Time-Dependent Changes in Excitability After One-Trial Conditioning of *Hermissenda*

TERRY CROW AND VILMA SIDDIQI

*Department of Neurobiology and Anatomy, The University of Texas Medical School, Houston, Texas 77225*

Crow, Terry and Vilma Siddiqi. Time-dependent changes in excitability after one-trial conditioning of *Hermissenda*. *J. Neurophysiol.* 78: 3460–3464, 1997. The visual system of *Hermissenda* has been studied extensively as a site of cellular plasticity produced by classical conditioning. A one-trial conditioning procedure consisting of light paired with the application of serotonin (5-HT) to the exposed, but otherwise intact, nervous system produces suppression of phototactic behavior tested 24 h after conditioning. Short- and long-term enhancement (STE and LTE) of excitability in identified type B photoreceptors is a cellular correlate of one-trial conditioning. LTE can be expressed in the absence of STE suggesting that STE and LTE may be parallel processes. To examine the development of enhancement, we studied its time-dependent alterations after one-trial conditioning. Intracellular recordings from identified type B photoreceptors of independent groups collected at different times after conditioning revealed that enhanced excitability follows a biphasic pattern in its development. The analysis of spikes elicited by 2 and 30 s extrinsic current pulses at different levels of depolarization showed that enhancement reached a peak 3 h after conditioning. From its peak, excitability decreased toward baseline control levels 5–6 h after conditioning followed by an increase to a stable plateau at 16 to 24 h postconditioning. Excitability changes measured in cells from unpaired control groups showed maximal changes 1 h posttreatment that rapidly decremented within 2 h. The conditioned stimulus (CS) elicited significantly more spikes 24 h postconditioning for the conditioned group as compared with the unpaired control group. The analysis of the time-dependent development of enhancement may reveal the processes underlying different stages of memory for this associative experience.

**INTRODUCTION**

The analysis of one-trial conditioning of *Hermissenda* has contributed to the identification of several possible cellular mechanisms of short-and long-term memory. Conditioning, consisting of pairing a light conditioned stimulus (CS) with the application of serotonin (5-HT) to the exposed but otherwise intact nervous system produces long-term behavioral suppression (Crow and Forrester 1986) and short-term and long-term enhancement (STE and LTE) in sensory neurons of the CS pathway (Crow and Forrester 1991, 1993). Enhancement in sensory neurons is expressed by an increase in the amplitude of generator potentials, an increase in excitability to extrinsic current, an increase in input resistance, modification of Ca$^{2+}$-dependent and voltage-activated currents, and a time-dependent increase in protein phosphorylation (Acosta-Urquidi and Crow 1993, 1995; Crow and Bridge 1985; Crow et al. 1991, 1996; Falk-Vairant and Crow 1992; Farley and Auerbach 1986; Farley and Wu 1989; Grover et al. 1989; Yamoa and Crow 1995, 1996). We previously showed that STE and LTE may be independent, parallel processes because they can be dissociated based on the role of mRNA synthesis, protein synthesis, and the contribution of protein kinase C (Crow and Forrester 1990, 1991, 1993; Crow et al. 1997). In the present study we examined the time-dependent development of STE and LTE, to further elucidate the temporal characteristics and independence of these different phases of memory detected after one-trial conditioning.

**METHODS**

One-trial conditioning procedure

The one-trial conditioning procedure was previously described in detail (Crow and Forrester 1986, 1990, 1991). Adult *Hermissenda* were maintained in closed artificial seawater (ASW) aquaria at 14 ± 1°C on a 12 h light:12 h dark cycle. Conditioning and electrophysiological procedures were carried out during the light phase of the light:dark cycle. Animals were placed in chambers containing ASW and the dorsal surface of the exposed nervous system was visualized in infrared illumination. After a 12 min period of dark adaptation the conditioned group received one-trial of light (∼10$^{-4}$ W/cm$^2$) paired with 5-HT (0.1 mM) applied to a region of the cerebropleural ganglion where 5-HT immunoreactive varicosities and processes were previously identified (Land and Crow 1985). Animals remained in the light and 5-HT for 5 min followed by an ASW rinse and 12 additional min of dark adaptation. Unpaired control groups received 5 min of light followed by 5 min of dark adaptation before the application of 5-HT to the same region of the nervous system. Serotonin was applied in the dark (infrared illumination) for the unpaired control group and remained in the ASW bath for 5 min followed by the application of normal ASW. All groups were returned to the ASW aquaria after the conditioning and control procedures.

