Pavlovian Conditioning of *Hermissenda*: Current Cellular, Molecular, and Circuit Perspectives

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The less-complex central nervous system of many invertebrates make them attractive for not only the molecular analysis of the associative learning and memory, but also in determining how neural circuits are modified by learning to generate changes in behavior. The nudibranch mollusk *Hermissenda crassicornis* is a preparation that has contributed to an understanding of cellular and molecular mechanisms of Pavlovian conditioning. Identified neurons in the conditioned stimulus (CS) pathway have been studied in detail using biophysical, biochemical, and molecular techniques. These studies have resulted in the identification and characterization of specific membrane conductances contributing to enhanced excitability and synaptic facilitation in the CS pathway of conditioned animals. Second-messenger systems activated by the CS and US have been examined, and proteins that are regulated by one-trial and multi-trial Pavlovian conditioning have been identified in the CS pathway. The recent progress that has been made in the identification of the neural circuitry supporting the unconditioned response (UR) and conditioned response (CR) now provides for the opportunity to understand how Pavlovian conditioning is expressed in behavior.

The analysis of learning in several vertebrate and invertebrate nervous systems has generated a number of candidate mechanisms of Pavlovian conditioning involving changes in both cellular excitability and synaptic strength. In general, studies of associative learning in these model systems have been dominated by the search for mechanisms of Pavlovian conditioning that provide an explanation for temporal contiguity between the conditioned stimulus (CS) and unconditioned stimulus (US). However, a comprehensive analysis of associative learning requires an understanding of all aspects of the associative process, including the generation of behavior, in addition to providing insights into mechanisms of temporal contiguity. The issue of how learning is expressed in behavior may be effectively addressed by studying conditioning from a cellular and synaptic perspective in the relatively simple nervous system of invertebrates. The analyses of learning in several model systems have used a combination of cellular and synaptic physiology in conjunction with a neural circuit analysis to examine how Pavlovian conditioning is expressed in behavior, or how learning results in the generation of a conditioned response (CR). Some invertebrate preparations are especially attractive for this type of analysis, as the neural circuitry supporting behaviors involving muscular contraction, respiration, locomotion, and feeding is known in considerable detail (for review, see Sahley and Crow 1998).

One animal that has contributed to an understanding of the physiology of learning and memory at a cellular, synaptic, and systems level of analysis is the nudibranch mollusk *Hermissenda crassicornis*. Associative learning in *Hermissenda* has been extensively examined using a Pavlovian conditioning procedure. The *Hermissenda* central nervous system is relatively simple, consisting of identifiable neurons in the neural circuitry that supports conditioning. Identified neurons in the CS pathway have been studied in detail using biochemical, biophysical, and molecular techniques. The two sensory structures mediating the CS and US are central, and thus, their synaptic projections remain totally intact after surgical isolation of the nervous system. Mechanisms of CS-US contiguity have been identified and have been the focus of biophysical, biochemical, and molecular analyses. Moreover, because the neurons that contribute to the neural circuitry supporting the unconditioned responses (URs) and conditioned responses (CRs) have been recently identified, and can be studied in semi-intact nervous systems, an explanation of how conditioning is expressed in the generation of behavior is now feasible.

Pavlovian Conditioning

The same Pavlovian conditioning procedure in *Hermissenda* results in the acquisition of two different CRs. Pavlovian conditioning produces both light-elicted inhibition of normal positive phototaxis (Crow and Alkon 1978, 1980; Crow and Offenbach 1983; Crow 1985a) and CS-elicited foot-shortening (Lederhendler et al. 1986). The description of the conditioning paradigm and the two CRs are summarized in Figure 1. The conditioning procedure consists of pairing light, the CS with high-speed rotation, or orbital shaking, the US. Two URs are elicited by rotation, a reduced rate of forward locomotion and foot-shortening (Alkon 1974; Crow and Alkon 1978; Lederhendler et al. 1986; Matzel et al. 1990b). Conditioned inhibition of phototaxis is expressed by a light-dependent inhibition in the initiation of locomotion (Crow and Offenbach 1983) and a reduced rate of forward locomotion in light (Farley and Alkon 1982; Matzel et al. 1990b). The two CRs are proposed to develop independently (Matzel et al. 1990b), and the URs may involve different components of the neural circuit responsible for foot contraction and ciliary locomotion (Crow and Tian 2003a,b). Retention of conditioned behavior persists for several days to weeks, depending upon the number of conditioning trials used in initial acquisition (Crow and Alkon 1978; Alkon 1983; Harrigan and Alkon 1985). Conditioning of phototactic inhibition can be extinguished with the presentation of nonreinforced CSs (Richards et al. 1984). Conditioned inhibition of phototactic behavior also exhibits CS specificity, as conditioned animals exhibit suppressed locomotor behavior in the presence of the CS; however, their locomotor behavior in the dark is not significantly changed (Crow and Offenbach 1983).

