Table 1. Kinetics of acetylcholine synthesis and hydrolysis in sartorius muscle in normals and myasthenics (nanomoles of ^{14}C product formed per milligram of protein per 20 minutes, *p*H 6.8, 37°C, that is, units of activity).

	CAT		AchE	
	Units of activity	Mean ± S.D.	Units of activity	Mean ± S.D.
General anesthesia controls $n = 2$	22.6 ± 1.9 21.3 ± 1.2	22.0 ± 2.3	182 ± 23 180 ± 11	181 ± 24
Propoxycaine controls $n = 4$	12.3 ± 2.2 11.7 ± 0.8 11.2 ± 0.8 11.4 ± 1.5	11.6 ± 0.4	195 ± 7 228 ± 1 253 ± 23 161 ± 9	209 ± 42
Myasthenics* $n = 3$	9.7 ± 1.8 8.2 ± 1.3 8.0 ± 0.1	8.6 ± 0.8	229 ± 9 245 ± 18 330 ± 32	268 ± 55

* $P < .005$ between propoxycaine controls and myasthenics for CAT specific activity.

sartorius muscle biopsies were obtained from nonmyasthenic patients undergoing hip surgery under general anesthesia.

Our data demonstrate a significant partial decrease in CAT activity per milligram of protein in myasthenic skeletal muscle as compared to controls. No significant differences in AchE activity were found between normal and myasthenic skeletal muscle (Table 1). Serum and skin fibroblasts grown in cell culture were assayed for CAT, but no significant differences were noted between normal and myasthenic tissues.

Each muscle homogenate was assayed for CAT activity at four concentrations of protein in duplicate. There was an average 26 percent reduction in CAT specific activity in myasthenic homogenates as compared to propoxycaine control homogenates ($P < .005$).

The Michaelis constant K_m (acetyl coenzyme A) for a propoxycaine control, a general anesthesia control, and for two myasthenic homogenates was $7.35 \times 10^{-5} M$ when the choline iodide concentration was kept at $2.5 \times 10^{-3} M$. Substrate concentration curves indicated that noncompetitive inhibition seemed to be occurring in a myasthenic homogenate compared with a normal homogenate for the maximum rate of product formed (V_{max}). There was a 54 percent reduction in myasthenic activity, that is, 5.9 units (a unit is the number of nanomoles of [1-^{14}C]acetylcholine formed per milligram of protein per 10 minutes at *p*H 6.8 and 37°C) compared to 12.7 units for a propoxycaine control, and 19.2 units for a general anesthesia control (Fig. 1). A mixing experiment with equal volumes of myasthenic and normal muscle homogenates produced intermediate levels of activity. Product identification in all samples showed [1-^{14}C]acetylcholine.

The enzyme activities in muscle reported here most likely reflect metabolism at the nerve terminal. Sartorius is a muscle in which it is thought that end plates occur diffusely throughout its length and depth (*8*). There is no cholinergic innervated structure within skeletal muscle in the lower extremity other than the muscle fiber itself at the end plate. The parasympathetic nervous system does not innervate smooth muscle of blood vessels of the leg (*9*).

It is postulated that a noncompetitive endogenous inhibitor might be present in myasthenic muscle, as is suggested by the substrate concentration curves. If this is true, the autoimmune findings in myasthenia gravis may be related to the defect in neuromuscular transmission. The impairment in acetylcholine synthesis with a normal rate of hydrolysis, however, represents only one factor causing the clinical symptoms in this disease. Hypoplastic motor end plates with a reduced nerve terminal surface area, antibodies to muscle, and postjunctional membrane insensitivity to acetylcholine must also be considered (*3, 4, 10*).

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22 March 1971; revised 11 May 1971

Neuronal Circuit Mediating Escape Responses in Crayfish

Abstract. *The neuronal circuit underlying rapid abdominal flexion in response to phasic tactile stimulation comprises identified afferents, interneurons of two orders, a decision unit, and several motor neurons. The circuit is organized hierarchically as a "cascade" in which electrical synapses predominate at higher levels. Behavioral habituation results from lability at chemical junctions early in the pathway.*

One objective of work with the restricted nervous systems found in invertebrates (*1*) is to construct circuits of identified cells that are responsible for a specific behavioral event. Such circuits logically begin with sensory elements and end with muscles. Those described most fully to date include segmental shortening reflexes in annelids (*2*) and withdrawal responses in mollusks (*3*). We report here the elucidation of a more complex response of exteroceptive origin. It comprises a population of sensory fibers, three layers of central interneurons, and a duplex motor pathway; a variety of synaptic mechanisms are employed at the different kinds of junctions involved.

