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Habituation of an invertebrate escape reflex due to modulation by higher centers rather than local events

(crayfish/lateral giant fiber/learning/synaptic plasticity/inhibition)

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ABSTRACT **Learning is widely thought to result from altered potency of synapses within the neural pathways that mediate the learned behavior. Support for this belief, which pervades current physiological and computational thinking, comes especially from the analysis of cases of simple learning in invertebrates. Here, evidence is presented that in one such case, habituation of crayfish escape, the learning is more due to onset of tonic descending inhibition than to the intrinsic depression of circuit synapses to which it was previously attributed. Thus, the altered performance seems to depend at least as much on events in higher centers as on local plasticity.**

Since the writings of Cajal, learning has been conjectured to be due to alterations of synapses. Currently, it is widely supposed that learned changes in behavior are specifically due to changes in the intrinsic potency of synapses within (i.e., "local" to) the neural pathways that mediate the behavior. This belief forms the basis for much contemporary physiological and computational thinking (e.g., refs. 1 and 2).

Evidence for such local intrinsic change comes largely, though not entirely, from work on simple forms of nonassociative and associative learning in invertebrates (3, 4). These studies on well-delineated systems have largely displaced older work (e.g., refs. 5–8) suggesting that at least some kinds of learning, such as habituation, might be due to modulation of mediating circuitry by higher centers. However, we now present evidence that habituation of an invertebrate behavior that provided some of the first clear evidence for intrinsic local change *is* in fact substantially due to extrinsic modulation.

Tail flip escape responses mediated by the lateral giant fibers (LGs) of the crayfish habituate when the mechanosensitive primary afferents of the reflex are repeatedly activated (9). In acute experiments, transmitter release from the initial, cholinergic (and sole chemical) synapses of the circuit readily diminishes with repeated stimulation of afferents, providing a plausible basis for the behavioral habituation (10–13) (Fig. 1*B*). A descending γ-aminobutyric acid (GABA)-ergic "tonic inhibitory" pathway that projects onto the LGs has been extensively studied (15–19), but a role for it in habituation has been discounted because severance of the cord between thorax and abdomen, which removes the influence of the inhibitory pathway on initiation of LG escape, has seemed to have little effect on habituation (9). Thus, intrinsic, homosynaptic depression of the first-order synapses has been accepted as the basis for habituation in this system.

However, the sensory interneurons whose responses are diminished due to intrinsic depression must provide sensory information for behaviors other than escape (20). Therefore, if intrinsic depression were truly the major cause of the habituation, other behaviors utilizing the same sensory channels would tend to be altered as well. By contrast, tonic inhibition, which is focused directly on the LG command neurons (15, 16, 18), is well suited to suppress escape selectively. A reexamination of the role of tonic inhibition in escape thus seemed warranted.

METHODS

General. Freely behaving crayfish (*Procambarus clarkii*, obtained from various suppliers) were given daily training/testing sessions consisting of a sequence of 0.2-msec cathodal shocks via chronically implanted electrodes to nerve roots 2–4 of the last abdominal ganglion, which contain afferents from the tail fan (Fig. 1 *A* and *B*). Electrodes were stainless steel 00 insect pins (Wards) insulated except for a small gap where the electrodes crossed nerve roots or connectives to be stimulated or recorded (see ref. 21 for details of implantation). A computer adjusted the voltage of the shocks from trial to trial so as to keep them near the threshold for firing of the LGs, whose action potentials were detected by a chronic recording electrode resting on the dorsal surface of the nerve cord connective between the third and fourth abdominal ganglia. An indifferent electrode was located off the cord in the next more rostral segment.

On alternate trials weaker shocks were used to keep track of the threshold of sensory interneuron A (Fig. 1*A*), whose firing was also detected by the LG recording electrode. The threshold of this interneuron, which tends to fire consistently at the stimulus frequencies used here, provided a check on the effectiveness of test shocks in recruiting afferents. Test shocks were limited to 8 × the threshold for A.

Implantation of electrodes and, where necessary, cord severance or cannula insertions were done several days prior to beginning of testing with the crayfish submerged in 5°C Ringer solution. After surgery, the animals were kept in air at 5°C for 10 min to allow blood clotting before return to fresh water.

For statistical evaluation two-tailed *U* or signed ranks tests were used, as appropriate.

