

rate was less than 3 pmole/hr. Although HIOMT has been thought to be the rate-limiting enzyme in melatonin synthesis (1), these observations suggest that dibutyryl cyclic AMP may control melatonin production in this experimental model through another mechanism. Dibutyryl cyclic AMP may primarily stimulate the acetylation of serotonin and control melatonin production through substrate availability for HIOMT.

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11. J. Axelrod and H. Weissbach, *J. Biol. Chem.* **236**, 211 (1961); D. C. Klein and S. Lines, *Endocrinology* **84**, 1523 (1969). The HIOMT reaction mixture includes *S*-adenosyl methionine ( $3 \times 10^{-6}M$ ), *N*-acetylserotonin ( $10^{-4}M$ ), 100 nc of [ $^{14}C$ -methyl] *S*-adenosyl methionine (Amersham Searle), and 40 percent of the homogenate of one gland in a total of 0.3 ml of 0.5M sodium phosphate buffer (pH 7.9).
12. During review of this paper, Shein and Wurtman [*Science* **166**, 519 (1969)] reported that dibutyryl cyclic AMP increases the formation of [ $^{14}C$ ]melatonin and [ $^{14}C$ ]serotonin from [ $^{14}C$ ]tryptophan. Our results agree with these because to increase synthesis of melatonin from tryptophan the synthesis of (the intermediate) serotonin may be increased. However, studies using the inhibitor of serotonin synthesis *p*-chlorophenylalanine (*p*CPA) seem to indicate that the effect of dibutyryl cyclic AMP in stimulating melatonin production is not dependent on prior elevation of serotonin production. We have found that whereas *p*CPA (1.0 mM) inhibits the effect of dibutyryl cyclic AMP on conversion of [ $^3H$ ]tryptophan to [ $^3H$ ]melatonin to  $35 \pm 20$  percent of the normal stimulation, the effect of dibutyryl cyclic AMP on [ $^{14}C$ ]serotonin conversion to melatonin is not reduced by *p*CPA, but is slightly enhanced ( $125 \pm 20$  percent). This suggests that a specific site of action of dibutyryl cyclic AMP involved in stimulating melatonin production is at a metabolic step that does not depend on the new synthesis of serotonin, and will take place when an exogenous source of serotonin is provided, as in the experiments presented here.

## Habituation and Dishabituation of the Gill-Withdrawal Reflex in Aplysia

**Abstract.** *A behavioral reflex mediated by identified motor neurons in the abdominal ganglion of Aplysia undergoes two simple forms of short-term modification. When the gill-withdrawal reflex was repeatedly evoked by a tactile stimulus to the siphon or mantle shelf, the amplitude of the response showed marked decrement (habituation). After a period of rest the response showed spontaneous recovery. The amplitude of a habituated response was facilitated by the presentation of a strong tactile stimulus to another part of the animal (dishabituation). Many characteristics of habituation and dishabituation in Aplysia are similar to those in vertebrates.*

The analysis of the neural mechanisms of learning and similar behavioral modifications requires an animal whose behavior is modifiable and whose nervous system is accessible for cellular analysis. In this and the subsequent two papers (1, 2) we have applied a combined behavioral and cellular neurophysiological approach to the marine mollusk *Aplysia* in order

to study a behavioral reflex that undergoes habituation and dishabituation. We have progressively simplified the neural circuit of this behavior so that the action of individual neurons could be related to the total reflex. As a result, it is possible to analyze the locus and the mechanisms of these behavioral modifications. We now describe behavioral parameters of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*.

Habituation and dishabituation are simple behavioral modifications often considered to be the most elementary forms of learning (3-5). Habituation is the decrement of a behavioral response that occurs when an initially novel stimulus is repeatedly presented. Spontaneous recovery of the decremented response occurs if the stimulus is withheld for a period of time. Dishabituation, the restoration of a previously decremented response, occurs following a change in the stimulus pattern, such as the presentation of another, stronger stimulus (4).

