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# ACTIVITY AND HABITUATION IN THE BRAIN OF THE POLYCLAD FLATWORM *FREEMANIA LITORICOLA*

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SUMMARY

1. A variety of spontaneously active units was measured in the brain of the polyclad flatworm *Freemania litoricola*. Following application of $MgCl_2$ there was both a decrease in number of active units and a decrease in frequency of firing of those cells which persisted in their activity.

2. Receptors which respond to vibration stimuli evoke potentials in the posterior part of the brain. Repetitive stimulation leads to habituation, the extent of which is dependent on both the number of times stimulated and the strength of the stimulus. Weaker stimuli habituate more rapidly than strong stimuli. Habituated responses can be dishabituated by tactile stimuli and also by stronger intensity stimuli of the same modality. The vibration-evoked potentials appear to occur in at least second-order cells, since vibration responses are abolished by the application of $MgCl_2$.

3. Tactile responses can also be elicited from the posterior portion of the brain when the stimulus is applied to the periphery of the animal. These responses are insensitive to $MgCl_2$.

4. Both vibration and tactile evoked responses are able to evoke further barrages of spike activity.

5. The presence of a dual sensitizing and inhibitory system during habituation is discussed.

## INTRODUCTION

The platyhelminthes occupy a strategic position in the evolution of structural complexity, as this is the first phylum to possess organ systems. Consequently, it is here that one first finds an anterior brain. The rest of the nervous system has many structural features which reflect the primitive nature of its organization; these include a plexiform network of strands making up the nervous system and the absence of ganglia. An understanding of the functions of the primitive brain should elucidate some of the factors underlying the evolution of this structure. Until now these kinds of studies have had to rely on behavioural observations and comparisons between normal and decerebrate animals (Gruber & Ewer, 1962; Koopowitz, 1970; Koopowitz & Silver, in preparation) as conventional neurophysiological methods could not be used. The major technical problems in handling these organisms are their fragility and mobility. These have now been solved, and this report describes

some of the initial observations and experiments designed to elucidate the neurophysiological organization of the brain in these animals. Certain units in the posterior regions of the brain appeared to be very sensitive to vibrations set up in the fluid surrounding the animal, and these have been used to investigate central habituation.

Habituation is the simplest example of behavioural plasticity that can be found and it occurs throughout the animal kingdom. It is apparent from recent reviews (Corning & Kelly, 1973; Eisenstein & Peretz, 1973; Wyers, Peeke & Herz, 1973) that even behavioural aspects of habituation in the flatworms have received little attention. Applewhite & Morowitz (1966, 1967) reported on habituation to mechanical tactile stimuli in the rhabdocoel *Stenostomum*. Habituation to these stimuli could occur in the absence of the brain (Applewhite, 1971) and hence one could not conclusively determine if this was a central or peripheral event. In the polyclad *Freemania litoricola* tactile stimuli evoke both central and peripheral events, which prove to be rather complexly interrelated. Surprisingly few examples of habituation in planarians have been reported (Westerman, 1963) and some studies even describe failure in attempting to habituate planarians (Brown, 1964; Bennett & Calvin, 1964). Groves & Thompson (1970, 1973) have suggested that behavioural habituation actually reflects a dual process of sensitization or facilitation as well as an habituation or waning of responsiveness. Sensitization appears to have received little attention from workers on invertebrate preparations. In *Freemania* it is possible to demonstrate that sensitization also occurs and hence that the dual process may be of general occurrence in the animal kingdom.

## METHODS

*Freemania litoricola* is a common intertidal polyclad flatworm beneath the rocks in protected areas of Puget Sound, Washington, U.S.A. The animals used in this study were between 2 and 3 cm long and 1 cm wide. They were maintained in the running sea-water tanks at Friday Harbor Laboratories and have also been kept for a number of weeks in stationary non-aerated sea water at the University of California, Irvine. They seem able to tolerate considerable fluctuations in ambient temperature, but all experiments were performed at $18{\cdot}5 \pm 1$ °C.

Polyclad preparations are difficult to handle and some general comments may help other workers who might wish to use these animals. The only reversible anaesthetic I have found is $MgCl_2$. The most effective concentration was 30% 0·36 M-$MgCl_2$ in 70% sea water. This immobilizes an animal within 1 h and the organisms can be kept in it for at least 12 h. Dissection is performed in this medium. A 10% 0·36 M-$MgCl_2$ solution in sea water may take 3 or 4 h to immobilize a worm. In stronger concentrations of $MgCl_2$, several hours for recovery may be required. Some species cannot tolerate such high concentrations, but *Notoplana acticola*, a common Californian flatworm recovers from 100% 0·36 M-$MgCl_2$ without apparent ill effects.

Anaesthetized animals are pinned on to a transparent base such as Sylgard so that they can be viewed with transmitted light. Under these conditions the nervous system tends to stand out, being a ruddy colour. The finest pins, such as 000 insect pins or minute pins, hold the animals best. Pins of wider diameter are not effective since the animal's body wall tends to disintegrate around these and when returned

to sea water the animals will tear themselves free. Further help in immobilizing the animals can be obtained by cutting off the marginal rim of the worm. The margin contains a high concentration of both sensory and adhesive cells and the organisms appear unable to grip the substrate if it is removed. It is not necessary to emarginate the entire animal; cutting off the anterior margin is most effective. In most of the preparations used here the anterior third of the animal was used with the margin excised. The brain can be dissected completely free from the animal, but this does not make a very viable preparation. The exposed brain will function for at least 3 days if the anterior portion of the animal is left attached. The epidermis overlying the brain can be cut with fine iridectomy scissors and then pulled off with fine forceps. Muscle overlying the brain can be teased off with an insect pin. The sheath surrounding the brain is very tough. If necessary, it can be torn open by sucking a large diameter (400 $\mu$m) suction electrode on to the sheath and leaving it in position for a few hours. The amount of pressure needed to rupture the sheath must be determined by experimentation.