**Measurement of short- and long-term enhancement**

Intracellular recordings were collected from identified lateral type B photoreceptors between 1 and 24 h after the conditioning trial. Experiments were conducted in ASW having the following composition (in mM): 460 NaCl, 10 KCl, 10 CaCl$_2$, 55 MgCl$_2$, buffered with 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and brought to pH 7.6 with NaOH. The temperature of the ASW in the recording chamber was maintained at 15 ± 0.5°C. Before intracellular recordings and stimulation, the isolated nervous system received a brief incubation in a protase solution (Sigma type VIII, 0.67 mg/ml, 5–6 min) to facilitate microelectrode penetration of type B photoreceptors. Action potentials were elicited from lateral type B photoreceptors by passing extrinsic current of different amplitude and duration through a balanced bridge circuit. Excitability was assessed by examining changes in spike frequency elicited by the passage of depolarizing
extrinsic current relative to spontaneous baseline activity. Three different levels of membrane depolarization (5, 10, and 20 mV) and two different durations of stimulation (2 and 30 s) were used. Excitability was first assessed with a 30-s, 20-mV depolarizing current step followed by independent replications at 3 h and 24 h using 2-s, 5-, and 10-mV depolarizing steps. Averages were determined by dividing the total number of action potentials by the duration of the different extrinsic current pulses. In addition, average CS-elicited and current-elicited spike frequency was derived from the mean of the interspike interval histogram generated by computer analysis of the output of a window discriminator.

Statistical analysis

A two-way analysis of variance (ANOVA) was used to assess main effects (SigmaStat, Jandel Sci.). After the determination of overall significant effects, paired comparisons consisted of the Newman-Keuls procedure. Differences between the conditioned groups and unpaired controls in excitability measured with short-duration current pulses and CS-elicited spike activity 24 h postconditioning involved t-tests for independent groups.

RESULTS

Excitability exhibits a time-dependent enhancement

Excitability in identified sensory neurons was examined using 30-s, 20-mV depolarizing extrinsic current pulses at different times after the conditioning trial. The results of the ANOVA revealed a significant effect of conditioning as compared with the unpaired controls ($F_{1,108} = 275.1; P < 0.001$) and a significant overall effect of the time after the conditioning trial ($F_{13,108} = 6.5; P < 0.001$). The interaction between the treatments (paired vs. unpaired) and time was also significant ($F_{13,108} = 4.3; P < 0.001$). Comparisons between the conditioned and unpaired groups after condi-

![Figure 1](image_url)