Experiment 1. Habituation of intact crayfish and crayfish whose nerve cords had been severed between thorax and abdomen to remove the influence of descending tonic inhibition of LG escape circuitry were compared. Cord severance was done with attention to sparing of the ventral artery. Lateral giant training/test shocks were given in three epochs: (I) 30 at 1 per 2 min, (II) 60 at 1 per min, and (III) 60 at 2 per min. The terminal epochs with LG test stimuli at 2 per min were included in an attempt to promote between-day habituation, but they were not used for data analysis, because intrinsic depression was expected to be substantial at this stimulation rate (10).

In some animals ongoing behavior at 3 sec before certain test trials was sampled. We scored for the occurrence of (*a*) defense posture, (*b*) backward walking, (*c*) climbing of aquarium sides, (*d*) forward walking, (*e*) manipulation of gravel, and (*f*) total apparent quiescence. Items *a–c* are known or thought to

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Abbreviations: EPSP, excitatory postsynaptic potential; LG, lateral giant fiber; PTX, picrotoxin; GABA, γ-aminobutyric acid.

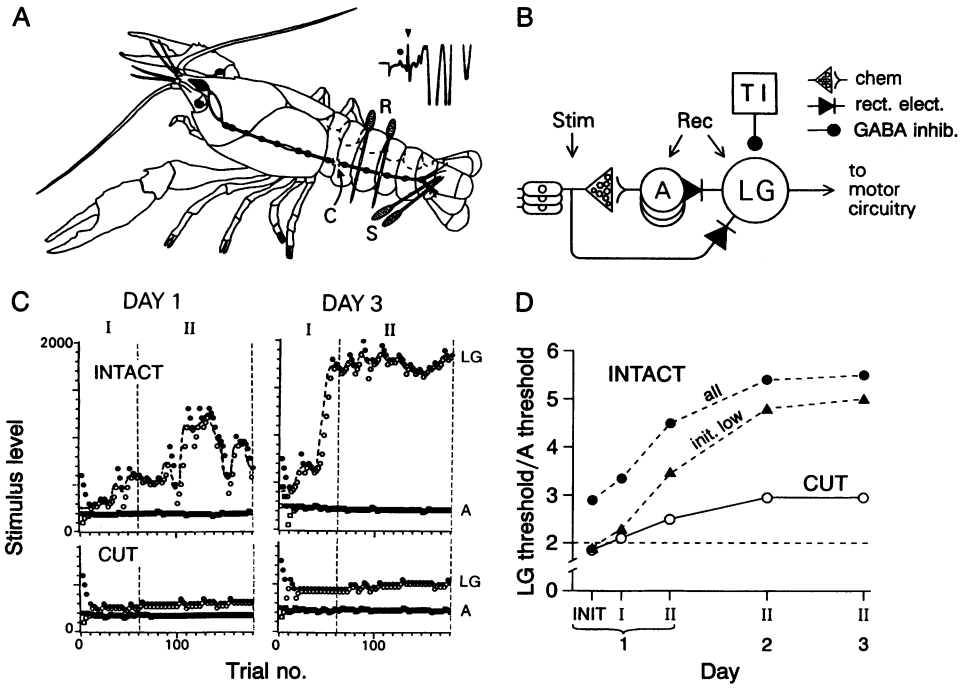

FIG. 1. Effect on habituation of severing the nerve cord at the entrance to the abdomen. (*A*) Location of chronic electrodes (S, stimulating; R, recording), level of cord severance (C), and recorded interneuron A (dot) and LG (arrowhead) responses to a voltage pulse. (*B*) Afferent portion of LG reflex circuit. Transmission at the rectifying electrical (rect. elect.) synapses of primary afferents on LGs appears to have a small chemical (chem) component (14). A, interneuron A; TI, rostral origin of tonic inhibition. (*C*) First and third days of habituation training/testing [for epochs I and II (10)] in illustrative intact and cut animals. Each point represents a trial; responses are indicated by solid symbols and nonresponses by open symbols. The dashed lines are interpretations of the threshold based on the data points. In each graph the LG curves lie above those for interneuron A. (*D*) Average thresholds (means across subjects of means over trials) for the periods indicated.

inhibit LG escape (ref. 22 and unpublished observations), whereas items *d* and *e* are common behaviors that could perhaps do so.