The experimental conditions that produce Pavlovian conditioning have been investigated in some detail. Conditioning is dependent upon the temporal association of the CS and US in-
that conditioning in butions to conditioning are quite different, it has been proposed temporal characteristics of associative and nonassociative contri-
tion US within that context. More recently, it was reported that an excitatory context produced by presenting unsignaled USs (rotation) in a context of chemosensory stimuli blocked later conditioning (Farley et al. 1997). Studies have also shown that a chemosensory CS, when paired with rotation, suppresses bite-strike responses normally elicited by the chemosensory CS prior to conditioning (Farley et al. 1990a). Rogers and Matzel (1996) reported that an excitatory context produced by presenting unsignaled USs (rotation) in a context of chemosensory stimuli blocked later conditioned foot-shortening produced by a light CS paired with rotation US within that context. More recently, it was reported that explicitly unpaired presentations of the CS and US produced conditioned inhibition expressed by increased phototactic behavior (Britton and Farley 1999). Behavioral studies of conditioning in Hermissenda consisting of light (CS) paired with rotation (US) results in two conditioned responses; CS elicited foot shortening and CS-elicited inhibition of locomotion. (A) Foot length in the dark before presentation of the unconditional stimulus (US). (B) Footshortening the unconditioned response (UCR) elicited by rotation (US) of the animal in the dark. (C) Foot length measured in the dark following Pavlovian conditioning before presentation of the light (CS). (D) Conditioned response (CR), foot-shortening elicited by presentation of the CS. The area enclosed by the dashed lines in B indicates foot length before the presentation of the US. The area enclosed by the dashed lines in D represents foot length before the presentation of the CS. (A–D, artwork based on data from Lederhendler et al. [1986]). (E) Light elicited locomotion toward a light source assessed before conditioning. (F) Suppression or inhibition of light-elicited locomotion detected after Pavlovian conditioning. Random or pseudorandom presentations of the CS and US do not result in the development of either suppression of light-elicited locomotion or CS-elicited foot-shortening.

Figure 1  Pavlovian conditioning of Hermissenda consisting of light (CS) paired with rotation (US) results in two conditioned responses; CS elicited foot shortening and CS-elicited inhibition of locomotion. (A) Foot length in the dark before presentation of the unconditional stimulus (US). (B) Footshortening the unconditioned response (UCR) elicited by rotation (US) of the animal in the dark. (C) Foot length measured in the dark following Pavlovian conditioning before presentation of the light (CS). (D) Conditioned response (CR), foot-shortening elicited by presentation of the CS. (A–D, artwork based on data from Lederhendler et al. [1986]). (E) Light elicited locomotion toward a light source assessed before conditioning. (F) Suppression or inhibition of light-elicited locomotion detected after Pavlovian conditioning. Random or pseudorandom presentations of the CS and US do not result in the development of either suppression of light-elicited locomotion or CS-elicited foot-shortening.

