Crayfish Escape Behavior and Central Synapses. II. Physiological Mechanisms Underlying Behavioral Habituation

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THE PREVIOUS PAPER (58) outlined the circuit responsible for exciting the lateral giant neuron and initiating single tail flips in response to phasic mechanical stimuli to the tail of crayfish. It was shown that the excitation of some tactile interneurons by tactile afferents antifacilitates extensively at low repetition rates. This phenomenon must be presumed to contribute to the habituation of the response. It is not clear, however, that this is the only phenomenon responsible for generating lability in the behavior. Receptor fatigue, variable properties of the excitable membranes of the lateral giant or the tactile interneurons, or labile properties of the circuit efferent to the giant are additional possibilities. One other point of lability has in fact been found. The strength of transmission at the neuromuscular junction between the motor giant neuron and the phasic flexor muscles is very sensitive to stimuli recurring only once per minute; this junction rapidly ceases to transmit activity after only a few stimuli (6). The motor giant is sometimes excited by the lateral giant (20, 39), and so it appears that this is a source of declining response strength in the efferent limb of the neural circuit mediating escape. However, this loss of transmission is adequately compensated by the continued activation of several nongiant flexor motoneurons, whose neuromuscular junctions facilitate (33, 39). Furthermore, electrical stimulation of the lateral giant axon at frequencies up to 5 Hz can elicit up to 50 apparently normal tail flips (unpublished observations; see also ref 33, 36), so the labilities in the circuit efferent from the lateral giant cannot contribute importantly to habituation of escape behavior.

In this paper, various possible sources of lability in the afferent limb of the circuit are explored. It appears that only presynaptic depression in the afferent to interneuron synapse can account for the habituation. Furthermore, there is a quantitative correspondence between the properties of the interneuronal excitatory postsynaptic potential (EPSP), the nature of the circuit, and the properties of the behavior.

METHODS

Crayfish (*Procambarus clarkii*) were maintained and prepared for experiments as described in the previous paper (58). The abdomen was pinned ventral side up in cold saline, and the nerve cord was exposed and illuminated with transmitted light. Single axons of interneurons were dissected from the nerve cord for stimulation and recording with suction electrodes, and their activity was monitored by a suction electrode placed on the nerve cord. The afferent activity was monitored with suction electrodes placed on ganglionic roots. Stimuli were applied by brushing tactile hairs on the carapace or shocking an afferent root through an additional suction electrode. Microelectrode recordings were made from the ventrolateral dendrites of the lateral giant or the axons of interneurons near their dendritic trees, using the procedures described in the previous paper. The stimulating and recording apparatus was as described earlier.

For experiments with single tactile receptors, the animals were prepared differently (31). The nerve roots to all but the last abdominal gan-

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glion were severed, and the telson and uropods, together with the sixth ganglion and the nerve cord, were separated from the abdomen, and pinned dorsal side up onto a Sylgard (Dow Corning Corp., Midland, Mich.) block in the preparation chamber. Tactile hairs were excited by mechanical pulses generated by a piezoelectric "cutter" crystal (Brush Development Co., Cleveland, Ohio), driven by 1-msec square pulses, provided by a Tektronix pulse generator. Afferent discharges were recorded from the fourth root with a suction electrode, and displayed and photographed conventionally. Successive-interval plots, used to represent adaptation, were computed manually using measurements from the film.

RESULTS

Physiological processes responsible for behavioral habituation

RECEPTOR FATIGUE. The first possible source of response lability in a reflex arc lies in the response properties of the receptor cells. An initial attempt at determining their response characteristics to repeated sensory stimulation is illustrated in Fig. 1. In five crayfish, the responses of tactile afferents were monitored in a second root, the responses of tactile interneurons were recorded in the nerve cord, and the intracellular depolarization in the lateral giant was observed, to repeated phasic tactile stimulation. The stimuli were provided by air bubbles blown from a pipette to strike against a pleural plate once every 2 sec. The average strength of the afferent volley remained constant, while the responses in the giant cell and interneurons waned. It appears that the receptors report the constant-intensity stimulus faithfully.

It might be argued that fatigue is actually occurring in small afferents whose spikes cannot be distinguished in peripheral root recordings. To meet this objection, the properties of a representative population of tactile hairs known to excite the lateral giant mono- and polysynaptically were investigated in five animals. A readily accessible population of such hairs occurs on the dorsal surface of the telson (31, 60). Using a semi-isolated nerve cord (see METHODS), single dually innervated hairs (3, 43) were excited tonically or phasically with a mechanical vibrator, and the response characteristics of one or both of the afferent neurons were determined. Adaptation was measured by lowering the stimulator probe onto a tactile hair for a few seconds, while monitoring the afferent activity in the fourth root. The response was characteristic for each afferent, and their properties fell along a continuum between rather tonically responding cells to very phasic cells which fired a burst only at the beginning of the stimulus (Fig. 2). All receptors studied, regardless of their adapting tendencies, showed constant response magnitudes or response probabilities to phasic mechanical stimuli repeated at 0.5 Hz. No receptors could be found that displayed any significant fatigue

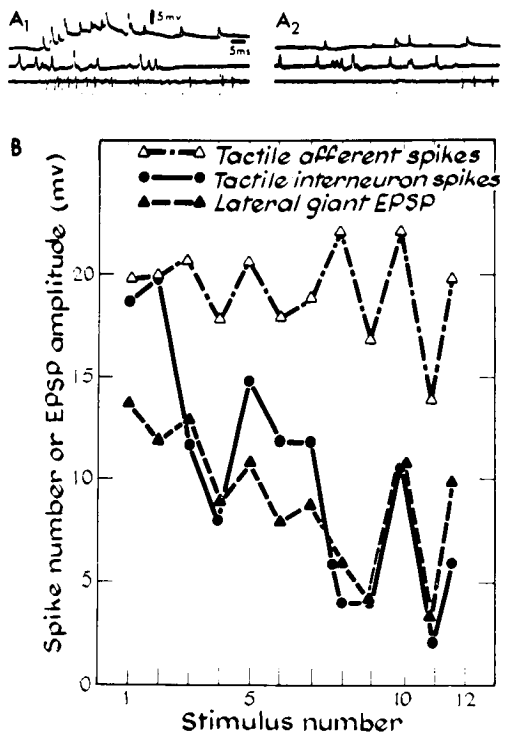

FIG. 1. Tactile afferents do not fatigue to repeated phasic stimulation. Twelve air bubbles were made to strike pleural plate hairs at 0.5 Hz, while recording the habituating responses of the lateral giant intracellularly in the third abodminal segment (top trace in *A*) and the activity of tactile interneurons in the nerve cord in the 2/3 connective (bottom trace in *A*). The afferent volley (middle trace) remains constant to repeated stimulation. A_1 and A_2 illustrate the first and ninth responses, respectively; *B* shows the amplitude of the lateral giant EPSP and the total number of afferent and interneuron discharges to each stimulus.