**FIG. 1.** A: time-dependent changes in excitability of lateral type B photoreceptors assessed after one-trial conditioning. Group data depicting mean frequency (±SD) in spikes/s minus spontaneous baseline activity elicited by 30-s, 20-mV extrinsic current pulses for conditioned animals (●) (total n = 65) and unpaired control groups (□) (total n = 71). Sample sizes for paired and unpaired groups at each time point (paired presented first) were 1 (n = 5, 7), 2 (n = 4, 7), 2.5 (n = 4, 8), 3 (n = 5, 4), 3.5 (n = 5, 4), 4 (n = 4, 4), 5 (n = 5, 5), 5.5 (n = 5, 4), 6 (n = 4, 4), 16 (n = 4, 4), 18 (n = 6, 4), 20 (n = 4, 4), 22 (n = 5, 5), and 24 (n = 7, 5) (* P < 0.05). Different times for measuring excitability are independent; e.g., excitability was assessed at only one time for each preparation. B: examples of enhanced excitability examined in lateral B photoreceptors from a different group of animals than shown in A, 3 h after one-trial conditioning. Extrinsic current pulses (2 s, 5 and 10 mV) from a membrane potential of -60 mV elicit more action potentials in cells from the conditioned animal (B1 and B2) as compared with the unpaired control (C1 and C2). C: group data showing mean (±SE) firing in spikes/s 3 h postconditioning for conditioned groups ∆5 mV (mean = 9.2, n = 3) and ∆10 mV (mean = 13.8, n = 3) and for unpaired controls ∆5 mV (mean = 5.2, n = 3) and ∆10 mV (mean = 7.9, n = 3).
conditioning revealed statistically significant differences at all times with the exception of 1 h (q = 2.5; NS), 5 h (q = 2.5; NS), 5.5 h (q = 3.5; NS), and 6 h (q = 2.5; NS). Excitability was significantly greater (P < 0.05) for the conditioned groups as compared with the unpaired controls at 2 h (q = 9.5), 2.5 h (q = 10.4), 3 h (q = 11.3), 3.5 h (q = 9.1), 4 h (q = 5.1), 16 h (q = 5.9), 18 h (q = 6.0), 20 h (q = 6.5), 22 h (q = 5.8), and 24 h (q = 7.1). The nonassociative component characterized by increased excitability in the unpaired group was maximal at 1 h and exhibited a rapid decline at 2 h postconditioning. This observation is consistent with the results of previously published behavioral data in *Hermissenda* showing that nonassociative components are expressed shortly after conditioning and rapidly decrement over time (Crow 1983). The unpaired group showed significant enhancement (P < 0.05) 1 h postconditioning as compared with 2 h (q = 6.6), 2.5 h (q = 6.7), 3 h (q = 5.2), 3.5 h (q = 7.1), 4 h (q = 6.4), 5 h (q = 5.8), 5.5 h (q = 6.6), 6 h (q = 6.4), 18 h (q = 5.6), 20 h (q = 5.1), 22 h (q = 4.9), and 24 h (q = 5.5). At times from 2 to 24 h there were no statistically significant differences in excitability for the unpaired groups. As shown in Fig. 1A the time course of the development of ST and LTE followed a biphasic pattern. Excitability increased following conditioning, reaching a peak at 3 h postconditioning, followed by a decline at 5–6 h postconditioning and the development of a plateau phase 16–24 h postconditioning. Enhancement was significantly greater (P < 0.05) for the conditioned group 3 h postconditioning as compared with the groups tested at 1 h (q = 4.8), 3.5 h (q = 5.1), 4 h (q = 7.3), 5 h (q = 9.8), 5.5 h (q = 9.2), 6 h (q = 9.8), 16 h (q = 5.9), 18 h (q = 5.6), 22 h (q = 5.9), and 24 h (q = 6.0). Times near the peak of enhanced excitability also showed significant differences as compared with groups tested between 5 and 6 h postconditioning. The group tested at 2 h was significantly different (P < 0.05) from the groups tested at 5 h (q = 6.4), 5.5 h (q = 5.8), and 6 h (q = 6.6). In addition, the group tested 2.5 h after conditioning was significantly different (P < 0.05) from the groups tested at 4 h (q = 4.7), 5 h (q = 7.0), 5.5 h (q = 6.4), and 6 h (q = 7.2). Excitability for the conditioned group assessed at times in the plateau phase was significantly different from excitability between 5 and 6 h postconditioning. The conditioned groups were significantly different (P < 0.05) at 18 h (q = 4.2), 20 h (q = 5.1), 22 h (q = 4.5), and 24 h (q = 5.1) as compared with the conditioned group examined 6 h postconditioning. The conditioned groups were also significantly different (P < 0.05) at 20 h (q = 4.8), 22 h (q = 4.2), and 24 h (q = 4.7) as compared with excitability measured 5 h postconditioning.

To assess a possible voltage dependency in the expression of LTE, we examined changes in excitability in sensory neurons from paired and unpaired groups by using short current pulses (2 s) at two levels of depolarization (ΔS and Δ10 mV) 3 h and 24 h postconditioning. As shown in the examples of raw data in Fig. 1, B and C, collected 3 h postconditioning, brief extrinsic current pulses at both levels of depolarization elicited more spikes in the conditioned group than the unpaired control group. The analysis of the group data shown in Fig. 1D revealed a significant difference between the conditioned group and unpaired controls at Δ5 mV (t4 = 3.48; P < 0.02) and Δ10 mV (t4 = 3.97; P < 0.01). Similar results were detected 24 h postconditioning. As shown in Fig. 2A and 2B, sensory neurons from the conditioned group exhibited greater excitability at both 5 and 10 mV depolarizations than did the unpaired controls. The analysis of the group data shown in Fig. 2C showed statistically significant differences between conditioned and controls at Δ5 mV (t10 = 2.85; P < 0.01) and Δ10 mV (t10 = 6.83; P < 0.001).
Enhancement is expressed by changes in CS-elicited excitability

