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Short- and Long-Term Modification of Reflex Function During Learning and Metamorphosis in *Manduca*

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However much one may disparage the stolid abdominal crawling behavior of caterpillars, the fact remains that they possess probably the most versatile abdomens in the animal kingdom. . . . The abdomen is to the caterpillar what arms are to a person.

. . . caterpillars are such schizophrenic beasts. At one and the same time they must be perfect caterpillars and potential moths. They must be prepared to meet all the challenges that life presents to a slow-moving, earthbound grazer while beginning the construction of the fragile furred body and gossamer wings that will offer them the freedom of the air.

—Dethier (1980)

Introduction

In his book, *The World of the Tent-Makers,* from which the above quotations are taken, V. G. Dethier chronicles the lepidopteran life cycle in an absorbing blend of lyric prose, philosophy, and astute observation of animal behavior. As detailed in this book, the insect's life is marked by countless challenges and opportunities to which the nervous system must respond in an adaptive manner. Although the basic neural circuits underlying many behaviors are preassembled in the egg, these behaviors must be modified to accommodate the exigencies of the real world. Furthermore, during metamorphosis, the neural circuits for larval behaviors must be replaced with those for pupal and adult behaviors. This review describes our investigations into how the nervous system strikes a balance between constancy and flexibility in generating behavioral output.

Our experimental animal is the tobacco hornworm, *Manduca sexta,* which transforms from egg to moth in approximately 6 wk. *Manduca* has proven to be ideal for investigating the neural mechanisms of behavior, as well as how hormones—the ecdysteroids and juvenile hormone (JH)—reorganize the nervous system during metamorphosis (reviewed by Weeks and Levine, 1992). This review focuses on a neural circuit that mediates a simple defensive withdrawal reflex of the larval proleg of *Manduca,* and describes how reflex function is modified in two contexts: during the dismantling of the circuit at pupation and during nonassociative learning in the larval stage. The signals for these two forms of plasticity differ, as do the associated electrophysiological changes that underlie the behavioral changes. Even in this simple reflex circuit, a variety of neural mechanisms contribute to behavioral modifications that occur on time scales from seconds to days and permit the insect to interact adaptively with the world around it.

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The Proleg Withdrawal Reflex

Manduca larvae have 8 pairs of legs: 3 pairs of segmented legs on the thorax and 5 pairs of prolegs on the abdomen (see Fig. 2). Our studies have focused on the prolegs located on abdominal segments three through six (A3–A6). Returning to Dethier's quotation above, if the caterpillar's abdomen is like a person's arms, then the prolegs are the hands on those arms: they are used to reach for and grasp objects, to crawl, and to sense disturbances. The tip of each proleg bears an array of mechanosensory hairs, the planta hairs (PHs; Weeks and Jacobs, 1987). Stimulation of the PHs evokes ipsi- or bilateral retraction of the prolegs in the stimulated segment—the proleg withdrawal reflex (PWR; Fig. 1A). The major features of the neural circuit for the ipsilateral PWR have been identified (Fig. 1B): the sensory neurons that innervate PHs excite proleg retractor motoneurons *via* mono-