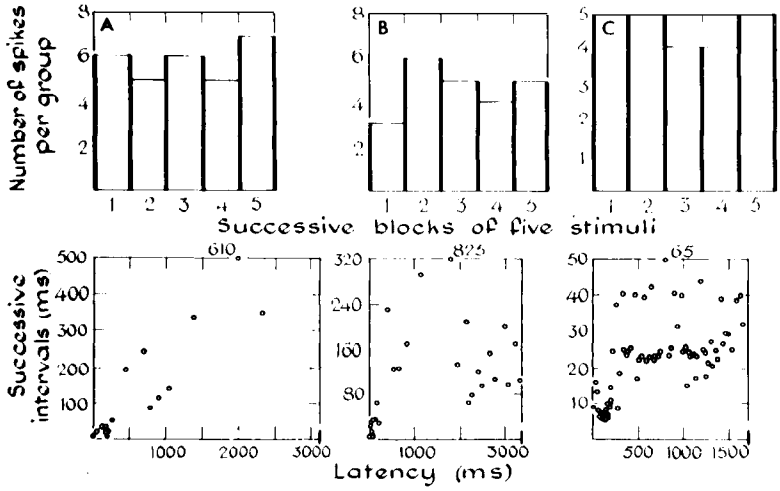

FIG. 2. Properties of single tactile hairs on the telson, known to excite tactile interneuron A chemically and the lateral giant electrically (60). For each of three hairs, 25 phasic stimuli were applied at .05 Hz and the number of impulses to successive groups of five stimuli are plotted in the top row. This is a measure of fatigue; each hair shows a constant level of excitability. In the bottom row are shown responses of the same hairs to tonic stimuli lasting for the length of the abscissa. Successive spike intervals (inverse frequency) are plotted on the ordinate as a function of the latency of each spike from the stimulus on the abscissa. This is a measure of adaptation. *A* shows a hair responding very phasically to a stimulus lasting 3 sec; *B* presents a receptor with prominent phasic and tonic phases to its response; while the hair in *C* responds more tonically to a maintained stimulus.

in response to this stimulus. On the other hand, similar stimuli repeatedly delivered to tactile receptors invariably elicit a rapidly habituating escape response. It is concluded that receptor fatigue does not contribute significantly to habituation of this behavior. This conclusion is consistent with the result that habituation can be elicited just as easily by peripheral root stimulation as by phasic mechanical stimulation.

ACCOMMODATION AND REFRACTORINESS IN LATERAL GIANT NEURON. The process of excitation of spikes in active membranes involves several time-dependent phenomena which can contribute to a lability in the process. Most changes in the responsiveness, excitability, or threshold of neurons are due to phenomena, like sodium-conductance inactivation, which decay in tens of milliseconds or less (18, 22, 50), and could not contribute much to a response decrement occurring over many seconds or even minutes. However, many nerve cells respond only phasically to constant excitation, and show other slow changes in their electrical responsiveness. These threshold changes can be classified as two basic types: *1*) refractoriness resulting from impulse firing, which may be relative, prolonged, and accumulating (17, 19, 28, 55); and *2*) prolonged accommodation or adaptation, brought about by persistence of the stimulus (10, 16, 48).

Two tests were made for accommodation in the lateral giant. The experiments were specifically designed to reveal neural phenomena with properties which might contribute to behavioral habituation. If the lateral giant becomes less excitable as a consequence of being subjected to repeated excitatory depolarization, then its threshold should increase after a series of stimuli which cause habituation. Figure 3 illustrates one of five experiments in which a microelectrode in the lateral giant dendrite was used to excite the fiber directly by passing long current pulses just above rheobase. The latency of the spike is a very sensitive measure of threshold changes under these circumstances. The figure shows that the lateral giant threshold was the same before and after 10 shocks to an afferent root, during which the tactile interneuron response (one convenient index of habituation) was reduced significantly. This test of threshold reveals no evidence for accommo-

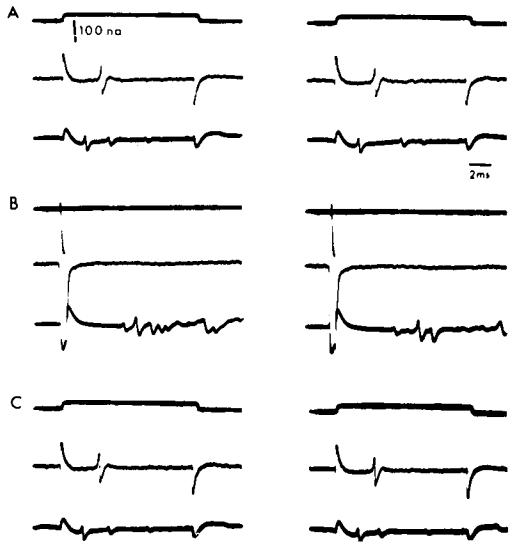

FIG. 3. Test for long-lasting accommodation in the lateral giant as a factor in habituation of escape behavior. In each record, the upper trace monitors the current injected into the lateral giant dendrite in the third ganglion, the middle trace shows the response recorded extracellularly from the lateral giant axon in the 5/6 connective, and the lowest trace interneuron activity in the ventral nerve cord in the 2/3 connective. In *A*, responses to the first and last of 10 current pulses delivered at 1 Hz are shown. One second after the last pulse, the second root was shocked 10 times at 1 Hz, to elicit habituating responses in tactile interneurons and the giant fiber; *B* shows interneuron responses on the bottom trace to the first and last stimuli. One second after the last root shock, a new sequence of current pulses to the giant was initiated, with the same intensity as in *A*. The 1st and 10th responses of the lateral giant are shown in *C*, at a latency unchanged from that obtained in *A*, indicating that the threshold is unchanged after habituation to tactile stimulation. The constancy of the threshold to trains of stimuli in *A* and *C* also indicates the absence of long-term refractoriness in the lateral giant.

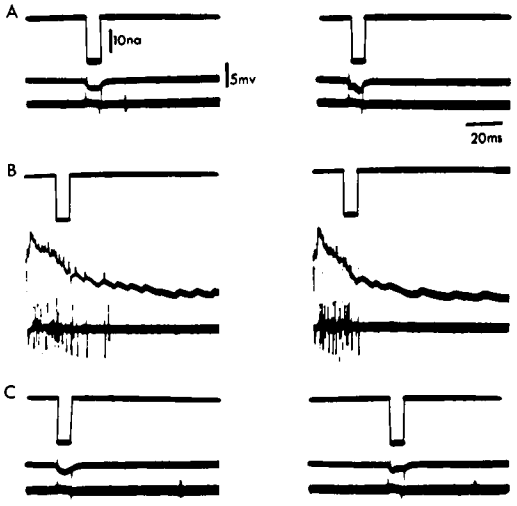

FIG. 4. Lateral giant input resistance before, during, and after a sequence of stimuli leading to habituation. Traces and electrodes as in Fig. 3, except that the middle trace records the intradendritic potentials of the giant neuron. The experimental design was the same as for Fig. 3: a series of 10 current pulses at 0.5 Hz was used to measure the input resistance of the lateral giant. *A* shows the first and second of the 10 resistance measurements preceding by 2 sec 10 root-two shocks delivered at 0.5 Hz. Responses to the first two of these shocks is shown in *B*. This sequence was followed after 2 sec by another series of 10 resistance measurements, the first 2 of which are shown in *C*. Throughout this experiment, the lateral giant dendrite showed only random resistance fluctuations of less than 50 kilohms. (Fast transients of current pulses were photographically retouched.)

dation which persists long enough to account for habituation.