In the experiments described here, polyethylene suction electrodes with fairly wide diameter tips (100–200 $\mu$m) were used with differential recording and a.c. pre-amplifiers (Tektronix 122). The record was displayed on an oscilloscope (Tektronix 565) and permanent recordings were made using a Brush chart recorder. The chart recorder pens were not fast enough to follow the spikes faithfully and there appears to be a 50% reduction in amplitude of some of the faster action potentials.

The stimuli administered were of two types, vibration and tactile. Vibrations were induced with puffs of air. A polyethylene tube with a tapered tip (opening 2 mm) was fixed at an angle of 30° some 8 cm above the surface of the bathing medium and pointed to the side away from the brain. The tube was connected to a 12 ml syringe by a length of polyvinyl tubing. Puffs of air were produced by rapidly depressing the plunger in the syringe. By administering the same volume of air, roughly comparable stimuli could be administered. Stimulus intensity could be changed, albeit rather crudely, by changing the volume of air and rate of plunger-depression. Other methods of producing vibrations such as dropping known weights known distances onto the bench did not seem very effective. Tactile stimuli were produced by prodding the margin of the animal with a polyethylene probe with a tip diameter of 0·5 mm.

## RESULTS

### *Spontaneous electrical activity*

Little spontaneous activity can be recorded from the anterior dorsal portion of the brain but a variety of units can be picked up from posterior and lateral regions. Activity varies from a more or less tonic background firing in some preparations (Fig. 1*a*) to more phasic and unpredictable bursts of action potentials in other preparations (Fig. 1*b*). A few preparations were more or less silent. Closer analysis indicates that there are at least four kinds of potentials which can be recorded at any one site. There are positive-going and negative-going monophasic potentials and biphasic potentials with either positive-going or negative-going phases leading (Fig. 1*c*). Any real significance to these differences remains to be ascertained. Spike amplitudes, measured on the CRO, varied in amplitude up to 500 $\mu$V. Most biphasic

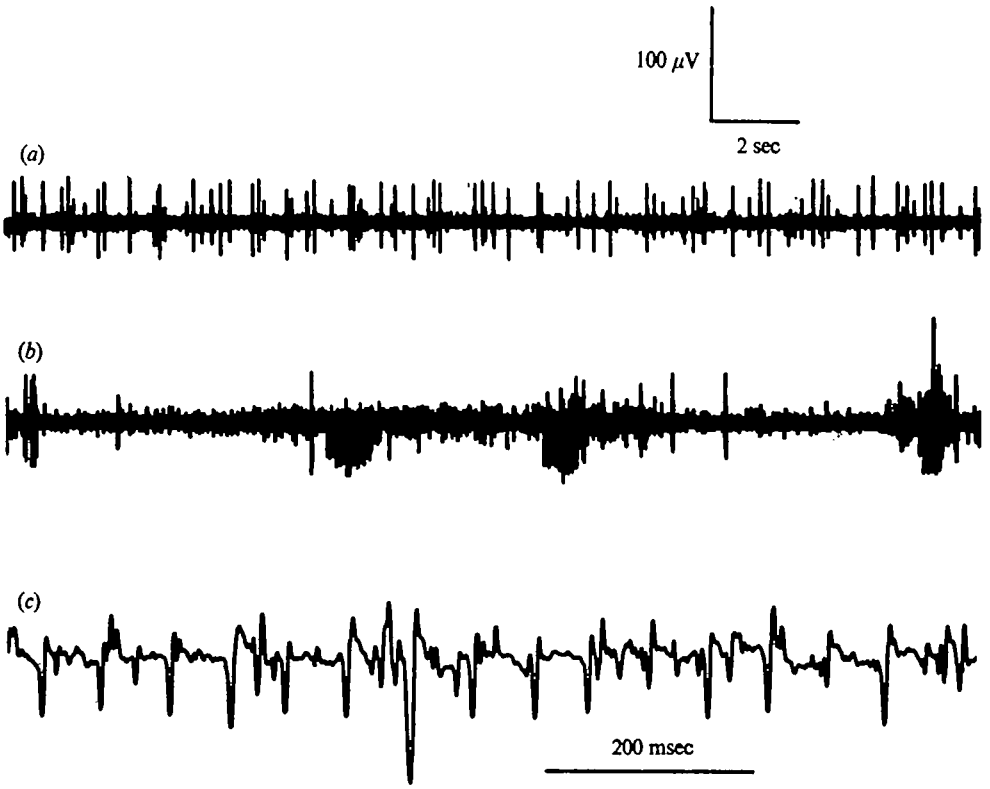


Fig. 1. Spontaneous activity recorded from the brain of *Freemania*. (*a*) Tonic spontaneous activity. (*b*) Phasic and irregular bursts of spikes. (*c*) A variety of units recorded during tonic spontaneous activity.

potentials measured between 40 and 200 $\mu$V, peak to peak. Monophasic negative potentials are generally less than 50 $\mu$V, but purely positive potentials have been measured up to 100 $\mu$V.

