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Light paired with serotonin mimics the effect of conditioning on phototactic behavior of *Hermissenda*

(classical conditioning/neuromodulation/associative learning)

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ABSTRACT **A conditioning procedure consisting of pairing-specific stimulation of the eyes and gravity-detecting statocysts in *Hermissenda* results in a long-term modification of normal positive phototactic behavior. The learning is expressed by a significant suppression of the initiation of locomotion in the presence of light. We now report that an analogue of the classical conditioning procedure, consisting of light paired with serotonin (5-HT) applied directly to the exposed circumesophageal nervous system of otherwise intact animals, mimics the effect of conditioning on long-term changes in phototactic behavior. The effect of the conditioning analogue on behavior shows some specificity with 5-HT since light paired with dopamine or octopamine does not significantly affect phototactic behavior. The conditioning analogue exhibits pairing specificity since unpaired light and 5-HT and 5-HT applied in the dark do not produce behavioral suppression. Animals that initially received unpaired light and 5-HT do show behavioral suppression after receiving paired light and 5-HT. These results indicate that light (the conditioned stimulus) paired with the putative transmitter of the unconditioned stimulus pathway (5-HT) is sufficient to produce long-term phototactic suppression.**

A long-term suppression of normal positive phototactic behavior in the Pacific nudibranch *Hermissenda crassicornis* is produced by a classical conditioning procedure (1–4). The conditioning procedure involves pairing illumination [conditioned stimulus (CS)] with high-speed rotation [nominal unconditioned stimulus (US)] of individual animals on a turntable. A complete understanding of the mechanism(s) underlying this example of associative learning in *Hermissenda* requires the identification of the neural circuit mediating the interaction between the CS and US and the analysis of the pharmacology of transmitter actions at the synapses that support the conditioning. The synaptic interaction of neurons, within the two primary sensory organs and between the two sensory pathways that mediate the CS and US, has been examined in some detail (5–11). The neurotransmitter of the primary sensory neurons of the CS pathway has been identified (12, 13); however, little is known about the action of putative neurotransmitters or modulators in neurons of the US pathway. Since serotonin (5-HT) has been shown to act as a neurotransmitter and neuromodulator in a number of invertebrate preparations (14–26) and to contribute to behavior and synaptic plasticity (27–34), it is attractive to hypothesize a role for 5-HT in conditioning of *Hermissenda*. Recent evidence has shown that 5-HT can modulate photoresponses in *Hermissenda* (14, 15). Application of 5-HT results in an increase in the amplitude and duration of light-evoked responses in type-B photoreceptors (14). In addition, 5-HT effects voltage- and Ca^{2+}-dependent K^+ conductances in putative motor neurons (21) and light-evoked current in B photoreceptors of *Hermissenda* (14). The evidence for serotonergic innervation of photoreceptors in *Hermissenda* was provided by recent immunohistochemical studies that revealed 5-HT-immunoreactive neurons and processes in the cerebropleural ganglia that terminate on the optic nerve and in the synaptic region of the neuropil near the photoreceptor terminals (35). Taken collectively, these results provide evidence for a possible physiological role for 5-HT in conditioning of *Hermissenda*; however, the effect of 5-HT on behavior and specifically learning has not been previously investigated.

In this paper we examined the hypothesis that 5-HT may contribute to conditioning in *Hermissenda* by substituting direct application of 5-HT for normal stimulation of the US pathway. The analogue of conditioning consisted of the normal CS (light) paired with 5-HT applied to the exposed circumesophageal nervous system of otherwise intact animals. We have found that light paired with 5-HT mimics the effects of conditioning on phototactic behavior in *Hermissenda*. The effects of 5-HT exhibit some specificity since control experiments in which light was paired with dopamine or octopamine did not produce significant overall suppression of phototactic behavior. A within-group comparison consisting of the application of the conditioning analogue to a control group that initially received unpaired light and 5-HT showed significant phototactic suppression 24 hr after the light was paired with 5-HT.