Parametrically similar forms of short-term habituation, which last from several minutes to several hours, have been demonstrated for a variety of behavioral responses in all animals which have clearly developed central nervous sys-

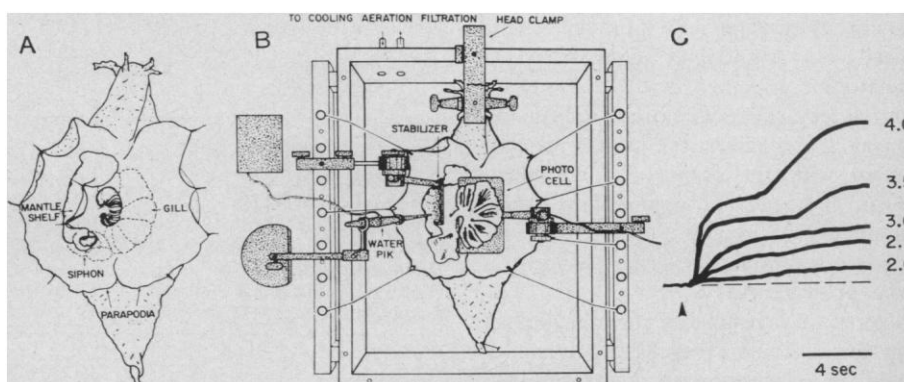


Fig. 1. (A) Dorsal view of an intact animal showing a fully contracted gill. Normally the parapodia and mantle shelf obscure the view of the gill, but they have been retracted to allow direct observation. The relaxed position of the gill is indicated by the broken lines. The tactile receptive field for the gill-withdrawal reflex includes the siphon and the edge of the mantle shelf. (B) The animal was immobilized in a small aquarium containing cooled and aerated circulating seawater. The edge of the mantle shelf was pinned to a substage, and a constant and quantifiable tactile stimulus consisting of a brief jet of seawater was delivered by a Water Pik (a commercially available oral hygiene apparatus). The stimuli were controlled by a Grass S-8 stimulator and were usually 800 msec. The gill contractions were monitored with a photocell placed under the gill. The output of the photocell was linearly related to the area uncovered as the gill contracted and was recorded on a polygraph. (C) Gill responses to individual tactile stimuli of different intensities. The stimuli were separated by very long intervals of time. The intensity of the stimulus could be adjusted anywhere from a very light touch (1.0, arbitrary units) to a very intense pressure (5.0). The weakest stimulus (2.0) evoked only a small gill contraction which consisted of a simple, short-latency withdrawal. Stronger stimuli (2.5, 3.0) evoked bigger and longer lasting gill responses of similar short latency but, if strong enough (3.5, 4.0), could bring in a second, longer latency component. The latency for this second component was quite variable, and with the strongest stimuli it sometimes merged with the first component.

tems (3-6). The behavioral similarity across species suggests that there may be common neuronal mechanisms of short-term habituation.

We have examined habituation and dishabituation of a behavioral reflex controlled by the abdominal ganglion of *Aplysia*. This ganglion offers a number of advantages for the cellular neurophysiological analysis of behavioral mechanisms. It contains a small number of nerve cells, all of which are large enough to be penetrated with microelectrodes for recording synaptic potentials and for direct stimulation. Many of these cells have been identified as unique individuals or as members of functional clusters (7). The connections of some of the cells with each other (8) and with peripheral sensory and motor structures (9) have also been specified.

The specific behavior that we have chosen for analysis is a gill-withdrawal reflex that occurs as part of a larger defensive withdrawal response that is triggered by a potentially noxious tactile stimulus (Fig. 1A). Analogous defensive escape and withdrawal responses are present in other invertebrates as well as in vertebrates. Probably because defensive reflexes must be fast to be effective, the neural circuitry of these reflexes is usually relatively simple, often involving only a few synaptic relays. In addition, defensive reflexes typically habituate quite readily (6).

