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Habituation in *Aplysia*: The Cheshire Cat of neurobiology

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ABSTRACT

The marine snail, *Aplysia californica*, is a valuable model system for cell biological studies of learning and memory. *Aplysia* exhibits a reflexive withdrawal of its gill and siphon in response to weak or moderate tactile stimulation of its skin. Repeated tactile stimulation causes this defensive withdrawal reflex to habituate. Both short-term habituation, lasting <30 min, and long-term habituation, which can last >24 h, have been reported in *Aplysia*. Habituation of the withdrawal reflex correlates with, and is in part due to, depression of transmission at the monosynaptic connection between mechanoreceptive sensory neurons and motor neurons within the abdominal ganglion. Habituation-related short-term depression of the sensorimotor synapse appears to be due exclusively to presynaptic changes. However, changes within the sensory neuron, by themselves, do not account for more persistent depression of the sensorimotor synapse. Recent behavioral work suggests that long-term habituation in *Aplysia* critically involves postsynaptic processes, specifically, activation of AMPA- and NMDA-type receptors. In addition, long-term habituation requires activity of protein phosphatases, including protein phosphatases 1, 2A, and 2B, as well as activity of voltage-dependent Ca^{2+} channels. Cellular work has succeeded in demonstrating long-term, homosynaptic depression (LTD) of the sensorimotor synapse in dissociated cell culture and, more recently, LTD of the glutamate response of isolated motor neurons in culture ("hemisynaptic" LTD). These in vitro forms of LTD have mechanistic parallels to long-term habituation. In particular, homosynaptic LTD of the sensorimotor synapse requires elevated intracellular Ca^{2+} within the motor neuron, and hemisynaptic LTD requires activity of AMPA- and NMDA-type receptors. In addition, activation of group I and II metabotropic glutamate receptors (mGluRs) can induce hemisynaptic LTD. The demonstration of LTD in vitro opens up a promising new avenue for attempts to relate long-term habituation to cellular changes within the nervous system of *Aplysia*.

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1. Introduction

Habituation, apparently ubiquitous in the animal kingdom (Christoffersen, 1997), is widely regarded as one of the simplest, if not the simplest, form of learning. Habituation of the gill- and siphon- withdrawal reflex in *Aplysia* was first described in 1970 (Pinsker, Kupfermann, Castellucci, & Kandel, 1970). Although habituation had been documented in a variety of invertebrate and vertebrate organisms before then (Christoffersen, 1997), its demonstration in *Aplysia* represented a major advance. This was because the relative simplicity of the nervous system of *Aplysia*, as well as the large size and accessibility of its central neurons to intracellular electrophysiological recording, offered significant advantages for a comprehensive cellular analysis of habituation. Indeed, it seemed possible at the time that a more-or-less complete neurobiological explanation for habituation would be readily achievable in *Aplysia*. But at present we have only an incomplete understanding of the cellular mechanisms underlying habituation of the withdrawal reflex. Therefore, the promise of *Aplysia* as a preparation in which the neurobiological complexities of habituation can be unraveled remains unfulfilled. Like the famous grin of the Cheshire Cat in Lewis Carroll's *Alice in Wonderland*, this promise hovers tauntingly before us. Nonetheless, recent behavioral and cellular work, summarized in this chapter, offers new insights into the old problem of habituation in *Aplysia*. In this chapter I will review current knowledge about the cellular basis of habituation in *Aplysia*, with particular emphasis on those underlying long-term habituation. For a detailed explanation of our current understanding of the mechanisms of short-term habituation, the interested reader should consult the chapter by Gover and Abrams in this volume.

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2. Early synaptic model of short-term habituation in *Aplysia*: presynaptic depression

The first synaptic correlate of habituation of the withdrawal reflex was reported in two papers published back-to-back, Kupfermann, Castellucci, Pinsker, and Kandel (1970); Castellucci, Pinsker, Kupfermann, and Kandel (1970). In the first of these papers the experimenters made use of a semi-intact preparation; they recorded with intracellular electrodes from the identified gill motor neuron, L7, while the reflex underwent habituation due to repeated tactile stimulation of the siphon or mantle. The polysynaptic response evoked in the motor neuron gradually decremented as the response habituated. In the second paper the experimenters succeeded in further isolating an electrophysiological correlate of habituation. The abdominal ganglion, which contains the sensory and motor neurons that innervate the gill, siphon and mantle, was removed from the animal, except for a portion of the siphon nerve, which was left connected to a small piece of the siphon skin. During habituation training the siphon skin was repeatedly stimulated with a weak mechanical or electrical stimulus. The weak stimulus evoked an excitatory postsynaptic potential (EPSP) in L7, and this EPSP, which consists of mono- and polysynaptic components (Antonov, Kandel, & Hawkins, 1999; Trudeau & Castellucci, 1992), decremented with repeated stimulation of the siphon skin. Importantly, there was no change in the input resistance of the motor neurons that could account for the decrement in the EPSP. The experimenters then identified sensory neurons within the abdominal ganglion activated by the stimulation of the skin. They impaled the sensory neurons with sharp electrodes and fired them with brief injections of positive current. When a sensory neuron was repeatedly fired, the EPSP evoked in the motor neuron depressed, just as had the EPSP produced by the mechanical/electrical stimuli applied to the siphon skin. These results suggested that synaptic depression of the sensorimotor connection was a mechanism of habituation of the withdrawal reflex.