If the excitability of the giant neuron changed during habituation of the escape response, one might expect to see a correlated change in the input resistance. This would be true, for example, if a threshold elevation due to a prolonged delayed rectification were to occur. In four experiments (see Fig. 4) the resistance of the lateral giant dendrite was measured before, during, and after a series of stimuli leading to a waning of the lateral giant EPSP and the responses of tactile interneurons. No change in the resistance was measurable with a technique sensitive to changes of 25 kilohms. Since the input resistance of the dendrite was often 250 kilohms, a change of less than 10% would not have been detected. The results also demonstrate that there is no measurable conductance increase during the EPSP, shown by other criteria to be electrical (58).

Krasne (34) reported that the late component of the lateral giant EPSP declines regardless of whether the neuron fires or not, so it seems unlikely that any long-term refractoriness is contributing to the habituation. Furthermore, Fig. 3*A* and *C* shows examples of a constant threshold in the giant to each of 10 suprathreshold stimuli applied at 0.5 Hz, indicating no accumulated refractoriness. It must be concluded that the waning of the response of the lat-

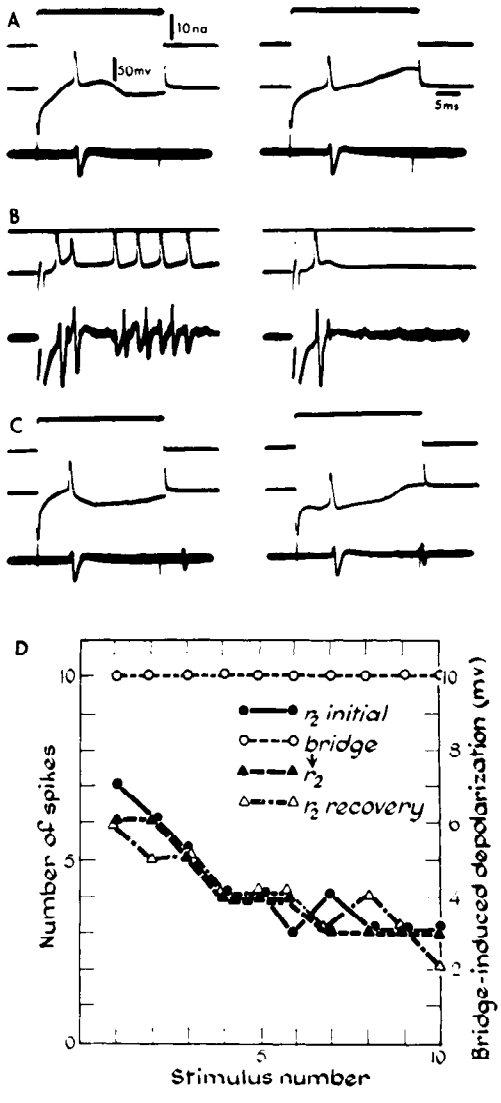

FIG. 5. Tests for long-lasting accommodation in a tactile interneuron as a factor in habituation of its response to repeated stimulation. The oscilloscope records illustrate an experiment identical in all respects to that of Fig. 3, except that the middle trace now shows the responses recorded intracellularly from interneuron C, and thus indicates the input resistance of the cell, as well as the spike threshold. Stimulation interval: 2 sec. The habituating stimulation sequence (1st and 10th responses shown in *B*) has no consistent effect on these properties (1st and 10th measurements before habituation shown in *A*; after habituation in C). In *D*, the number of impulses in interneuron C in another preparation in response to sequences of 10 second-root (r_2) shocks delivered at 0.2 Hz are shown. Responses to such shocks presented alone are marked "r_2 initial." They are compared to responses to a similar sequence of shocks preceded by 5 sec by a conditioning sequence of intracellular bridge-induced depolarizing pulses delivered at 0.2 Hz. This conditioning-test paradigm is indicated as "bridge → r_2." Pulses were adjusted to produce depolarizations similar to the compound EPSP evoked by root stimuli, just below threshold for eliciting spikes (pulse parameters: 4 na, 30 msec). If habituation were due in part to interneuron accommodation, responses to these conditioned shocks (marked "r_2") should be weaker than for shocks presented alone. The two sequences were separated by a 5-min rest. Any decline in response strength could simply be due to an insufficient rest period. This variable was controlled by following the conditioned sequence of shocks with another sequence of root shocks alone (marked "r_2 recovery") after another 5-min rest. Incomplete recovery is reflected by a progressive slight decline in the responses to the three sequences. If accommodation were involved in habituation, responses to the middle sequence would be the weakest.

eral giant is due to a decline in the presynaptic excitation to this cell during habituation.

ACCOMMODATION AND REFRACTORINESS IN TACTILE INTERNEURONS. Since the monosynaptic and polysynaptic EPSPs in the lateral giant occur without amplitude change at input frequencies up to 400 Hz and the receptors show no fatigue, the polysynaptic pathways must contain the source of response lability. It is shown below that the waning of the compound EPSP in the giant cell follows the waning of the activity in the tactile interneurons, so the possible sources of interneuronal lability must be considered.

First it will be shown that in tactile interneurons no changes in threshold are demonstrable which last long enough to account for behavioral habituation. This possibility cannot be ignored, since Takeda and Kennedy (48) reported the presence of both accommodation and delayed refractoriness in unidentified tactile interneurons lasting at least tens of milliseconds. The question considered here is whether these effects last long enough to contribute to habituation to stimuli occurring every few seconds. Figure 5*A*, *B*, and *C* illustrates one of five experiments on interneuron C,[1] in which both the threshold and input resistance were measured before and after a sequence of stimuli which leads to a habituated response in the interneuron. No

[1] Interneurons B and C are multisegmental tactile interneurons described in the first paper (58).

changes in the measured properties are observed.

In Fig. 5*D*, the responses of interneuron C to an initial test sequence of shocks delivered at 0.2 Hz to part of the second root were compared to the later responses to an identical stimulation sequence preceded by a conditioning sequence of subthreshold bridge-induced depolarizations. The two experiments were separated by a 5-min rest, and the initial test sequence of stimuli was repeated 5 min after the conditioned sequence to test whether the rest was long enough for the interneuron response to recover from habituation. The three sequences of habituating responses are indistinguishable. Six similar experiments demonstrate that interneuron accommodation is not involved in habituation, and cannot be used to induce it.

The results from two other types of experiment bear directly on the possibility that interneuron refractoriness contributes to habituation. Stimulation of any segment in an interneuron's receptive field leads to impulse generation, and the impulses propagate throughout the neuron. If long-term refractoriness contributed to habituation, one would expect habituating stimuli delivered to one segment to leave the neuron less responsive to stimuli delivered to another segment than the neuron would be in the absence of the conditioning habituating sequence.