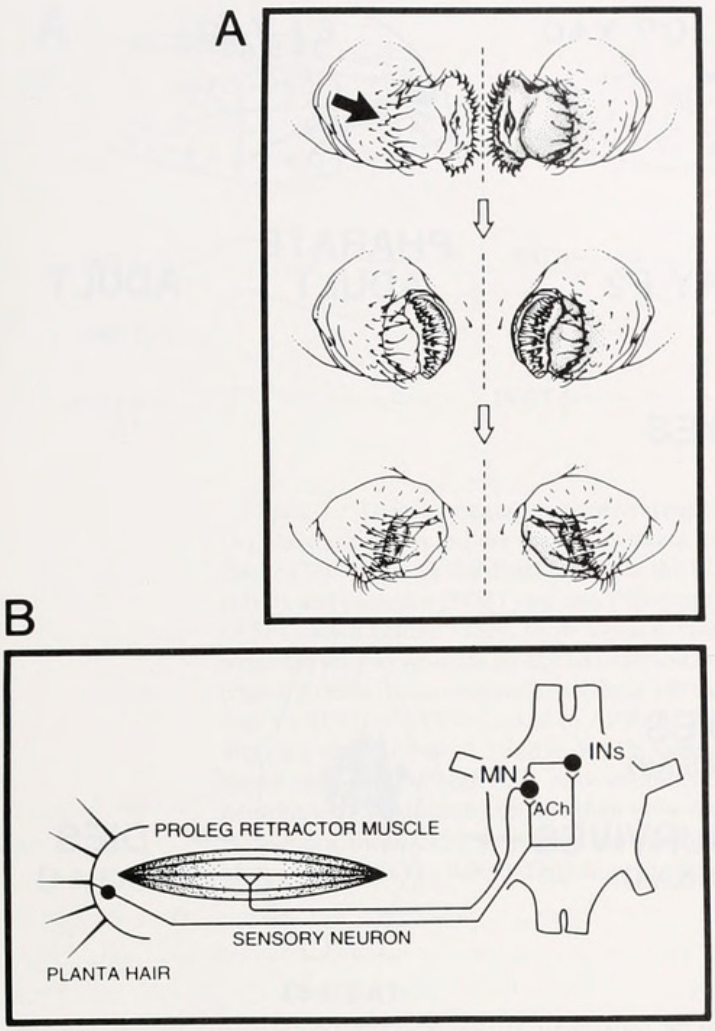

Figure 1. PWR behavior and neural circuit. (**A**) The PWR. A pair of prolegs is shown in ventral view with the ventral midline marked by a dashed line. Tactile stimulation of PHs (filled arrow) evokes proleg withdrawal, as shown in the successive drawings (open arrows). (**B**) PWR circuit. The proleg tip (left), a proleg retractor muscle (middle), and the ganglion of the same abdominal segment (right) are shown schematically. Neurons are indicated by filled circles. Each PH is innervated by a single sensory neuron with a cell body in the proleg epidermis and an axon that projects to the ganglion. PH sensory neurons excite ipsilateral PPR and APR motoneurons (MN) *via* monosynaptic, cholinergic (ACh) synapses, and polysynaptic pathways through interneurons (INs). The diagram shows the neural circuit for the left proleg only.

synaptic, cholinergic connections and by polysynaptic pathways involving unidentified interneurons (Weeks and Jacobs, 1987; Peterson and Weeks, 1988; Trimmer and Weeks, 1989, 1991; Jacobs and Weeks, 1990; Sandstrom and Weeks, 1991; Streichert and Weeks, 1995). The interneuronal pathway includes excitatory and inhibitory interneurons, but the excitatory components predominate. All of the necessary neural components for the PWR are located in the ganglion of the same segment as the stimulated proleg. Studies of plasticity in PWR function have focused on two identified motoneurons, PPR and APR (Fig. 2), which innervate the principal planta retractor muscle (PPRM) and the accessory planta retractor muscle (APRM). Each proleg has one PPRM and one APRM. One PPR innervates each PPRM, whereas each APRM is innervated by a pair of similar APRs (Weeks and Truman, 1984; Sandstrom and Weeks, 1996). These motoneurons and muscles represent the output pathway of the PWR reflex circuit.

Long-Term Modification of the PWR Circuit During Metamorphosis

Dismantling of the PWR circuit

Over the final few days of larval life, the prolegs collapse and stiffen, most of their retractor muscles degenerate (see below), and the PWR can no longer be elicited. Loss of the behavior results in part from the weakening of central excitatory pathways in the circuit, as indicated by a developmental decrease in the ability of electrical stimulation of the proleg sensory nerve to evoke reflexive firing in proleg motoneurons (Jacobs and Weeks, 1990). The decreased excitation could involve changes in sensory neurons, interneurons, motoneurons, or all three, but a previous finding that the dendrites of PPR and the APRs regress dramatically over the same period that the PWR disappears (Fig. 2) suggested that they might be a key locus of change. As reviewed elsewhere (Weeks *et al.*, 1996), dendritic regression is triggered by a prepupal peak of ecdysteroids in the hemolymph.

Because dendrites are the sites of synaptic input, we hypothesized that the regression of motoneuron dendrites would weaken their inputs from sensory neurons and interneurons. To test this idea, we stimulated the proleg sensory nerve while recording the amplitude of the compound excitatory postsynaptic potential (cEPSP) evoked in PPR and the APRs. The cEPSP reflects both the mono- and polysynaptic components of the PWR. During the larval-pupal transformation, cEPSP amplitude decreased by greater than 50% despite an increase in motoneuron input resistance (Jacobs and Weeks, 1990; Streichert and Weeks, 1995). To better define the contribution of different neurons to this decrease, we took advantage of a unique characteristic of *Manduca:* the ability to manipulate the levels of ecdysteroids and JH to generate *heterochronic mosaic* neural circuits composed of neurons in different developmental states (*e.g.*, Levine *et al.*, 1989). It is then possible to determine whether developmental changes in particular neurons are necessary or sufficient for the developmental change in circuit function. For this experiment, we generated heterochronic mosaic insects in which the PH sensory neurons on one proleg were retained in their larval form while the central components of the PWR circuit underwent pupal development. Notably, motoneuron regression in mosaic hemisegments was the same as in unmanipulated controls. In the mosaic hemisegments, cEPSP amplitude

