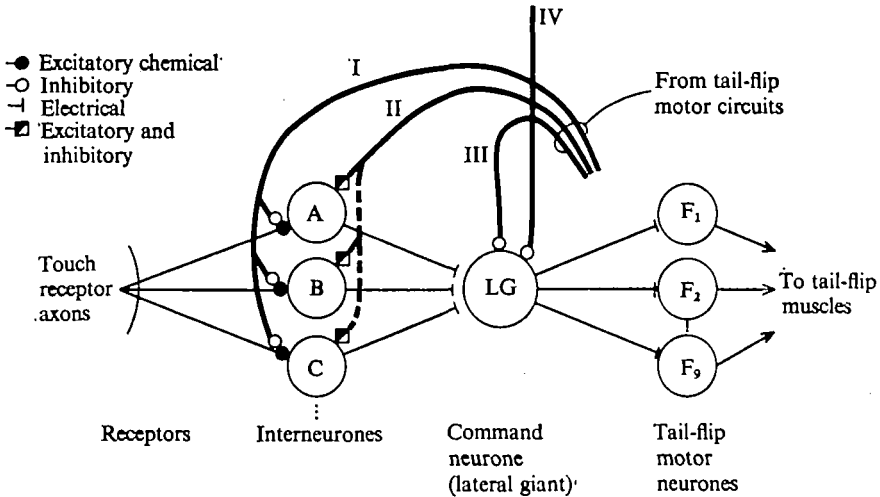
Fig. 1. Excitatory and inhibitory pathways in the lateral giant fibre (LG) escape response circuit showing the excitatory chemical synapses of receptors onto interneurones, and inhibitory pathways I, II, and III (from motor circuitry) and IV (from rostral CNS). Some pathways not relevant to this paper have been omitted. (After Krasne & Bryan, 1973; Krasne & Wine, 1975; Roberts, 1968; Zucker, Kennedy & Selverston, 1972).

mechanism involves homosynaptic depression at the first central synapse between afferents and sensory interneurones (Zucker, 1972a, and Fig. 1.)

In this paper we examine the decrement of the LG-mediated escape response in intact, restrained crayfish. A brief report of some of these results appeared earlier (Wine & Krasne, 1969).

METHODS

General procedures

Procambarus clarkii measuring approximately 7 cm from rostrum to telson and of either sex were obtained from a local supplier. Animals were cooled to near freezing prior to surgery. Pairs of silver-ball electrodes, for recording or stimulation, were slipped through small slits in the ventral integument of the abdomen so as to make contact with an abdominal connective and the fast flexor muscles. In some animals the thoracic-abdominal or first abdominal connectives were exposed and then severed, the ventral artery being left intact. Experimental animals were maintained individually in well-aerated, 19 l aquaria, each being suspended from a rigid support to which they were pinned through the posterior portion of the gill chamber on each side (Fig. 2). During testing their abdomens were extended by a thread to permit precise stimulation. One or two days were allowed for recovery before testing for thresholds. Lateral giant mediated tail flips were evoked by tapping the side of an abdominal pleural plate (Wine & Krasne, 1972). In Experiment I the taps were produced by pulling a flexible metal rod a measured distance away from the side of the animal and then releasing it, the output from a microphone taped to the top of the rod triggering the oscilloscope. In Experiment II the excursion of the rod was controlled by a solenoid with a spring return.