The behavior is a simple one. When crayfish are given a phasic mechanical stimulus to an abdominal segment, they often respond with a sudden contraction of the abdominal flexor muscles. Such a contraction, which propels the animal rapidly backward, is brought about by activity in nine large motor neurons located in each abdominal half-segment. One, the motor giant cell, is excited by the central giant fibers at an electrotonic junction located near its

exit from the central nervous system (*4*); this neuron innervates all the fast flexor muscles in its half-segment (*5*). The other eight motor neurons synapse with certain of the central giant fibers, as well as with other elements in the ganglionic neuropil, by branching in stereotyped fashion (*1, 6*). Each of these eight motor neurons innervates a specific, restricted part of the flexor musculature (*5*). A dual motor pathway thus connects central giant fibers to phasic flexor muscles.

The escape response elicited by tactile stimulation of the abdomen is best seen in young animals and habituates rapidly (*7*). Nearly always it involves the lateral giant fibers (*8*). These cells are segmental interneurons, and each contains a cell body with a system of dendrites in each abdominal ganglion (*9*). The axons of adjacent segments are connected end-to-end by septal electrotonic junctions and are cross-connected as well (*10*).

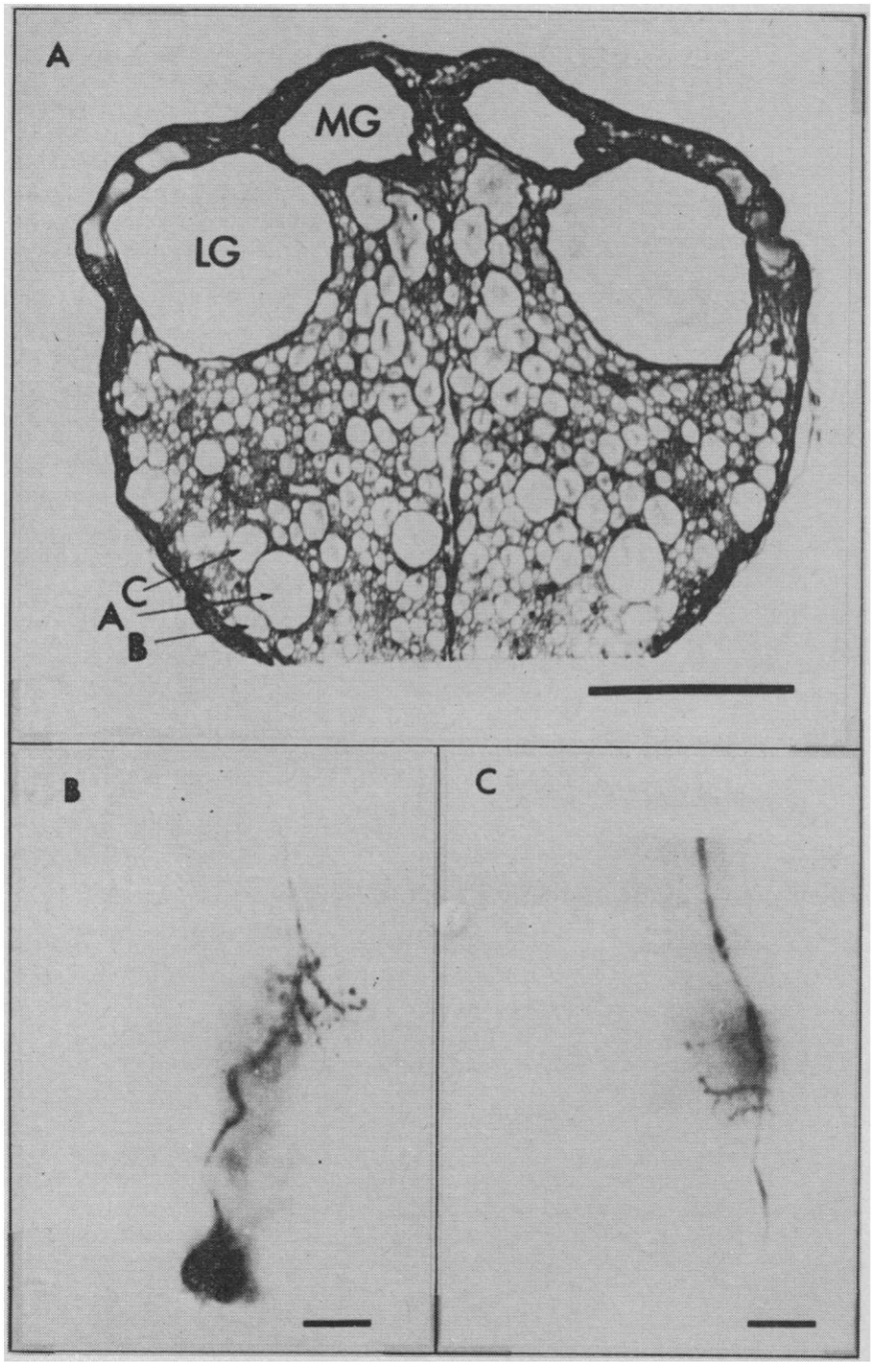

Fig. 1. Position and morphology of identified interneurons in the abdominal nerve cord of the crayfish. (A) Cross section of the ventral nerve cord between ganglia 3 and 4; *LG*, lateral giant axon; *MG*, median giant axon; *A*, *B*, and *C*, interneurons discussed in text (10-μm paraffin section, Masson's trichrome). (B) Interneuron A in sixth ganglion. (C) Interneuron B in fifth ganglion. (B) and (C) are both from whole mounts of ganglia in which the cell was injected with Procion yellow through the isolated axon after the latter had been identified physiologically. Calibration lines: 200 μm.

Both the lateral giant fibers and other identified interneurons receive input from abdominal tactile receptors. Among the latter are three axons shown in the cross sections of Fig. 1A. Cells B and C [probably equivalent to cells A63 and A64 (*11*)] are activated by hairs located on the dorsum of several abdominal segments; cell A [A6 (*11*)] is excited by similar receptors on the tail fan. The first two are known to receive synaptic connections separately in each of several segments, whereas A connects with afferent fibers only in the sixth ganglion. Reconstructions of these cells have been made by means of axonal injections of the fluorescent dye Procion yellow M4RS (*12*) (Fig. 1, B and C).

The afferent fibers that supply excitation to the interneurons originate in exoskeletal hairs. These structures, which are often dually innervated (*13*) are located in shallow pits on the dorsal and lateral exoskeleton of each abdominal segment and on the tail appendages. The conduction velocities of the afferent fibers range between 1.5 and 6.0 m/sec; larger cells tend to be more phasic and to have higher thresholds.