Experiment 2. To evaluate the effects on habituation of picrotoxin (PTX) antagonism of tonic inhibition, PTX mixed in 15 mM Hepes-buffered crayfish Ringer solution adjusted to pH 7.2 was injected via a chronic, rostrally pointing PE-10 cannula in the ventral sinus beside the 1–2 abdominal connective. Injections were made in three 0.1-ml aliquots of PTX (12 μg/ml) spaced 2 min apart to minimize behavioral agitation; dye dilution studies indicated that this produced a final concentration in blood of $\approx$0.6 μM PTX. This caused the legs to rigidly extend.

The excitation of LGs produced by root shocks is initially pure, but beginning at about 1.5 msec after the start of excitation it is mixed with feed-forward inhibition (23). To avoid confounding tonic inhibition with this feed-forward inhibition, the effects of PTX were assessed by selectively tracking the threshold for short-latency LG firings (<2.8 msec at our 3–4 connective recording site).

PTX injections were given on day 1 of the experiment, when habituation and inhibition were expected to be minimal, and then again when significant habituation (judged subjectively) had occurred. Stimulating electrodes were implanted bilaterally. On day 1, LG tests were given at 90-sec intervals alternating between sides. The side showing less initial inhibition as judged by the lesser threshold drop in response to PTX was selected for further experimentation. These initial tests were kept as short as possible (usually about 1.5 hr) to minimize habituation at this stage of the experiment. Once an electrode was selected for experimentation LG tests occurred every 90 sec, and training/testing sessions were about 2.5 hr long. On alternate LG tests (i.e., every 180 sec) thresholds were adjusted so as to track the LG threshold for responses at any latency (as opposed to short latency), but these data were not used.

Experiment 3. To evaluate generalization of habituation to contralateral stimuli, animals were implanted with stimulating electrodes bilaterally (see Fig. 5*A*). Each day 60 LG test stimuli at 1 per min (epoch I) followed by 60 at 2 per min (epoch II), alternating with interneuron A tests, were delivered first to one side of the animal to produce habituation and then to the other to test contralateral generalization. The side trained/tested first was alternated from day to day. As in experiment 1, the 2-per-min-trial data (epoch II) were not analyzed.

RESULTS

Experiment 1: Removal of Descending Inhibition by Cord Severance. As shown in Fig. 1 *C* and *D* and Fig. 2 the pattern of habituation was very different in intact crayfish (n = 21) and crayfish with the influence of tonic inhibition removed by cord severance (n = 19). For animals in both groups the LG threshold rose across days ($P < 0.01$ for percent increases of the ratio LG threshold/A threshold from the first to last point of Fig. 1*D* for both cut and intact animals) as well as within

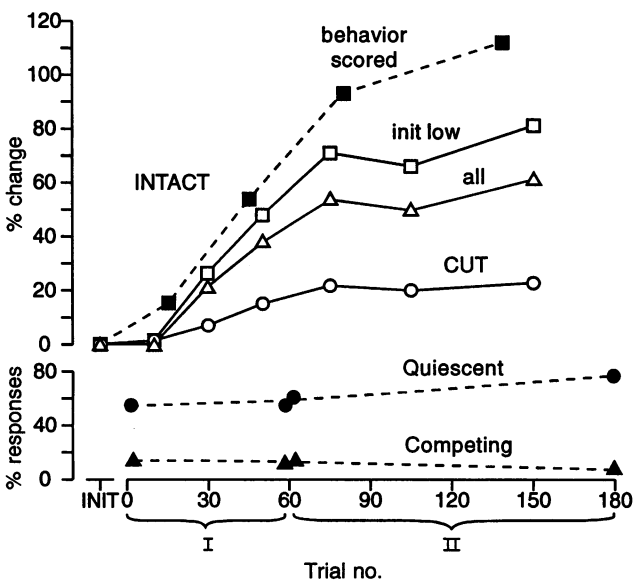

FIG. 2. Changes of escape and other behaviors within sessions. Solid curves show changes of threshold within session for the data of animals of Fig. 1 averaged over their 3 days of habituation training (and over thirds of epochs). The upper dashed curve is for a similarly treated group of animals (average over 4 days and halves of epochs) in which behavior just prior to selected LG test trials; the lower graph gives the frequency of two types of behavior (see *Methods*).