Nerve and muscle potentials were recorded on magnetic tape or displayed on a
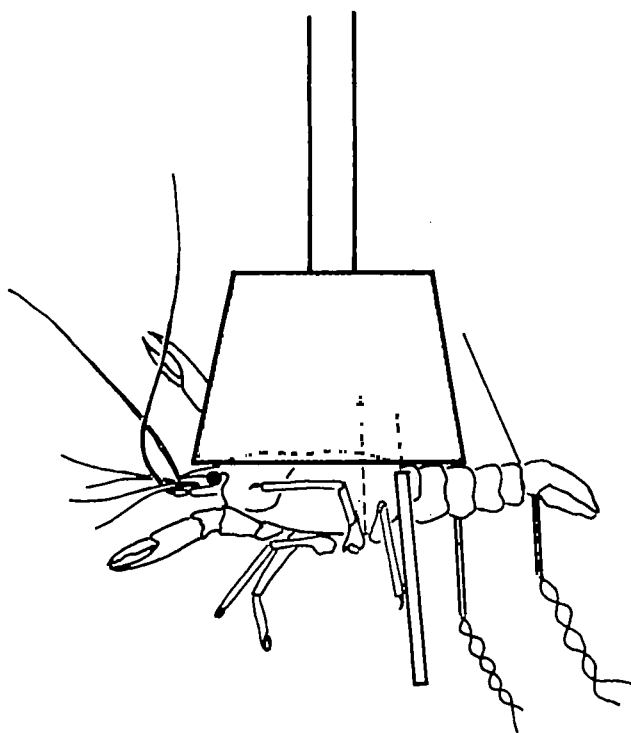
Fig. 2. Experimental procedure for stimulating and recording. See text for details.

storage oscilloscope for immediate analysis. Large amplitude spikes produced at short latency (4–15 msec) by tap stimuli to the abdomen, and directly followed by fast flexor muscle potentials, were presumed to be LG spikes (Wine & Krasne, 1972). This was checked in several preparations by comparing reflex-elicited spikes with those produced by just supra-threshold, direct shocks to the caudal connectives. The LGs, which are the largest ($>$ 100 μm) axons in the caudal connectives, tend to be selectively fired by such shocks. Fig. 3 compares direct and reflex-produced spikes in three animals and demonstrates that they are very similar in size, shape and direction of travel, supporting the assumption that they are LG spikes.

Approximate thresholds for LG responses to taps were determined for each animal. A maximum of 10 stimuli were given, at 1 per 5 min intervals, to minimise habituation. We started with an intermediate intensity and then adjusted the intensity to find the minimum level adequate to elicit a response at least twice; this level was then used consistently in subsequent sessions. Testing began 1–2 days after threshold determination.

Experimental design

Two experiments were run. Experiment I involved three groups of animals. Group I (normals, habituated, n = 6). Intact crayfish were stimulated as follows. (*a*) Initial test: 10 taps at a rate of 1 per 5 min to determine initial excitability. (*b*) Habituation to criterion: further taps at 1 per min in blocks of 10 separated by 10 min rests until completion of a block without any responses. (*c*) Post-tests: 10 taps at 1 per 5 min
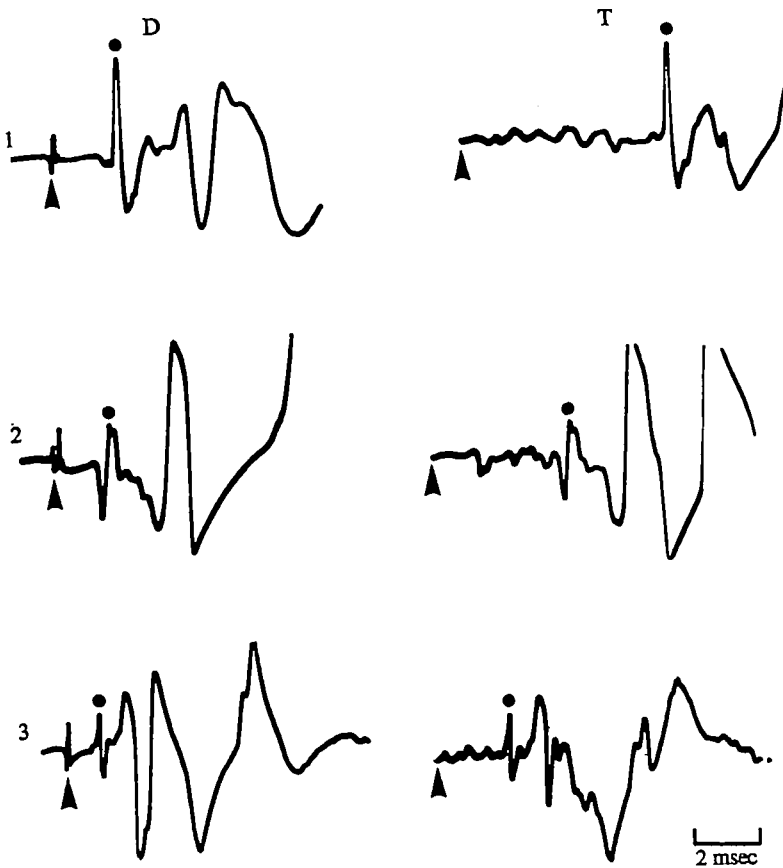
Fig. 3. Comparison of reflexly- and directly-elicited giant fibre action potentials in 3 preparations. Triangles indicate either just suprathreshold cord shock (D) or a tap to the pleural plates (T). Both kinds of stimuli elicit large, rostrally travelling spikes of near identical form (dots) that precede fast flexor muscle potentials.