We have studied the synaptic relationships among these elements by recording, in units at each level, the results of activity in every identified antecedent neuron. Intact preparations of crayfish (*Procambarus clarkii*) abdomens were arranged (*14*) so that the entire nervous system was left connected to the periphery and the ganglia and connectives could be viewed by transmitted light. Interneurons were isolated from connectives by fine dissection so as to leave them intact at both ends; they were identified by location and receptive field. We then searched the neuropil of the appropriate ganglion with fine (15 to 40 megohm) micropipettes filled with 3*M* KCl until we penetrated a process of the isolated cell, as confirmed by its response to antidromic stimulation. Afferent fibers, or other identified interneurons, were stimulated or recorded from with fine suction electrodes (*15*). In some experiments, the axons of in-

terneurons were injected with Procion yellow after recording.

Cell A receives input from about 100 primary tactile afferents in the sixth abdominal ganglion. Figure 2A illustrates some properties of the unitary excitatory postsynaptic potentials (EPSP's) they produce in A. The EPSP's are judged to be chemical on the basis of their duration (> 5 msec for all penetration sites), their estimated synaptic delay (> 0.5 msec), and their antifacilitation at repetition rates of over 10 hz. The EPSP's are known to be unitary because each amplitude class at low frequency is associated with an impulse in a single afferent (Fig. 2B). They are also judged to be monosynaptic because their latencies are constant at all effective frequencies and are usually too short (0.6 to 1.2 msec) to permit an intervening interneuron. Within the portion of a receptive field served by a single ganglionic root, afferent fibers associated with proximomedial hairs showed faster conduction velocities and higher thresholds than those serving distolateral hairs. The large, proximomedial fibers are more likely than small, distolateral ones to innervate interneuron A, but no correlation was found between conduction velocity and synapic efficacy as measured by unitary EPSP amplitude. Since all unitary EPSP's in A are depolarizing and could be associated with impulses in tactile afferent fibers, A may be classified as an exclusively primary interneuron receiving only excitatory input.

The multisegmental interneurons B and C are also primarily chemically excited by tactile afferents in each segment of their receptive fields. These synapses invariably show dramatic antifacilitation, with both unitary and compound EPSP's declining to one-fourth their initial magnitude after a few stimuli delivered once every 2 to 5 seconds (Fig. 2B). Lesser decrements were observed even to stimuli delivered only once every 2 minutes. These EPSP's also increased in amplitude upon membrane hyperpolarization and decreased upon depolarization when current was injected through the recording microelectrode by means of a bridge circuit (*16*).