However, the results left open the question of whether the synaptic depression was due to pre- or a postsynaptic changes. To address this important question, Castellucci and Kandel (1974) used the technique of quantal analysis. This statistical technique was first developed by Bernard Katz and his colleagues for understanding chemical synaptic transmission at the vertebrate neuromuscular junction (Del Castillo & Katz, 1954a, 1954b; Fatt & Katz, 1952). In principle, quantal analysis allows one to dissect out the relative contributions of pre- and postsynaptic changes to a change in the strength of transmission at a synapse. Based on their quantal analysis of short-term, habituation-related synaptic depression at the sensorimotor synapse, Castellucci and Kandel (1974) concluded that depression was due to a decrease in the number of quanta of presynaptic transmitter released per action potential in the sensory neuron, and not to a change in the sensitivity or number of postsynaptic receptors.

A later study that used sensorimotor synapses in dissociated cell culture provided strong support for the idea that short-term depression was due to an exclusively presynaptic change. In this study Armitage and Siegelbaum (1998) repeatedly stimulated the presynaptic sensory neuron of sensory neuron-L7 cocultures at a low frequency (once per min); this rate of stimulation produced significant short-term depression of the sensorimotor EPSP (homosynaptic depression). To test whether homosynaptic depression requires activation of postsynaptic glutamate receptors, Armitage and Siegelbaum activated the sensory neuron at rate of stimulation that produced homosynaptic depression in the presence of the drug DNQX, an antagonist of AMPA/kainate-type glutamate receptors. The blockade of glutamate receptors during the low frequency stimulation did not affect homosynaptic depression. From this result Armitage and Siegelbaum concluded that the induction of short-term homosynaptic depression of the sensorimotor synapse was due to a presynaptic mechanism (see Gover and Abrams, in this volume).

3. Long-term habituation: early physiological and morphological data

Long-term habituation of the gill- and siphon-withdrawal reflex was reported in 1972 (Carew, Pinsker, & Kandel, 1972). Four days of spaced habituation training, in which animals received 10 weak siphon stimuli (jets of water, 30-s interstimulus interval [ISI]) per day, produced habituation of the reflex that persisted for up to 3 weeks. By contrast, animals that received massed habituation training—40 consecutive siphon stimuli on a single day—were not significantly different from an untrained control group when tested 1 week after training (although the responsiveness of the massed-trained animals was somewhat less 24 h after training than it had been prior to training). This result is consistent with results from behavioral studies across a wide range of species and learning tasks that show that spaced training yields more persistent memory than massed training (Dudai, 2002).

Just as short-term habituation was accompanied by short-term depression of the synaptic connection between the sensory and motor neurons in the abdominal ganglion that mediate the withdrawal reflex (Castellucci & Kandel, 1974; Castellucci et al., 1970), long-term habituation was found to be accompanied by long-term depression of the sensorimotor synapse. Carew and Kandel (1973) developed a modified spaced training protocol for inducing long-term habituation; animals received four training sessions of 10 trials each, with only 90 min between sessions. This training regimen induced habituation that persisted for 1 week. These experimenters then examined changes in the excitatory synaptic input to the motor neuron L7 in a cellular analog of the behavioral protocol. Here, one of the afferent nerves to the abdominal ganglion (either the siphon or branchial nerve) was electrically stimulated using a stimulus regimen that mimicked the behavioral training (four blocks of 10 stimuli with 90 min separating the blocks), and the complex PSPs produced by the nerve stimulation were recorded intracellularly in L7. Carew and Kandel found that the habituation-related electrical training produced significant depression of the complex PSP in L7 24 h after training compared to that in control preparations. This electrophysiological correlate of long-term habituation was extended to the monosynaptic component of the reflex by Castellucci, Carew, and Kandel (1978). They first gave animals 5 days of habituation training, which produced significant long-term habituation of the withdrawal reflex, as assessed after the end of training. 24 h later the trained animals were dissected, and the experimenters measured the amplitude of the monosynaptic EPSPs between the sensory neurons and L7 using intracellular electrophysiological recording. They observed that the mean percentage of sensorimotor synapses with detectable (>50 μV) EPSPs was significantly less in the trained animals than in animals that were untrained. (Interestingly, the mean amplitude of the detectable monosynaptic EPSPs in the habituated and control animals did not differ.) This result represented one of the first instances, if not the first instance, in which a specific form of long-term memory could be accounted for in terms of a long-term change in the strength of identified synaptic connections.

The morphological basis of the long-term synaptic change that underlay long-term habituation in *Aplysia* was subsequently explored in studies by Bailey and Chen (Bailey & Chen, 1983). These investigators used electron microscopy to examine synapses between siphon sensory neurons in the abdominal ganglion and follower cells in animals subjected to long-term habituation training.

Sensory neurons were labeled for electron microscopy through intracellular injection of an electron dense marker (horseradish peroxidase). Bailey and Chen observed that the number of terminal varicosities—the sites of presynaptic release—on the axonal branches of labeled sensory neurons in habituated animals were significantly reduced in number compared to those in control animals. Moreover, the number and area of the active zones, as well as the number of presynaptic vesicles associated with each active zone, were significantly reduced in habituated animals. That the number of presynaptic varicosities is reduced in animals that have undergone long-term habituation was confirmed in a later study (Bailey & Chen, 1988).