starting at 0, 3, and 28 h after habituation to criterion. This entire procedure was repeated three times for each animal with several days separating one run from the next.

Group 2 (operated individuals, habituated, $n = 8$). Animals whose abdomens had been neurally isolated from the rostral CNS by cutting the thoracic-abdominal connectives were treated in the same way as Group 1. The rostral nervous system of the crayfish can suppress escape behaviour via a descending pathway (Fig. 1, and Wine & Krasne, 1975). This group controls for the influence of such inhibition on habituation.

Group 3 (controls, $n = 6$). Normal crayfish were treated as in Group 1 except that habituation to criterion and the 0 and 3 h post-tests were omitted (the final test was actually run at 24 rather than 28 h in this group). This group thus provides a control to check for changes in reflex excitability which are unrelated to habituation 'training'.

The means of the three replications carried out in each animal were entered as single scores for that animal in data plots and statistical tests.

Experiment II was designed to determine if the response decrement demonstrated in Experiment I is due primarily to central changes rather than to peripheral effects such as receptor damage. The control experiment was based on the recent discovery that activation of the tail flip circuits inhibits, presynaptically, the labile synapse between tactile afferents and first-order interneurones in the LG circuit (see Fig. 1). During the period of inhibition (about 90 msec) the labile synapses are protected from use-induced decrement (Krasne & Bryan, 1973). The above conclusions are based on findings in acute preparations where shocks to segmental nerves were used as stimuli. By applying the same procedure to chronic animals where the stimulus is a tap, the central synapses can be protected while the receptors continue to be used. In this way any contribution of receptor changes to the decrement seen in chronic animals can be parcelled out.

In Experiment II, all subjects had the 1–2 connectives (i.e. those between the 1st and 2nd ganglia) severed, the stimulating electrodes being close to 2–3 connectives, and the recording electrodes adjacent to 4–5 connectives. Animals were mounted and maintained as in Experiment I, but stimuli were delivered automatically by a solenoid tapper to the side of the second abdominal segment. Two groups of animals were run.

Group 1 (protection $n = 10$). The testing procedure for this group consisted of a control run followed 48 h later by a 'yoked' experimental run. The control was as follows. (*a*) Habituation to criterion: taps being given at 1 per min, in blocks of 10 separated by 10 min rests, until a weak criterion of habituation was met. For about half the animals this criterion was five successive failures to respond during a single block. For the rest of the animals the criterion was the development of a block with at least three failures, followed directly by a block with at least seven failures. (*b*) 2 h post-test: 10 taps at a rate of 1 per 5 min, 2 h after completion of habituation to criterion. The subsequent experimental run was identical in all respects to the preceding control run, except that (i) 30 msec before each tap a direct cord shock fired the giant fibres, and (ii) the series of habituation taps, instead of being to the criterion, was identical in number and sequence to that during the preceding control run. This entire procedure was repeated an average of 2 times for each animal; two days separated one replication from the next. Each animal was represented on graphs and in statistical tests by its mean score taken over these replications.

Group 2 (control, $n = 5$). In this group reflex activation was directly followed by activation of the giant fibres. The rationale for this control group will be explained more fully below. The procedure was identical to that in Group 1 except that during the habituation sequences of experimental runs the direct activation of the giant fibres via cord shocks *followed* taps by 10–15 msec.

Statistical significance was assessed with either signed ranks or *t*-tests, both two-tailed.