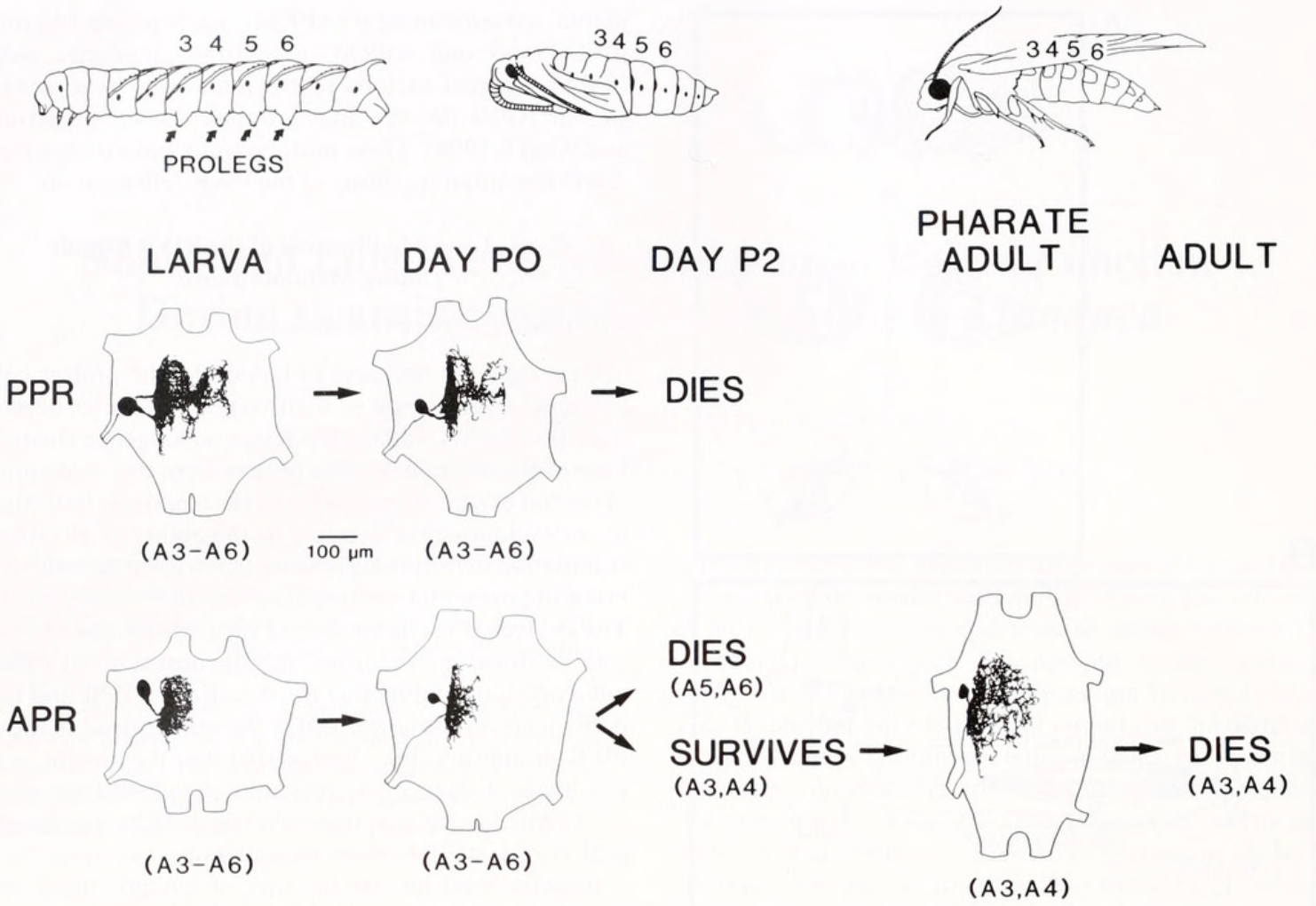

Figure 2. Life history of proleg motoneurons. (**Top**) Drawings show a larva, pupa, and adult *Manduca* with abdominal segments 3, 4, 5, and 6 indicated. (**Bottom**) Camera lucida drawings of cobalt-stained PPRs and APRs are shown on day L3 (final instar larva before the onset of metamorphosis), day P0 (first day of pupal life), day P2 (third day of pupal life), pharate adult (the final day of pupal life), and in the adult moth several days after emergence. The segmental locations of the neurons are indicated in parentheses. PPRs in all segments regress by day P0 and die by day P2. APRs in all segments regress by day P0, but only those in segments A5 and A6 die by day P2. The APRs that survive in segments A3 and A4 show dendritic regrowth during the pupal stage and die after adult emergence. Data from Weeks and Ernst-Utzschneider (1989).