Analysis of a variety of recognizable units from within a particular preparation and from a number of preparations indicates a variety of frequencies. Some units appear to be quite irregular while others are fairly rhythmic. It should be noted, however, that very precise rhythms have not been found (Fig. 2). They always appear to be somewhat irregular. This corresponds with findings of irregular spontaneous muscular contractions in other flatworm preparations (Koopowitz & Ewer, 1970; Koopowitz, 1973*a*). The origins of this activity are of interest. If $MgCl_2$, isotonic with sea water, is added to the bathing medium to make up a 10% solution, nearly all activity disappears within 25 sec, leaving a few pacemaker units which fire at low frequencies, often with several seconds elapsing between spikes (Fig. 2). The frequency of spike production by these units is usually quite imprecise. As most of the frequencies recorded in normal sea water are an order of magnitude faster, it seems that only a few pacemakers are unaffected by interactions with other cells.

*Responses to vibration stimuli*

Units which respond to vibration stimuli appear to be localized in the dorsal posterior regions of the brain. The response to a puff of air on a naive preparation

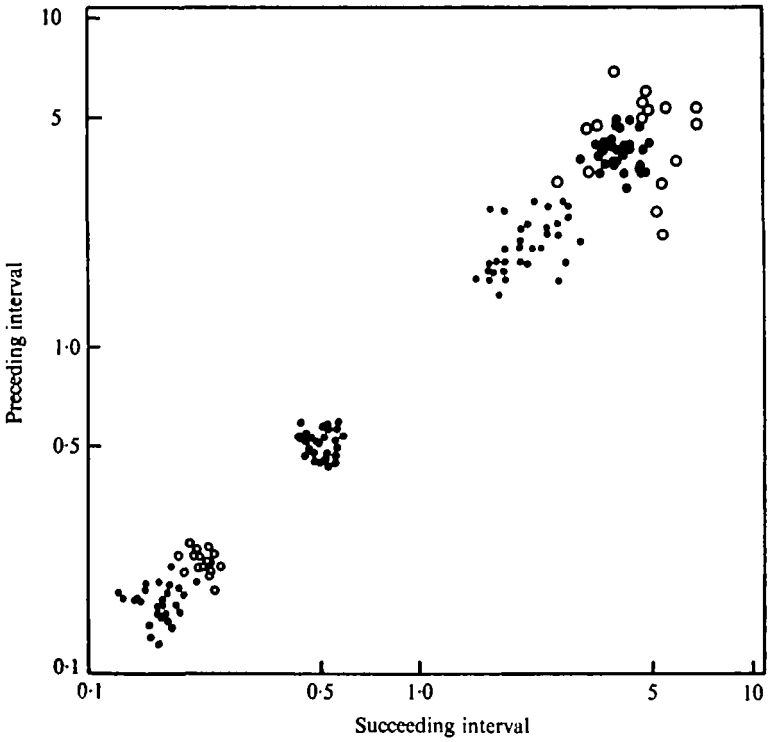


Fig. 2. Joint interval histogram of spontaneous activity recorded from recognizable individual units from six different preparations. The large open circles and the filled circles in the upper right-hand corner of the graph were taken from preparations under $Mg^{2+}$ anaesthesia. The axis is the time interval preceding a spike and the abscissa is the time interval following a spike. Time is in sec.

is a prolonged discharge of action potentials, usually produced by several units, Fig. 3. The burst can be divided into two components – an initial discharge associated directly with the stimulus and a subsequent more tonic discharge which can last for over 1 min. The latter component can be rapidly suppressed (Fig. 3) and usually disappears following a second or third stimulus. The response, as counted by the number of spikes during the 2 sec including and succeeding the stimulus, is, to a certain extent, dependent on the intensity of the stimulus (Fig. 4). Because of problems in quantifying the stimulus it is difficult to determine the real relationship between intensity and response other than that an increase in one leads to an increase in the other. The sites of response-initiation and of the receptor cells have not yet been determined. The cells from which the recordings have been made appear to be at least second- or even higher-order cells. The addition of 10% $MgCl_2$ to the bath around the preparation abolishes the response rapidly, though reversibly. During the attempts to localize the receptor site, experiments have been performed in which the peripheral margin, the tentacles with eye clusters and the dorsal epithelium have been successively removed without affecting the response. Since cleaning off all material from the sheath around the brain does abolish the vibration response, it is possible that the vibration receptors occur in this mixture of muscle and mesenchyme. Statocysts, as such, do not appear to occur in these polyclads.