volving both contiguity (Crow and Alkon 1978) and contin-
gency, the predictive relationship between the CS and the US (Farley 1987a,b). Extra CS and US presentations inserted into a sequence of CS–US pairings attenuates conditioning (Farley 1987a). However preconditioning exposure to the CS (latent inhibition) or US pre-exposure does not impair subsequent conditioning (Farley 1987a). Conditioned inhibition of phototaxis can be enhanced by compound conditioning in both overshadowing and blocking paradigms (Farley et al. 1997). Potentiation of phototactic suppression is produced by the addition of a chemosen-
sory stimulus, scallop extract, although second-order conditioning and sensory preconditioning have not been demonstrated (Farley et al. 1997). Studies have also shown that a chemosensory CS, when paired with rotation, suppresses bite-strike responses normally elicited by the chemosensory CS prior to conditioning (Farley et al. 1990a). Rogers and Matzel (1996) reported that an excitatory context produced by presenting unsignaled USs (rotation) in a context of chemosensory stimuli blocked later conditioned foot-shortening produced by a light CS paired with rotation US within that context. More recently, it was reported that explicitly unpaired presentations of the CS and US produced conditioned inhibition expressed by increased phototactic behavior (Britton and Farley 1999). Behavioral studies of conditioning in Hermissenda have also shown that sensitization is not an important contributor to conditioned inhibition of positive phototactic behavior. Nonassociative contributions to phototactic behavior are expressed in the initial trials of each conditioning session and decrement rapidly following the termination of multtrial conditioning sessions (Crow 1983). Because the magnitude and temporal characteristics of associative and nonassociative contributions to conditioning are quite different, it has been proposed that conditioning in Hermissenda is not an elaboration or potentiation of the mechanisms responsible for nonassociative learning (Crow 1983).

Conditioning in the two different behavioral response sys-
tems supporting the two CRs is sensitive to both CS-US contigu-
ity and forward interstimulus-interval manipulations (Matzel et al. 1990c). Moreover, both conditioned foot-shortening and conditioned inhibition of phototaxis involve the development or emergence of a new response to the CS, not the potentiation, through US presentations of an already existing response to the CS referred to as reflex potentiation (e.g., Schreurs 1989; Sahley and Crow 1998). In both of the CRs, there is a transfer of functional aspects of the response-evoking properties of the US to the CS (Crow and Alkon 1978; Lederhendler et al. 1986; Matzel et al. 1990b). This feature probably accounts for the increased complexity of the circuit supporting the CS and US, and the multiple sites of CS–US pathway convergence in the nervous system, and multiple synaptic interactions within the neural network support ing behavior. In addition to multiple-trial conditioning of suppression of light-elicited locomotion and foot-shortening, one-trial conditioning also inhibits light-elicited locomotion (Crow and Forrester 1986). Pairing the CS with the direct application of one of the proposed transmitters of the US pathway (5-HT, nominal US) to the exposed nervous system of otherwise intact Hermissenda produces suppression of light-elicited locomotion when the animals are tested 24 h following the one-conditioning trial. In addition, procedures for in vitro conditioning of the isolated nervous system have been developed. In vitro conditioning involves pairing the CS (light) with stimulation of the statocyst produced by mechanical perturbations (US). In vitro conditioning involving several conditioning trials produces similar cellular correlates in type B photoreceptors as found following in vivo procedures (Farley and Alkon 1987; Matzel et al. 1996; Gandhi and Matzel 2000).

Anatomy of the CS and US Pathways

The two sensory structures that are stimulated by the CS and US have been described in detail by Alkon and colleagues (Alkon and Fuortes 1972; Alkon 1973a,b; Alkon and Bak 1973; Detwiler and Alkon 1973). In addition, the convergence sites providing for synaptic interactions between the CS and US pathways have been identified (Alkon 1973a,b; Alkon et al. 1978; Akaike and Alkon 1980; Crow and Tian 2000, 2002a,b, 2003a, 2004).
Photoreceptors
Each eye of Hermissenda contains five photoreceptors, three classified as type B and two as type A. The general classification of photoreceptors can be identified further on the basis of their location within the eye. There are medial and lateral A and B photoreceptors and one central B photoreceptor. The synaptic connections between the type B photoreceptors and between type B and type A photoreceptors are in the neuropil of the cerebropleural ganglion and are mutually inhibitory (Alkon and Fuortes 1972; Alkon 1973a; Crow et al. 1979; Sefit et al. 1982; Frisztak and Crow 1993). Light produces a depolarizing generator potential and an increase in spike activity in both type A and B photoreceptors (Dennis 1967; Alkon and Fuortes 1972).