decreased to the same extent as in normal pupae, suggesting that metamorphic changes in PH sensory neurons are *not necessary* and metamorphic changes in central neurons (motoneurons and interneurons) are *sufficient* for the weakening of excitatory pathways in the PWR circuit (Jacobs and Weeks, 1990; Streichert and Weeks, 1995).

One limitation of these experiments was that the cEPSP is a mixture of mono- and polysynaptic inputs to the motoneurons and hence does not reveal how individual synapses change during metamorphosis. To test more explicitly the hypothesis that dendritic regression disconnects synaptic inputs to the motoneurons, we measured the amplitude of monosynaptic EPSPs produced in APR by individual PH sensory neurons in larvae and heterochronic mosaic pupae. At both stages, EPSP amplitude was correlated with the position of the PH on the proleg (Fig. 3A), but the key finding was the EPSP amplitude decreased significantly in all regions of the PH array during the larval-pupal transformation (Fig. 3B). Analysis of EPSP shape indices indicated that the decreased EPSP amplitude in pupae resulted from a decrease in synaptic current, consistent with a reduction in the number of synaptic contacts between the sensory neurons and motoneurons as the motoneurons regress (Streichert and Weeks, 1995).

In summary, these experiments revealed a specific mechanism that contributes to the dismantling of an outmoded neural circuit at the entry into a new life stage: the hormonally driven regression of motoneuron dendrites "sheds" sensory synapses that constitute the input pathway for the reflex. In a broader context, steroid hor-