4. Problems with the homosynaptic depression model of long-term habituation

A serious difficulty for the original model of long-term habituation emerged from a study of synaptic depression in sensorimotor cocultures. Montarolo and colleagues (1988) attempted to demonstrate long-term homosynaptic depression of sensorimotor synapses in sensory-L7 cocultures. In these experiments sensory and motor neurons in vitro were impaled with sharp electrodes, and sensory neurons were stimulated at a low frequency. Importantly, the protocol for sensory neuron stimulation was designed to mimic the expected activity of sensory neurons in vivo during long-term habituation training (Carew & Kandel, 1973; Carew et al., 1972). Sensory neurons were given four blocks of stimulation at a rate of one block per 90 min; in each block there were 10 trials at a 30-s intertrial interval. In some experiments the sensory neuron was activated a single time each trial, whereas in others the sensory neuron was fired four times at 9 Hz. These two stimulation protocols simulated the range of effects on sensory neurons of moderate tactile stimulation of the skin (Byrne, Castellucci, Carew, & Kandel, 1978). The sensorimotor EPSP was recorded from the postsynaptic L7 neuron during the stimulation protocols and 24 h later. Although the sensory neuron stimulation produced significant short-term depression of the EPSP, surprisingly, there was no long-term depression. Thus, paradoxically, stimulation of sensory neurons using a protocol designed to mimic their expected activity during long-term habituation training failed to induce long-term synaptic depression, in striking contrast to the effect of the behavioral training itself (Castellucci et al., 1978).

Interestingly, Montarolo, Kandel, and Schacher (1988) were able to produce long-term depression of the in vitro sensorimotor synapse by repeated applications of the endogenous inhibitory neuropeptide, FMRFamide (Piomelli et al., 1987). Based on this result, and on the inability of homosynaptic stimulation to produce long-term depression, it is possible that behavioral training recruits FMRFamide-containing interneurons. If so, perhaps it is the repeated release of this neuropeptide onto sensorimotor synapses that induces long-term depression, rather than the repeated activation of the synapse. While plausible, this idea also faces difficulties. Homosynaptic stimulation clearly produces robust short-term depression of the sensorimotor synapse, and one would anticipate that the sensory-neuron-to-interneuron synapse would undergo similar depression during habituation training (see Cohen, Kaplan, Kandel, & Hawkins, 1997; Frost et al., 1997) (but also see Stopfer & Carew, 1996). If so, as the behavioral training progressed the amount of FMRFamide released within the abdominal ganglion would be expected to decrease. It is conceivable, however, that the amount of the neuropeptide released during the initial stages of habituation training would be sufficient to induce long-term depression of the sensorimotor synapse. Another factor to consider in evaluating the potential contribution of heterosynaptic modulation to long-term habituation is activity-dependent enhancement of heterosynaptic inhibition (or activity-dependent inhibition). Small and colleagues (1989) showed that pairing sensory neuron activation (five spikes at 10 Hz) with FMRFamide application produced greater short-term depression of in vitro sensorimotor connections than did unpaired stimulation or FMRFamide alone. It has not yet been demonstrated that pairing sensory neuron activation with FMRFamide can result in enhanced long-term depression of the sensorimotor synapse. At present, however, a plausible mechanism for long-term habituation is activity-dependent inhibition due to the repeated occurrence of homosynaptic sensorimotor activity in conjunction with release of FMRFamide from heterosynaptic pathways. Nonetheless, the involvement of activity-dependent inhibition in long-term habituation remains to be shown.

5. Evidence that postsynaptic glutamate receptor activation mediates long-term habituation in *Aplysia*

While work on the role of homosynaptic depression in long-term habituation of the withdrawal reflex has reached something of a cul-de-sac, recent work indicates that long-term habituation, in contrast to short-term habituation, involves postsynaptic mechanisms. This insight comes from experiments performed initially in my laboratory by Youssef Ezzeddine (Ezzeddine & Glanzman, 2003). We used a reduced preparation, comprising the gill, siphon, much of the body wall, and the central nervous system (CNS), together with the peripheral nerves connecting the CNS to the gill and siphon (Fig. 1). The siphon was stimulated with implanted electrodes. In some experiments electrodes were implanted in only one side of the siphon, whereas in others electrodes were placed into both sides of the siphon, and each side was stimulated independently (within-preparation protocol). The gill contraction in response to siphon stimulation was measured with a force transducer; the gill was attached to the transducer via a fine silk suture. In addition, the siphon artery was cannulated to allow drugs to be infused into the abdominal ganglion and not into other central ganglia. Long-term habituation training consisted of four blocks of trials with 90 min separating the blocks. (Note that in later experiments five blocks of training trials were used.) Each block comprised 30 trials (ITI = 30 or 60 s). During the initial experiments a between-preparation design was used. Only one side of the siphon was stimulated; experimental preparations received both the test and training stimuli, whereas the control preparations received only the test stimuli. We found that the four blocks of training produced habituation that lasted for at least 6 h (Fig. 2),

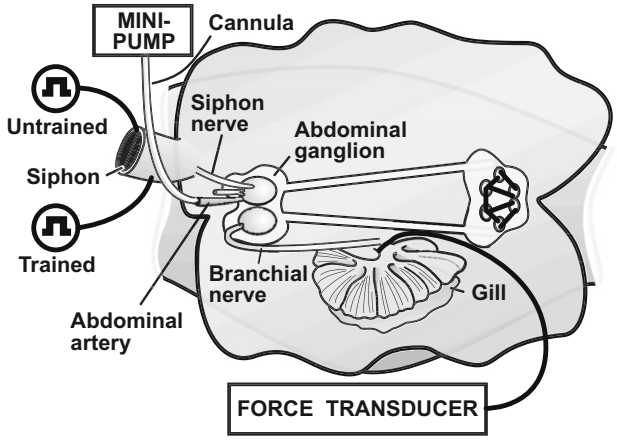

Fig. 1. Reduced preparation of *Aplysia* used for experiments investigating habituation of siphon-elicited gill withdrawal. The abdominal ganglion is shown artificially enlarged relative to the other central ganglia. From Ezzeddine and Glanzman (2003).