Hair Cells
The sensory structures stimulated by the US are the two central gravity detecting statocysts (Alkon and Bak 1973; Detwiler and Alkon 1973; Detwiler and Fuortes 1973; Alkon 1975). Each statocyst contains 13 hair cells, whose cell bodies are located around the perimeter of the statocyst. Hair cells that are located in opposite positions in the statocyst are mutually inhibitory (Detwiler and Alkon 1973). Statocyst hair cells contact calcium carbonate particles, referred to as statoconia, by interactions with motile cilia that project into the lumen of the statocyst from the apical region of the somas (Alkon 1975). Rotation or gravity causes the statoconia to press against the motile cilia of cells in front of the centrifugal or gravitational force vector, resulting in a depolarizing generator potential and an increase in spike activity (Alkon 1975). Hair cells in back of the centrifugal force vector hyperpolarize in response to rotation.

Optic Ganglion Cells
Second-order neurons in the visual system are located in the optic ganglion (Alkon 1973a; Tabata and Alkon 1982) and cerebropleural ganglion (Akaike and Alkon 1980; Crow and Tian 2000, 2002a). Type B photoreceptors, but not type A photoreceptors, inhibit ipsilateral optic ganglion cells. The 14 optic ganglion cells have been classified into multiple types referred to as C, D, E, and S (Alkon 1973a; Tabata and Alkon 1982). The type E optic ganglion cell is presumed to be electrically coupled to the S ganglion cell (S–E complex), and produces EPSPs in all ipsilateral type B photoreceptors, but not type A photoreceptors, and IPSPs in ipsilateral cephalic hair cells. All of the other cells within the same optic ganglion do not have synaptic interactions, however, type C optic ganglion cells inhibit contralateral type D optic ganglion cells. Alkon (1973a) has proposed that the synaptic interactions between C and D optic ganglion cells would enhance the contrast between the cells’ responses to illumination of each of the two eyes, which would signal the approach of a moving shadow or light.

The synaptic convergence between the CS and US pathways involving the optic ganglion is complex and a potential role in plasticity is poorly documented in conditioned animals. Type B photoreceptors and cephalic hair cells inhibit the S–E optic ganglion cell complex. The S–E cell produces positive feedback by exciting type B photoreceptors and cephalic hair cells, and inhibiting cephalic hair cells (Tabata and Alkon 1982). Therefore, the S–E cell complex can produce direct and indirect excitation of ipsilateral type B photoreceptors. The indirect excitation of B photoreceptors is the result of inhibition of cephalic hair cells that inhibit type B photoreceptors and excitation of cephalic hair cells that inhibit cephalic hair cells. Cephalic and cephalic hair cells are mutually inhibitory (Detwiler and Alkon 1973). On the basis of the interaction between hair cells, photoreceptors, and optic ganglion cells, it has been proposed that an increase in the frequency of S–E optic ganglion cell generated EPSPs detected in type B photoreceptors following light and rotation could contribute to the prolonged depolarization and increased input resistance of type B photoreceptors observed in conditioned animals (Tabata and Alkon 1982). The synaptic feedback to photoreceptors that is the result of the synaptic interactions between photoreceptors, hair cells, and optic ganglion cells could potentially contribute to the acquisition of conditioning correlates detected in type B photoreceptors. However, it is unlikely that the optic ganglion contributes to either the generation of the CR or the induction of intrinsic plasticity recently detected in other components of the CS pathway. Optic ganglion cells have not been reported to interact synaptically with neurons other than primary sensory neurons, for example, photoreceptors and hair cells. Moreover, there is no evidence that any of the synaptic connections between optic ganglion cells and sensory neurons exhibit plasticity with conditioning. In contrast, synaptic connections between sensory neurons and identified interneurons with synaptic projections to motor neurons have been well documented (Akaike and Alkon 1980; Goh and Alkon 1984; Crow and Tian 2000, 2002a, 2003a). In addition, synaptic facilitation of monosynaptic postsynaptic potentials (PSPs) in type Ic and Ie interneurons elicited by single type B spikes in conditioned animals does not involve any contribution from optic ganglion cells (Crow and Tian 2002b). Moreover, the facilitation of complex PSPs in type Ie interneurons of conditioned animals that contribute to the inhibition of ciliary locomotion is most likely the result of intrinsic changes at two CS–US convergence sites, the photoreceptors and type Ie interneurons (Crow and Tian 2002b, 2003b).

