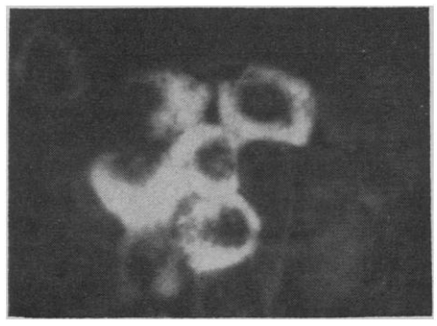

Fig. 1. Photomicrograph of a section of the anterior pituitary of an adult female rhesus monkey treated with guinea pig antiserum to SPRO followed by application of fluorescein-conjugated globulin prepared from antiserum to guinea pig globulin. The group of fluorescent cells in the center of the field as well as the weakly fluorescent cell in the upper left hand corner of the field are carminophils; the nonfluorescent cells to the right of center are all orangeophils. Other nonfluorescent cells in this field are either basophils or chromophobes. Magnification, ×1000.

consisting of either large cells frequently found in groups or clusters, or relatively small cells randomly distributed and occasionally present near the periphery of the section. The orangeophils were similar in size to the small carminophils and were not found in groups or clusters.

The orangeophils considerably outnumbered the carminophils in all but two female pituitaries, one of which was the lactating female, wherein nearly three-fourths of all cells were carminophils. The well-known function of prolactin during lactation (*10*) in all likelihood accounted for the large number of carminophils observed in the pituitary from the lactating animal. Since the state of the reproductive cycle in the other female monkeys was unknown, correlations could not be made with the relative numbers of carminophils present in their pituitaries. There was little doubt, however, that the pituitary of the lactating monkey showed the greatest absolute as well as relative numbers of carminophils.

The indirect technique was used in the fluorescent antibody studies, in which the same guinea pig antiserum to SPRO was employed as was used in the agar diffusion studies, followed by the application of fluorescein-conjugated globulin prepared from rabbit antiserum to guinea pig globulin (Nutritional Biochemical Corporation). The yellow-green fluorescence was clearly localized within both large and small types of cells (Fig. 1) which were identified as carminophils in the same section stained by Brookes' technique. Fluorescence did not localize within the orangeophils, basophils or chromophobes. Conversely, when guinea pig antiserum to human GH was used, followed by application of the same fluorescein-conjugate as used above, only the cells identifiable as orangeophils fluoresced. The latter was demonstrated in sections from three different pituitaries including one from a male and two from female monkeys. Various tests were performed as controls for the specificity of fluorescence, among which were: (i) incubation of the guinea pig antiserum to SPRO with unconjugated antiserum to guinea pig gamma globulin before its application to the section, followed by application of the fluorescein-conjugate; (ii) examination of the unstained, untreated section; and (iii) treatment of the section with guinea pig antiserum to human GH followed by application of the fluorescein-conjugate used above. None of the control tests showed fluorescence in the carminophils. The last of these procedures showed fluorescence only in the orangeophils and served as an experimental procedure, as previously mentioned, as well as a control.

Since only the carminophils fluoresced when pituitaries were treated with antibodies to SPRO and only the orangeophils fluoresced when treated with antibodies to GH, the results indicate that there is a substance in the primate pituitary that is immunochemically both related to SPRO and distinguishable from primate GH. This substance is presumably primate PRO. Our findings by no means disregard the biologic properties of PRO associated with preparations of purified human GH and monkey GH (*3*). These properties may well be intrinsic to the primate GH molecule, but the results of our investigation strongly suggest the existence of a separate PRO molecule in primate pituitaries.

D. C. HERBERT
T. HAYASHIDA
Department of Anatomy and
Hormone Research Laboratory,
University of California,
San Francisco 94122

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11. We thank Miss Eleanor Lasky for technical assistance and the Endocrinology Study Section (NIH) and Dr. Maurice Raben for gifts of ovine prolactin and human GH, respectively. Supported by grants from the PHS (AM 03550 and HD 04063), the University of California School of Medicine, and the Graduate Division of the University of California, San Francisco.