METHODS

A total of 95 adult *Hermissenda crassicornis* was used in the experiments. *Hermissenda* were obtained from Sea Life Supply (Sand City, CA). The animals were maintained in an artificial sea water (ASW) aquarium at 14 ± 1°C on a 12-hr light:12-hr dark cycle. Animals were fed small pieces of scallops each day during the experiments. Prior to the application of the conditioning analogue, phototactic behavior was measured to establish baseline preconditioning responses. The automated behavioral testing apparatus has been described in detail in previous reports (1–4, 36) and will be described only briefly in this report. The animals were tested in sea water-filled glass tubes that were attached to clips on the top surface of a turntable enclosed inside an incubator that maintained temperature at 14 ± 1°C. During the period of dark adaptation the animals were confined to the starting ends of the glass tubes by a small foam plug inserted into an aperture in the tube 4 cm from the end. After 12 min of dark adaptation the foam plugs were removed and phototactic behavior was assessed by measuring the time taken by individual animals to initiate locomotion toward a focused light source (9.3×10^{-4} W/cm^2 at 510 nm) projected onto the center of the turntable. The behavior was measured

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Abbreviations: 5-HT, serotonin; ASW, artificial sea water; CS, conditioned stimulus; US, unconditioned stimulus.

by detecting the movement toward the light source when animals moved between an infrared emitter and a phototransistor located at the starting end of the glass tube. The output of the phototransistors was amplified and connected to a 20-channel event recorder. After the behavior was recorded the animals were anesthetized with 0.25- to 0.50-ml injections of isotonic $MgCl_2$ and a small incision was made overlying the circumesophageal nervous system. Following the surgery, the animals were placed into a chamber containing 50 ml of normal ASW. The exposed nervous system was visualized in infrared illumination provided by a 45-W tungsten/halogen lamp and an infrared filter (Schott model RG-850). A dissecting microscope formed an image of the nervous system upon a Dage MTi video camera connected to a video monitor and time-lapse video recorder.

In the first experiment, after behavioral testing and exposure of the nervous system the animals were dark adapted 12 min followed by the application of one of four different experimental treatments involving a candidate neurotransmitter or a control procedure. The different treatments consisted of light paired simultaneously with the application of 0.5 ml of dopamine, octopamine, 5-HT, or ASW. The putative transmitter was applied to the region of the cerebropleural ganglion where previous immunocytochemistry revealed 5-HT-reactive processes near the optic nerve and photoreceptor terminals in the neuropil (35). The concentration of the transmitter that was tested was adjusted to yield a final concentration in the ASW bath of 0.1 mM. The concentration of 0.1 mM was selected for the initial studies since previous electrophysiological results showed that B photoresponses were enhanced and prolonged in the presence of 0.1 mM 5-HT (14). The animals remained in the light for 5 min in the presence of the transmitter followed by an ASW rinse and were then returned to the home cages in the ASW aquarium. The animals were not sutured after the treatment since the wound margins typically close within a few hours after surgery. Twenty-four hours after the application of the four treatments phototactic behavior was again measured using the same procedure described for the preconditioning test.

To test if the effect of light and 5-HT on behavior is dependent upon pairing a second series of experiments was conducted. The following procedures were used for experiments comparing the effect on phototactic behavior of paired light and 5-HT with unpaired light and 5-HT. Following behavioral testing and surgical exposure of the nervous system animals were dark adapted for 12 min followed by the presentation of light (CS) focused on the nervous system and paired simultaneously with 0.5 ml of 5-HT applied to the exposed nervous system. The light remained on for 5 min followed by 12 min of additional dark adaptation before returning the animals to the cages in the ASW aquarium. Unpaired controls received 5 min of light followed by 5 min of dark adaptation before the application of 0.5 ml of 5-HT viewed under infrared illumination. An additional control group received 0.5 ml of 5-HT in the dark without prior illumination (under infrared illumination). All groups received 12 min of dark adaptation following the 5-HT treatments before returning the animals to their home cages in the ASW aquarium. For within-group comparisons animals that had initially received unpaired light and 5-HT were retested after the application of light paired with 5-HT. The animals that previously received unpaired light and 5-HT received light paired with 5-HT within 7 days of the application of the initial control procedure. Changes in phototactic behavior following the different experimental treatments were compared to preconditioning baseline responses in the form of suppression ratios (A/A + B), where A represents the preconditioning start latency and B indicates the start latency 24 hr after the treatment. Suppression ratios <0.50 indicate an increase in the post-test latency following conditioning, ratios >0.50 indicate a decrease in the post-test latency, and 0.50 signifies no change from the pre-test latency.