Interneurons in the UCR Pathway
Statocyst hair cells project to optic ganglion cells and three identified types of cerebropedal interneurons (Akaike and Alkon 1980; Tabata and Alkon 1982; Crow and Tian 2004). As shown in the diagram of Figure 2, statocyst hair cells form monosynaptic connections with type Ic and Ie interneurons. Type Ic and Ie interneurons project polysynaptically to type III, interneurons that inhibit VP1 and VP3 ciliary activating motor neurons (Crow and Tian 2003a). Rotation, the US depolarizes hair cells that produce excitation of type Ic interneurons, which results in excitation of type III inhibitory interneurons and a decrease in the spike activity of VP1 and VP3 ciliary motor neurons. An increase in the spike activity of type III interneurons results in inhibition of ciliary locomotion mediated by inhibition of VP1 and VP3 motor neurons. The second pathway shown in Figure 2 mediates contraction of the foot elicited by hair-cell stimulation. This pathway involves polysynaptic connections with interneurons that have not been identified. Activation of the proposed circuit involves depolarization of hair cells by rotation and excitation of type Ie interneurons through polysynaptic pathways. Excitation of type Ie interneurons directly excite pedal ventral contractile motor neurons (VCMNs), and posterior foot contraction motor neurons that collectively produce foot contraction (Crow and Tian 2004). In summary, rotation produces a depolarizing generator potential in identified statocyst hair cells, and by way of monosynaptic and polysynaptic connections with identified interneurons, the elicitation of foot contraction and inhibition of ciliary locomotion, the two UCRs.

Convergence of the CS and US Pathways
As summarized in Figure 3, one site of convergence between the CS and US is at the primary sensory neurons of the CS and US pathways. Synaptic projections from statocyst hair cells to the photoreceptors are both monosynaptic and polysynaptic (see Fig. 3). Hair cells and photoreceptors form reciprocal monosynaptic inhibitory connections (Alkon 1973b). Caudal hair cells inhibit photoreceptors and cephalic hair cells are inhibited by type B photoreceptors.
that pharmacological agents that affect 5-HT neurotransmission (imipramine, bufotenine, and 5,7-DHT) attenuate in vitro conditioning correlates in type B photoreceptors (Grover et al. 1989). In addition, 5-HT modulates generator potentials and membrane conductances in type B photoreceptors, modifications that have been identified as neural correlates of Pavlovian conditioning (Crow and Bridge 1985; Farley and Wu 1989; Crow and Forrester 1991; Acosta-Urquidi and Crow 1993; Rogers and Matzel 1995; Yamoah and Crow 1995, 1996). A computational model of the type B photoreceptor used to investigate the contribution of different ionic conductances modulated by 5-HT to the enhanced excitability produced by 5-HT suggested that changes in $I_h$, $I_{Ca}$, or $I_o$ (Yamoah et al. 1998) would produce excitability changes comparable to experimental findings (Cai et al. 2003). One-trial conditioning studies also have provided evidence for a role for 5-HT in conditioning. Light (CS) paired with 5-HT application to the exposed, but otherwise intact circumesophageal nervous system is sufficient to produce long-term phototactic suppression (Crow and Forrester 1986).

The synaptic organization of the secondary components of the visual pathway and graviceptive pathway of *Hermissenda* have now been characterized and described in considerable detail (Alkon et al. 1978; Crow et al. 1979; Akaike and Alkon 1980; Crow and Tian 2000, 2002a, 2003a, 2004). These studies have identified type Ie and Ii cerebroleural interneurons as an additional site of convergence between the CS and US pathways (see Fig. 3). Photoreceptors and statocyst hair cells form monosynaptic excitatory connections with type Ii interneurons and monosynaptic inhibitory connections with type Ie interneurons (Akaike and Alkon 1980; Crow and Tian 2000). The third site of convergence...
between the CS and US pathways is between statocyst hair cells and recently identified type Ib interneurons (Crow and Tian 2004). As summarized in Figure 3, statocyst hair cells form polysynaptic excitatory connections with type Ib interneurons, and photoreceptors exhibit variable and weak excitatory polysynaptic connections with type Ib interneurons.