The configuration of input to these multisegmental interneurons is more complex than that to unisegmental interneurons exemplified by A. It was previously shown (*17*) that a synchronous afferent volley delivered in one segment to such a cell provides excitation to that interneuron in adjacent ganglia, as well as in the ganglion of entry. Either branching sensory axons or interposed central interneurons therefore must distribute excitation up and down the nerve cord. We have now demonstrated that unisegmental interneuron A produces EPSP's in unidentified multisegmental interneurons in the sixth ganglion, and generates small

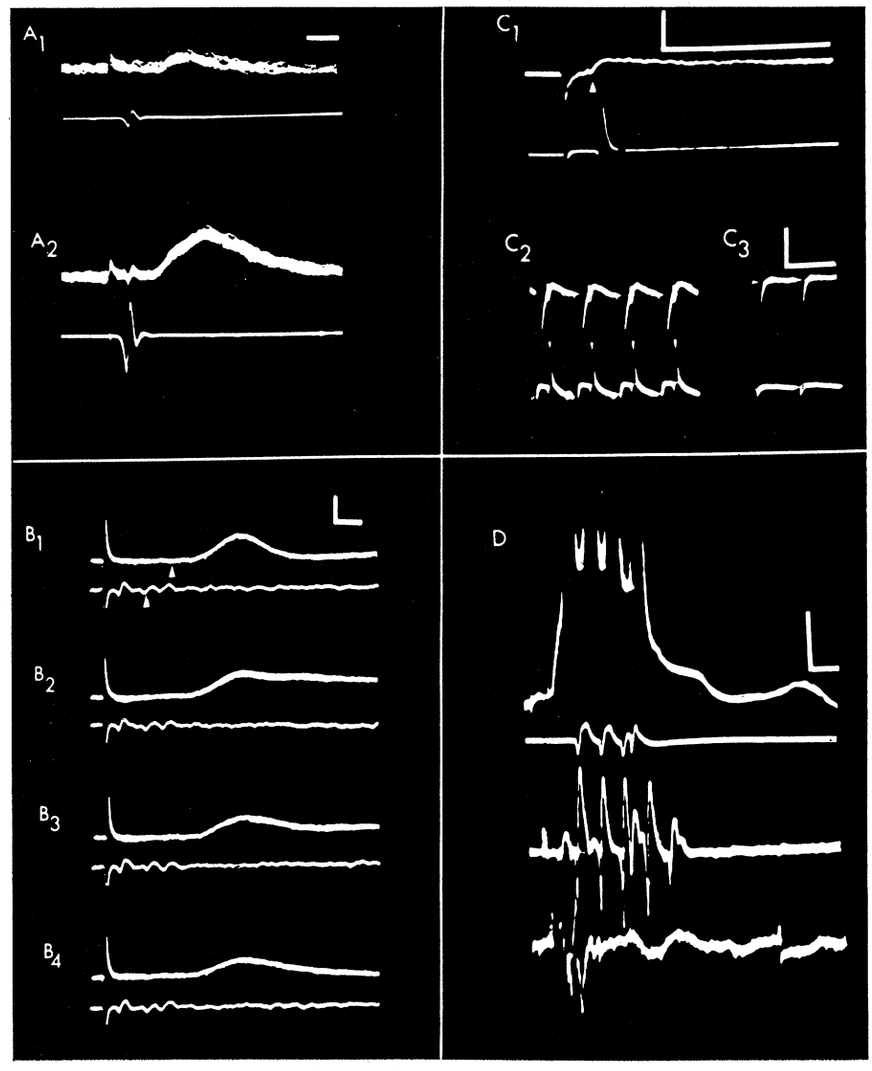

Fig. 2. Inputs to tactile interneurons. (A) Unitary EPSP in interneuron A in the sixth ganglion, as a result of stimulation of a single tactile afferent fiber in the fourth root (monitored on lower trace). Successive responses to stimuli repeated at 30 hz, recorded with an a.c.-coupled amplifier, are superimposed photographically (upper trace). The arrival of the presynaptic afferent discharge appears as a small focal potential. In A_2, stimulation of several afferent axons generated a compound EPSP. (B) Unitary EPSP in interneuron C recorded intracellularly in the fourth ganglion. The EPSP was uniquely associated with activity in the tactile afferent whose second root impulse is marked by a triangle in the lower trace. The calculated arrival time of the presynaptic discharge is indicated by a triangle on the upper trace in this and later figures; B_1, B_2, B_3, and B_4 represent the second, fourth, sixth, and eighth of a series of stimuli at 0.5 hz. Late portions of the intracellular trace are contaminated by tiny EPSP's from other low-threshold afferents which have already declined to a steady state. (C) Intracellularly recorded response of interneuron C to an impulse in interneuron A (monitored on lower trace). A single shock is delivered to the isolated axon in C_1 and repeated at 200 hz in C_2. (C_3) The artifacts generated by subthreshold shocks. (D) Intracellularly recorded response in interneuron C, fourth ganglion, to a maximal second root volley (upper trace). The axon was isolated in the connective between the fourth and fifth ganglia (4-5 connective) (second trace), and its impulses are also recorded by a gross electrode placed on the 3-4 connective (third trace). The bottom trace monitors the second root volley. The short latency of the response indicates a small electrical component to the mainly chemical afferent excitation. The first three spikes arise from the fourth ganglion, and the fourth from the fifth ganglion. Calibration lines: 2 mv, 2 msec.