RESULTS

Experiment I. Habituation of moderate duration

Reflex excitability, in both normal controls and operated groups, is depressed to about 50 % of its initial value after only 10 stimuli presented at 5 min interstimulus intervals (Figs. 4 and 5; first vs. last two trial blocks, normals* P < 0·01; operated animals, P < 0·01). Further stimulation at 1 per 5 min to the criterion of 10 successive failures drove reflex excitability to less than 10 % of its initial value (see 0 h test of Fig. 5; initial test vs. 0 h post-test: P < 0·01 for each group).

After 2 h of rest, recovery was negligible and the depression was still significant (Fig. 5, inset A, initial test vs. 3 h post-test: P < 0·01 for each group). However, during a 24 h rest following the 3 h post-test, considerable recovery occurred (Figs. 4, 5, and inset B) 3 h vs. 28 h post-test: P < 0·01 for each group. This recovery suggests that the profound depression seen at the earlier intervals in not due to general deterioration with time in the experimental situation.

Although the recovery at 28 h is considerable, it is not complete (Fig. 5, inset B) in either group (initial vs. final tests: P < 0·01, Groups 1 and 2). Such persistent depression could be due to either long-term habituation or to deterioration of excitability which is unrelated to the habituation training. A comparison of responsiveness at the terminal post-tests between normal, habituated, animals (Group 1) and the control animals that received an initial test but no intervening stimulation (Group 3) suggests that the effect might be due to habituation (Fig. 5). However, this difference is not statistically significant. It should be noted in this context that individuals in Group 3 might still retain after 24 h some effects of the habituation produced during the initial tests.

Virtually all of the characteristics of habituation and recovery seen in our tests apply equally to the normal and operated animals. However, as would be expected (Krasne & Wine, 1975), the excitability of the escape reflex in the operated animals was higher than in the normal ones. We attempted to equalize initial response probabilities in this study by determining stimulus thresholds individually for each animal, but this manoeuvre was not entirely successful in compensating for excitability differences, for as seen in Fig. 4, the responsiveness of the operated group was still somewhat greater than that of normal animals. When we further corrected for this difference, by expressing responsiveness during the various tests as percentages of initial responsiveness (as was done in Fig. 5), the behaviour of the two groups seems quantitatively very similar, particularly at the 0 and 3 h post-tests.

Experiment II. Protection against habituation

The prevention of short-term habituation, by presynaptic inhibition of the labile synapses between tactile afferents and sensory interneurones (Kranse & Bryan, 1973), can be used to analyse the response decrement observed in chronic animals. If the response decrement demonstrated in chronic animals can also be prevented by central inhibition, then the participation of peripheral changes in the response decrement is extremely unlikely. On the other hand, if the response decrement cannot be prevented,

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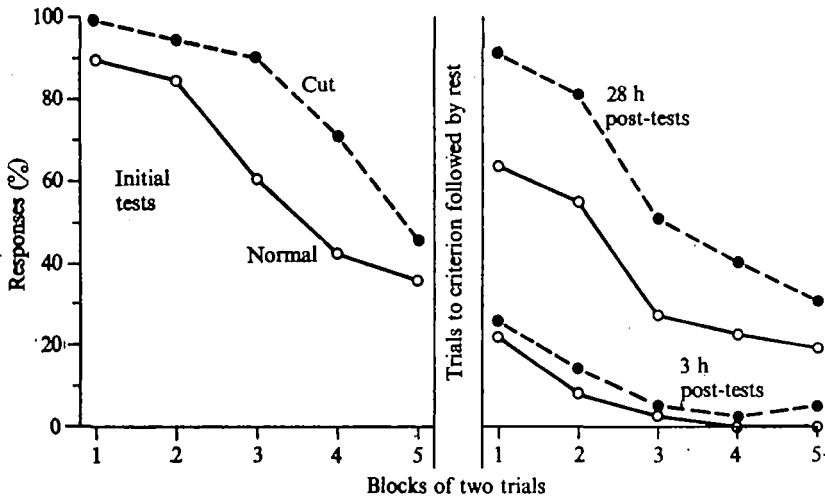* Habituation and 24 h controls were pooled for this test.