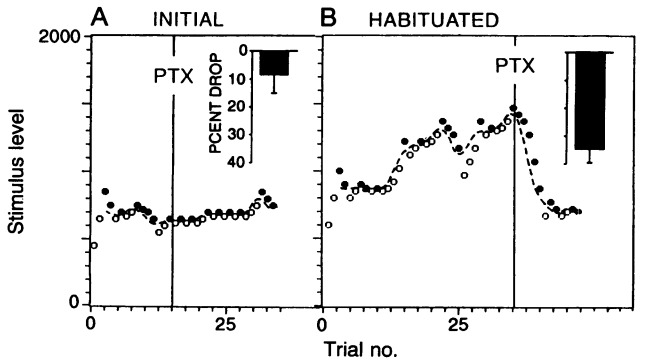

FIG. 3. Effects of PTX on fresh and habituated animals. (*A*) Response to PTX on day 1 in an illustrative animal. (*B*) Response to PTX after development of habituation. (*Insets*) Percent threshold drops (mean and SEM) produced by PTX for all animals.

sessions ($P < 0.01$ for percent threshold increases from first to last points in Fig. 2 for animals in both groups). However, these increases were more extreme in the intact animals [$P < 0.05$ (Fig. 1*D*) and $P < 0.01$ (Fig. 2) for percentage increases (as above) in ("all") intact vs. cut animals].

The within-session increases of the intact animals were often quite erratic (Fig. 1*C*), as might be expected if descending inhibition were being irregularly applied as habituation progressed; by contrast, the threshold increases of the cut animals were usually comparatively regular, consistent with habituation due to progressively developing synaptic depression. However, a few cut animals did sometimes show large, erratic threshold changes.

In cut animals, and in the majority of intact ones, the LG threshold at the beginning of the experiment was about twice that of the A threshold. But some of the intact animals had high thresholds from the outset, suggesting the preexisting operation of tonic inhibition. When the eight animals whose initial LG threshold was ≥3× the A threshold were removed from the analysis, the difference between the intact and cut animals increased in reliability [$P < 0.01$ (Fig. 1*D*) and $P < 0.003$ (Fig. 2) for differences of increases in cut ($n = 19$) vs. initially low intact ($n = 13$) animals].

To evaluate whether the threshold increases seen here might be due to an increase in behavior patterns known to inhibit LG escape, behavioral responses occurring 3 sec before each of the first and last 5 trials of periods I and II were scored in a group of intact animals run exactly as above (see Fig. 2). In fact, behavior patterns known or thought to inhibit LG escape (labeled "competing" in Fig. 2) did not increase within a test session, and animals actually became less active overall ($P <$ 0.02 for the increase in occurrences of "total quiescence" between the first and last points of Fig. 2 *Lower*).

Experiment 2: Antagonism of Inhibition with PTX. If habituation in intact animals is really due to the onset (or an increase) of tonic inhibition, then it should be reversed by the application of PTX, which blocks the GABA-linked chloride channels that mediate it (19). In nine animals PTX was injected on the first day of the experiment to assess its effect before development of habituation and then again on the first day where a conspicuous degree of habituation was seen. The PTX had little effect initially (Fig. 3*A*) but a sizable one once habituation had developed (Fig. 3*B*), consistent with a substantial contribution of tonic inhibition to the habituation process ($P < 0.02$ for percent threshold drop in initial vs habituated sessions). Of the nine animals in this experiment, six of them, like the one in Fig. 3, showed no discernable response to PTX on the initial (unhabituated) test, while others showed small to moderate responses presumably indicating the preexisting operation of tonic inhibition. On some occasions additional PTX tests were done before significant habituation had developed; in these instances PTX was without effect. The effect of PTX in an animal developed (or in the case of animals that showed some preexisting inhibition, increased) only when thresholds rose due to habituation. The threshold drop due to PTX was sometimes, as in Fig. 3, to the initial level for that animal but sometimes was incomplete, presumably because of some contribution of intrinsic depression to habituation or because of incomplete suppression of inhibition by the PTX.

In life, and in most experimental studies, habituation is manifest as a decrease of responding to repetition of a constant stimulus. However, in the threshold tracking procedure utilized here responses are forced out of the animal by continually increasing stimulus strength as habituation develops; it is conceivable that inhibition is invoked by the nervous system only when other habituation mechanisms do not lead to a cessation of energetically costly responding. To evaluate this possibility we habituated four animals to 1-per-min presentations of stimuli set 30% above initial the LG threshold. When applied after cessation of responding, PTX always restored responsiveness (Fig. 4).