20 April 1970

Habituation and Dishabituation in the Absence of a Central Nervous System

Abstract. *Habituation and dishabituation have been observed in a semi-intact* Aplysia *preparation in which the central nervous system is removed. The amplitude of withdrawal responses in the gill decreases in proportion to the rate of water drops applied (one drop per 0.5 minute to one drop per 2.5 minutes at 15°C). The effects of habituation last for at least 2 hours. A dishabituated response is elicited by stopping the water drops or electrically stimulating the preparation. Furthermore, the gill contains nerve cell bodies, and habituation and dishabituation appear to be properties of these peripheral neurons.*

Habituation has been defined as a progressive reduction in response to repeated stimulation (*1*). The reduction can be reversed (dishabituation) by stopping or changing the stimulus (*2*). It is a process characteristic of the central nervous system of vertebrates and invertebrates (*1*, *3*, *4*) and is described as an elemental form of learning in that an animal has learned not to respond to a particular stimulus (*1*). Habituation also has been reported in semi-intact invertebrate and in spinalized vertebrate preparations (*2*, *5*, *6*). Thus, the intact central nervous system is not necessary for the process to occur. I report that habit-

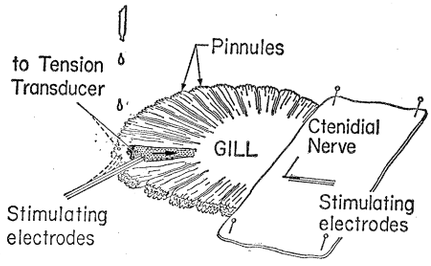

Fig. 1. The experimental arrangement of the deganglionated gill preparation. The pinnule's (stippled) withdrawal movement is indicated by the direction of arrow. The mantle was reflected away from the gill so that there was no contact between them. The water drops were released from a height of 14 cm.

uation and dishabituation are exhibited in an *Aplysia* preparation from which the entire central nervous system has been removed.

In an earlier study (*7*), electrical stimulation of the branchial and ctenidial nerves in the deganglionated gill preparation elicited patterned gill movements. The movements closely resembled those occurring when these nerves are left intact between the gill and the abdominal ganglion (*8*). Histological study revealed that several types of nerve cells in the gill (*7*) could account for patterned movements. These results permit one to ask whether the gill's neural organization is alone sufficient to carry out behaviors in addition to reflex withdrawal and patterned movement—

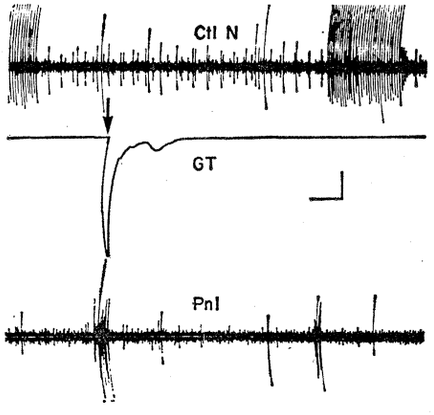

Fig. 2. Simultaneous recordings from the ctenidial nerve (*CtlN*), of pinnule movement (*GT*), and of electrical activity from the pinnule (*Pnl*). They are shown before, during (arrow), and after a water drop is applied. Considerable afferent activity is shown to be conducted by the nerve from the gill. Much of the activity recorded in the pinnule is not correlated with movement, suggesting the presence of active neural elements in the periphery. Calibration: 10 μv, 10 seconds.

behaviors such as habituation and dishabituation.

In the semi-intact gill preparation the cerebral, buccal, pedal, pleural, abdominal, and genital ganglia were excised, and the upper half of the animal was cut away. The gill, with its attachment to the mantle region, was left intact (Fig. 1). The preparation was suspended so that the gill moved freely in a chamber filled with seawater at 15°C (*8*). A thread connected one gill pinnule to a force transducer, and the amplitude of the pinnule movements, in response to water drops, was recorded on a polygraph. A suction electrode was positioned in the region in which water drops touched the pinnule. This electrode was used to stimulate or to record electrical activity from the pinnule. Another of these electrodes was placed over the ctenidial nerve [one of two nerves innervating the gill (*8*)] and used for electrical stimulation or for recording neural activity.

Neural activity was recorded from the ctenidial nerve in the deganglionated gill preparation (Fig. 2). The activity was composed of spontaneous and rhythmic discharges and could be observed for at least 12 hours. Recordings from the pinnule showed spontaneous electrcal activity not associated with movement, assumed to be from neural elements, as well as activity evoked by the water drops (Fig. 2). During spontaneous gill movements discrete discharges were correlated with the movements.