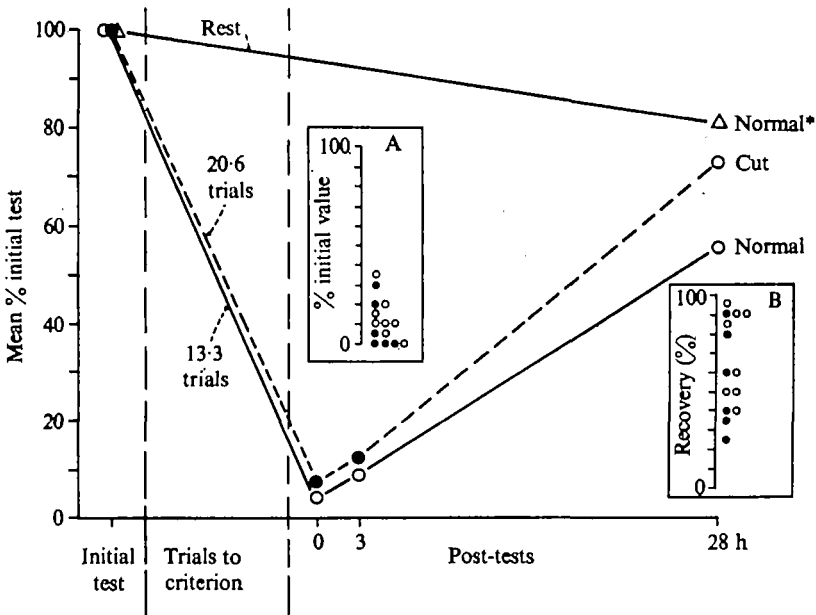Fig. 4. Habituation and recovery of normal and cord-transected crayfish, plotted in blocks of two trials.

Fig. 5. Habituation and recovery of normal and cord-transected crayfish, plotted as mean percent of initial test. Inset A shows distribution of scores, at 3 h post-test, of normal (dots) and cord-transected (circles) animals. Inset B shows the distribution of scores at the 28 H post-test. The asterisk on the upper curve indicates that the second test for the normal controls was at 24 rather than 28 h.
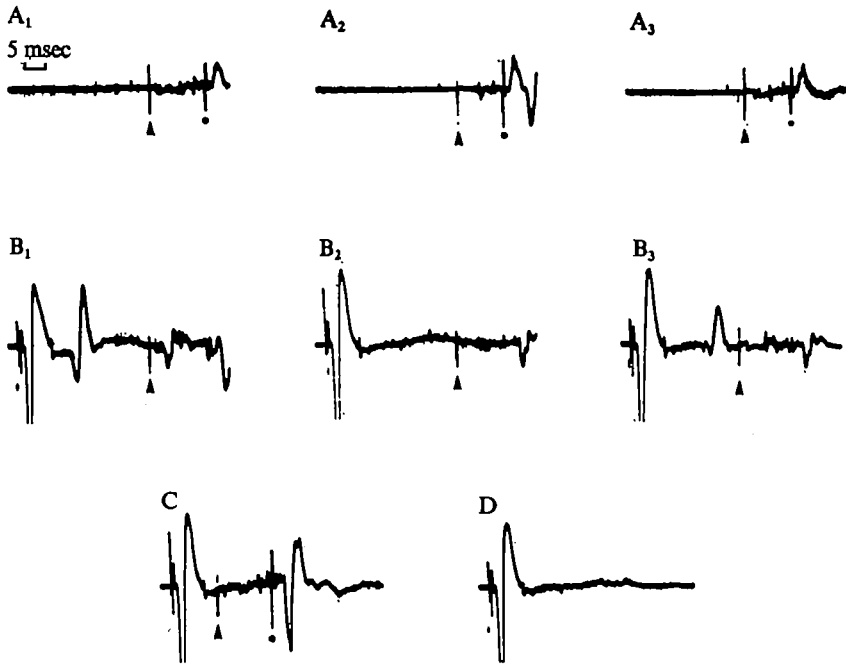
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Fig. 6. Inhibition of LG reflex by prior activation of tail flip motor circuitry. Strong tail taps, indicated by solid triangles, were given at 5 min intervals throughout the experiment. A_1–A_3 show responses to the 6th, 10th and 20th taps. LG responses, which occurred to each tap stimulus are marked by dots; muscle potentials follow the giant fibre spikes. In trials B_1–B_3 tap stimuli were preceded by shocks to the nerve cord which directly fired one or more cord giants at the start of each trace. B_1–B_3 were trials immediately adjacent to the corresponding trials in A. Note that the taps, which followed the directly-elicited responses by 25–30 msec, did not evoke LG spikes. However, C (the 12th tap of the series) shows that a tail tap given only 10 msec after a directly-evoked spike produces a reflex LG spike, presumably because inhibition was not developed fully at 10 msec. In D a direct shock to the cord was given by itself for comparison. The different sizes of LG spikes were due to small movements of the recording electrodes and to variations in the degree of synchrony between spikes in the two LGs.