Figure 6*A* shows that there is no such intersegmental spread or generalization of habituation. The experimental design is the same as in Fig. 5*D:* a sequence of shocks delivered to a branch of the second root of the ganglion penetrated by the microelectrode leads to a waning in the response of the interneuron to successive stimuli. After a 5-min rest, the same stimulation sequence was preceded by an identical conditioning sequence of shocks to the second root of the next posterior ganglion. The response magnitude and rate of decline to the conditioned test stimuli were similar to the responses to the unconditioned stimuli. A final sequence of test stimuli presented after a second 5-min rest shows nearly complete recovery. Apparently, habituation is segment specific, that is, the response of interneuron C declined to successive stimulation of either segment, but immediately following habituation of responses to one segment, the responses to stimulation of the next seg-

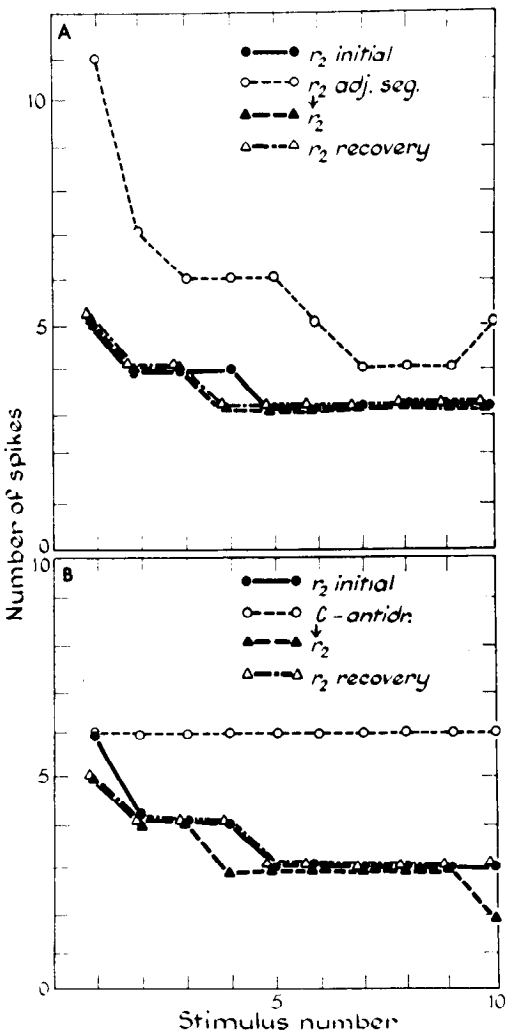

FIG. 6. Tests for long-lasting refractoriness as a factor in habituation in tactile interneurons. *A* illustrates results of an experiment identical to that of Fig. 5*D*, except that here the conditioning stimulus consists of a sequence of shocks delivered to the second root of the fourth ganglion ("r_2 adj. seg."); test stimuli ("r_2 initial," "r_2," and "r_2 recovery") were delivered to the third ganglion. Cross habituation does not occur. In *B*, the same experimental design is used to test the effect of a sequence of 10 bursts of six spikes separated by 5 msec, induced in tactile interneuron C by stimulation of the isolated axon in the 4/5 connective ("C-antidr."). These bursts mimic the typical response of the interneuron to root stimulation. If refractoriness were involved in habituation, responses to the middle sequence of test shocks would be the weakest; as in Fig. 5*D*, this is not seen.

ment started at a nonhabituated level and declined at the normal rate.

In Fig. 6*B*, a similar experiment was performed, using a sequence of bursts of antidromic spikes in interneuron C for the conditioning stimulus. Each burst, lasting 30 msec and containing six spikes, was very similar to the average burst evoked by root shocks in interneuron C. Again, this conditioning stimulus did not influence or mimic habituation in the interneuron. These experiments were repeated 4 times with the same results, and it is concluded that refractoriness does not last long enough to contribute to the behavior.

It might be objected that antidromic spikes, or those arising from an adjacent segment, do not invade the same regions of an interneuron as do orthodromic spikes, and therefore cannot be expected to have similar effects. A better procedure might be to look for interactions between spikes arising in the same segment by using for conditioning stimuli either suprathreshold depolarizations induced via the microelectrode, or shocks applied to the other half of the second root. These experiments have been performed several times, but unfortunately the results are ambiguous. In three of the seven preparations tested, it was possible to demonstrate a small but statistically significant difference ($P < 0.005$ for intercepts, analysis of covariance; see ref 13) between the responses to test shocks to one-half of the second root, which were or were not preceded by an identical sequence of shocks to the other half of the root. In other words, in these few preparations, a small amount of intrasegmental generalization or transfer of habituation occurred between two separate populations of afferents; thus habituation may not always be perfectly afferent specific. In one out of three experiments, it was possible to mimic this effect by using bridge-induced spikes in the interneuron for the conditioning sequence. This result suggests the presence of some long-term refractoriness induced at the input region of the interneuron in each segment, but not induced by spikes arising in another segment. However, some long-term interaction between compound subthreshold EPSPs from the two groups of afferents may also be present. In two of five experiments, a sequence of habituating interneuronal EPSPs from one set of afferents was slightly depressed by a conditioning sequence of weak stimuli to other afferents, whose EPSPs also failed to elicit spikes. Although this long-term interaction between EPSPs was not statistically significant, its apparent occasional occurrence complicates the interpretation of the intrasegmental cross habituation. It is possible, then, that some weak, local, intrasegmental long-term refractoriness persists for some seconds after firing, and/or that some heterosynaptic depression is present, and contributes slightly to behavioral habituation in some animals. The important point of these results is that the effects observed were small, difficult to repeat, and often not statistically different from chance fluctuations.

CHEMICAL AFFERENT SYNAPSE ONTO TACTILE INTERNEURONS IS LOCUS OF HABITUATION. The decline in the number of poststimulus spikes in tactile interneurons parallels the waning of the lateral giant compound EPSP recorded simultaneously (Fig. 7) and thus seems to account for it. If one measures the area under the lateral giant EPSP instead of the amplitude, the waning follows more closely the decline in interneuron response (Fig. 8). This result is expected, since the EPSP is generated by a dispersed group of electrical components from several tactile interneurons (58). The amplitude of the compound EPSP would be expected to reflect accurately the magnitude of the input only if the input consisted of synchronous activity in all elements.

Figure 8 also compares the above measures of habituation to the antifacilitation of a unitary afferent EPSP recorded from interneuron C in a different experiment. This EPSP is depressed at the same rate and to the same fraction of its original magnitude as are the lateral giant EPSP and the suprathreshold response of a tactile interneuron. All other sources of lability having been nearly completely eliminated, it appears that the behavioral habituation is entirely due to antifacilitation of this class of synapse between afferents and tactile interneurons, which is the most common type of input to the multisegmental tactile interneurons (58).

In their behavioral investigations, J. J. Wine and F. B. Krasne have found (personal communication) that tail flips known to be mediated by the lateral giants can be reliably elicited by stimuli presented once per 2 min, but that the response probability begins to wane to stimuli presented once per minute, approaching zero after about 20 stimuli. More rapid stimulation results

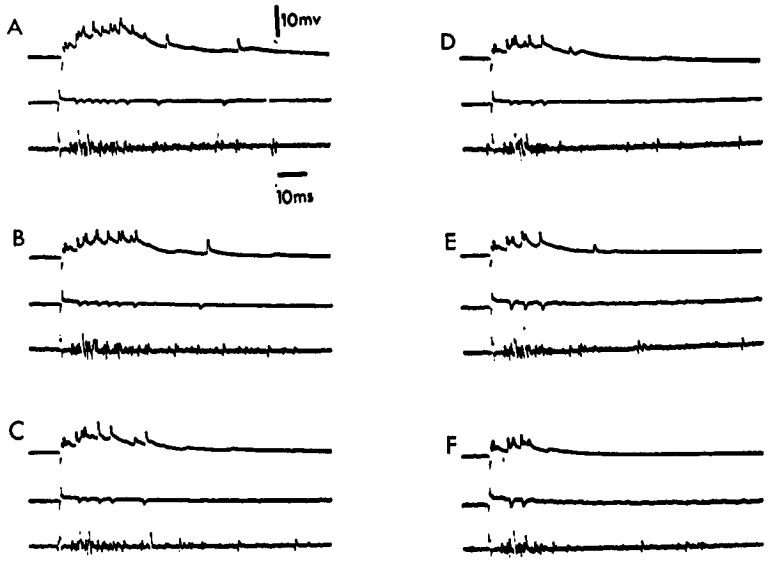

FIG. 7. Decline of the lateral giant EPSP to repeated stimulation is due to waning of the interneuron response. *A* through *F* show the 1st, 2nd, 3rd, 4th, 10th, and 30th responses to second-root stimuli applied at 1 Hz to the fourth segment. Lateral giant EPSPs are recorded intracellularly in the fourth ganglion in the top trace; interneuron B spikes are monitored in the 5/6 connective in the middle trace; and the bottom trace represents nerve cord discharges in the 3/4 connective. A particular unitary EPSP in the lateral giant is seen to be generated by spikes in interneuron B.