Water drops, applied to the surface of an individual pinnule, elicited contraction and movement toward the mantle cavity (Fig. 1). Other tactile stimuli also were restricted to the same region, but strong stimuli elicited movements over larger gill areas. The response occurred in both deganglionated gill preparations and in those in which the gill was still attached to the abdominal ganglion.

In the deganglionated preparation, with water drops presented at regular intervals, the amplitude of the withdrawal movements decreased with time (Fig. 3). In eight preparations tested, the intervals between drops ranged from 0.5 to 2.5 minutes. In Fig. 3 results from one preparation tested with three series of stimuli are shown. A rate of one drop per 0.5 minute was used in series *a* and *b*, which was twice the rate in *c*. Thirty minutes elapsed between *a* and *b* and 2 hours between *b* and *c* during which time no stimuli were applied. The results were as follows.

1) The rate of decrement occurs more rapidly (fewer stimuli) in *b* than in *a*, although the initial response in *b* shows full recovery. The first series has a pronounced shortening effect on the second series in spite of the rate of water drops being the same for both.

2) The same effect is not observed when one compares *b* to *c* where the drop rate in *b* was twice that in *c*. The same number of stimuli was used in both series. The rate of decrement in *b*

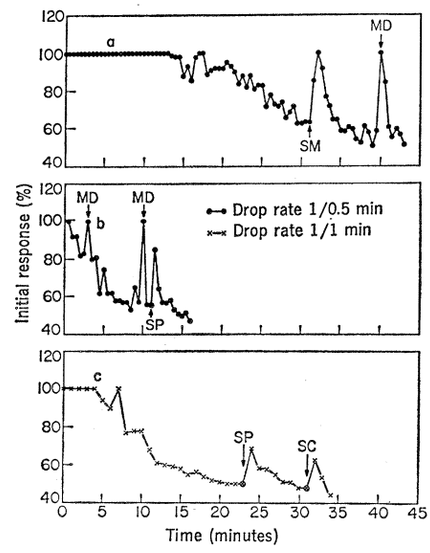

Fig. 3. Response decrement is shown as a function of time during which the water drops are applied to the gill at a constant rate. The response amplitude is plotted as the percentage of the amplitude ratios of successive responses to the first response. Curve *a* shows the time course of the habituation in an untested preparation, drop rate of one per 0.5 minute. After 37 minutes 50 percent of the initial value is reached. Reversal of the response decrement occurs after spontaneous gill movements (*SM*) and to two water drops in succession (*MD*). The effects of these two events carry over to several successive trials. The *b* stimulus series was taken 30 minutes after *a* at the same rate, one drop per 0.5 minute. After 11 minutes 50 percent of the initial value was reached. Here again two water drops elicited a full response. Electrical stimulation of the pinnule (*SP*) dishabituates the response for at least two trials. The *c* stimulus series was taken 2 hours after *b*; however, the stimulus rate was slower, one drop per 1 minute; *c* is about twice as long as *b* in reaching 50 percent of initial value. Compare *b* and *c* from time zero to *SP* (11 compared to 23 minutes). Electrical stimulation of both the pinnule and the ctenidial nerve give rise to dishabituated responses to water drops. *MD*, two water drops in rapid succession; *SC*, ctenidial nerve electrically stimulated prior to testing gill's responsiveness to water drop. (Electrical stimulus, 8 pulses per second for 1 second.)

is faster than in *c*, and the habituation is proportional to the drop rate.

3) The effects of the previous series are still in evidence in *c*. The rate of response decrement in *c* was still faster than in *a*; fewer stimuli were applied in *c* than in *a* (74 compared to 23) to bring the decrement to 50 percent. The effects of previous stimulus series (*a* and *b*) on *c* appear to linger in the deganglionated preparation in the absence of any external stimulus. All of the above findings were observed for other intervals.

Recovery of the full response (dishabituation) was achieved by (i) cessation of the water drops and (ii) change in the quality of the stimulus. After 30 minutes (10 minutes in some cases) during which no drops were presented, resumption of the stimulus elicited a full response (spontaneous recovery) to the first stimulus, but subsequent drops elicited reduced amplitudes of response and the rate of decrement was more rapid (compare *a* and *b* in Fig. 3). When two drops were applied in rapid succession (within 2 seconds, Fig. 3, *MD*) the amplitude of the response returned to that of the initial response and was sometimes greater. Two other manipulations restored, in part, the response amplitude. Electrical stimulation of the pinnule's surface and of the ctenidial nerve elicited partial return of responsiveness (Fig. 3, *SP*, *SC*). Spontaneous movements of the gill also temporarily restored the pinnule's responsiveness to water drops (Fig. 3, *SM*). In fact, the aftereffects of the electrical stimulation and spontaneous movements persisted for several presentations of water drops (Fig. 3). Dishabituation by the above manipulations, especially by the application of two water drops in rapid succession, argues against receptor adaptation, or effector fatigue, or both.