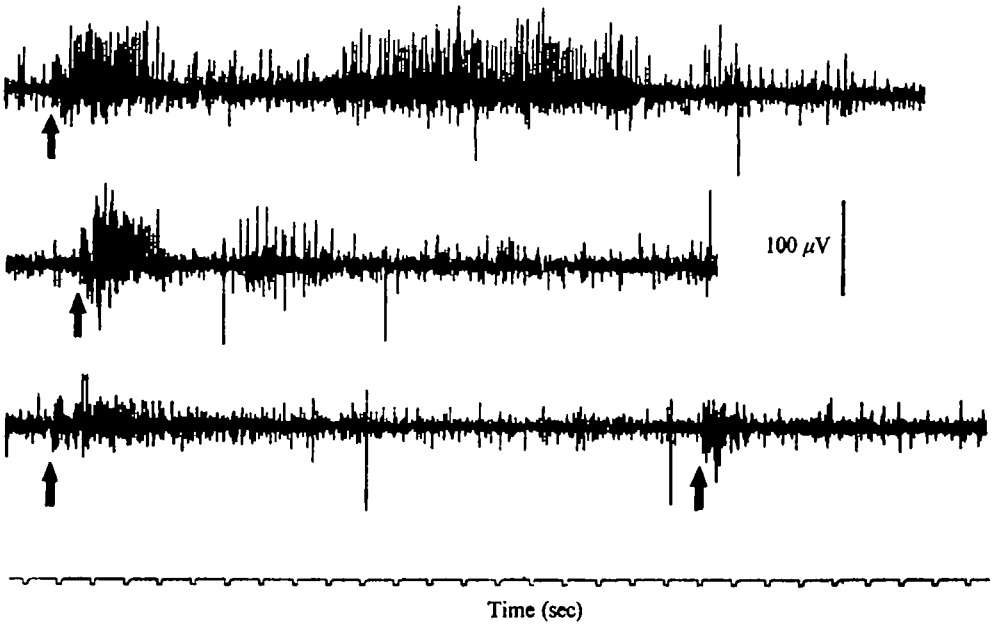


Fig. 3. Habituation of evoked potentials to a vibration stimulus. The three traces are a continuous recording reading from the top down. A stimulus was administered at each arrow. Note the rapid waning of the barrage of activity following the initial burst of evoked spikes.

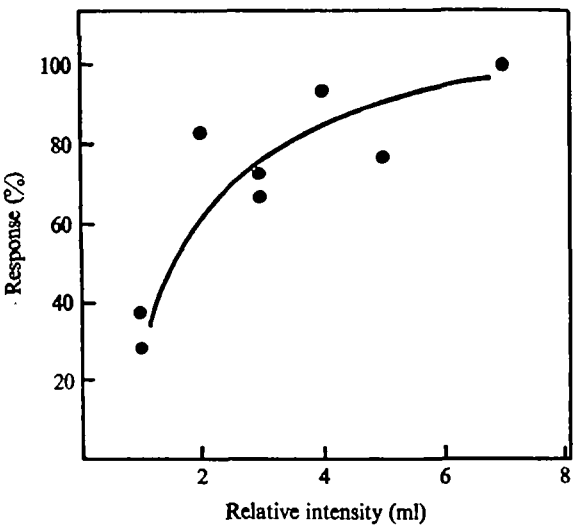


Fig. 4. The relationship between vibration stimulus intensity and the response evoked during 2 sec following the stimulus. The axis is the percentage of the number of spikes produced by the most intense stimulus and the abscissa is the relative stimulus intensity as ml of air used to produce the stimulus.

*Habituation of the vibration response*

Repeated application of stimuli not only leads to a waning of the second component but also of the initial discharge. There is a rapid drop in the total number of spikes evoked by succeeding stimuli (Fig. 5*a*). When the action potentials were broken