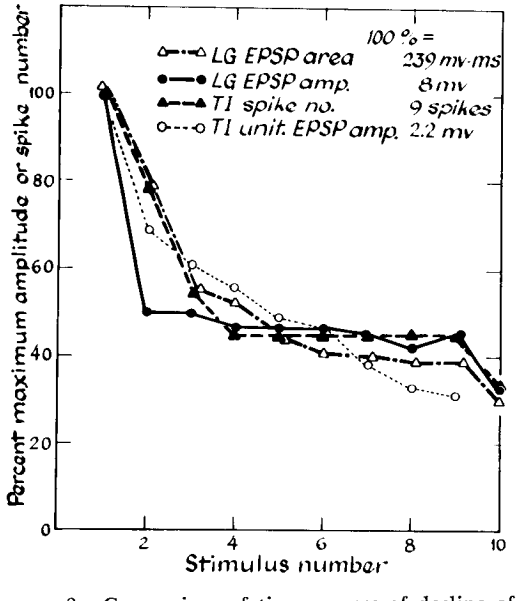

FIG. 8. Comparison of time courses of decline of the amplitude ("amp.") and area under the lateral giant EPSP ("LG EPSP area"), the number of spikes in interneuron B from the experiment of Fig. 7 ("TI spike no."), and the amplitude of a unitary EPSP in a tactile interneuron from a different experiment ("TI unit. EPSP amp."). Stimulation rate: 1 Hz. Responses expressed as percent of maximum.

in habituation after fewer stimuli. It seemed important to show some waning in the responses in the elements of the circuit exciting the lateral giant to such very low frequency stimulation. Figure 9 shows that the area under the lateral giant EPSP and the number of spikes in interneuron C or two other tactile interneurons taken together (whose spikes were indistinguishable) all decrease at the same rate and to the same extent during 1/min root stimulation. Further stimulation at higher frequencies caused an additional waning of these physiological indexes of habituation. Like the behavior, the measures recovered after a 35-min rest. Similar results were obtained from a second stable long-lasting preparation. A similar record for a unitary interneuronal EPSP would be desirable; unfortunately, stable penetrations of interneurons could not be maintained for the tens of minutes required for such experiments. It seems, nevertheless, that the properties of the behavior are accounted for by the antifacilitating properties of these synapses.

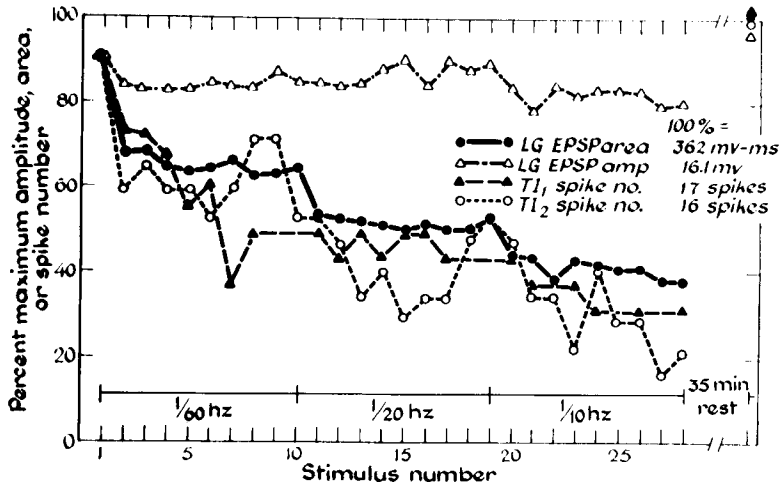

FIG. 9. Physiological signs of habituation to very infrequent stimulation. The LG EPSP was recorded in the third ganglion, and spikes of several interneurons could be distinguished in the 4/5 connective. The amplitude and area under each EPSP is plotted along with the number of spikes in interneurons C (TI_1) and two other interneurons (TI_2), to second-root shocks delivered at 1/60, 1/30, and 1/10 Hz. Responses after a 35-min recovery are also shown. Responses expressed as percent of maximum.

Physiological mechanisms underlying antifacilitation and habituation

One way to distinguish among alternative hypotheses concerning antifacilitation is to make use of the quantal nature of transmitter release (29). In several neuromuscular and neuronal junctions, it has been demonstrated that synaptic transmission is accomplished by the release of discrete quanta in response to presynaptic depolarizations; the number of quanta released during each such depolarization is distributed according to Poisson's law in response to presynaptic depolarization (4, 5, 12, 14, 37, 42). If the same processes underlie afferent excitation of interneurons, then a statistical analysis of the EPSP amplitudes may be used to distinguish changes in quantum size or efficiency from changes in quantal content or number as different sources of EPSP variation. More specifically, receptor desensitization and postsynaptic inhibition would diminish the size of EPSPs by reducing the quantal efficiency. Presynaptic inhibition, or a presynaptic failure of the synthesis, mobilization, or release of transmitter would diminish EPSPs by reducing the quantal number.

A statistical analysis of interneuronal EPSP amplitude fluctuations was therefore undertaken. Three serious drawbacks limit the precision of the analysis. First, it was not possible to observe miniature EPSPs in neuropil recordings, so the method of direct estimation of quantal number from the ratio of miniature to full EPSP amplitude (27, 41) could not be applied. Second, it was not possible to reduce the EPSPs much by Mg^{++} poisoning or Ca^{++} deprivation (58), so the method of failures could not be used, because failures were rarely observed. Finally, in order to obtain a reasonable signal-to-noise ratio, it was necessary to stimulate several afferents and work with a compound interneuron EPSP, so the average quantal number was large and the distribution of EPSP amplitudes displayed no peaks. Thus the method of reconstructing a sum of Poisson distributions could not be applied. This leaves only the weakest technique, the variance method, for estimating the quantal content and efficiency of EPSPs.

It should be noted that compound EPSPs generated by a constant number of afferents will vary in amplitude according to Poisson's law in the same manner as the component unitary EPSPs from each afferent. This is a consequence of the fact that a sum of Poisson random variables is itself a Poisson variable (27). Normally, this sort of statistical analysis is used for a unitary

EPSP which is the sum of a number of discrete potentials generated at several points of contact between the many independent Poisson-operating release sites of the presynaptic terminal arborization and the postsynaptic receptor sites. It is just as legitimate to apply a Poisson analysis to a compound EPSP which is a sum of unitary EPSPs.