Statistical analysis of the data consisted of a nonparametric one-way analysis of variance to assess overall significance followed by two-group comparisons using Mann–Whitney U tests (37). The within-group comparison used the Wilcoxon matched-pairs signed-ranks test (37).

RESULTS

Light Paired with 5-HT Produces Phototactic Suppression. To examine the possible role that various candidate neuromodulators may play in conditioning in *Hermissenda* animals whose nervous systems were surgically exposed received a conditioning analogue consisting of light paired with the application of 0.5 ml of either dopamine, octopamine, or 5-HT. If any of the putative neurotransmitters/neuromodulators were released by stimulation of the US pathway by the natural US (rotation), then direct application to cells in the CS pathway should produce similar effects on behavior. Twenty-four hours after the various treatments phototactic behavior was tested as described in *Methods*. The results of the analysis of phototactic behavior following the conditioning analogue revealed that light paired with 5-HT produced significant phototactic behavioral suppression (Fig. 1). The overall statistical analysis using the Kruskal–Wallis one-way analysis of variance (37) was significant [$H_{3df} = 11.5$ (where df = degrees of freedom); $P < 0.01$]. Individual two-group comparisons between the various treatments and the ASW control group using the Mann–Whitney U test (37) revealed that the group that received light paired with 5-HT ($n = 13$) was significantly different from the light paired with octopamine group ($n = 9$) ($U = 21$; $P < 0.01$), the light paired with dopamine group ($n = 8$) ($U = 19$; $P < 0.01$), and the ASW controls ($n = 9$) ($U = 25.5$; $P < 0.025$). The groups that received light paired with dopamine or light paired with octopamine were not different from each other or the ASW control group (see Fig. 1). These results indicate that pairing light, the natural CS, with a putative neuromodulator, 5-HT, mimics the effect of the conditioning procedure

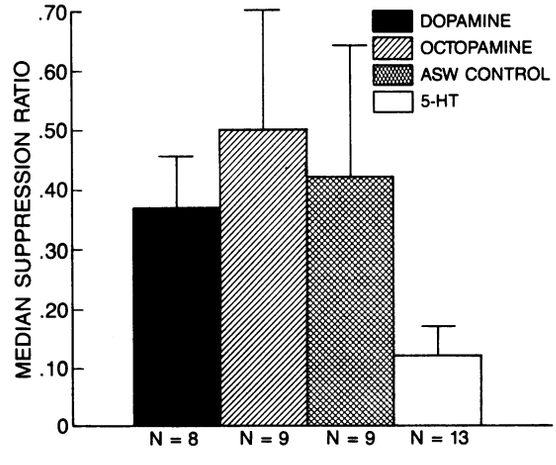

FIG. 1. Effects of light (CS) paired with several putative neurotransmitters/neuromodulators (US) on the behavioral response to light in *Hermissenda*. Experiments examined the effect of light paired with dopamine ($n = 8$), octopamine ($n = 9$), 5-HT ($n = 13$), and ASW controls ($n = 8$) on phototactic behavior 24 hr after the application of the conditioning analogue. The group data are expressed as median suppression ratios (± semi-interquartile range) in the form of (A/A + B), where A represents preconditioning scores and B indicates posttreatment scores. Only the group that received light paired with 5-HT exhibited significant suppression of phototactic behavior ($P < 0.01$).

on suppression of phototactic behavior. The conditioning analogue, light paired with 5-HT, and conditioning of intact animals, light paired with rotation, produce significant long-term changes in phototactic behavior.

Effect of Light and 5-HT Is Pairing Specific. Previous research has shown that a few conditioning trials produce a rapidly decrementing nonassociative effect on phototactic behavior in *Hermissenda* (3). Since the conditioning analogue used in this study involves one training session, it is necessary to assess any nonassociative contribution to the change in phototactic behavior. To rule out the possible contribution of nonassociative factors such as sensitization to the observed effects of the conditioning analogue on behavior of *Hermissenda*, additional experiments were conducted to examine nonassociative factors and to replicate the initial observation. In these experiments, control groups received either 5-HT applied in the dark (infrared illumination) or explicitly unpaired light and 5-HT. The controls were compared to a different group that received light paired with 5-HT. The procedure for the unpaired group consisted of 12 min of dark adaptation followed by 5 min of illumination. After the 5-min period of illumination the preparation was dark adapted for an additional 5 min before applying 5-HT under infrared illumination (see *Methods*). As described in *Methods*, the paired group was dark adapted for 12 min, followed by light paired with the application of 5-HT. After pairing light and 5-HT the animals were dark adapted for 12 min before being returned to the ASW aquarium. An additional control group received 5-HT under infrared illumination at the end of the 12-min period of dark adaptation.