Experiment 3: Generalization to Contralateral Stimuli. If habituation were entirely due to intrinsic depression of synapses of primary afferents, then there would be no generalization of habituation to an afferent channel not stimulated during habituation training. However, if tonic inhibition contributes to habituation, some generalization of habituation might be expected. To evaluate this possibility, we initially performed experiments in which animals were implanted with stimulating electrodes bilaterally (Fig. 5*A*), and the side on which LG test stimuli were given alternated from trial to trial. It was anticipated that sudden threshold changes such as those shown by the intact animal illustrated in Fig. 1*C*, and presumed to be due to onset and offset of tonic inhibition, might be synchronized bilaterally. Previous work had established that tonic inhibition can occur unilaterally (13, 17), but we thought that it might turn on bilaterally during habituation training. However, this was not the case; in a few animals there were some synchronous changes, but for the most part the two sides seemed to change independently.

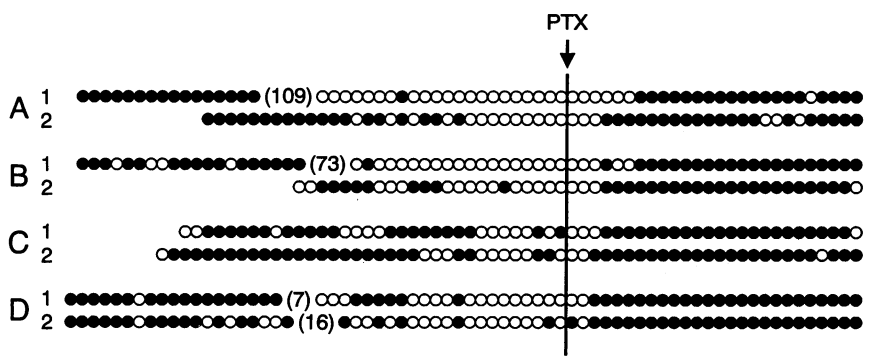

FIG. 4. Effect of PTX on animals habituated with constant stimuli. Responses (●) and nonresponses (○) for each trial are indicated. The first and second habituation tests for each of four animals (*A–D*) are given. The start of PTX infusion (as above) is marked.