The data used were obtained from two long series of equal-intensity second-root stimuli which evoked compound subthreshold EPSPs in interneuron C. Records of the second-root volley verified that an invariant number of afferents were activated by all stimuli. About 80 stimuli were applied in two series, the first at 0.5 Hz, and the second at 5 Hz. After 25 stimuli, the EPSP reached a stationary plateau or steady-state level of antifacilitation, so only the last 50 responses of each series were used.

The quantal content was estimated from the square of the inverse of the coefficient of variation of the amplitudes of the compound EPSP in each series. Details of the procedure are described elsewhere (27, 41). Several corrections were used in the calculations. The variance of the signal was reduced by the variance of the noise-level voltage fluctuations. The latter was calculated directly from 50 measurements of the membrane potential between stimuli. In addition, the estimate of quantal content was corrected for the effects of the nonlinear relation between synaptic conductance changes and postsynaptic potentials. This correction requires an estimate of the EPSP equilibrium potential. This was provided (see ref 8) by multiplying the EPSP reversal potential, found earlier to be 40 mv (58), by $e^{-X/\lambda}$, where λ is the space constant of the axon and dendrite and X is the distance of the electrode from the synaptic site. X was assumed to be equal to 0.2 λ as estimated in the previous paper (58). In addition, the EPSP amplitudes were increased by $e^{X/\lambda}$ to correct for their decrement between generation and recording sites. Finally, the quantal content was corrected for the coefficient of variation of the quantal size or efficiency, assumed to be 0.25. An estimate of quantal efficiency was obtained as the quotient of the average EPSP amplitude divided by the quantal number. The results are presented in Table 1, along with estimates for the standard error of each estimated quantity. The standard error of the quantal content, m, is given by Martin (41) as

$$SF_m = \left(\frac{4(1/m) + 2}{N} \right)^{1/2}$$

where N is the number of observed EPSPs. I derived the following expression for the standard error of the quantal efficiency (see ref 30, p. 231–235):

$$SF_q = \left(\frac{(1/m) + 2}{N} \right)^{1/2}$$

The results may be summarized as follows: when the stimulation rate increased from 0.5 to 5 Hz, the average EPSP amplitude declined by 25%, the quantal content declined by 48%, and the quantal efficiency increased by 49%. Using Welch's generalization of the t test for populations having different variances (ref 13, p. 105), these

TABLE 1. *Estimated corrected quantal content and quantal efficiency for steady-state compound EPSPs in tactile interneuron C*

Stimulus Frequency, Hz	Measured Steady-State EPSP, mv	Est Corrected Quantal Content, no. of Quanta	Est Corrected Quantal Efficiency, μv
0.5	4.133 ± 0.220	439.7 ± 88.2	9.40 ± 1.88
5	3.166 ± 0.236	227.0 ± 45.6	13.95 ± 2.79
	Tests for differences between means		
t	21.20	15.16	9.55
df	102	74	87
P	<0.005	<0.0005	<0.0005

Values are means ± SE.

differences were all statistically significant ($P < 0.0005$).

The results at first appear somewhat anomalous. The decrease in quantal content is significantly greater than the drop in EPSP amplitude, leading to an apparent increase in quantal efficiency. These results can be explained if it is realized that for a compound EPSP consisting of unitary EPSPs each following Poisson release statistics, the measured m is the sum of quanta released by all of the afferent synapses excited, and the estimated quantal efficiency is a weighted average of the quantal efficiencies of the n separate synapses (i):

$$q = \frac{\sum_{i=1}^{n} q_i m_i}{m}$$

(see ref 27, p. 137–138). Suppose antifacilitation were to occur by a parallel and equal decline in the quantal content (m_i) of each synapse. Then the percentage decrease in m would equal the percentage decrease in average EPSP amplitude ($\bar{v}$) and q would be unchanged. Suppose, on the contrary, that some synapses antifacilitated more than others and, in particular, that in this instance the most effective synapses changed least. Then m would change more than $\bar{v}$, and q would increase. This is what was observed.

This explanation seems rather ad hoc, and is unsatisfactory without supporting evidence. Figure 10 shows the results of three experiments in which different size EPSPs in tactile interneurons declined to different fractions of their initial amplitudes during repetitive root stimulation. In Fig. 10*A*, the compound EPSPs generated by stimulating the two halves of the second root are compared. In Fig. 10*B*, weak shocks of opposite polarity to the second root excited different afferents, as determined by examination of the volley monitored by a second-root electrode. The EPSPs associated with the two types of stimulation are compared. In Fig. 10*C*, the intensity was adjusted to evoke the first EPSP and the stimulus repeated until the response was nearly completely fatigued. Then the intensity was increased to recruit the next afferent, and the stimulus repeated until it, too, was fatigued. Three more EPSPs were recruited and studied in like manner, and the residual depolarization from the incompletely fatigued EPSP was subtracted from each next EPSP recruited. All amplitudes in Fig. 10 were corrected for the nonlinear relation between conductance and potential (41), so that they represent more precisely the strengths of excitation.

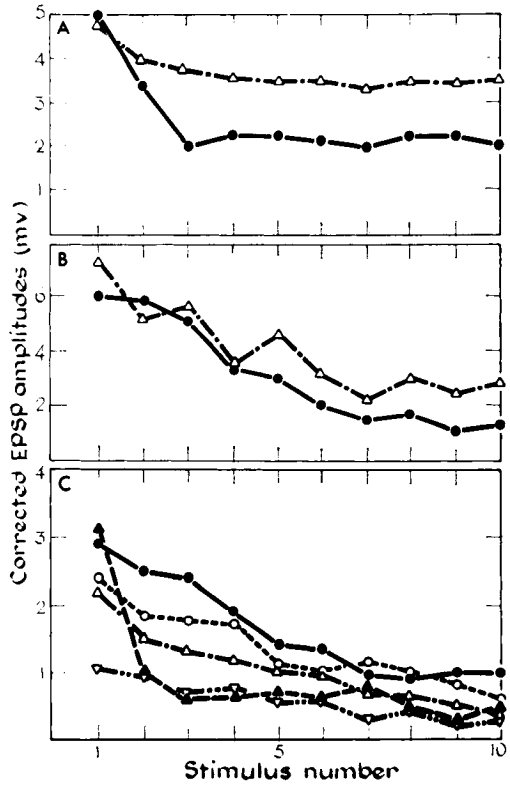

FIG. 10. Different rates and extents of decline in EPSPs recorded from tactile interneurons in three separate experiments. *A* shows responses of interneuron B to stimulation of each of the two halves of a split second root. *B* illustrates interneuron C responses to submaximal stimulation of the second root with opposite polarities. *C* shows responses of interneuron C to different afferents in the second root. In all cases, stimuli were delivered to the third segment every 2 sec, responses were recorded extracellularly from the interneuron axon dissected out of the nerve cord, and the afferent volley was monitored on the second root. EPSP amplitudes were adjusted to be proportional to incremental excitatory conductance changes.

All of these experiments indicate that different EPSPs antifacilitate at slightly different rates, and to somewhat different percentages of initial amplitude. Thus the above explanation of the results of the statistical analysis is now more acceptable.

The results support the hypothesis of presynaptic antifacilitation, and are inconsistent with a basis in postsynaptic inhibition or pharmacological receptor desensitization.