Testing phototactic behavior 24 hr after the different procedures revealed significant overall differences between the three groups ($H_{2df} = 14.61$; $P < 0.001$). The group that had received light paired with 5-HT ($n = 18$) exhibited significant phototactic suppression (Fig. 2). Individual two-group comparisons showed that the paired group ($n = 18$) was significantly different from the unpaired group ($n = 19$) ($U = 71$; $P < 0.001$) and the group that received 5-HT in the dark ($n = 19$) ($U = 68.5$; $P < 0.001$). The two control groups were not significantly different from each other.

These results show that the effect of the conditioning analogue on phototactic behavior is pairing specific and that light paired with 5-HT can mimic the effects of the conditioning procedure on phototactic behavior in *Hermissenda*. As an additional control for nonassociative effects, animals from the second replication of the control group that had initially received unpaired light and 5-HT ($n = 7$) were tested following the application of light paired with 5-HT. Prior to the application of the conditioning analogue the animals were tested to verify that the unpaired procedure did not alter phototactic behavior as compared to preconditioning baseline responses and behavior assessed 24 hr after the unpaired procedure. The animals were anesthetized, the nervous systems were exposed, and light was paired with the application of 0.5 ml of 5-HT to the exposed nervous system. The analysis of behavior 24 hr after the conditioning analogue revealed significant behavioral suppression ($P < 0.025$) as compared to the behavioral results of the same animals after the unpaired control procedure (Fig. 3). These results indicate that unpaired light and 5-HT did not significantly alter phototactic behavior; however, when the same animals received light paired with 5-HT the effect on phototactic behavior was similar to the other groups that had initially received light paired with 5-HT (see Fig. 3).

DISCUSSION

We have found that an analogue of conditioning consisting of light paired with direct application of 5-HT to the exposed circumesophageal nervous system of otherwise intact *Hermissenda* produces behavioral suppression in the presence of light when the animals are tested 24 hr after the treatment. The component of phototactic behavior that is modified by the conditioning analogue has been shown previously to exhibit long-term suppression following 3 days of behavioral conditioning (2–4). This finding provides evidence that the exogenous application of 5-HT can effectively serve as an US to produce long-term behavioral changes that are expressed in the intact animal. Based upon these results, it is attractive to hypothesize that the interaction between light-dependent processes and 5-HT may provide a mechanism for encoding information concerning the temporal association between the CS and US during conditioning in *Hermissenda*. There is now a considerable amount of evidence indicating that conditioning produces several cellular correlates that are intrinsic to the type-B photoreceptors in *Hermissenda* (for a review, see

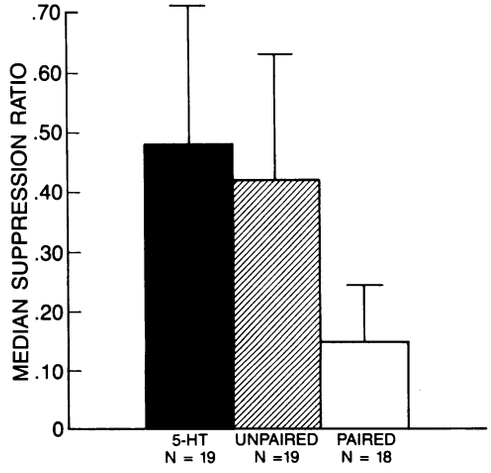

FIG. 2. Pairing-specific effects of light and 5-HT. Effect of light (CS) paired with 5-HT ($n = 18$), 5-HT applied in the dark ($n = 19$), and unpaired light and 5-HT ($n = 19$) on phototactic behavior. The group data are expressed as median suppression ratios (± semi-interquartile range). The group that received light paired with 5-HT exhibited significant behavioral suppression compared to the unpaired group ($P < 0.001$) and the group that received 5-HT in the dark ($P < 0.001$).