The gill system of *Aplysia* offers further advantages for a neural analysis of behavior. First, most of the motor neurons that control gill contractions have been identified (9). Second, the gill reflex can be effectively studied in a restrained, but otherwise intact, animal. Third, reflex withdrawal of the gill can be elicited from stimulation of a receptive field that does not include the gill itself, thereby minimizing the contribution of local peripheral reflexes. Finally, gill movements occur both reflexly, as a result of sensory stimulation, and spontaneously, as a result of the endogenous activity of neurons within the ganglion (9). The gill system therefore also serves as a potential model for a variety of more complex behavioral processes that involve stimulus pairing or pairing of a spontaneous response with a reinforcing stimulus.

A major problem in studying behavioral responses in *Aplysia* is to restrain these soft-bodied animals with minimum damage and to apply reproducible stimuli to their peripheral sensory re-

ceptors. We accomplished this in the apparatus shown in Fig. 1B. The animal was immobilized in a small seawater aquarium, and gill contractions were monitored with a photocell placed under the gill. The gill-withdrawal reflex can be evoked by a tactile stimulus within a receptive field that is centered on the siphon and mantle shelf and falls off sharply in the surrounding regions. The area along the dorsal edge of the mantle shelf was pinned to a substage, and a tactile stimulus was delivered by means of a brief jet of seawater (10).

We first examined the responses of the animal to individual stimuli that

were presented to the same spot on the skin and differed only in intensity (Fig. 1C). The weakest stimulus evoked only a small gill contraction which consisted of a simple, short-latency withdrawal. Stronger stimuli evoked bigger and longer lasting gill responses of similar short latency, but, if strong enough, these stimuli also brought in a second component that usually had a much longer latency.

In order to simplify the behavioral analysis of habituation we adjusted the stimulus intensity (Fig. 1C) to obtain a short-latency component in the absence of a superimposed late component. In addition, we further restricted