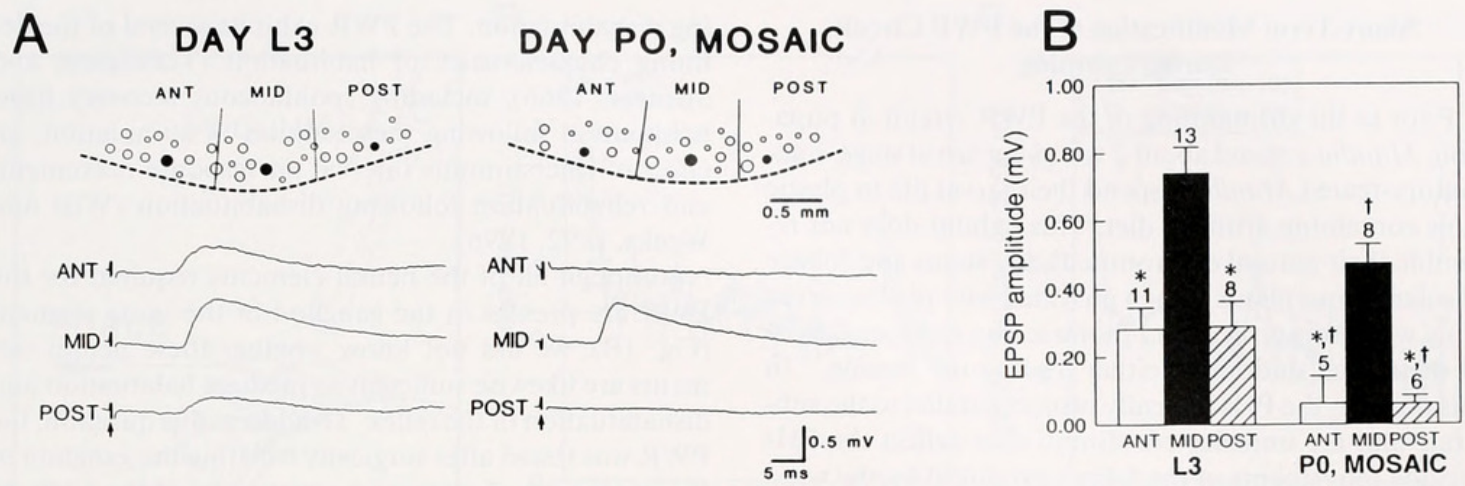

Figure 3. Developmental changes in synaptic connections between PH sensory neurons and APR. In (**A**), the drawings at top are maps of sockets (circles) corresponding to individual PHs in the PH array. Dashed line indicates the distal border of the PH array, and dotted lines indicate anterior (ANT), middle (MID), and posterior (POST) regions. Filled circles indicate the sockets stimulated (at 1 Hz) to produce the EPSPs shown below. Traces show signal-averaged responses recorded in an APR while stimulating the indicated sensory neurons on day L3 (left) and, in another preparation, on day P0 in a mosaic hemisegment (right). Arrows indicate stimulus artifacts. (**B**) Comparison of EPSPs on days L3 and P0. The mean amplitude (± SEM) of EPSPs evoked in APRs by PH sensory neurons located in the three regions of the PH array are shown on day L3 and in day P0 mosaics. The number of PH sensory neurons tested is indicated above each bar. On both days of development, EPSPs produced by anterior and posterior PH sensory neurons were significantly smaller than those from middle sensory neurons (* indicates $P < 0.001$). In day P0 mosaics, mean EPSP amplitude was significantly smaller than on day L3 in all three regions of the PH array († indicates $P < 0.001$). Data from Streichert and Weeks (1995).

mones cause simultaneous changes in neuronal structure and behavior in many animals (*e.g.,* Forger and Breedlove, 1987; Woolley and McEwen, 1993; Smith *et al.,* 1995), but it is typically difficult to test whether the relationship is causal or merely correlational. At present, the PWR of *Manduca* is the best-documented example in which a causal relationship has been demonstrated using electrophysiological methods. Progress is also being made in a number of other systems, both vertebrate and invertebrate (reviewed in Weeks and Levine, 1995).

Fates of leftover components of the PWR circuit in pupae

What happens to members of the PWR circuit after their larval function is obsolete? The PH sensory neurons die (Jacobs and Weeks, 1990; Streichert and Weeks, 1995), and the fates of the interneurons are unknown. The proleg motoneurons exhibit two pupal fates: death or respecification. All of the PPRs, the APRs in segments A5 and A6, and their target muscles die in response to the prepupal peak of ecdysteroids (Fig. 2; reviewed in Weeks *et al.,* 1996). Recent experiments utilizing cell culture indicate that the ecdysteroids act directly on proleg motoneurons to activate an intrinsic program of cell death (Streichert and Weeks, 1994; reviewed in Levine and Weeks, 1996). The APRs that survive in segments A3 and A4 grow new dendritic arbors, are respecified for new functions, and die after adult emergence (Fig. 2). In segment A4, the APRs' larval target muscle, APRM, degenerates and the APRs appear to innervate a new abdominal extensor muscle (Weeks and Ernst-Utzschneider, 1989). In segment A3, the larval APRMs persist in reduced form during the pupal stage. After the prolegs degenerate, these APRMs insert on the ventral abdominal wall at a location that is covered externally by the wings of the developing adult moth (see Fig. 2). At pupation, the APRs and APRMs in segment A3 begin to be driven in a rhythmic motor pattern that circulates hemolymph within the lumen of the developing wing (Sandstrom, 1993; Lubischer *et al.,* 1995 and unpub. data). This is a remarkable example of functional respecification: returning to Dethier's analogy, the neuromuscular system of the hands (prolegs) has been respecified to function like a heart! We plan to investigate the ontogeny of synaptic inputs that drive APRs in the new circulatory motor pattern that, intriguingly, begins to be expressed when the APRs' dendrites are maximally regressed. Studies of how the APRs are incorporated into this new circuit will complement our previous work examining how the APRs are phased out of the PWR circuit at the end of larval life.

Short-Term Modification of the PWR Circuit During Learning

Prior to the dismantling of the PWR circuit at pupation, *Manduca* spend about 2 wk in the larval stage. Laboratory-reared *Manduca* spend their larval life in plastic cups containing artificial diet. This habitat does not resemble their natural environment: the stems and foliage of solanaceous plants. When provided with plants, larvae cling with their prolegs and thoracic legs and spend most of their time stationary, either feeding or "resting." In this posture, the PHs typically project parallel to the substrate and are undeflected: stimuli that deflect the PHs include movements of the foliage produced by the wind or the activity of other insects. In a cinematographic analysis of *Manduca* larvae, Reinecke *et al.* (1980) noted that feeding can be interrupted by external disturbances, potentially affecting growth rate. Therefore, it may be advantageous for larvae to disregard routine stimuli that are nonthreatening. This issue has not been addressed in a natural setting but, under more controlled conditions, we have now shown that PWR exhibits the two simplest forms of nonassociative learning: habituation and dishabituation. Habituation is a decrease in the amplitude of a response to a repeated stimulus, whereas dishabituation is an increase in a decremented (habituated) response after presentation of a novel or noxious stimulus (Thompson and Spencer, 1966). These forms of plasticity serve to filter out mundane sensory stimuli while leaving the system sensitive to novel, and thereby potentially significant, stimuli. Below we describe behavioral and electrophysiological experiments that investigate these short-term modifications of PWR function in *Manduca*.