The gill response displays other characteristics of habituation as generally discussed in the literature (*2*, *3*, *5*, *9*). That is, the response decrement appears to follow a negative exponential function; the more rapid the stimulation rate the faster habituation occurs (Fig. 3, *b* and *c*); successive series of stimuli bring about response decrement more quickly (compare *b* and *c* to *a* in Fig. 3); recovery of response occurs after water drops are withheld; dishabituation is exhibited when the quality of the stimulus is changed, two drops being presented instead of one; electrical stimulation of gill or nerve dishabituates the withdrawal response.

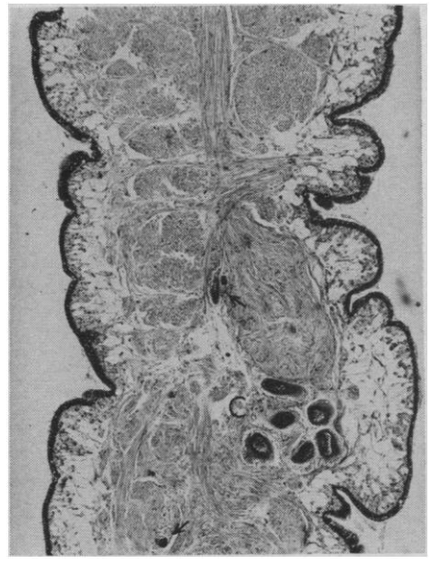

Fig. 4. Longitudal section through pinnule showing cluster of neurons (*C*) at its base. These nerve cells are found around the nerve trunk passing into the pinnule. Smaller nerve cells are seen in muscle masses (arrows). Nissl substance has been observed in nerve cells in the gill. The histological findings suggest that three neural elements (sensory neuron, interneuron, and motoneuron) are possibly involved in the habituated withdrawal response. This is analogous to the neural circuit proposed for the flexion reflex which also habituates and dishabituates in spinalized cats (*13*).

Histological study of the gill has revealed a variety and a considerable number of neurons which could be responsible for habituated responses (*7*). Clusters of nerve cells with processes are found at the base of the pinnules (Fig. 4). Cells with axon-like processes are located along the epithelial margin of the pinnules; they may very well be first-order sensory neurons. Other nerve cells similar to those seen in the clam adductor muscle (*10*) are found in the gill musculature (7). The recordings (Fig. 2) in the ctenidial nerve and pinnule could be the activity of these peripheral neurons, thus making it possible to study activity in the gill during habituation.

Gill movement appears to be a necessary prior condition for rapid dishabituation to occur. Reversal of response decrement to electrical stimulation of the ctenidial nerve suggests that activity passing from the central nervous system to the gill, in part, has a dishabituating influence. Habituation of the gill to tactile stimuli can easily occur because it is in contact with the mantle flap and the floor of the mantle cavity much of the time, and because there is debris and particulate matter passing through the pallial cavity. The periodic spontaneous gill movements under central control may be, in addition to their other purposes (*8*, *11*), dishabituating the gill's response to tactile stimulation. However, why the gill does not habituate to the periodic activity passing from the abdominal ganglion is as yet undetermined.

These results indicate that habituation and dishabituation are perhaps characteristic of a particular neural organization independent of its location and are not necessarily central processes. The *Aplysia* gill, with its abundance of nerve cell bodies, can be used to determine minimum organization necessary for exhibiting habituation and dishabituation and the neural mechanisms involved in these processes.

Note added in proof: After this paper was submitted, three reports (*12*) emphasized that habituation in *Aplysia* is a central process. Further investigation is needed to show the relationship between peripheral and central neurons.

BERTRAM PERETZ
Department of Physiology and Biophysics, University of Kentucky College of Medicine, Lexington 40506

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13. R. F. Thompson, *Foundations of Physiological Psychology* (Harper and Row, New York, 1967), pp. 608–619. It is possible that the dishabituation is not simply a reversal of habituation but the expression of another process, sensitization, as Sharpless (personal communication) has suggested.
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