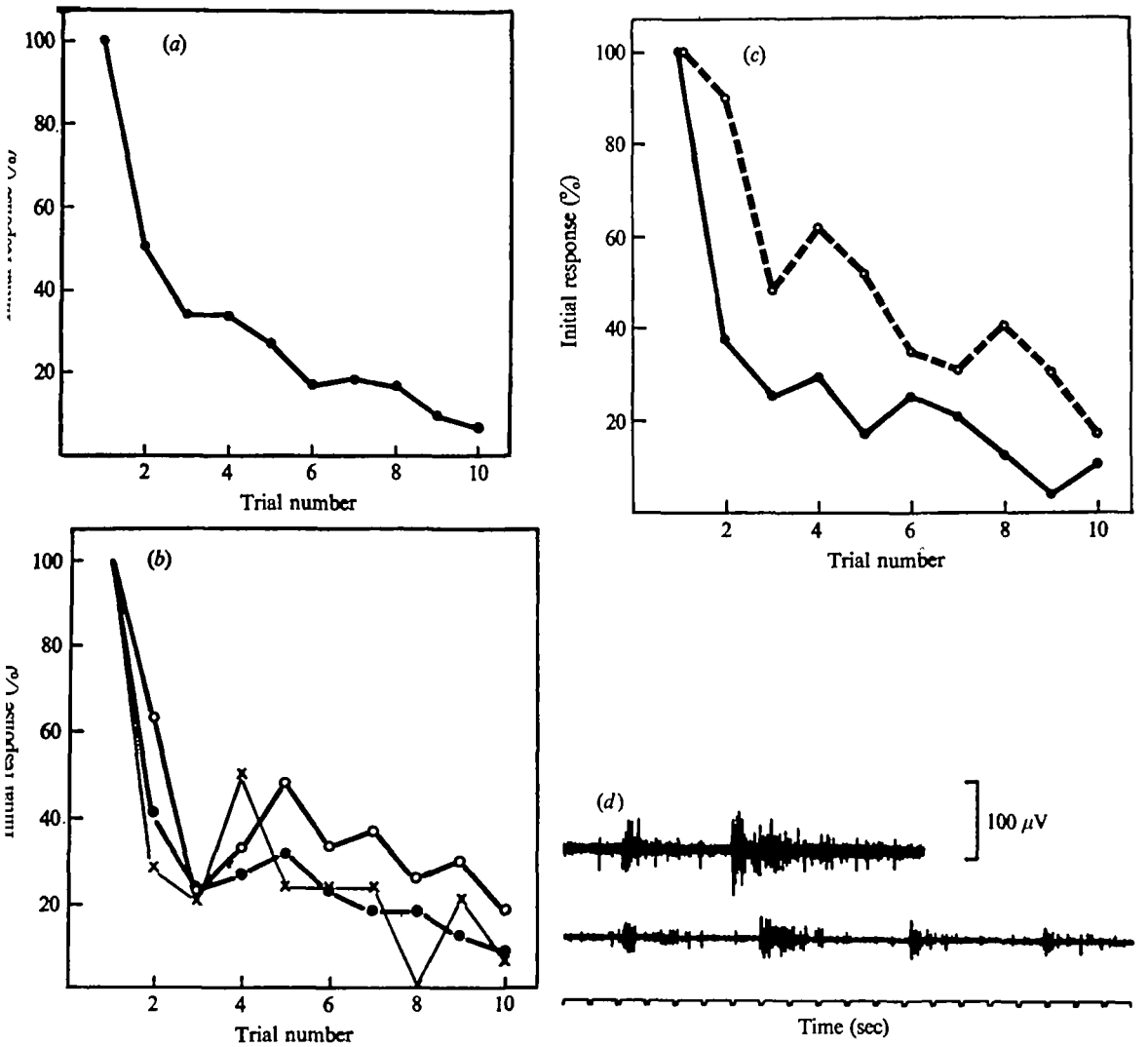


Fig. 5. Habituation to vibration stimuli. (*a*) Habituation to a stimulus administered every 5 sec. Axis is the total number of spikes as a percentage of the initial response and abscissa the trial number. (*b*) Habituation of various components making up the response. Axis is the percentage of the initial number of spikes for each category and the abscissa the trial number. Each category probably contained a number of units and as measurements were made from chart recorder tracings, absolute amplitudes in microvolts cannot be assigned to any category. ○, Category containing the smallest amplitude potentials; ●, intermediate values; and ×, the largest spikes. (*c*) Habituation to different intensity stimuli. Axis is the percentage of the initial response and abscissa the trial number. ○, Series where the stimulus was produced by a 7 ml air puff; ●, produced by 2 ml air puffs. (*d*) Increasing responsiveness to the second stimulus. In the upper trace the stimulus was a 5 ml air puff, in the lower trace the stimuli were 2 ml. Traces were from different preparations.

down into categories dependent on spike amplitude, no pattern of habituation among the various units constituting the burst was found although all individual units did habituate (Fig. 5*b*). Occasionally, however, very large spikes habituated more rapidly than others, but this was not consistent from preparation to preparation.

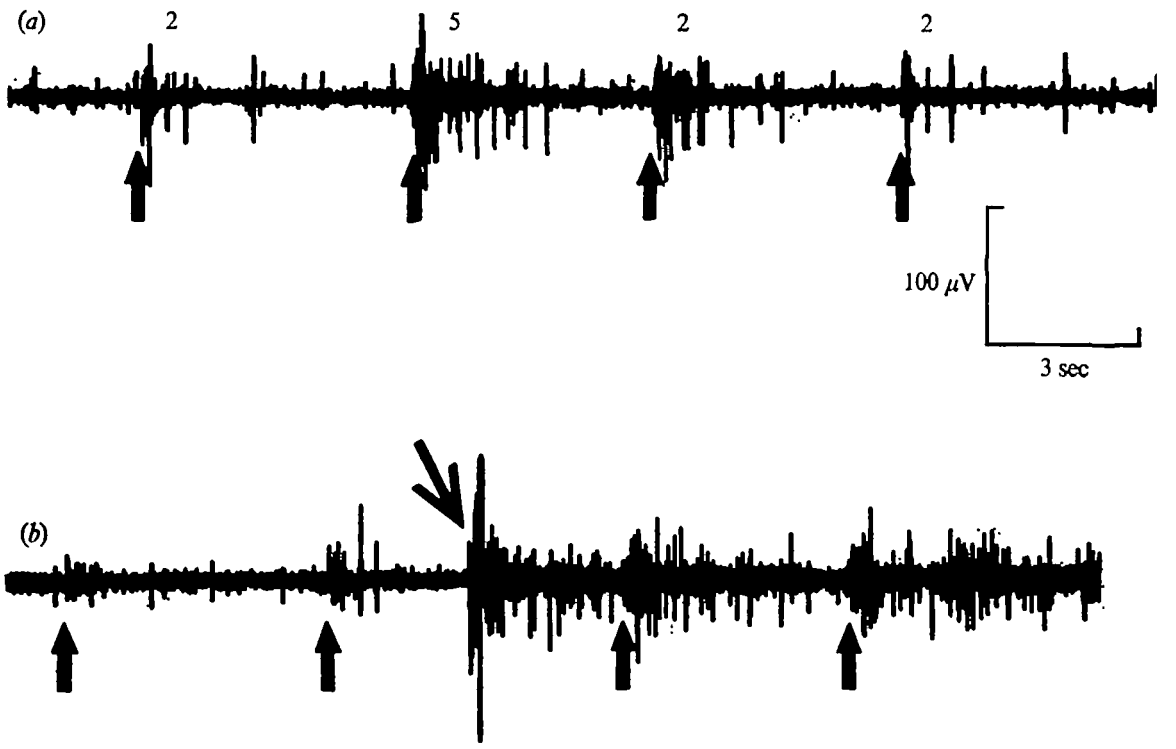


Fig. 6. Dishabituation. (*a*) Dishabituation by a stronger stimulus of the same modality. The arrows indicate when the stimuli were administered. The numerals give the relative amplitude of the stimulus. (*b*) Dishabituation by a different modality. A tactile stimulus was administered (large arrow) between two vibration stimuli. Traces were taken from different preparations. In both cases the preparation had been subjected to a series of stimuli so that the initial arrow at each trace indicates a habituated response.