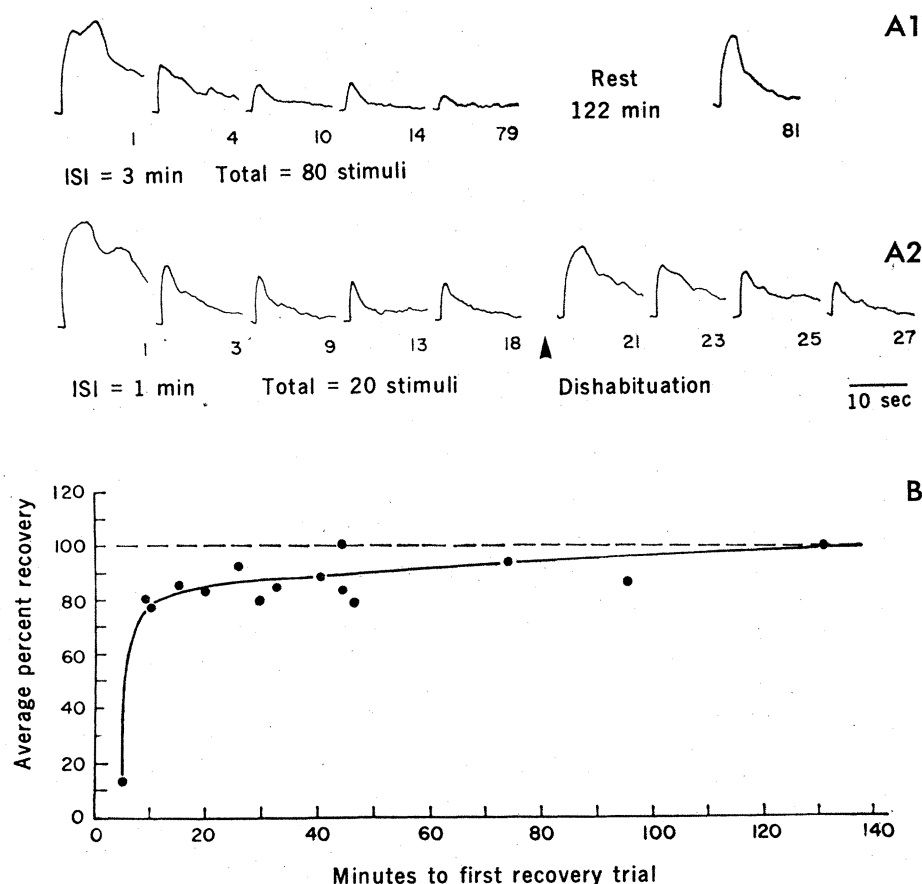


Fig. 2. Habituation, spontaneous recovery, and dishabituation of the gill-withdrawal reflex. (A) Records from two response habituations in a single preparation. The interval between stimuli (ISI) and total number of habituating stimuli are indicated. Part 1 shows decrement of the response with repetition of the stimulus. Following a 122-minute rest the response was almost fully recovered. Part 2 shows a later experiment from the same preparation. After rehabituation of the response a dishabituation stimulus consisting of a strong and prolonged tactile stimulus to the neck region was presented at the arrow. Successive responses were facilitated for several minutes. (B) The time course of recovery was estimated by habituating individual animals with repeated stimuli and testing for the percent of recovery by presenting a single stimulus after different intervals of rest. The curve is based on 44 separate habituation and recovery runs in 27 different animals. Each point is the average of three measures (last point based on only two) taken at roughly the same interval. In longer experiments, later responses would often recover beyond the initial control level (for example, compare the first response in A1 with that in A2). For the purpose of this figure all responses equal to or greater than the control response for that run were assigned a value of 100 percent. The shortest time in which full recovery occurred was 10 minutes, whereas the longest time in which the response was not fully recovered was 122 minutes.

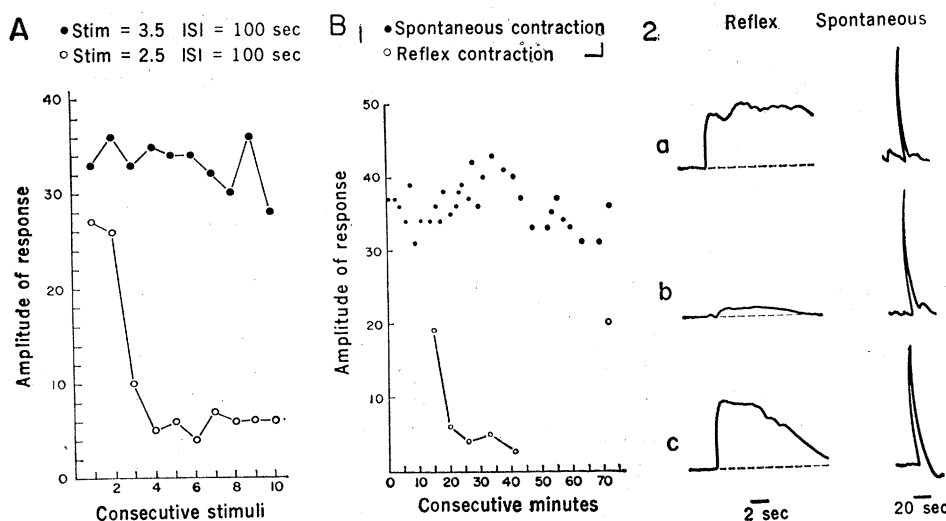


Fig. 3. (A) Habituation with weak and strong tactile stimuli. Responses to the stronger stimulus (filled circles) are initially larger and show less decrement with repetition than responses to the weaker stimulus (open circles). (B) Spontaneous contractions and reflex contractions of gill. Part 1, spontaneous gill contractions (filled circles) remain constant in amplitude while reflex contractions (open circles) produced by tactile stimuli presented at 5-minute intervals show habituation and then recovery with a 30-minute rest. Part 2, sample records from this experiment to compare amplitude of reflex and spontaneous contractions (a) before reflex habituation, (b) during maximum habituation, and (c) following recovery with rest. The spontaneous contraction shown is the one occurring closest in time, either before or after the reflex contraction. Note the difference in time calibration.