Habituation and dishabituation of PWR behavior

Habituation and dishabituation of the PWR were tested in isolated larval abdomens, which show fewer spontaneous movements and greater stereotypy of the PWR than do intact insects (Weeks and Jacobs, 1987). The stimulus, a brief deflection of a single PH, was delivered by an automated apparatus (Fig. 4A). The response, measured as the evoked force of proleg withdrawal, was measured by an isometric force transducer attached to the proleg tip. Dishabituating stimuli were delivered by pinching the body wall dorsal to the experimental proleg with a pair of forceps.

Figure 4B shows a representative example of habituation and dishabituation of the PWR. The PH was deflected for 500 ms once every 60 s for 25 trials. The evoked force of proleg withdrawal decreased significantly between trials 1 and 20, reflecting habituation of the PWR, and pinching the body wall between trials 20 and 21 caused a significant recovery on trial 21, demonstrating dishabituation. The PWR exhibits several of the defining characteristics of habituation (Thompson and Spencer, 1966), including spontaneous recovery from habituation following the cessation of stimulation, an effect of interstimulus interval on response decrement, and rehabituation following dishabituation (Wiel and Weeks, 1992, 1996).

Although all of the neural elements required for the PWR are present in the ganglion of the same segment (Fig. 1B), we did not know whether these neural elements are likewise sufficient to mediate habituation and dishabituation of the reflex. To address this question, the PWR was tested after surgically isolating the ganglion of the experimental segment from the rest of the CNS. In these neurally isolated body segments, PWR habituation was robust but dishabituation was negligible (Wiel and Weeks, 1996; Wiel, 1995). Thus, habituation can be produced within a single body segment, whereas dishabituation involves intersegmental processes.

Neural correlates of PWR habituation

We next sought to identify the locus or loci within the PWR circuit that are responsible for response decrement during habituation. Peripheral mechanisms such as sensory adaptation or muscle fatigue could contribute, as could central changes within the PWR circuit. A semi-intact electrophysiological preparation consisting of an abdominal ganglion and attached proleg tip (Trimmer and Weeks, 1989) was used to test whether central neural changes were involved. To eliminate the possible contribution of sensory adaptation, a train of electrical pulses that mimicked the normal pattern of PH sensory neuron activity during a 500-ms deflection was applied to a PH socket to activate its sensory neuron. The PH sensory neuron was activated in an identical pattern on each stimulus trial. To eliminate the possible contribution of muscle fatigue, the response to each stimulus was measured as the number of action potentials (spikes) evoked in the ipsilateral proleg motor nerve. In response to the same stimulation protocol used in isolated abdomens (20 trials, 60 s interval), the motoneurons exhibited a significant response decrement, representing a central neural correlate of habituation (Wood *et al.*, 1994, and in preparation; Wiel, 1995). Moreover, following a 30-min rest after trial 20, the response recovered spontaneously to prehabituation levels, indicating that the response decrement resulted from repeated stimulation rather than nonspecific factors. Thus, changes in the central neural circuit for the PWR contribute to habituation.

Where in the circuit do these changes occur? The monosynaptic excitatory connections from PH sensory neurons to proleg motoneurons (Figs. 1B, 3A) were likely sites of plasticity for two reasons. First, homosyn-