With increases in stimulus strength there was generally an increase in the number of trials needed to reach a particular level of habituation (Fig. 5*c*). However, this was not always obvious unless one examined the extremes of the intensity range used. Similar relationships between stimulus-strength and response have been noted in other invertebrate preparations. In a naive preparation, initial trials do not always lead to a waning of the response. On the contrary, in a number of preparations an increase in the number of spikes produced has been noted (Fig. 5*d*). This appeared to occur only where medium-to-weak-strength stimuli were used.

*Dishabituation of the vibration response*

Often the recording electrode was in a fortuitous position where one could record both vibration and tactile responses. If a tactile stimulus was interposed during a train of habituating vibration stimuli (Fig. 6*b*), an increased responsiveness to the succeeding vibration stimulus occurred. Habituation, however, was not completely abolished and rapidly reappeared.

In another series of experiments it was possible to dishabituate the system using the same modality stimulus as that causing habituation. The observations in Figs. 5(*c*) and (*d*) suggested that changes in response might be due to the interactions of two systems, one being facilitating (sensitization is the term used by Groves &

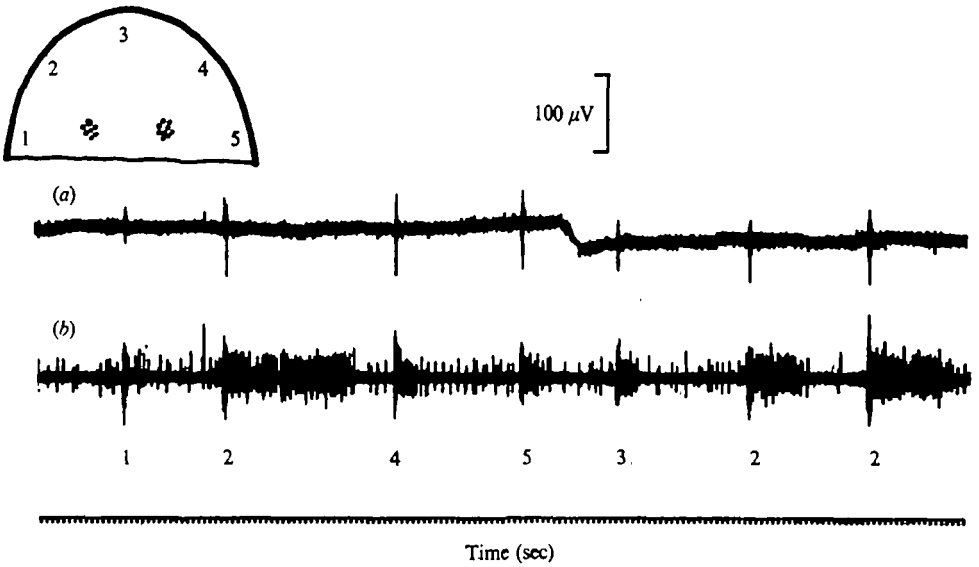


Fig. 7. Recording tactile responses from the brain. (*a*) Recordings from the dorsal anterior portion of the brain and (*b*) simultaneous recordings from the dorsal posterior portion. The numerals refer to positions along the margin which are portrayed on the inset. The inset is the anterior third of this particular preparation.

Thompson, 1970) and the other inhibitory. This was tested as follows: a train of 2 ml air puffs was administered at 5 sec intervals. When it was apparent that the response had waned, a 5 ml air puff was administered and this was then followed by a 2 ml air puff. As can be seen (Fig. 6*a*), the response was dishabituated, suggesting that the more intense stimulus was able to increase the responsiveness of the system.

*Responses to tactile stimuli*

In a previous study, using behavioural criteria (Koopowitz, 1973*a*), a dual tactile sensory system was demonstrated in *Notoplana*. There appeared to be both a system carrying information along labelled lines and a more diffuse conducting system. These two systems also appear to exist in *Freemania*. Tactile stimuli administered along the margin of the animal evoke a short burst of activity in the brain. Recording from both a posterior and anterior portion of the brain simultaneously (Fig. 7) revealed activity in both sectors. The activity recorded in the anterior section was very brief (one suspects this was involved with information coming into the brain). In the posterior sector, the tactile potentials appear to evoke a secondary discharge of spikes. Sometimes the secondary barrage lasted for several minutes. Observations on the preparation during the barrage often revealed locomotory movements, but the barrage does not represent the final motor output of the system because substantial barrages can also be recorded while the preparation is quiescent. This suggests that the final motor pattern may have to be evoked in the peripheral system. Once a site in the brain has been found which is responsive to tactile stimuli, then activity can be evoked by stimulating any part of the margin of the animal. Generally, however, barrages are observed only when specific sites are stimulated (Fig. 7).