Besides antifacilitating EPSPs, other phenomena are involved in the activation of

tactile interneurons and complicate the above analysis. It is known that some of the subthreshold depolarization to afferent stimulation is due to branch spikes in these interneurons (32, 48), and that there are likely to be present small electrical EPSPs from other tactile interneurons, when moderate intensity stimuli are applied (58). These factors would tend to distort the simple relation that is assumed in the statistical analysis between the amplitude of the EPSP and the conductance change induced by afferent chemical synapses. Furthermore, some compound EPSPs show complex nonmonotonic changes to repeated stimulation (Fig. 11), and an early or late phase of facilitation is occasionally observed. In summary, the input to multisegmental tactile interneurons is predominantly, but not purely, chemical and antifacilitating.

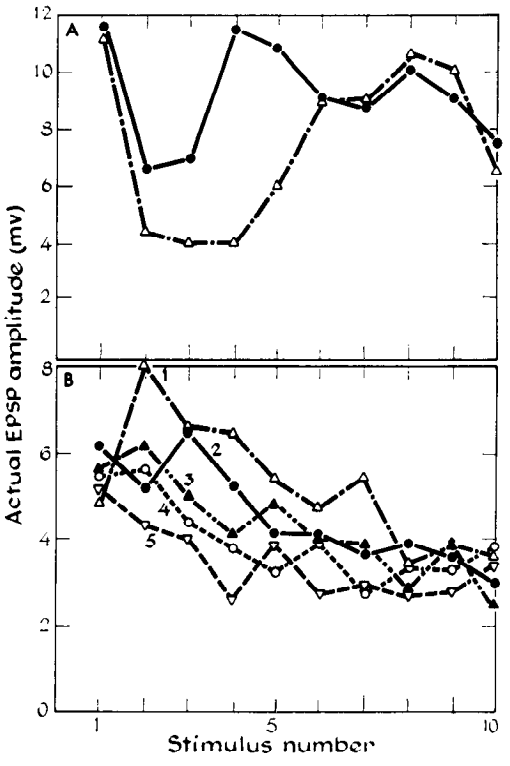

FIG. 11. Complex temporal behavior of some EPSPs recorded in the third ganglion from tactile interneuron C in response to repeated second-root stimulation. In *A*, an early phase of antifacilitation seems to be overcome, perhaps by an independent and slowly developing process of facilitation. The two lines connect data from different preparations. In *B*, the EPSP amplitudes to successive sequences of 10 stimuli are shown. Sequences are separated by 5-min rests. In the first few sequences, an early and rapidly saturating phase of facilitation appears to be superimposed on the usual gradual antifacilitation. In later sequences, this early phase is not evident, perhaps because the facilitation has remained at some plateau throughout the rest periods, or because the facilitation process is exhausted. Stimulus frequency: 0.2 Hz; except upper curve of *A*, 0.5 Hz.

DISCUSSION

Locus of physiological lability responsible for behavioral habituation

The results of early experiments (33, 36, 53) demonstrated clearly the relative reliability of transmission from the giant fibers to the flexor muscles via some of the phasic flexor motoneurons. The present results demonstrate similarly that receptor fatigue and active membrane processes in the interneurons and the decision fiber are unable to provide means for any response labilities lasting several seconds. The pit hairs simply do not fatigue to stimuli repeated that slowly. The presence of slow accommodation or delayed refractoriness in the lateral giant was sought by looking for signs of these processes following natural habituating stimuli or bridge-induced spikes. No changes in threshold or input resistance were detectable. The measurement of the latency of a spike to a barely suprathreshold depolarizing stimulus is particularly telling, because this measure is very sensitive to even slight changes in threshold, resistance, or time constant. The same tests yielded negative results in the major tactile interneurons of the neural circuit mediating escape. Furthermore, attempts to mimic or enhance waning of the interneuron responses to repetitive stimulation by conditioning sequences of subthreshold depolarizing resembling EPSPs or antidromic bursts of impulses, or afferent volleys from different segments, all failed. Only occasionally could response waning in an interneuron to a sequence of stimuli be enhanced slightly by stimuli delivered to other afferents to the same ganglion, or by bridge-induced bursts in the interneuron, and the possibility of some weak long-term refractoriness could not be eliminated. In addition, sequences of EPSPs from different afferents occasionally appeared to interfere with each other mildly,

but these effects are seen only rarely. Some long-term heterosynaptic depression must be acknowledged as possible. However, these phenomena can account for only a very small fraction of the response waning in interneurons.

On the other hand, the properties of the antifacilitation of the afferent-to-interneuron excitatory synapse nicely matched the response waning of spikes in interneurons and the decline of the lateral giant EPSP area. Furthermore, these electrophysiological signs of habituation share the properties of the behavior (J. J. Wine and F. B. Krasne, personal communication; and unpublished observations). In particular, repeated stimulation results in a decrement in response probability and in the magnitude of the physiological measures (Figs. 5, 6, 8, 9). The form of the decline is similar to that of a negative exponential. If the stimulus is withheld, the behavioral response and the electrical signs tend to recover progressively with time (Fig. 9). In successive series of habituating sequences of stimulation, the habituation proceeds more rapidly, apparently because of incomplete recovery (Fig. 5*B*). If the frequency of stimulation is increased, habituation occurs more rapidly, and is more pronounced (Figs. 8 and 9). If the intensity of stimulation is increased, habituation proceeds more slowly, and the electrical signs may wane more gradually (Fig. 10*C*). Response recovery is slowed when stimulation is continued beyond the point of zero response probability; this property has not been studied quantitatively for the electrical signs, but casual observations indicate that it is true for the afferent-to-interneuron EPSP and all consequent manifestations. These properties are characteristic of the majority of habituating behaviors (21, 49), and serve to qualify the response decrement of crayfish escape behavior as a bona fide example of habituation.

Certain properties of some other habituating behaviors are not shared by the crayfish escape response. For example, it has not been possible to find a stimulus which causes dishabituation of escape behavior to phasic mechanical stimulation of the tail. Similarly, electrical shocks to any afferent root or the nerve cord, interpolated into a habituating stimulation sequence, do not restore the responsiveness of any electrical sign to the habituating stimulus. Second, habituation appears to be quite stimulus specific. Crayfish habituated to anterior abdominal light taps will respond to a similar tap to the telson or uropods, and vice versa. The physiological results reveal no interganglionic generalization of habituation (Fig. 6*A*) and only occasionally a very weak intraganglionic generalization which is apparently not expressed behaviorly. These two properties of habituation, dishabituation and generalization, are frequently absent in other behaviors which habituate (21), and do not serve to remove this response decrement from the category of habituation. Thus the characteristics of the behavior are well represented by the properties of the excitatory synapses to which habituation is attributed.

The above cursory description of the behavior applies only to tail flips elicited by phasic abodminal tactile stimulation and mediated by the lateral giant neuron. An earlier report attributed somewhat different properties to this behavior (36). However, these investigators used abdominal compression as a stimulus, and it has recently been shown that such stimuli elicit nongiant mediated escape responses that cannot be compared to the behavior treated here (57). The only published study of the present behavior (56) is very incomplete, but does agree generally with the above description. One minor point of disagreement is that Wine and Krasne found slower recovery in crayfish escape behavior than I observe behaviorally or physiologically. The discrepancy is probably due to the fact that they overstimulate and carry habituation much further into saturation than I have.