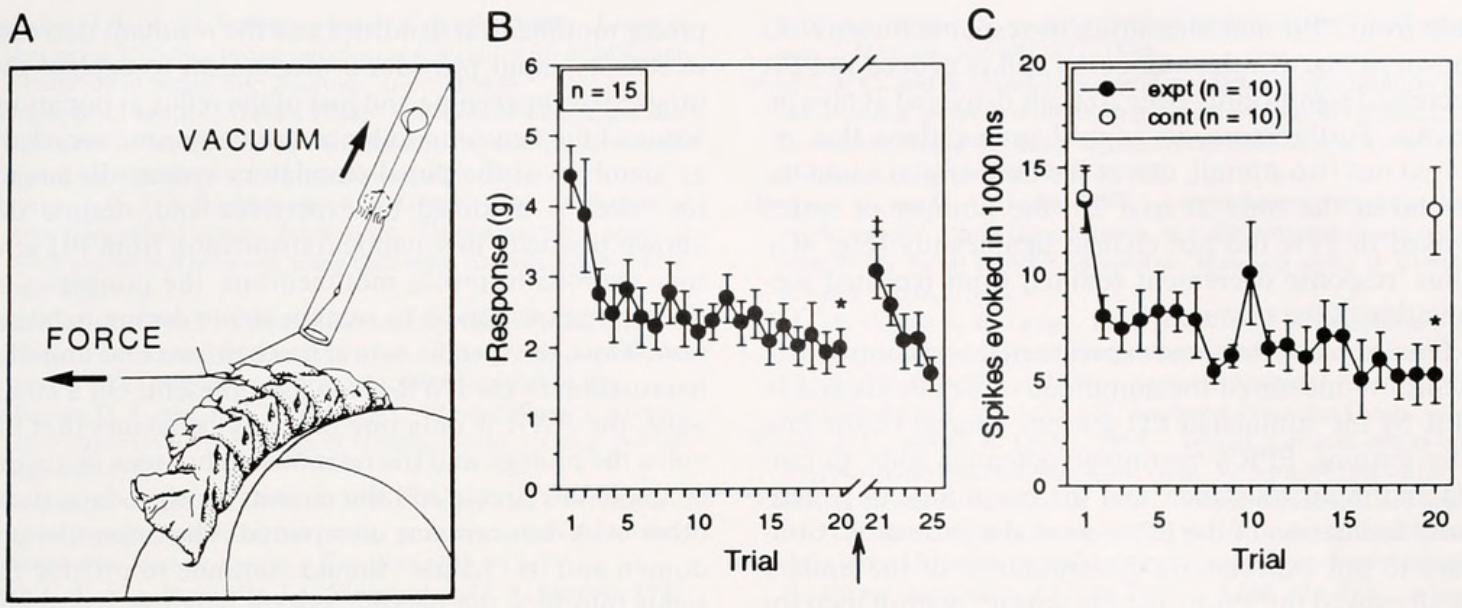

Figure 4. Habituation and dishabituation of the PWR. (**A**) Behavioral preparation. An isolated abdomen was glued to a curved platform and a single PH was deflected by attachment to a small styrofoam ball that was sucked up a pipette by controlled negative pressure (VACUUM). The force of proleg withdrawal was measured (in grams) by an isometric force transducer (FORCE) attached to the proleg tip. (**B**) Habituation and dishabituation in isolated abdomens. A PH was deflected for 500 ms at 60-s intervals for 25 trials, and the evoked force of proleg withdrawal was calculated as the difference between the peak force during the PH deflection minus baseline force before the deflection. Thirty seconds after trial 20, the body wall dorsal to the experimental proleg was pinched with forceps (arrow). Significant habituation occurred between trials 1 and 20 (* indicates $P < 0.01$), and significant dishabituation occurred between trials 20 and 21 (‡ indicates $P < 0.01$); $n = 15$ abdomens. (**C**) Decrease in activity evoked in PPR by repeated PH sensory neuron stimulation. The preparation consisted of an isolated proleg tip and the ganglion of the same segment. An intracellular recording was made from the ipsilateral PPR. A train of electrical pulses that mimicked the normal pattern of sensory neuron activity during a 500-ms PH deflection was applied to a PH sensory neuron. Experimental preparations (expt) received 20 trials at a 60-s ISI, whereas control (cont) preparations received 2 trials at a 19-min ISI. The response to each stimulus was measured as the number of spikes evoked over baseline in PPR, during the 1000 ms following stimulus onset. PPR's response decreased significantly between trials 1 and 20 in experimental ($n = 10$; * indicates $P < 0.01$) but not in control ($n = 10$; $P > 0.05$) groups. Values in (**B**) and (**C**) are mean ± SEM. Data from Wiel and Weeks (1992, 1996), Wiel *et al.*, (1995, and in preparation) and Wiel (1995).