then we must assume that short-and moderate-term decremental processes involve different mechanisms, including the possibility that peripheral changes contribute to the longer lasting decrement.

Inhibition of tap-produced responses. The above argument rests on the assumption that the phasic inhibition of the LG circuit that follows an escape command in dissected preparations can also be reliably produced in chronic animals. Fig. 6 illustrates that LG firing induced by supra-threshold tap stimuli was inhibited following the firing of the giant fibres. This inhibition was demonstrable in mild form at 10 and 20 msec after a giant fibre spike, and was strong at 30 and 50 msec. Thirty msec was chosen as the interval for testing for protection from habituation. (The inhibition in the chronic preparations seems to reach its maximum some 10–20 msec later than does that studied in reduced (primarily de-efferented) acute preparations. The basis for this difference is not understood.)

Protection experiments. When taps are preceded by directly-elicited giant fibre spikes, the normally observed response decrement does not occur (Fig. 7). The dashed line in Fig. 7 shows the extent of habituation observed during control runs. The average
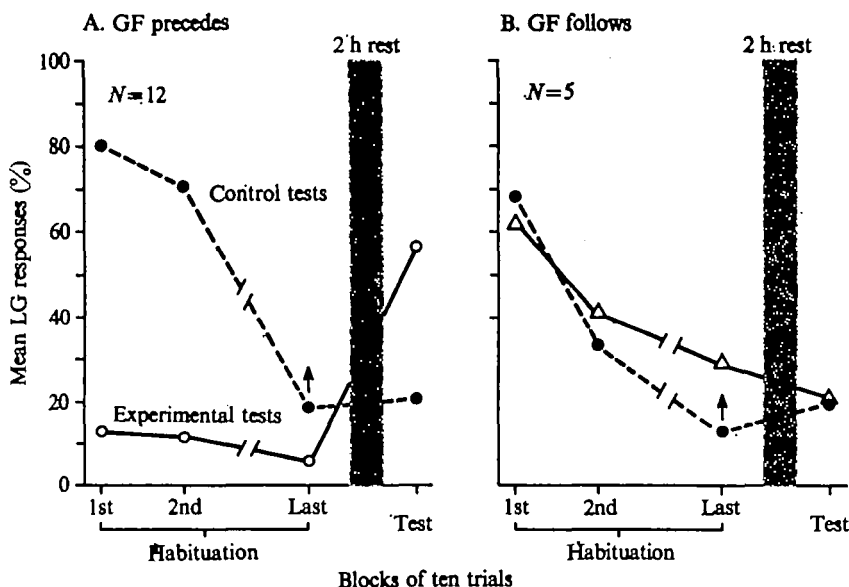
Fig. 7. Protection against habituation. (A) Control test, dashed line: Tap stimulation alone decreases responsiveness on a 2 h post-test to about 25 % of initial value. Experimental test, solid line: When taps are *preceded* by directly elicited giant fibre spikes, responsiveness is reduced to near zero, while responsiveness during 2 h post-test to taps alone is significantly greater than control. (B) No difference occurs between tests to taps alone (dashed line) and taps *followed* by electrically elicited giant fibre spikes.

response rate falls from 80%, initially, to 20% following habituation and a 2 h rest. Since each control run was continued until a specified criterion of response failure was reached, the percentage responses plotted for the last block of habituation trials probably underestimates the true response probability during this trial block (as indicated by arrows in Fig. 7).