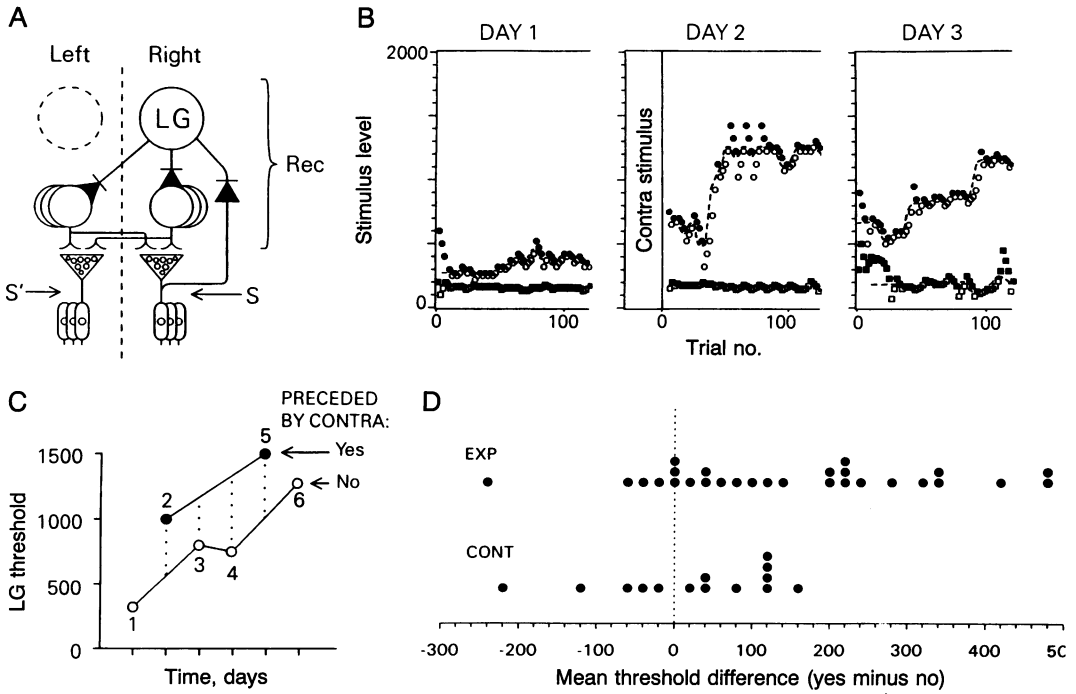

FIG. 5. Stimulus generalization of habituation. (*A*) Sources of input to the right LG. Stimulation at S′ should have no effect on primary afferent synapses excited by stimulation at S (see text). Primary afferents have a little contralateral innervation not depicted in the diagram (24), but this would provide no basis for generalization of habituation between S and S′. (*B*) First three test days (only epoch I is shown) for the left side of an illustrative animal; on day 2 habituation was increased by prior contralateral stimulation. (*C*) Mean LG threshold for epoch I for all sessions of above animal (see text). The mean threshold difference (the average of the difference between points on the two curves at the dotted lines) is 486 stimulus units. (*D*) Scatter plots of mean threshold differences for all electrodes tested. EXP, experimental; CONT, control (see text).

We therefore evaluated the possibility that habituation to stimuli on one side might increase the likelihood or extent of habituation to contralateral stimuli. To test this possibility, animals were each day trained/tested first on one side (to establish habituation) and then on the other (to test generalization); the side tested first was alternated from day to day. Fig. 5*B* shows the first three test days on the left of one animal. On the first and third days the left side was trained/tested at the start of the session, but on the second day the other side was trained/tested first. The effect of this preceding contralateral stimulation was to increase the amount of habituation on the left. Fig. 5*C* shows the mean LG threshold over the epoch I of each training/test session on this animal; on average these means were 486 (arbitrary) stimulus units higher when the side shown was tested second than when it was tested first. The similarly determined threshold differences for 28 similarly tested electrodes are given in the upper scatter plot of Fig. 5*D*, which shows that generalization of habituation contralaterally is a fairly consistent phenomenon [$P < 0.001$ for test that mean threshold differences in experimental condition (top points) of Fig. 5*D* are, as a group, different from zero].

To control for nonspecific factors a group of animals was implanted with bilateral electrodes as above, but the sensory roots of one side were severed and the LG testing stimuli to that side were fixed at 1500 stimulus units (near the maximum used in this experiment). The lower scatter plot of Fig. 5*D* shows that prior stimulation of the side with severed roots had little effect on habituation of the normal side. ($P < 0.2$ for test that mean differences of the control condition differed from zero; also $P < 0.05$ for test of experimental vs. control differences).

DISCUSSION

A role for higher centers in the establishment of habituation is obviously suggested by experiment 1, which establishes that removing the influence of higher centers on LG circuitry reduces the tendency of the LG threshold to rise during habituation training. By itself, this finding could result from removal of a constant inhibitory input that had served to attenuate the amplitudes of excitatory postsynaptic potentials (EPSPs). Intracellular experiments have shown that LG EPSPs increase as a negatively accelerated function of stimulus strength (ref. 10 and unpublished observations); thus, enhancement of EPSPs by removal of constant inhibition would diminish the stimulus strength needed to produce EPSPs above firing level and would thereby bring stimulation into a range where larger increases of EPSP amplitude were produced by smaller increases of stimulus strength. Consequently, a given percent EPSP reduction due to habituation would cause a smaller rise in LG threshold than would be the case were constant inhibition still operative. However, intact animals whose initial LG threshold indicated no initial inhibition still habituated more readily than did cut animals. This suggests that habituation training actually causes the onset of descending inhibition.

Inhibition that is not constant is also suggested by erratic changes of threshold during development of habituation in intact animals. However, such erratic changes were also sometimes seen in cut animals. This is puzzling but could indicate that the responsible inhibitory neurons are intrinsic to the abdomen and *can* be excited by events there though *usually* recruited by descending input from higher centers. Further work will be needed to clarify this.

Experiments with PTX provided more direct evidence that habituation is due to the fresh development of inhibition during habituation training, for in a number of animals in which habituation was largely reversed by PTX, this agent initially had no visible effect on LG threshold.