Another result obtained by these authors is relevant to an assumption implicit in my preparation. I use only isolated abdomens and compare physiological results to behaviors elicited in an intact crayfish. This comparison is only legitimate if the entire circuit mediating escape behavior is contained in the abdomen, and if it is not influenced by higher nervous centers in any way that significantly changes its response characteristics. Wine and Krasne (56) tested these assumptions explicitly, and confirmed them. The time courses of escape habitua-

tion and recovery in intact animals and in animals with the nerve cord transected at the thoracoabdominal connective were indistinguishable.

Mechanism responsible for antifacilitation of synapses causing habituation

A statistical analysis of the afferent-to-interneuron compound EPSP revealed that antifacilitation was associated with a decrease in quantal number that more than accounted for the EPSP reduction. The concurrent increase in quantal efficiency was explained by an unequal degree of antifacilitation occurring in the different unitary components composing the compound EPSP. This complication could be avoided by recording only a single unitary EPSP in different states of depression. Unfortunately, stationary long-term recordings of a single uncontaminated EPSP were not obtained in these experiments (cf. Figs. 15 and 16, ref 58), and this refinement must await future work. It was concluded, nevertheless, that postsynaptic processes alone may be excluded as being responsible for antifacilitation, and that the phenomenon was due to some intrinsic presynaptic reduction in the synthesis, mobilization, or release of transmitter.

Failure of presynaptic spikes to invade terminals is unlikely, because recent anatomical studies (11, 60) indicate that crustacean synapses are formed by presynaptic axon cylinders coursing through postsynaptic dendrite ramifications, where the presynaptic spike does not have to invade a fine terminal to be effective. Presynaptic inhibition was shown not to be primarily responsible for synaptic depression because antifacilitation is observed in unitary EPSPs evoked by the stimulation of a single primary afferent (58). Presynaptic inhibition of long duration may possibly play a part in causing an occasionally observed heterosynaptic depression between EPSPs from different populations of afferents. A separate bit of evidence suggesting that inhibitory processes are not involved in habituation comes from the discovery (35) that picrotoxin does not affect the time course of EPSP decline in the lateral giant. Of course, picrotoxin may not block all central inhibitory processes, and there may be a diffusion barrier protecting the synapses from interference by this drug (see ref 58).

This analysis depends on the untested assumption that the afferent release of excitatory transmitter is a Poisson process. Without the ability to see miniature EPSPs, or to test explicitly the distribution of quanta released by presynaptic spikes at single synapses, this assumption must be treated with great caution. Several workers (1–3, 37) have seriously questioned the evidence for a precise Poisson description of the statistics of release of transmitter quanta. This weakens the credibility of the assumptions, therefore, and the results must be considered as only qualitatively indicative of the mechanism involved in EPSP antifacilitation. Nevertheless, even if the statistics of transmitter release are not strictly Poisson, so long as transmitter is released in quanta according to any similar statistical process, the change in the coefficient of variation of the EPSP amplitude means that antifacilitation consists primarily of a change in the statistics of release of quanta. Precautions employed in the data analysis eliminated artifactual contributions to the variance of the EPSP amplitude from voltage noise, nonlinear EPSP summation, and the probable variation in quantal size. Thus the suggestion that presynaptic mechanisms are responsible for the synaptic depression is still tenable.

Comparison of escape circuit to other circuits mediating behaviors

Only two other animal behaviors have been explained so far in terms of explicit connections between identified neurons. These are shortening reflexes in leech (44) and the gill-withdrawal response in the sea slug (9, 38). Both of these responses are mediated by simple monosynaptic reflex arcs between receptors and motoneurons, and are thus much simpler than the circuit described for crayfish escape behavior. In the leech, the synapses onto motoneurons were chemical or electrical, depending on the adequate stimulus to which the receptor responded best. It is not reported whether leech segmental shortening habituates. In *Aplysia,* chemical connections were described between sensory neurons and puta-

tive motoneurons (but see ref 45). Like crayfish escape responses, the gill-withdrawal response habituated, and the lability was partially attributable to antifacilitation of the afferent-to-motoneuron EPSP. The mechanism of synaptic depression in *Aplysia* remains to be elucidated.

The circuit described for crayfish differs from the above in that it involves several hierarchical orders of interneurons between the receptors and the motoneurons. The circuit is organized to evoke a complex stereotyped response to certain types of disturbances (58–60). There may also exist monosynaptic connections from afferents to motoneurons, but these alone are never strong enough to elicit tail flips, because tail flips evoked by phasic abdominal tactile stimuli must always be preceded by lateral giant impulses (57). The circuit is more similar in its structure and complexity to the sorts of connections that are envisioned in the vertebrate spinal cord and mediate spinal reflexes (15, 40, 51). These circuits, like the one discussed here, also display response decrements. Decrements in spinal cord (47, 52), and other nervous structures (23, 24, 26, 46, 54) usually can be observed in the consecutive responses of sensory interneurons, which may habituate so dramatically that they are called newness or novelty detectors. The striking input specificity of the response decrement has often encouraged speculation that the process was due to a depression of the synapse between afferents and primary interneurons. This has never before been proved, however. In addition, there have been few experiments to determine explicitly the physiological mechanisms underlying such synaptic depressions; discussions up until now have been mainly speculative in nature (1, 6, 7, 9, 25). If these speculations about the locus and nature of habituation are correct, it may be that the type of circuit presented here, and the mechanisms implicated, will turn out to mediate a large variety of adaptive behaviors.

The positive adaptive nature of habituation itself might be questioned. The answer lies in the natural history of the animal, the nature of its interactions with the environment, its sensitivity to these interactions, and the nature of the behavior which habituates. In the case of escape responses in crayfish, the utility of habituation to monotonously repeated stimulation is evident. Some of the tactile receptors which elicit tail flips are very sensitive, and respond to objects breaking the air-water interface. Quiescent animals respond at first to such stimuli (57), but soon habituate to successive stimuli. The utility of this behavior is easily demonstrated by letting water droplets fall onto the surface of a tank of young crayfish. Initially, they flip their tails, but soon they quiet down. Yet the animals still escape from novel disturbances. The circuit described in these papers and the consequent behavioral habituation thus permit crayfish to escape predators in a somewhat agitated environment.

SUMMARY

If a crayfish tail is tapped repeatedly, the animal produces single abdominal flexions and escapes from the first few stimuli; soon this all-or-none response ceases to appear, and the crayfish may appear to ignore the stimulus. The possible sources for behavioral habituation were explored.

It was already known that most of the neuromuscular and lateral giant-to-motoneuron junctions transmit reliably at frequencies greater than those needed to elicit habituation, and that the muscles do not fatigue; therefore, the lability responsible for habituation must be located in the afferent limb of the circuit.

Although the tactile receptors may vary in their adapting properties, none shows any fatigue to repeated phasic stimuli.

Neither the lateral giant nor the tactile interneurons show evidence of any long-lasting accommodation or refractoriness, which might contribute to habituation. These neurons have the same threshold and input resistance in both rested and habituated states. Furthermore, it is not possible to mimic habituation by subjecting these cells to sequences of depolarization or discharges which resemble their activity during repeated tactile stimulation.

The decline in the lateral giant compound EPSP and the number of spikes in tactile interneurons can be accounted for quantitatively by the decline in the ampli-

tude of the afferent-to-interneuron EPSPs during repeated tactile stimulation.

A statistical analysis of compound EPSPs in interneurons suggests that the primary, if not only, site of EPSP antifacilitation is presynaptic, and probably intrinsic to the afferent terminals.

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