aptic depression of synaptic transmission from sensory neurons to postsynaptic neurons is a primary mechanism of habituation in several other systems, including the gill- and siphon-withdrawal reflexes of *Aplysia* and the tail flip escape reflex of crayfish (reviewed by Krasne and Glanzman, 1995). Second, previous experiments involving PPR demonstrated that these connections exhibit considerable short-term plasticity. The EPSPs are cholinergic and mediated by nicotinic acetylcholine receptors (nAChRs) on PPR (Weeks and Jacobs, 1987; Trimmer and Weeks, 1989, 1993). There are also two classes of muscarinic cholinergic actions: activation of presynaptic muscarinic acetylcholine receptors (mAChRs) reduces EPSP amplitude (Trimmer and Weeks, 1989), whereas postsynaptic activation of mAChRs on PPR activates an inward cationic current that lowers PPR's spike threshold (Trimmer and Weeks 1989, 1993; Trimmer, 1994). In addition to the muscarinic effects, EPSP amplitude is affected by three types of activity-dependent plasticity—facilitation, depression, and post-tetanic potentiation—which come into play under different patterns of sensory neuron activity (Weeks and Jacobs, 1987; Trimmer and Weeks, 1991). These phenomena appear to be homosynaptic and caused by alterations in neurotransmitter release from sensory neuron terminals. Two forms of plasticity at the synapses—the activation of presynaptic mAChRs and activity-dependent homosynaptic depression—decrease EPSP amplitude and were thus possible candidates for mediating PWR habituation.

Before examining EPSP amplitudes, we first confirmed that PPR's evoked activity decreased during habituation training. This was suggested by the finding that evoked activity in the proleg motor nerve decreased during habituation training (see above) but, in these whole nerve recordings, PPR's spikes could not be distinguished from those of other motoneurons. Accordingly, we repeated this experiment while recording intracellu-

larly from PPR and measuring its response directly. As shown in Fig. 4C, the number of spikes evoked in PPR decreased significantly over 20 trials delivered at 60-s intervals. Furthermore, in control preparations that received just two stimuli, one at the time of trial 1 and the second at the time of trial 20, the number of spikes evoked in PPR did not change significantly (Fig. 4C). Thus, response decrement resulted from repeated presentation of the stimulus.

Finally, using the same experimental and control protocols, we measured the amplitude of EPSPs evoked in PPR by the stimulated PH sensory neuron before and after training. PPR's membrane potential, spike threshold and input resistance, and the magnitude of paired-pulse facilitation of the EPSP were also measured. Contrary to our expectation, the amplitude of the unitary EPSP evoked in PPR by the PH sensory neuron used for training did not change detectably in either experimental or control preparations (Wiel *et al.*, 1995, and in preparation; Wiel, 1995). Therefore, it appears unlikely that either of the two known decrementing forms of plasticity contribute to PWR habituation. Of the other parameters measured, PPR's spike threshold became significantly more hyperpolarized and input resistance significantly increased in both the experimental and control groups (Wiel *et al.*, 1995 and in preparation; Wiel, 1995). Because these values changed in both groups, they cannot account for habituation; furthermore, both of the changes would tend to make PPR more excitable and therefore *less* likely to exhibit a response decrement.

These data suggest that PWR habituation is mediated neither by changes in synaptic transmission from PH sensory neurons to motoneurons nor by changes in the intrinsic electrical properties of the motoneurons. The former finding was surprising, given the range of plastic mechanisms known to be in place at these synapses (see above). The functional role of plasticity at these connections therefore remains elusive. These findings indicate that we must look elsewhere in the circuit for the changes that mediate habituation. One possibility is suggested by studies of habituation of the *Aplysia* and crayfish reflexes mentioned above. In both cases, homosynaptic depression of sensory synapses occurs but, in addition, inhibitory activity in interneurons increases during training and causes a reduction in motor output (Fischer and Carew, 1993; Krasne and Teshiba, 1995). Further experiments will be required to investigate whether inhibition contributes to habituation of the PWR in *Manduca*.

Conclusions

The PWR of *Manduca* has been useful for investigating how a neural circuit can be modified under different circumstances. The ecdysteroid-mediated regression of proleg motoneuron dendrites and the resultant decrease in sensory input provides a mechanism to explain the progressive weakening and loss of the reflex at pupation. Some of the neuromuscular components are "recycled" as members of the pupal circulatory system. In larvae, the reflex is modified by experience and, despite the known plasticity in synaptic transmission from PH sensory neurons to proleg motoneurons, the properties of these synapses appear to remain stable during habituation. Thus, the specific neural mechanisms that underlie habituation of the PWR elude us at present. On a larger scale, the PWR is only one of many behaviors that involve the prolegs, and the relationship between elements of the PWR circuit and the circuits that produce these other behaviors remains unexplored. The caterpillar abdomen and its "hands" should continue to provide insights into how the nervous system balances constancy with change when generating behavior.

Acknowledgments

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