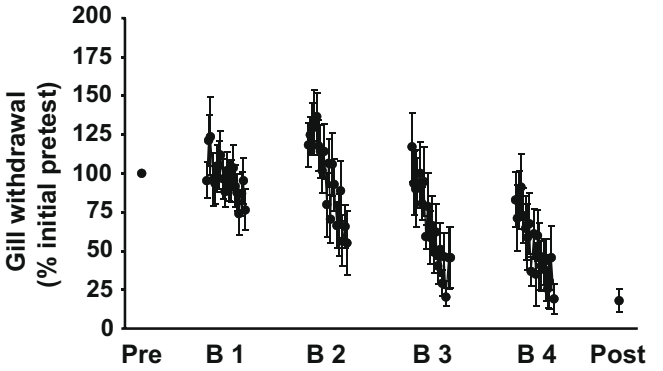

Fig. 2. Long-lasting habituation of gill withdrawal in a reduced preparation. Gill withdrawal in reduced preparations that received spaced blocks of habituation training. Only one site on the siphon was stimulated in these experiments. Habituation training in this and in the experiments presented in Figs. 3–5 consisted of four blocks of stimuli. The interblock interval was 90 min. Each block comprised 20 stimuli (weak siphon shocks, ISI = 30 s). Prior to the training the preparation received three pretests trials (ITI = 10 min). The mean score on the three pretests is shown. After the fourth block of habituation training there was a 6-h rest period, and then the preparation was given three posttests (ITI = 10 min). The mean posttest score shows that the habituation training produced significant long-term habituation.

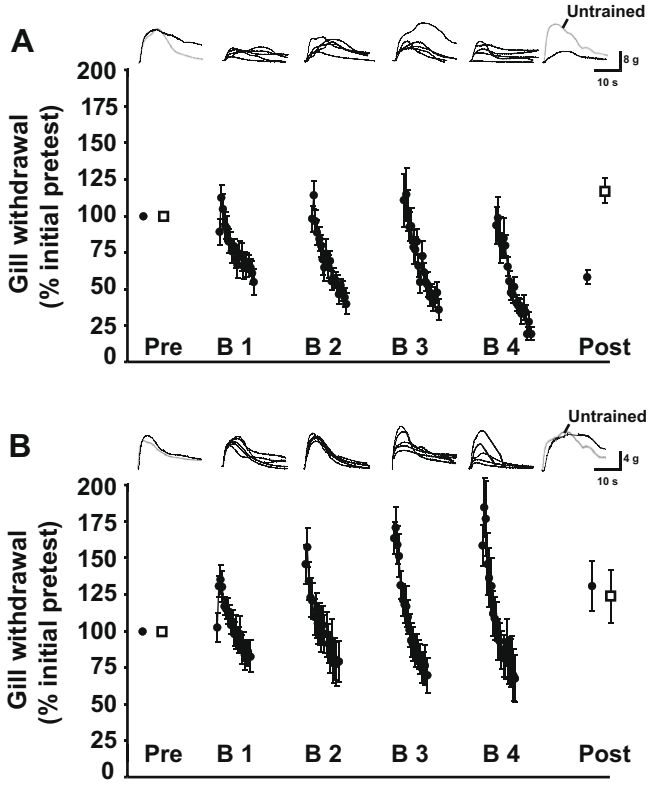

Fig. 3. Effect of inhibition of protein synthesis with anisomycin on long-term habituation. (A) Results from training in normal artificial seawater (ASW). In the experiments presented here and in Figs. 4 and 5 two sites on opposite sides of the siphon were stimulated (see Fig. 1). One site (untrained) served as the control site and received only the test stimuli; the other site received the habituating stimuli as well as the test stimuli. The side of the siphon chosen to be the trained site was alternated systematically between left and right sides. In the graphs in this figure and in Figs. 4 and 5 the gill responses to trained-site stimulation are represented by *filled circles*; responses to untrained-site stimulation are represented by *open squares*. Traces shown above the graph represent individual gill-withdrawal responses from a single experiment recorded using a force transducer. From left to right: the response to the first trained-site pretest and the response to the untrained-site pretest are shown superimposed. (Responses to trained-site stimulation are in black, and responses to untrained-site stimulation are in gray.) Next, five superimposed gill responses (those to stimuli 1, 3, 8, 13, and 20) from each of the four training blocks are presented sequentially. Finally, the response to the first trained-site posttest and the response to the untrained-site posttest are shown superimposed. The trained site initially received three pretests at a rate of one per 10 min. (The response was normalized to the mean of the three pretests.) Ten minutes after the third pretest to the trained site, the other side of the siphon (untrained site) received a single pretest stimulus. (The untrained response was normalized to the response value for the one pretest.) Fifteen minutes after the pretest stimulus to the untrained site (25 min after the third pretest to the trained site), the trained site received habituation training. There were four blocks of habituation training. The training protocol was identical to that used for the between-preparation experiments (refer to Fig. 2). In the within-preparation experiments, there was 60 min rest period between the final block of training stimuli and the onset of the posttests. Then the trained site received three posttests at one per 10 min. The mean normalized posttest response value is presented. Ten minutes later, the untrained site received a single posttest. (B) Results from habituation training in the presence of the protein synthesis inhibitor anisomycin (30 μM in ASW). Traces at top are the individual gill responses recorded during a single experiment. For details, see the legend in A. Notice that the reflex exhibited significant interblock sensitization during training, as indicated by the increase in withdrawal responses to the first several stimuli of each training block. From Ezzeddine and Glanzman (2003).