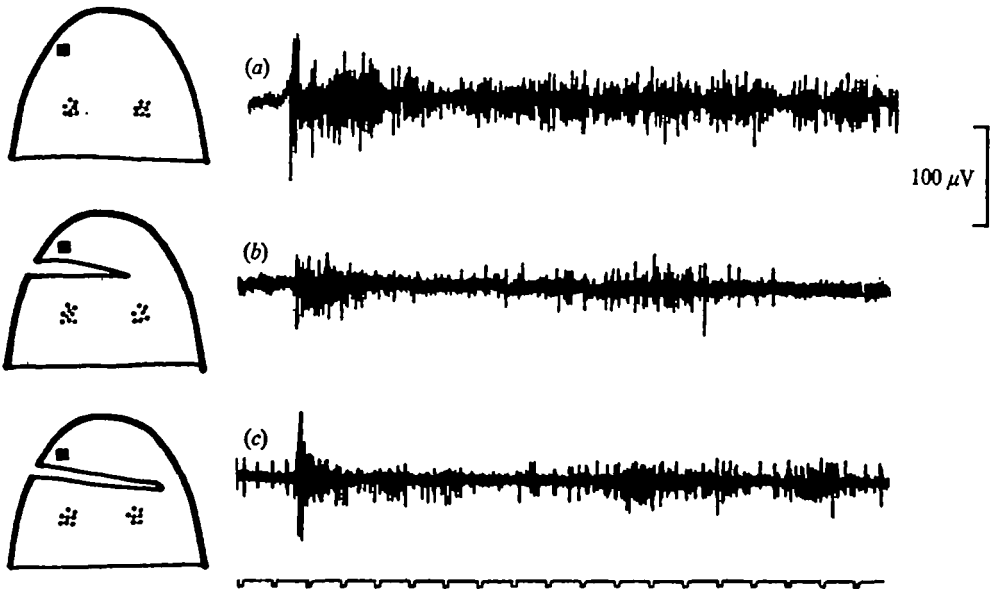


Fig. 8. Conduction of tactile information around lesions. Each trace corresponds to the response recorded from the same site on the posterior dorsal part of the brain. The insets portray stimulation site and extent of lesion. The brain is situated between the two clusters of eyes.

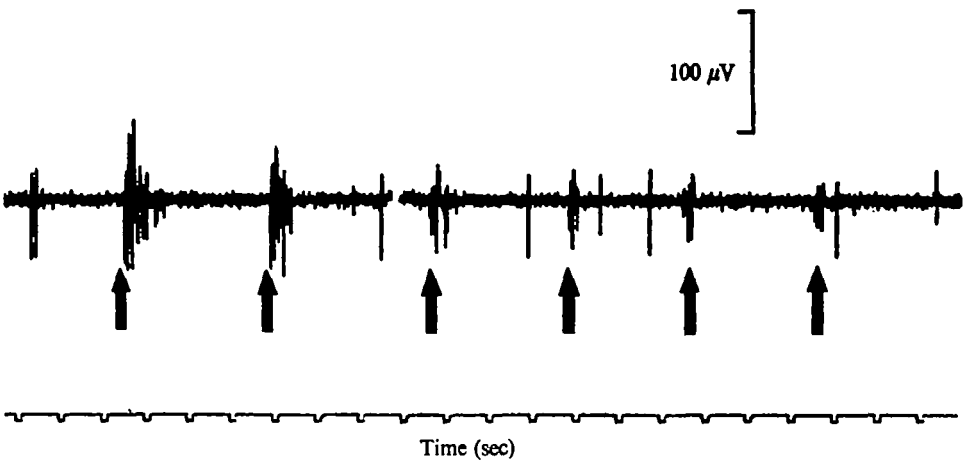


Fig. 9. Habituation to tactile stimuli, recorded from the brain.

The fact that one could record activity from so many sites suggested that perhaps one might be dealing with a diffusely conducting system. This was substantiated (Fig. 8) by recording from the brain after severing the major anterior tracts leading into the brain. Tactile stimuli on a partially isolated flap resulted in activity in the brain. The properties of this diffusely conducting nerve-net are described in an earlier paper (Koopowitz, 1975).

The initial tactile response also habituates (Fig. 9). There is also some evidence that the barrage can be habituated. Following a sustained barrage it is frequently difficult to elicit another, unless the preparation is allowed to recuperate; subsequent

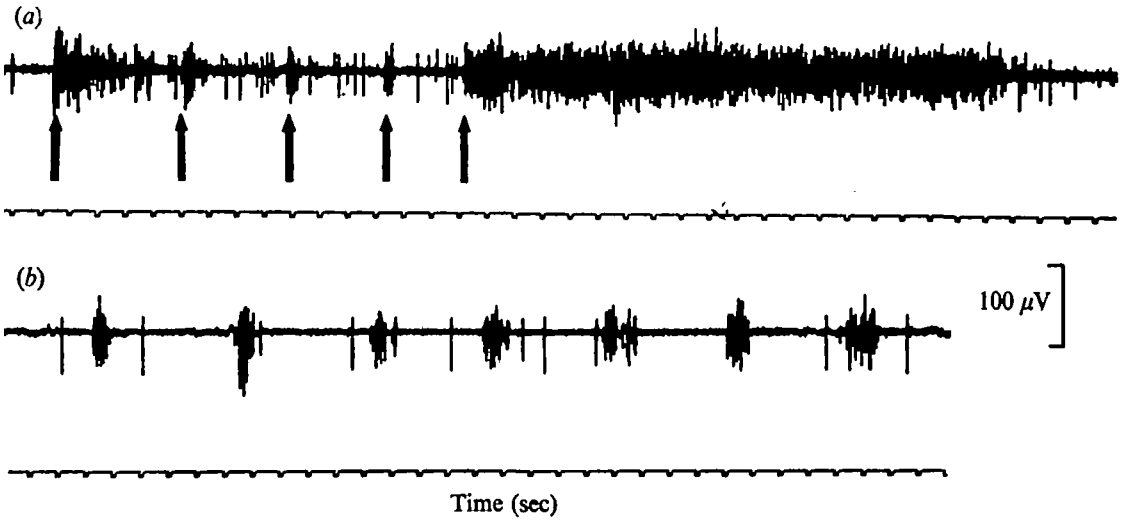


Fig. 10. (*a*) Simultaneous habituation of the initial evoked response to a tactile stimulus and the elicitation of a barrage of spikes. (*b*) Absence of habituation to tactile stimuli in the presence of $Mg^{2+}$. Records from two different preparations.