EPSP's in at least one identified multisegmental interneuron in the fourth ganglion (Fig. 2C). Multisegmental interneurons B and C were also found to be synaptically coupled. Such interneurons thus receive input from other interneurons, as well as by means of a monosynaptic route from afferents. These findings explain the duplex nature of EPSP's observed previously in such cells (Fig. 2D).

Microelectrode penetrations of the lateral giant cell's dendrites showed that a variety of central interneurons, including all of the identified elements discussed above, produced unitary EPSP's there. Figure 3A shows such responses correlated with the extracellularly recorded discharges of the presynaptic neurons that evoked them. These unitary EPSP's, and those produced by primary interneurons in multisegmental ones, shared the following properties (Figs. 2C and 3B): (i) they were fast, having falling times equal to or greater than the membrane time constant; (ii) they were temporally stable, showing no amplitude or latency change even at driving frequencies of several hundred hertz; and (iii) they had synaptic delays approaching zero (*18*). On the basis of these criteria, they must be designated electrical EPSP's. In addition, these EPSP's were essentially unaffected by mild membrane polarization, unlike typical chemical EPSP's.

The EPSP's evoked in the lateral giant fiber by electrical stimulation of the abdominal second roots exhibit two components, an early (α) response that is temporally stable and a depolarization of longer latency (β) that antifacilitates rapidly (*19*). Our results suggested that the first component might be attributed to monosynaptic excitation by tactile afferent fibers, and the second to polysynaptic excitation mediated by interneurons of lower order. Figure 3, C and D, prove this proposition. The α response is recruited in increments that correspond to the addition of unitary discharges from single afferent fibers in the second root. As judged by the criteria referred to above, these unitary EPSP's are electrical. The β component is composed mainly of similar fast postsynaptic potentials contributed by a variety of identified interneurons; these potentials drop out in unitary fashion upon repetitive stimulation, as a result of the antifacilitation of chemical synapses onto tactile interneurons and the consequent decline in number of discharges.

Since the afferent fibers from tactile hairs can produce chemical EPSP's in primary interneurons or electrical ones in interneurons of higher order, it seemed important to ask whether single elements can have both actions.