whereas the response of control preparations showed no decrement over the same period (data not shown). To begin to understand the cellular processes that underlie this form of memory, we performed pharmacological experiments using a within-preparation design. Here, one side of the siphon received the habituation training, while the other (control) side received only the test stimuli. Long-term habituation was assessed 60 min (120 min in later experiments) after the last training block. The habituation training produced significant habituation of gill withdrawal to posttest stimulation of the side of the siphon that was trained, but not to posttest stimulation of the control side (Fig. 3A). Thus, Ezzeddine and Glanzman were able to demonstrate site-specific habituation in a reduced preparation (see also Stopfer, Chen, Tai, Huang, & Carew, 1996). Moreover, this form of habituation meets the standard definition of long-term memory (Goelet, Castellucci, Schacher, & Kandel, 1986), because it depends both on protein synthesis (Fig. 3B) and on RNA synthesis (Ezzeddine, Pearce, & Glanzman, 2004). In other experiments it was shown that the long-term habituation requires protein phosphatase activity, because it was disrupted when training was carried out in the presence of okadaic acid, a selective inhibitor of protein phosphatases 1 and 2A (Ezzeddine & Glanzman, 2003). To test whether activation of postsynaptic AMPA-type receptors (Yung et al., 2002) was necessary for long-term habituation of the gill-withdrawal reflex, Ezzeddine and Glanzman (2003) perfused the abdominal ganglion with the AMPA/kainate receptor antagonist DNQX during training. Treatment with DNQX blocked the induction of long-term habituation (Fig. 4). This result implies that homosynaptic (presynaptic) depression is insufficient to account for long-term habituation in *Aplysia*, because the induction of homosynaptic depression is not blocked by DNQX (Armitage & Siegelbaum, 1998). In addition to activation of AMPA-type receptors, Ezzeddine and Glanzman found that activation of NMDA-type receptors (Dale & Kandel, 1993; Ha, Kohn, Bobkova, & Moroz, 2006) was also required for long-term habituation. Perfusion of the NMDA receptor antagonist APV during the experiment blocked long-term habituation (Fig. 5). In more recent experiments we have found that long-term habituation depends as well on activity of L-type Ca^{2+} channels and calcineurin (phosphatase 2B) (Ezzeddine, Pearce, & Glanzman, 2005, and unpublished data).

Although our training methods yield habituation in the reduced preparation that persists for at least 6 h after the end of training (Fig. 2), we have not yet demonstrated 24-h habituation memory using this preparation. This raises the question of the extent to which the memory that we have studied in the reduced prepara-

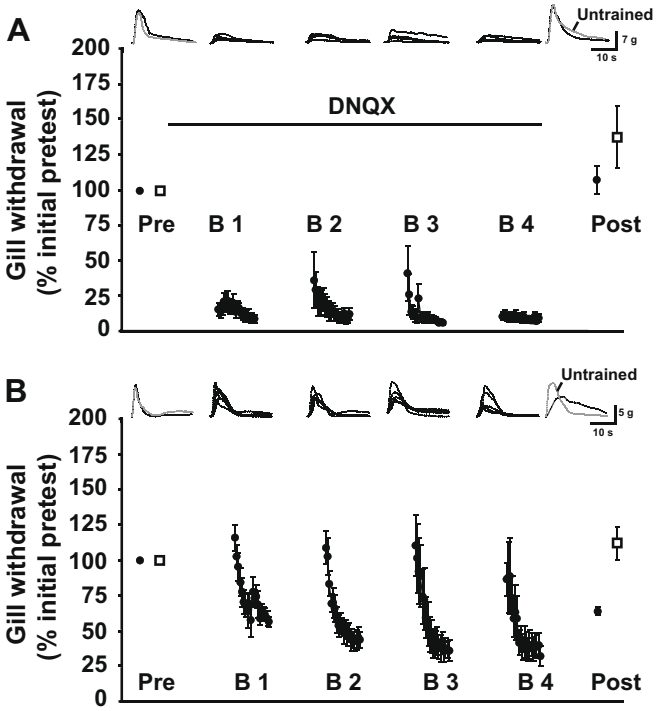

Fig. 4. Effect of the AMPA/kainate receptor antagonist DNQX on long-term habituation. (A) Results from training in the presence of DNQX (500 μM in ASW with 0.2% DMSO). The drug was present only during the training, as indicated by the black line. Notice that the responses evoked during training in the presence of DNQX were greatly reduced but not completely eliminated. (B) Results from habituation training in ASW plus DMSO without DNQX. Traces shown above the graphs in (A) and (B) are the individual gill responses recorded during a single experiment. The set of traces shown with each graph are from one preparation. For details, see the legend in Fig. 3A. From Ezzeddine and Glanzman (2003).