The solid line in Fig. 7 shows the behaviour of the same group of animals during experimental runs. As expected, responsiveness is low during habituation training trials, because the reflex is inhibited. On the other hand, the inhibition is not total, hence, we did not anticipate perfect protection from habituation. After the 2 h rest, when the first test trials without inhibition were given, the average response rate rose to about 60%. If habituation trials on experimental runs had been as effective in causing habituation as those on control runs, responsiveness during the terminal block of test trials should have been equal in the two conditions.

In Fig. 8, the difference between the number of responses during experimental and control test trials is given for each animal and each pair of control and experimental runs. The difference between the experimental and control tests is statistically significant ($P < 0.01$).

Control experiments. While the above results are predicted by the protection hypothesis, it is possible that repeated activation of the giant fibres either facilitates or dishabituates the escape reflex. This would increase responsiveness on test trials so as to mask, rather than prevent, habituation. It is also conceivable that the apparent habituation seen on control runs is a statistical artifact of having run animals to criterion.
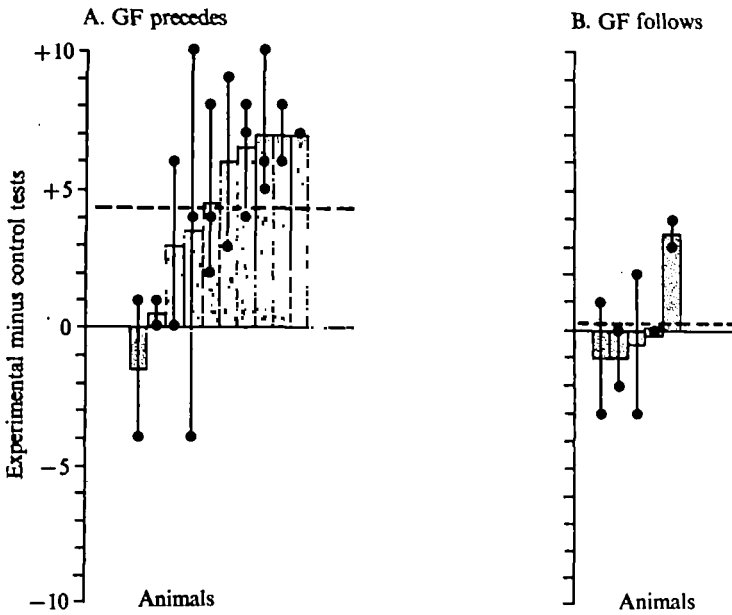
Fig. 8. Results from individual animals in protection experiment. For each condition (A, giant fibre shock precedes tap, and B, giant fibre shock follows tap) the mean responses given on experimental test trials minus the mean on control test trials is plotted as a shaded bar for each animal. The differences for each pair of experimental and control runs are also shown as solid dots; the distance between the dots along a given vertical line indicates the variability shown by individual animals on different runs. The bold dashed lines indicate grand mean differences.

This could be the case if the escape response were subject to fairly long cycles of spontaneous variation in levels of excitability. The procedure used on control runs could thus have merely run each animal until it spontaneously entered a phase of low excitability, which might be maintained until the 2 h post-test. On the other hand, since the testing schedule in experimental runs was not contingent on the animals current behaviour, these runs would not show this illusory habituation effect.

Both of the above possibilities were controlled for by Group 2 (Fig. 8B). The treatments of this group and Group 1 were identical in the features of direct activation of LGs on each habituation trial and a running schedule not contingent on current behaviour. The difference between the groups was in the relative timing of reflex and direct giant fibre activation. Figs. 7B and 8B show that there was no significant effect when direct LG activation *followed* tactile stimulation. The mean differences between paired control and experimental test runs in the two paradigms (Fig. 8, dashed lines) are significantly different (mean differences, GF precedes *vs.* GF follows, P < 0·02).