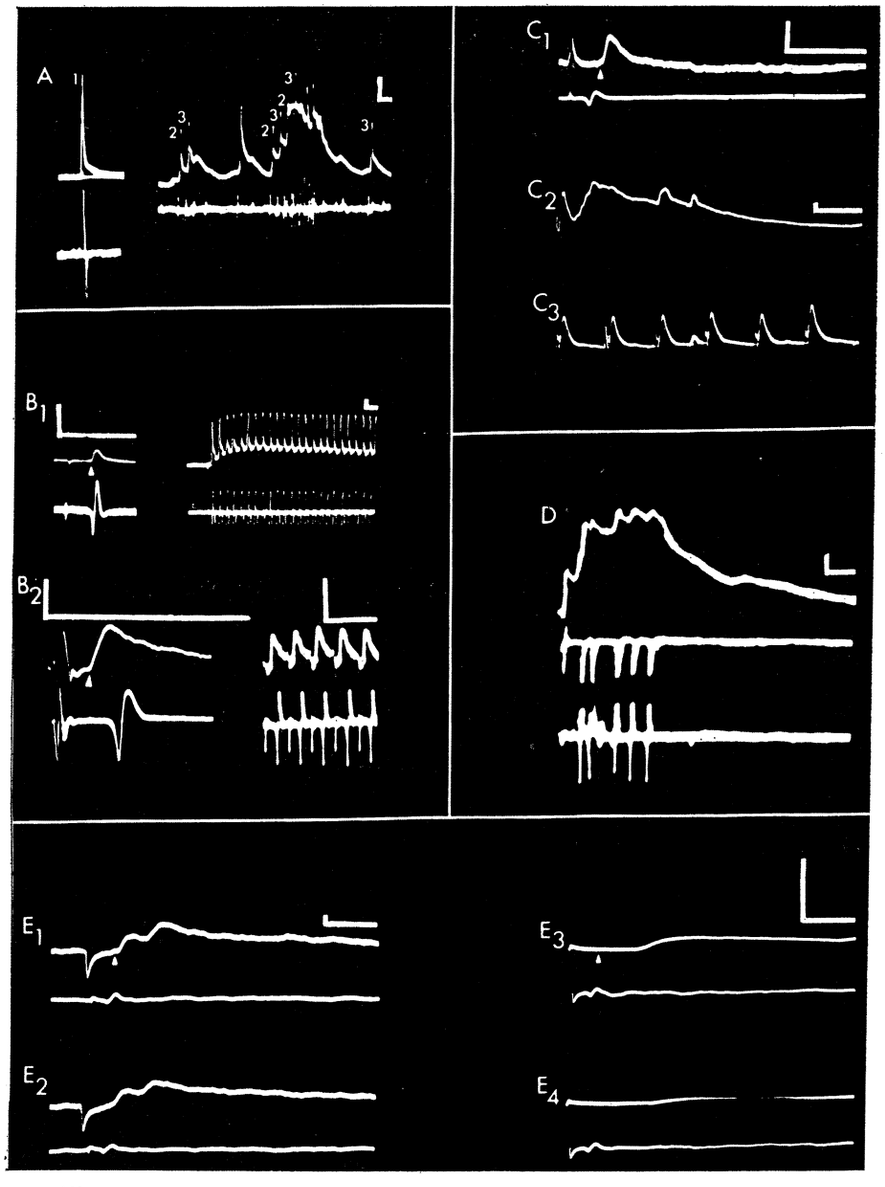

Fig. 3. Inputs to the lateral giant neuron. Intracellular recording from the lateral giant dendrite in the fourth ganglion on the upper trace in all cases except E_3 and E_4. Fast EPSP's in the lateral giant are produced by impulses in tactile interneurons (lower trace, monitored on the 4-5 connective). The interneuron generating each spike was identified by its receptive field. Spikes and EPSP's numbered 1, 2, and 3 correspond to interneurons A, C, and B, respectively. (B) Lateral giant responses to single shocks and high-frequency stimulation of interneuron A (B_1) and interneuron C (B_2). The lower traces record discharges in the identified interneurons. Vertical calibrations: (B_1) left, 20 mv; right, 2 mv. (C) Monosynaptic afferent excitation of lateral giant. (C_1) A unitary EPSP in the lateral giant begins just as the single tactile afferent impulse monitored in the second root (lower trace) arrives in the ganglion. When a maximal root stimulus (C_2) is repeated at 200 hz (C_3), only the early (α) component of the compound EPSP remains. (D) The late (β) component of the lateral giant response to a second root stimulus consists in part of five EPSP's generated by interneuron C, recorded alone from the 4-5 connective (middle trace) and in the 3-4 connective (lower trace). (E_1 and E_2) First and fifth lateral giant responses to stimulation of two tactile afferents at 0.5 hz. The first EPSP component is generated by the afferent discharge monitored in the second root on the lower trace. (E_2) and (E_4) are first and fifth responses recorded from an unidentified tactile interneuron in the fourth ganglion in the same experiment. The responses are associated with activity in the same tactile afferent fiber which is stimulated at 0.5 hz. Calibrations: 2 mv and 5 msec, except in B_1.

In Fig. 3E the arrangement of peripheral electrodes remained unchanged while recordings were made from successive penetrations of a multisegmental interneuron and the lateral giant. As judged from the waveform, amplitude, and latency of the recorded sensory discharges, the same afferent element responding near threshold can be identified in the two experiments and associated with unitary EPSP's in both postsynaptic elements. It may be concluded that some primary afferents evoke chemical EPSP's and electrical EPSP's in different cells (*20*). However, similar experiments have uncovered some afferent fibers which excite the tactile interneurons electrically, or do not apparently excite both interneurons and the lateral giant.

The motor giant neuron (*1*), as well as several of the other motor neurons (*21*) receive excitation from the lateral giant fibers in each abdominal segment. The junction from the lateral giant fiber to the motor giant neuron is known to be electrical. When recorded from somata of the other motor neurons, the highly decremented potential generated by the lateral giant has a relatively long central delay and appears to antifacilitate. For these reasons, chemical mediation of this connection seemed possible. This junction was explored further by the penetration of dendrites of motor neurons other than the motor giants, identified by their unique branching patterns in the third root. The approximate location of the dendrites is known from previous anatomical reconstructions (*1*). Repetitive stimulation of a motor neuron by a single giant fiber at 50 hz causes the potential to fragment into components which progressively drop out, leaving a small underlying potential of constant shape and amplitude and no measurable synaptic delay. We interpret these results to indicate an electrical synapse from the lateral giant fiber onto this motor neuron (*22*). The latter amplifies the synaptic potential with dendritic branch spikes (*21*). This interpretation also accounts for the soma potentials recorded earlier from this cell.