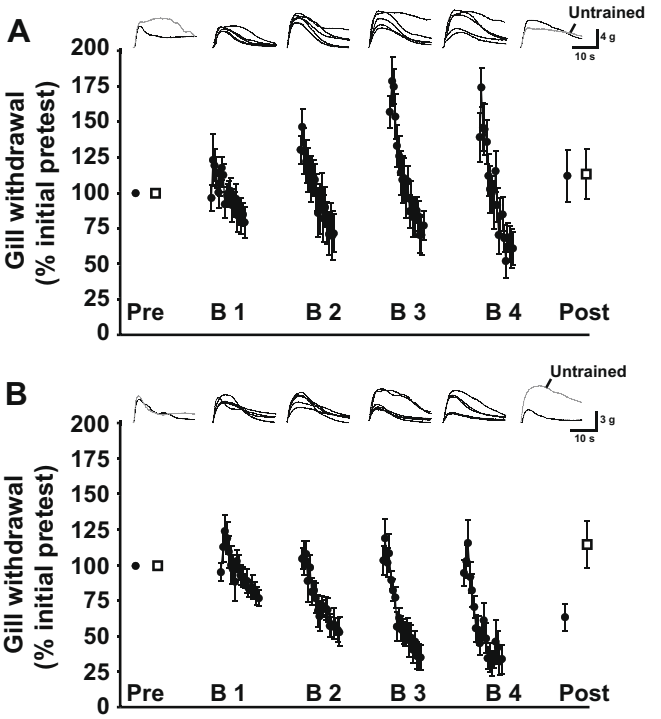

Fig. 5. Effect of APV on long-lasting habituation. (A) Results from training in the presence of the NMDA receptor antagonist APV (150 μM in ASW). The drug was present throughout the experiments. Notice that the reflex exhibited significant interblock sensitization during training. (B) Results from habituation training in normal ASW without APV. Traces shown above the graphs in (A) and (B) are the individual gill responses recorded during a single experiment. For details, see the legend in Fig. 3A. From Ezzeddine and Glanzman (2003).

tion is comparable to the long-term habituation originally demonstrated in the intact animal, which persisted for ⩾24 h (Carew et al., 1972). Thus far, all other forms of memory studied in *Aplysia* that depend on both protein synthesis and gene transcription have been shown to persist for ⩾24 h (Sutton & Carew, 2002). Therefore, I believe it is reasonable to term the habituation we have demonstrated in the reduced preparation "long-term". It is admittedly possible that mechanistic distinctions between 24-h habituation and the form of habituation described here will eventually be discovered. In future experiments we plan to use intact animals to confirm that the processes we have shown to be critical for habituation that lasts for 1–6 h after training in the reduced preparation are also critical for habituation that persists ⩾24 h in the intact animal. In these experiments pharmacological inhibitors will be applied via intrahemocoel injections prior to the onset of the testing/training (see Fulton, Condro, Pearce, & Glanzman, 2008).

6. Homosynaptic LTD of the sensorimotor synapse: a potential mechanism for long-term habituation

A type of plasticity that resembles long-term depression (LTD) of hippocampal synapses (Bear & Abraham, 1996; Malenka & Bear, 2004) has been described for the *Aplysia* sensorimotor synapse (Lin & Glanzman, 1996). Similar to LTD of synapses in the CA1 region of the hippocampus (Dudek & Bear, 1992; Mulkey & Malenka, 1992), LTD of the in vitro sensorimotor synapse can be induced by prolonged low-frequency stimulation (in the case of *Aplysia* synapses, 2 Hz for 15 min). This pattern of stimulation results in depression that persists for at least 75 min (Fig. 6). Furthermore, LTD of the sensorimotor synapse is similar to the low-frequency stimulation-induced LTD of hippocampal synapses (Mulkey & Malenka, 1992) in that its induction depends on elevated postsynaptic intracellular Ca^{2+}; chelating intracellular Ca^{2+} in the motor neuron (with BAPTA) prior to the application of 2 Hz stimulation blocks the induction of LTD (Lin & Glanzman, 1996). Interestingly, Lin and Glanzman observed that postsynaptic BAPTA did not interfere with synaptic depression for the first 30 min after the low-frequency stimulation. This result suggests that there is an early phase of depression that is independent of postsynaptic Ca^{2+}. Depression during this early phase may rely mainly on presynaptic processes.

A complication for the mechanistic analysis of LTD of the sensorimotor synapse is that the synapse readily depresses in response to very low frequency (< once per min) test stimulation (above). This short-to-intermediate-term depression appears to be due predominately, if not exclusively to presynaptic changes (Armitage & Siegelbaum, 1998; Castellucci & Kandel, 1974; Gover, Jiang, & Abrams, 2002; Royer, Coulson, & Klein, 2000b). In an *Aplysia* LTD experiment, therefore, this presynaptic depression to the test stimuli will be superimposed on the LTD induced by higher frequency (1–2 Hz) stimulation. To remove the contribution of presynaptic changes to LTD, we have recently begun to perform experiments using isolated siphon motor neurons in cell culture (Chitwood, Li, & Glanzman, 2001; Villareal, Li, Cai, & Glanzman, 2007). Here, the motor neuron is stimulated with brief pulses of glutamate, the putative excitatory transmitter of *Aplysia* sensory neurons (Dale & Kandel, 1993; Levenson et al., 2000), delivered by pressure ejection from a micropipette; the response of the motor neuron to the glutamate pulses (the Glu-EP) is recorded with a sharp electrode. Unlike the sensorimotor EPSP, the Glu-EP is stable at low rates of test stimulation (e.g., once per 10 s). To induce LTD of