DISCUSSION

Although casual observations and preliminary work clearly indicated some kind of response decrement of the LG tail flip, these are the first quantitative and controlled studies of behavioural habituation in this system. An early attempt to study waning of escape behaviour (Krasne & Woodsmall, 1969) was carried out before the discovery

of non-giant tail-flip pathways (Schrameck, 1970; Wine & Krasne, 1972); subsequent work has established that the stimuli used by Krasne & Woodsmall are highly selective for the non-giant system (Wine & Krasne, 1972). Hence that study, together with the experiments reported here, establish that both LG and non-giant mediated escape behaviour show habituation of at least several h duration.

Very little is known about the central pathways which mediate non-giant escape behaviour, whereas the circuit for the LG response has been established (Fig. 1). A decrement in the compound EPSP of the LG to repeated stimulation has been demonstrated (Krasne, 1969) and the site of the short-term decrement has been localized to the first central synapse (Krasne, 1969; Zucker, Kennedy & Selverston, 1971; Zucker, 1972b). The response decrement in both chronic and acutely prepared animals is similar, but the time course of recovery appears to be much slower in chronic animals. As far as we know, no additional sites for habituation exist in the abdomen. Furthermore, the additional mechanisms of effector fatigue, receptor adaptation and incremental descending inhibition, which might operate in chronic animals but not in physiological preparations, have been specifically excluded by these and previous experiments.

While the site of lability is almost certainly the same for habituation in both kinds of preparation, it is possible that additional factors operate to prolong the time course of recovery in chronic animals. For example, Farel & Krasne (1972) have shown in the chronic spinal frog that an habituated reflex can be maintained in a depressed state by infrequent stimulation – even though such stimulation rates do not appreciably influence an unhabituated response. In chronic, as opposed to acute crayfish, greater activity can be expected in afferent nerves because: (1) the temperature is higher in the chronic experiments (18–20 vs. 13 °C); (2) many of the afferents in the acute experiment are completely silent because their receptive fields have been removed, although these cells still participate in the electrically-elicited response; (3) water currents are much greater in the large, aerated tanks used for chronic experiments; and (4) the animal's own movements act as a source of disturbance.

It is also probable that at least part of the discrepancy between recovery times in the two situations is an artifact which results from the different stimulus strengths and estimates of responsiveness used. The time course of recovery from habituation is known to be, at least in part, a function of the estimate of responsiveness used. Thus, even though the initial response in a test series is equal to control values, habituation usually proceeds more rapidly during subsequent sessions (Thompson & Spencer, 1966), therefore any procedure that averages a number of trials, or compares the rates of decline seen in different sessions, provides a more sensitive measure of habituation than does a single trial. In some preparations, habituation can be shown to last for days or weeks when measured in this way (Farel, 1971; Carew, Pinsker & Kandel, 1972).

In previous electrophysiological studies, both Krasne (1969) and Zucker (1972b) used primarily subthreshold stimuli and measured the amplitude of a single EPSP in the LG. In behavioural experiments, on the other hand, it is necessary to use stimulus strengths that are initially suprathreshold, and then to average a number of trials to obtain a measure of responsiveness. In the electrophysiological experiment that most closely approximated the chronic ones, Krasne (1969, Fig. 8), gave strong shocks (100 % responding [LG spikes] on first 10 trials) at a rate of 1 per min. After 5 sessions

(15 stimuli per session separated by 15 min rest periods) the response rate was 30 % but declined to zero at the 6th session. There was no recovery in behavioural terms (i.e. no LG spikes) after 15 min.

Comparisons between behavioural and electrophysiological experiments are complicated by factors such as the deterioration of the reduced preparation. There is at present, however, no compelling evidence which suggests that a fundamentally different physiological mechanism mediates habituation in chronic animals. However, this possibility can only be conclusively eliminated by demonstrating and analysing habituation of moderate duration in electrophysiological preparations.

A final point of interest is the demonstrated interaction between inhibition and habituation. The protection from habituation seen in these experiments was considerable, which supports the contention that recurrent inhibition can protect labile synapses from habituation produced by the animal's own movements (Krasne & Bryan, 1973). Furthermore, the parallel between the effects of recurrent inhibition on habituation in the chronic and acute experiments adds to the circumstantial evidence that habituation, in both the electrophysiological and behavioural experiments, is achieved by a single mechanism: homosynaptic depression of the synapses between tactile afferents and sensory interneurones.

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