These lateral giant synapses onto motor neurons normally all operate with a high safety factor, so that impulses in central giant fibers are always adequate to secure transmission to several motor neurons. In the periphery the motor giant system fatigues rapidly, whereas the other motor pathway is temporally stable; repeated stimuli to central giant fibers therefore produce flexion reflexes that attenuate somewhat in strength (*5, 23*).

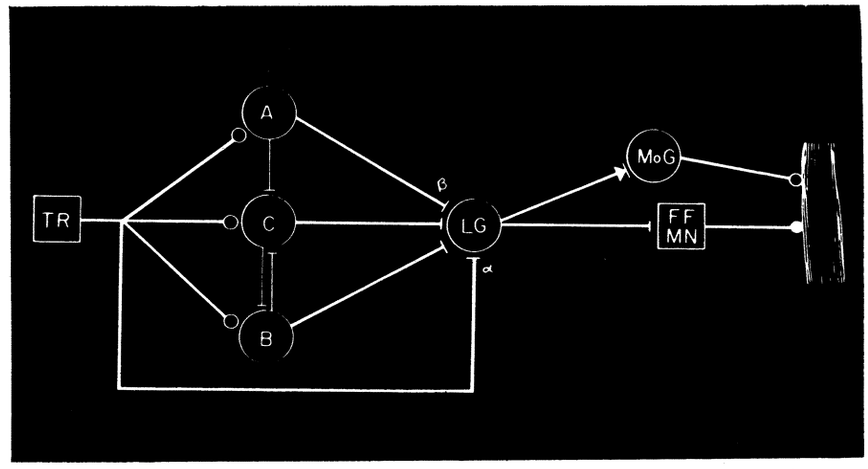

Fig. 4. Schematic diagram of identified neurons involved in the escape response circuit in the crayfish abdomen. Squares refer to populations of elements, whereas circles represent single neurons. *TR*, tactile receptors; *A*, an unisegmental tactile interneuron; *B* and *C*, multisegmental tactile interneurons; *LG*, lateral giant neuron; *MoG*, motor giant motor neuron; *FFMN*, fast flexor motor neurons exciting the phasic flexor musculature. Only excitatory connections are shown. Bars indicate electrical junctions, filled circles represent facilitating chemical synapses, and open circles are antifacilitating chemical synapses. Minor pathways are shown by thin lines.

The properties of the elements and junctions in the circuit are summarized in Fig. 4. Labile sites are located at the chemical junctions between primary afferents and primary interneurons, and between motor giant fibers and flexor muscles. In each case, temporally stable routes are available as alternatives to the labile ones, but motor output is reduced upon repetitive stimulation because the motor giant pathway drops out and because antifacilitation of the first, chemical synapses in the afferent limb of the response brings the total excitation supplied to the lateral giant fiber below its discharge threshold (*24*). The latter effect causes crayfish to cease to escape in response to repeated tactile stimulation, which constitutes a behavioral habituation. Every element at a given level in the circuit up to the lateral giant responds to elements at all levels below it. All input to primary unisegmental interneurons known so far is chemical, and all that to the lateral giant, the highest order interneuron and the effective decision fiber for the response, is electrical; in the latter case, but not the former, the size of the presynaptic element is positively correlated with synaptic efficacy. The multisegmental interneurons are intermediate, both in the hierarchy and in the nature of their inputs. They receive mostly chemical synapses from tactile afferents, which antifacilitate markedly when stimulated repetitively; they also receive small electrical inputs from other interneurons of lower or equal order.

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18. We have tried to distinguish chemical from electrical synapses by selectively blocking chemical junctions with solutions of high concentrations of Mg^{2+} or low concentrations of Ca^{2+}. Isosmotic saline containing eleven times the normal concentration of Mg^{2+} did not affect the strength of transmission between primary afferents and tactile interneurons, nor did it block large chemical inhibitory PSP's recorded intracellularly in other elements. Apparenty, Mg^{2+} does not block these chemical synapses and cannot be used to distinguish them from electrical junctions, perhaps because there is some diffusion barrier between the synapses and the external solution. Solutions containing 2 m*M* Ca^{2+}, instead of the normal 14 mmole/liter, often blocked axonal conduction and made stimulation of presynaptic elements difficult or impossible. This procedure thus also failed to distinguish chemical from electrical transmission.
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20. In addition, we have found that members of the same population of large and phasic tactile hair afferents located on the proximal margin of the uropod always excite interneuron A chemically, and the lateral giant electrically, in the sixth abdominal ganglion.
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24. We have compared the time courses of antifacilitation of the EPSP's produced by single tactile afferents upon tactile interneurons, the decline in the number of spikes in such interneurons, and the decrease in amplitude of the compound EPSP in the lateral giant and found them all to be similar. All these processes show sufficient decrements to prevent an escape response at frequencies similar to those used to demonstrate habituation behaviorally [see (*8*)].
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Defense of Phalangid: Liquid Repellent Administered by Leg Dabbing