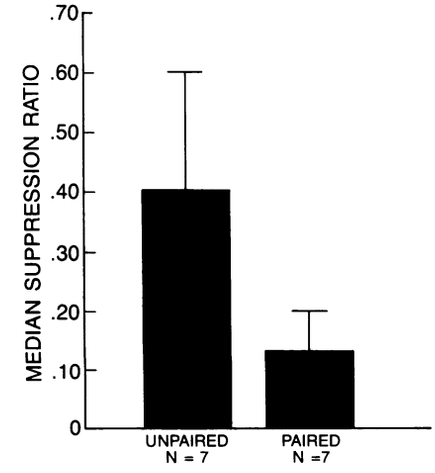

FIG. 3. Within-group comparison of the effects of unpaired light and 5-HT and paired light and 5-HT. The data are expressed as median suppression ratios (± semi-interquartile range). Unpaired represents the median suppression ratio for a control group that initially received unpaired light and 5-HT. When the group that had previously received the control procedure received paired light and 5-HT (paired) they exhibited significant behavioral suppression ($P < 0.025$).

refs. 38 and 39). These results indicate that one locus for the storage of the memory of the associative experience is in the primary sensory neurons of the pathway mediating the CS. Though a number of mechanisms have been proposed to explain the intrinsic changes in B photoreceptors of conditioned *Hermissenda*, the possibility that a neurotransmitter/neuromodulator released by activation of the US pathway may interact directly with the photoreceptors has not been seriously considered. As recently suggested, the reduction in the early K^+ current (I_A) and the Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$), which is the most common mechanism, may initially involve only depolarization of the B photoreceptors accompanied by elevation of intracellular Ca^{2+} (38). Moreover, it has been suggested that if a neuromodulator played a role in learning in *Hermissenda*, it would be to amplify conditioning-induced changes in the previously described membrane currents (38). One candidate for this role would be a catecholamine such as dopamine or norepinephrine since a previous histofluorescence study revealed a cell in the optic ganglion that showed green fluorescence by the Falck–Hillarp method (13). However, dopamine is not a good candidate for neuromodulation since it does not produce alterations in the photoresponse of type-B photoreceptors in *Hermissenda* (38). The α_2-agonist clonidine and norepinephrine produce a reduction in I_A and $I_{K\text{-}Ca}$ in B photoreceptors (38, 40). In addition, clonidine enhances the amplitude of the photoresponse (14). However, clonidine is not a transmitter and norepinephrine is not found in *Hermissenda* as well as other related gastropod molluscs (12). The evidence for a possible role for 5-HT in conditioning of *Hermissenda* is more compelling. First, biochemical and histofluorescence studies have demonstrated the presence of 5-HT in cell bodies and processes in the neuropil of the pedal and cerebropleural ganglia (12, 13) (P. W. Land and T.C., unpublished observations). 5-HT has been shown to enhance the B photoresponse (14) and alter voltage- and Ca^{2+}-dependent K^+ currents in photoreceptors and putative motor neurons in *Hermissenda* (15, 21). In addition, the results of a recent immunohistochemical study revealed 5-HT-immunoreactive fibers and varicosities on the optic nerve and in the synaptic region in the neuropil near the photoreceptor synaptic terminals (35). Although this observation provides a potential serotonergic pathway for direct interaction with the photoreceptors, we do not yet know the presynaptic source of the 5-HT or if the putative serotonergic interneurons receive input from statocyst hair cells.

The action of 5-HT as a modulator when coactivated with other direct inputs appears to be quite general since 5-HT has been shown to influence behavior and synaptic plasticity in a number of related molluscs (16–34). In *Aplysia*, 5-HT may be a facilitating transmitter involved in sensitization of the gill and siphon withdrawal reflex (28–33). In addition, 5-HT may play a central role in the proposed mechanism for classical conditioning in *Aplysia* (31–34).

It is clear that additional work must be done to demonstrate a physiological role for 5-HT in associative learning in *Hermissenda*. However, the results of this study showing that substitution of stimulation of the US pathway with 5-HT can mimic the effect of conditioning on behavior demonstrate the utility of this approach in analyzing the precise role of light-dependent processes interacting with transmitter actions in the acquisition of associative learning in *Hermissenda*.

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