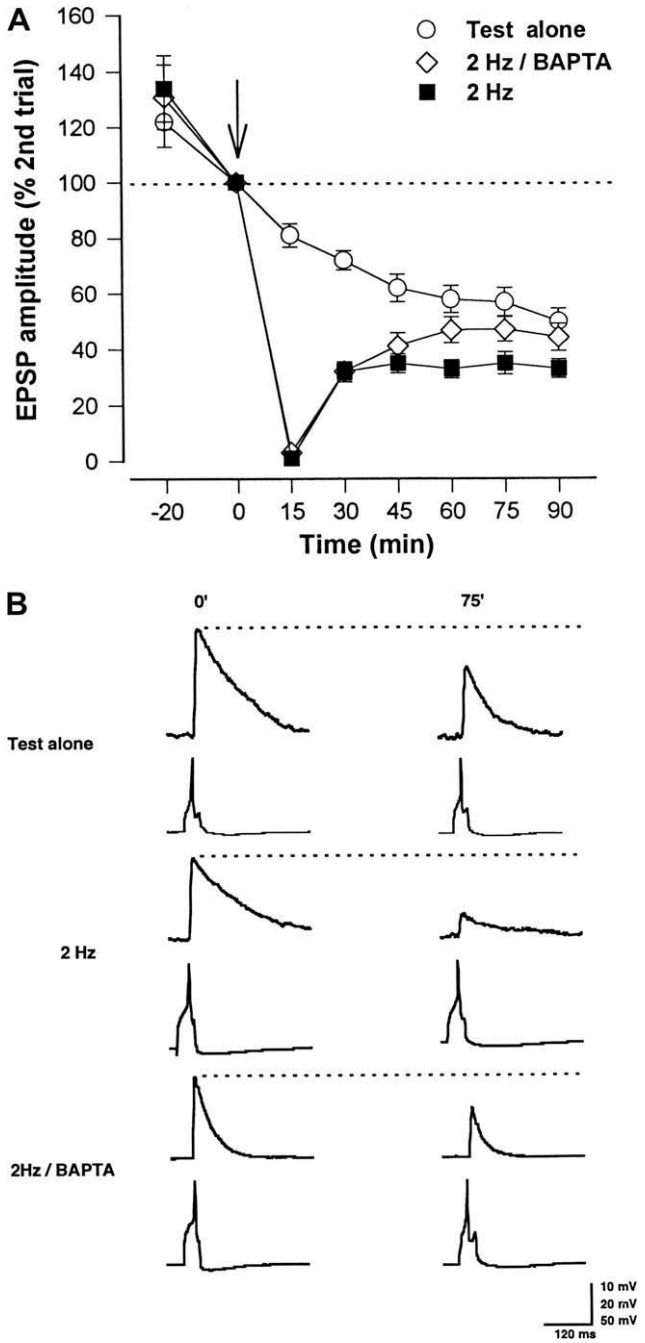

Fig. 6. Long-term, homosynaptic depression of isolated *Aplysia* sensorimotor synapses in dissociated cell culture. (A) Normalized amplitudes of excitatory postsynaptic potentials (EPSPs) for sensorimotor synapses that received only the test stimulation (test alone group); 2-Hz activation of the sensory neuron for 15 min (2-Hz group); or 2-Hz activation of the sensory neuron for 15 min with BAPTA present in the motor neuron (2-Hz/BAPTA group). The onset of the 2-Hz stimulation is indicated by the *arrow*. (B) representative electrophysiological records of sensory neuron action potentials (bottom row of traces for each group) and EPSPs evoked on the 0-min and 75-min trials for the three experimental groups. Vertical calibration bar, 10 mV for the test alone and 2-Hz EPSPs; 20 mV for the 2-Hz/BAPTA EPSPs; and 50 mV for the sensory neuron actions potentials. From Lin and Glanzman (1996).

the Glu-EP Mario Mata and colleagues delivered 900 pulses of glutamate to the motor neuron at 1 Hz. This stimulation protocol results in a 20–30% depression of the Glu-EP that persists for at least 45 min (Mata, Chen, Cai, & Glanzman, 2008). The LTD is blocked by the NMDA receptor antagonist APV. (Interestingly, blockade of LTD with APV reveals long-term potentiation [LTP] of the Glu-EP. Therefore, LTD and LTP appears to be coinduced by the 1 Hz stimulation, but the LTP is only expressed when the LTD is blocked.) LTD of the Glu-EP is also blocked by the AMPA receptor antagonist CNQX.

Thus far the results from our mechanistic analyses of homosynaptic and "hemisynaptic" LTD (LTD of the glutamate response in the isolated motor neuron) in *Aplysia* are reminiscent of those from our studies of long-term habituation (above). Both LTD and long-term habituation depend on activation of NMDA- and AMPA-type receptors. Moreover, the dependence of long-term habituation on activation of voltage-dependent Ca^{2+} channels and calcineurin (Ezzeddine et al., 2005) is reminiscent of the requirement of homosynaptic LTD for elevated postsynaptic intracellular Ca^{2+}. Of course, we did not determine the cellular locus of the required increase in intracellular Ca^{2+} for long-term habituation in our behavioral experiments. Nonetheless, the results from the behavioral and cellular studies have been mutually consistent so far. An important question that should be addressed in the near future is whether either homosynaptic or hemisynaptic LTD requires protein phosphatase activity and protein synthesis, as does long-term habituation (Ezzeddine & Glanzman, 2003). Interestingly, pharmacological activation of group I and II metabotropic glutamate receptors (mGluRs) also yields hemisynaptic LTD (Indersmitten, Mata, & Glanzman, 2007, M. Mata and D. L. Glanzman, unpublished data). It would therefore be valuable to investigate whether mGluR activity plays a role in long-term habituation.