We next examined spike activity of identified sensory neurons elicited by the presentation of the CS 24 h postconditioning. Because only the lateral B photoreceptor exhibits LTE after one-trial conditioning the synaptic inhibition from other photoreceptors could potentially block the expression of enhancement elicited by the CS. Such a result would suggest that LTE would not contribute to the behavioral changes observed 24 h after one-trial conditioning. As shown in the inter-spike interval histograms in Fig. 3A and B, the CS elicited more action potentials in the example from the conditioned group than in the example from the unpaired control. CS-elicited spike frequency was computed from the mean of the inter-spike interval histograms and depicted in the group data shown in Fig. 3C. The mean frequency for the conditioned group (mean = 5.39, n = 7) as compared with the unpaired control (mean = 3.73, n = 9) was significantly greater (t_{14} = 5.43; P < 0.001). These results show that LTE in the lateral B photoreceptors is also expressed by the CS after one-trial conditioning.

**DISCUSSION**

In our assessment of the time-dependent changes in excitability after one-trial conditioning we have provided evidence for a multiphasic process underlying memory retention. Our analysis involved the use of independent groups at each time point that was tested and thus should reflect excitatory processes that vary as a function of time and not the history of previous testing. These results are consistent with the view that memory consolidation involves multiple processes with unique time-dependencies and mechanisms. Behavioral, cellular neurophysiological, and pharmacological studies of memory consolidation in vertebrates and invertebrates have reported that multiple components of memory are expressed at different times after conditioning (Crow et al. 1991, 1996; McGaugh 1966; Rosenzweig et al. 1993; Yin et al. 1994). Cellular studies of time-dependent processes underlying memory consolidation suggest that a complex series of cellular signaling pathways are engaged as a result of conditioning (for review see DeZazzo and Tully 1995). These would include the activation of second messenger pathways, posttranslational modification of proteins, and the alteration of gene expression. Moreover, different cellular signaling pathways may support independent components of memory in a time-dependent manner. Indeed, the recent views of memory propose a system of stages that may be independent and organized in parallel rather than a series of sequential steps. The evidence to support this view of parallel stages comes from studies showing that different components of memory are differentially effected by inhibition of translation, transcription, and protein kinase activity (Allweis 1991; Crow and Forrester 1993; DeZazzo and Tully 1995; Freeman et al. 1995; Montarola et al. 1986; Rosenzweig et al. 1993). LTE in *Hermissenda* is dependent on both translation and transcription. Inhibition of translation and transcription block LTE, while leaving STE intact (Crow and Forrester 1990; Crow et al. 1997). In contrast, inhibitors of protein kinase C (PKC) and down-regulation of PKC block the induction of STE and normal LTE is expressed 24 h postconditioning (Crow and Forrester 1993). Transgenic *Drosophila* inhibited for PKC also show a disassociation between short-term and long-term memory (Kane et
and long-term synaptic facilitation can be expressed in the absence of short-term facilitation in Aplysia (Emptage and Carew 1993).

Of particular relevance to our findings is the report by Ghirardi et al. (1995) on two phases to 5-HT-induced synaptic facilitation in cultured Aplysia neurons. They reported an intermediate phase of facilitation that was dependent on translation, but not transcription (Ghirardi et al. 1995). Our results suggest that the early phase of enhancement expressed between 1 and 3 h postconditioning may also consist of a dissociable short-term and intermediate-term phase. This is supported by the recent finding that enhanced excitability examined 1.5 h postconditioning is dependent on translation, but not transcription (Crow et al. 1997). The analysis of the time-dependent development of LTE will help to identify basic mechanisms underlying memory consolidation.

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Address for reprint requests: T. Crow, Dept. of Neurobiology and Anatomy, The University of Texas Medical School, Houston, TX 77225.

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