barrages may also be of shorter duration. Habituation of the barrage and the initial response are not necessarily related. Fig. 10(*a*) shows habituation to a touch stimulus in which there is a progressively decreasing response to four stimuli, but the fifth stimulus elicits a sustained barrage of action potentials. Bathing the preparation in a 25% $MgCl_2$ isotonic solution in sea water appears to abolish both the barrages and the habituation of the initial response. A series of peripheral stimuli evoke responses in the brain which do not appear to habituate (Fig. 10*b*). These results suggest that both the barrages and habituation involve $Mg^{2+}$-sensitive synapses. One should not assume, however, that those units in $Mg^{2+}$-bathed brains that respond to peripheral tactile stimuli represent first-order cells. On the contrary, they appear to belong to a diffusely conducting $Mg^{2+}$-insensitive system (Koopowitz, 1975) which may be made up of first-order units.

## DISCUSSION

### *Spontaneous activity*

Spontaneous muscular contractions occurring at rather irregular intervals have been described in the polyclad flatworm *Planocera* (Koopowitz & Ewer, 1970; Koopowitz, 1974). Similar irregular contractions have also been described from the parasitic cestodarian *Gyrocotyle* (Koopowitz, 1973*b*). The brain recordings from *Freemania* showed both rhythmic and arrhythmic units. Although these may have nothing to do with muscular contractions, it is worth noting that even the rhythmic units tend to be imprecise. When spontaneous bursts of activity were recorded from the brain they also tended to occur at irregular intervals. The spike frequencies measured in unanaesthetized preparations are at least an order of magnitude different from those measured under the influence of $Mg^{2+}$ ions, and once again the rhythms tended to be irregular. There are two possible explanations. In the first case it is possible that cells were producing $Ca^{2+}$-dependent spikes, as have been described

in certain molluscan ganglia (Kerkut & Gardiner, 1967), and that the excess $Mg^{2+}$ then interfered with the normal pacemaker potentials. Alternatively, the higher-frequency cells may be driven by other units and $Mg^{2+}$ may abolish this influence by blocking synaptic activity. At present the second alternative is favoured, but the true explanation will have to await intracellular work.

*Habituation*

Perhaps the most important observation here is that habituation occurs at many levels. Already at this phylogenetic level the central nervous system involves integration activities at a number of points. In the vibration system one can get habituation of the initial burst as well as of the follower discharge. In the tactile system even the follower discharge can be blocked after leaving the brain. There is also the possibility that activity in the tactile units may undergo a certain amount of integration before the information reaches the brain (Koopowitz, 1975). Applewhite (1971) found that anterior halves, posterior halves, and entire animals of *Stenostomum* showed habituation to tactile stimuli. There appeared to be little difference between preparations containing the brain and the posterior pieces, which makes one suspect that perhaps the habituation he measured did not involve the brain. Analysis of gill-withdrawal habituation in the mollusc *Aplysia* (Black, Peretz & Moller, 1972; Peretz & Howieson, 1973) shows that habituation occurs peripherally, but is much more rapid in the presence of central ganglia. One might have expected some differences in the rhabdocoel too. Complex neuronal interactions occur in *Freemania*: whether or not these activities are reflected in the behaviour of the animal has still to be tested.

As has been pointed out by Groves & Thompson (1970, 1973), changes in responsiveness to repeated stimuli appear to involve two components. One is an increase in responsiveness, a sensitization to the stimulus, and the other, a waning or decrease in responsiveness. The net outcome of these two processes is usually a decrease in the amplitude of response which is considered to be habituation. As far as invertebrate preparations are concerned, little attention appears to have been given to sensitization. At a subneural level sensitization and decrement (i.e. habituation) may be quite independent. But both should be considered if one wishes to understand the neural basis underlying changing responsiveness to repeated stimulation. The main evidence for the presence of sensitization comes from experiments where one measures (1) increased responsiveness before response decrement occurs (Fig. 5*d*), (2) decreasing rates of habituation with increasing stimulus intensity (Fig. 5*c*) and (3) dishabituation by interposing a more intense stimulus of the same modality during a train of weaker ones (Fig. 6*a*). The neural basis of sensitization could be either postsynaptic facilitation or presynaptic inhibition of the inhibitory synapses that cause response-depression. Hopefully, future experiments involving intracellular recording will help distinguish between these and other possibilities. Dishabituation by the same modality of stimulus means that there must be positive as well as negative feedback, and/or feedforward mechanisms, within this neural pathway. The presence of positive feedback makes good biological sense in that it

would alert an organism to changes in an environmental parameter to which it might have already become habituated. Dishabituation by other modalities appears to be a universal phenomenon of obvious importance.

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