Abstract. *The phalangid* Vonones sayi *has a pair of exocrine defensive glands that secrete quinones (2,3-dimethyl-1,4-benzoquinone and 2,3,5-trimethyl-1,4-benzoquinone). When distributed, the animal emits the secretion, dilutes it with aqueous regurgitated fluid, and effects dosaged delivery of the mixture by brushing it on the assailant with the tips of its forelegs. Predators such as ants are effectively repelled.*

Many arthropods possess effective means of chemical defense (*1*). When attacked, they douse the predator with a repellent liquid and escape. The fluid is usually secretory or enteric in origin, being discharged either from special glands, or from the mouth or anus. An anomalous case is described here, involving the phalangid *Vonones sayi* (Arachnida: Opiliones), which employs secretory and enteric products in mixture, and administers them to the enemy in an unusual way.

The defense of *Vonones* (*2*) was first observed when the animals were seized by hand or in forceps. Typically, as soon as an individual is grasped and gently pinched, two spherical droplets, usually of similar size, appear at the anterolateral margins of the body (Fig. 1A). The droplets are clear and odorless at first, but a small amount of brownish additive is suddenly squirted into them, causing them to swirl and discolor as the components mix (Fig. 1B), and to become strongly odorous. The animal then dips the tips of its forelegs into the mixture and, with intermittent bursts of rapid strokes, brushes them against the offending agent. Transfer of fluid to the target is quick and effective (Fig. 1, C and D).

Observation with a stereomicroscope showed that the clear fluid initially discharged in an oral effluent, which seeps to the margins of the body along two linear clefts formed between the closely apposed and immovable bases of the first and second legs (Fig. 1A, arrow). Proof that the fluid has been regurgitated was provided by the finding that animals fed a dilute aqueous solution of a nonabsorbable dye (amaranth) produced defensive droplets that on formation were pink.

The brownish material secondarily discharged is a glandular product. It is emitted from two small pores located marginally on the carapace (Fig. 1E) at the sites of emergence of the droplets of regurgitated fluid. Additive delivery of secretion to the droplets is thus assured. The two glands, previously studied in related species (*3*), are of conventional design, each consisting of a small sac with a narrow outlet and terminal valve (Fig. 1F).

As judged from its characteristic odor and ability to tan human skin, the brownish secretion appeared to be quinonoid, as is the defensive fluid of many other arthropods (*1*). This was confirmed by analysis. We milked *Vonones* by holding them in forceps, causing them to discharge, and soaking up the effluent with small disks of filter paper. Extraction of the papers with methylene chloride, followed by analysis of the dried extract by infrared spectroscopy, gas chromatography, and mass spectrometry, showed the presence of two components. These were identified by comparison with authentic samples as 2,3-dimethyl-1,4-benzoquinone and 2,3,5-trimethyl-1,4-benzoquinone. An analysis was also made of pure regurgitated fluid, obtained by taking up in capillary tubes freshly emitted droplets that were still clear and untarnished by added secretion. As expected, the fluid proved to be mostly aqueous and free of quinones (*4*).

In order to obtain some quantitative estimate of the defensive potential of *Vonones*, measurements were made of the quinone content of the glands, the concentration of quinone in the regurgitated fluid, and the total volume of the latter that can be evoked from an animal. Pure contents of the glands were obtained from animals killed by freezing, a procedure that causes the secretion to be squeezed in its entirety from the glands (*5*) and to collect as frozen spherical pellets (*6*) at the openings.

Two animals killed in this fashion, after an undisturbed caged existence of several weeks, produced, in one case, frozen secretion in an amount (0.05 μl) (*7*) matching the estimated capacity of the glands, and in the other case, a lesser quantity (0.02 μl). Subsequent gas chromatographic analyses of the two samples showed them to contain, respectively, 48 μg and 18 μg quinones, indicating that the secretion is apparently a pure quinone mixture, free from water. The concentration of quinones in the regurgitated fluid varies, but within relatively narrow limits (0.9 ± 0.6 μg/μl; based on four samples). The total fluid that can be elicited from a replete animal by persistent stimulation is also relatively constant (1.65 ± 0.22 μl; based on seven deliveries from four animals) (*8*). From these calculations it follows that an animal with a full glandular reserve of quinones (~ 50 μg) possesses sufficient chemical ammunition for an approximate total of 56 μl of regurgi-