At first glance homosynaptic LTD may seem an unlikely mechanism of long-term habituation. After all, the induction of homosynaptic LTD of the sensorimotor synapse (or hemisynaptic LTD in the isolated motor neuron) requires rather prolonged (15 min) stimulation at 1–2 Hz. This is quite different from the pattern of stimulation typically used for habituation of the reflex. (Notice that we have not yet attempted to obtain homosynaptic LTD using longer interstimulus intervals, e.g., 30 s.) However, it should be recalled that the possibility that hippocampal LTP (Bliss & Lømo, 1973) might subserve memory was originally dismissed out of hand by many neuroscientists because the high frequency (~100 Hz) stimulation that was used to induce LTP did not resemble activity in the nondiseased brain. Eventually, of course, it was found that LTP could be obtained using patterns of electrical stimulation (such as paired or Hebbian stimulation) that more closely mimicked normal brain activity (Bliss & Collingridge, 1993). Furthermore, homosynaptic LTD in the mammalian hippocampus and cortex is thought by more than a few neuroscientists to represent an important mechanism of memory, even though to my knowledge no one believes that the pattern of synaptic activity induced during the typical LTD experiment occurs commonly in the brain. Additional work will be required to determine whether homosynaptic LTD of the sensorimotor synapse can be induced using other, more natural, patterns of stimulation. If this attempt succeeds, it will then be necessary to determine whether such LTD-inducing patterns of sensorimotor activity actually occur during long-term habituation. Fortunately, simultaneous synaptic electrophysiological and behavioral experiments can be readily accomplished in *Aplysia* using existing semi-intact preparations (Cohen et al., 1997; Ezzeddine & Glanzman, 2003; Stopfer & Carew, 1996). Finally, it will be important to establish that activity-induced LTD of the sensorimotor synapse can persist for more than 1–2 h. At present, the only known way to obtain depression of this synapse that endures for at least 24 h, other than through long-term behavioral training (Castellucci et al., 1978), is via repeated application of the neuropeptide FMRFamide (Montarolo et al., 1988).

7. Summary

Almost 40 years after its first formal description (Pinsker, Kandel, Castellucci, & Kupfermann, 1970), the cell biology of habituation of the gill- and siphon-withdrawal reflex remains incompletely understood. The greatest cellular progress has been made with respect to short-term habituation, where it seems clear that presynaptic depression of transmission at the sensorimotor synapse plays a significant role (Armitage & Siegelbaum, 1998; Castellucci & Kandel, 1974; Cohen et al., 1997; Frost et al., 1997; Gover et al., 2002; Royer et al., 2000b). More prolonged forms of habituation, however, are problematic. Presynaptic depression, by itself, is inadequate to account for long-term habituation. Furthermore, no pattern of synaptic activity has yet been shown to be capable of inducing depression of the sensorimotor synapse that persists for $\geqslant$24 h. Nonetheless, some recent progress has been made regarding the cellular mechanisms of long-term habituation in *Aplysia*. Similar to long-term habituation in the nematode *Caenorhabditis elegans* (Rose, Kaun, Chen, & Rankin, 2003), long-term habituation of the gill- and siphon-withdrawal reflex requires activity of AMPA-type receptors (Ezzeddine & Glanzman, 2003). In addition, long-term habituation of the withdrawal reflex depends on the activity of NMDA-type receptors, protein phosphatases, and L-type Ca^{2+} channels (Ezzeddine & Glanzman, 2003; Ezzeddine et al., 2005). It is important to point out, however, that the pharmacological evidence from the behavioral experiments provides no information about the necessary cellular sites for activity of AMPA receptors, NMDA receptors, protein phosphatases and L-type Ca^{2+}. An important question that must be addressed in the future is whether these critical processes operate at sensorimotor synapses, or at other cellular sites, such as within interneurons, or both.

Although an activity-dependent LTD-like mechanism is a plausible mediatory candidate for long-term habituation, it remains to be demonstrated that homosynaptic activity alone can induce 24-h LTD. Lin and Glanzman (1996) did not ascertain whether their 2-Hz homosynaptic stimulation could produced 24-h depression of the sensorimotor synapse. It is possible that such long-lasting depression of the sensorimotor synapse cannot be achieved without input from heterosynaptic modulatory pathways during habituation training (see Montarolo et al., 1988).

Bailey and Chen's (Bailey & Chen, 1983, 1988) studies raise further challenges for a cellular model of habituation in *Aplysia*. The results from those studies indicate that long-term habituation of the withdrawal reflex involves persistent structural changes in the sensory neurons that mediate the reflex, including a reduction in presynaptic varicosities and in presynaptic vesicles. To reconcile Bailey and Chen's findings with those from our studies of long-term habituation (Figs. 4 and 5), it would appear necessary to posit some form of retrograde signaling. Interestingly, retrograde signaling from the motor-to-sensory neuron has recently been shown to be critical for long-term facilitation of the sensorimotor synapse (Cai, Chen, & Glanzman, 2008).

Will neurobiologists ever understand habituation? Given the frustratingly slow progress on this most basic of learning phenomena during the last 40 years, one would be excused for suspecting that solving the problem of habituation might prove to be a millennium-long enterprise! (Grizzled veterans of the habituation campaign can only regard with wonder those younger colleagues who are eager to undertake the task of unraveling the neurobiology of cognition.) But such a suspicion would be unduly pessimistic. First, new potential synaptic mechanisms for both short-term (Gover et al., 2002; Royer, Coulson, & Klein, 2000a) and long-term (Indersmitten et al., 2007; Lin & Glanzman, 1996; Mata et al., 2008) have been described. Second, new molecular, genetic and optical tools have recently been developed (see, e.g., Luo, Callaway, & Svoboda, 2008; Zhang, Aravanis, Adamantidis, de Lecea, & Deisseroth, 2007) that promise new avenues of attack on the old problem of habituation. We therefore have reason to be optimistic that another 40 years will not be required before the Cheshire Cat of habituation is finally revealed in its entirety.

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