our analysis to the earliest peak of the response which is most closely associated with activation of the mono-synaptic pathway to be analyzed in neurophysiological experiments (2).

With repetition of the tactile stimuli at intervals that ranged from 30 seconds to 5 minutes, the gill responses habituated to an average of 25 percent of control amplitude (5 to 45 percent) (Fig. 2A). Habituation has sometimes been seen with intervals as long as 20 minutes between stimuli. Typically, the major part of the decrement was produced by the first 5 to 10 stimuli in a series (see Fig. 3, A and B). Periods of rest that ranged from 10 minutes to more than 2 hours were required for full recovery from habituation. To obtain a more quantitative measure of the rate of recovery we plotted percent recovery in 44 separate response habituations with a single stimulus given after different intervals of rest (Fig. 2B). The data suggest that there is a rapid phase lasting 10 to 20 minutes that accounts for about 75 to 85 percent of recovery, followed by a slow and highly variable return to the original response level and often beyond.

After habituation of the response, a single strong tactile stimulus presented to another part of the animal produced dishabituation (Fig. 2A, part 2). The previously decremented re-

sponse was facilitated, and successive responses remained elevated for several minutes. On occasion, a dishabituation stimulus facilitated the decremented response to an amplitude greater than the initial (unhabituated) control response.

As described above, a contraction similar to that evoked by tactile stimulation also occurs spontaneously. We used this spontaneous contraction to examine whether reflex habituation results from fatigue of the gill musculature. We found that spontaneous gill contractions that occurred before the onset of reflex habituation, during maximum response decrement, and after recovery of the reflex, were of similar amplitude (Fig. 3B), which indicated that gill fatigue is not a factor in habituation. This inference is further supported by the finding that an extrastimulus can dishabituate a habituated response and that strong stimuli (which are more likely to produce fatigue) produce less habituation than weak stimuli (Fig. 3A).

Thompson and Spencer (4) described nine parametric characteristics of behavioral habituation in vertebrates. Six of these characteristics have consistently been found in *Aplysia*. In addition to (i) response decrement, usually a negative exponential function of the number of stimulus presentations, (ii) spontaneous restoration with rest,

and (iii) dishabituation, we have typically found (iv) habituation of the dishabituation stimulus with repeated presentations, (v) greater habituation with short rather than long inter-stimulus intervals, and (vi) greater habituation with weak rather than strong stimuli. Three other parametric characteristics of habituation have sometimes been noted but do not appear to be characteristic of habituation in *Aplysia*. These features are (vii) greater habituation with repeated periods of habituation and recovery, (viii) generalization of habituation to a stimulus in another part of the receptive field, and (ix) prolongation of recovery following additional stimulation after the response has decremented to an asymptote. The existence of a satisfactory fit between many characteristics of habituation in *Aplysia* and in vertebrates suggests that it may be of general interest to analyze the underlying neuronal mechanisms, and this will be the object of the following two papers (1, 2).

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10. Before being placed in the test apparatus the animal was stimulated to cause release of ink from the purple gland and secretion from the opaline gland. After the animal was secured in the apparatus, at least 1 hour of complete rest was given before any data were collected. The animals in these experiments ranged in weight from 325 to 650 g. Although in most experiments stimuli were applied to the edge of the mantle shelf, qualitatively similar results were obtained from other parts of the receptive field. Habituation of the gill-withdrawal reflex was also obtained using brush strokes or brief electrical stimuli.
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