The possibility should be considered that PTX, which as a GABA antagonist can foster convulsive neural activity, might enhance escape excitability by increasing after-discharge of sensory interneurons or by causing behavioral arousal and attendant sensitization of LG escape (see ref. 21). However, in

either case PTX should reduce the LG threshold in unhabituated as well as habituated animals, which it did not. Moreover, behavioral sensitization is associated with drops in the threshold of sensory interneuron A as well as of LG (21), but in the present experiments PTX had no effect on the A threshold (data not shown).

It might be conjectured that PTX, whatever the basis for its action, fails to affect the LG threshold initially not because it is without effect but because in the unhabituated animal, where stimulus thresholds are low, EPSP amplitude increases as such a steep function of stimulus strength that enhancements of the EPSP resulting from PTX injection cause negligible changes in LG threshold (see discussion of experiment 1). In fact, however, as assessed from acute experiments, at the relatively high stimulus levels used to evoke short latency responses from unhabituated animals in PTX experiments, the slopes of (negatively accelerated) EPSP as a function of stimulus strength curves have dropped to about 8% of their slopes at the lower stimulus levels where EPSPs begin to rise. Moreover, in several cases PTX caused unambiguous drops in thresholds of habituated responses scored at "any" latency (see *Methods*), even when these were no greater than those of unhabituated short-latency responses. Finally, even though PTX never caused threshold drops in short-latency responses of cut (and therefore presumably noninhibiting) animals, octopamine, which increases LG EPSP amplitudes (25, 26) generally does do so (unpublished observation); therefore we would have expected to see threshold drops of short-latency responses in unhabituated animals if EPSPs had increased due either to reduction of tonic inhibition or to sensitization.

While the above discussion raises possible questions about particular experiments, the totality of the results presented here provides strong support for the hypothesis that habituation of LG escape in intact, freely behaving crayfish is in substantial measure due to an increase of inhibition that depends on the influence of centers rostral to the abdomen.

The onset of habituation within a session was never associated with an increase in the A threshold (though this did usually rise somewhat over many days of testing); thus it appears that the inhibition that contributes to habituation is directed specifically to the LGs. Since this inhibition shares target selectivity and PTX sensitivity with previously described "tonic inhibition" (15, 16, 18, 19), we assume as a working hypothesis that the same neurons produce both.

Intrinsic depression also appears to contribute to habituation but by itself to have only a modest impact on the animal's behavior. This means that habituation is under the control of higher centers rather than being an automatic consequence of local input derived solely from segmental stimulating events. This finding is in accord with common sense, which would seem to dictate that animals with encephalized nervous systems should not abdicate to low-level circuitry the control of something so important as habituation to potentially life-threatening stimuli. Obviously, if this is true for habituation in crayfish, then it would be expected to be true all the more for learning in animals with more encephalized control of behavior.

We believe that the present effects were not noticed in previous experiments (9) because habituation was evaluated by applying a constant stimulus set just above the initial LG threshold; under these circumstances the differences between intact and cut animals is small. The intact animals differ from the cut ones not in the rate at which habituation develops but in the degree to which thresholds rise.

One advantage to producing habituation by means of descending tonic inhibition of the LGs rather than intrinsic depression of first-order synapses is that the habituation is then specific to the escape response. Theoretically, such specificity could be achieved, even with intrinsic depression of the first-order synapses, if the escape-mediating interneurons whose responses were depressed were but a small proportion of those involved in producing responses whose habituation would be undesirable. However, this "parallel, distributed" processing approach (1) to achieving specificity seems not to play a major role in this system.

The use of descending inhibition of the LGs to produce habituation raises questions about the potential stimulus specificity of the habituation. However, the evidence indicates that the descending inhibitory pathway is at least capable of selectively inhibiting one side of a single segment (18).

Although the present findings show that habituation in crayfish is heavily dependent on the onset of descending inhibitory control, these experiments examined habituation during the relatively early stages of its development. It remains possible that at a later stage more profound local segmental changes might develop, for there is some evidence that local activity in conjunction with inhibitory events might be a condition for induction of some forms of intrinsic depression (27–29). Thus, it is conceivable that the participation of tonic inhibition in habituation demonstrated here is a transient condition that allows higher centers to control whether habituation occurs but that eventually results in establishment of local intrinsic changes that free the system from the need for constant descending control.

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