In addition to the identification of the sites of synaptic convergence between the CS and US pathways, most of the components of the network generating ciliary locomotion have now been identified (Crow and Tian 2000, 2002a, 2003a, 2004). This provides for the opportunity to investigate how modifications in a neural circuit produced by Pavlovian conditioning are expressed in the generation of a CR. Progress toward this goal was supported by recent studies showing that light inhibits the activity of VP1 ciliary motor neurons after conditioning. In contrast, light produced excitation of ciliary motor neurons in pseudorandom controls (Crow and Tian 2003b).

The development of a semi-intact preparation has provided some insights into the physiology of the motor system mediating the foot-shortening UCR and CR (see Fig. 4). Less is known about the circuitry supporting the foot-shortening UCR and CR, although some of the motor neurons mediating anterior and posterior foot contraction have now been identified (see Fig. 2). Studies of semi-intact preparations have led to the identification of type Ib interneurons that project monosynaptically to VCMNs and posterior foot contraction motor neurons. Mechanical stimulation of statocyst hair cells evokes a depolarizing generator potential and an increase in spike activity of type Ib interneurons and VCMNs. Depolarization of type Ib interneurons with extrinsic current is sufficient to produce contraction of the anterior and posterior foot. Moreover, extrinsic current depolarization of identified hair cells elicits EPSPs and complex EPSPs in type Ib interneurons, and complex EPSPs and spikes in VCMNs. The development of a semi-intact preparation and the identification of components of the neural circuitry supporting foot contraction and ciliary locomotion provides the opportunity to study the acquisition and expression of two CRs within the same nervous system.

**Cellular and Synaptic Plasticity at Convergence Sites of the CS and US Pathways**

An essential step in the analysis of Pavlovian conditioning is the identification of loci in the animal’s nervous system, in which memories of the associative experience are stored. Crow and Alkon (1980) identified the primary sensory neurons (photoreceptors) of the pathway mediating the CS as one of site for memory storage. Studies of neural correlates of conditioning in the primary sensory neurons of the CS pathway have identified cellular changes involving both enhanced excitability that is intrinsic to identified type A and type B photoreceptors and synaptic facilitation of connections between identified photoreceptors (Crow and Alkon 1980; Alkon et al. 1982, 1985, 1992; Farley and Alkon 1982; West et al. 1982; Crow 1985b, 1988; Frysztak and Crow 1993, 1994, 1997; Gandhi and Matzel 2000). Cellular correlates of conditioning in type B photoreceptors are expressed by enhancement of CS-elicted generator potentials and increased spike frequency, increased excitability to extrinsic current, modification of light-dependent, Ca2+-dependent, and voltage-activated currents, and increases in the phosphorylation of several proteins (Crow and Alkon 1980; Neary et al. 1981 1986; Alkon et al. 1982, 1985, 1992; Farley and Alkon 1982; Alkon 1984; Crow 1985b, 1988; Goh et al. 1985; Alkon and Nelson 1990; Farley et al. 1990b; Matzel 1990a; Frysztak and Crow 1993, 1994, 1997; Muzzio et al. 2001). Studies of identified type A photoreceptors have reported a decrease in the amplitude of light-elicited generator potentials, enhanced excitability to extrinsic current, increases in CS-elicted spike activity, and decreases in the magnitude of two K+ currents (Farley et al. 1990b; Farley and Han 1997; Frysztak and Crow 1993, 1994, 1997). In addition to changes in primary sensory neurons, facilitation of monosynaptic and complex PSPs in identified type Ib and I interneurons has been recently shown in conditioned *Hermissenda* (Crow and Tian 2002b; see Fig. 5).

Enhanced excitability in identified photoreceptors of conditioned *Hermissenda* is expressed by a significant increase in spike activity elicited by the CS or extrinsic current, an increase in the input resistance, an alteration in the amplitude of light-elicited generator potentials, decreased spike frequency accommodation, and a reduction in the peak amplitude of voltage-dependent (I(A), I(Ca)) and Ca2+-dependent (I(Ca)) currents (Alkon et al. 1982, 1985; Collin et al. 1988; for reviews see Crow 1988; Alkon 1989; Sahley and Crow 1998). Modification of light-induced potassium currents in type B photoreceptors has also been proposed to contribute to correlates of conditioning (Blackwell 2002b). Enhanced excitability, expressed by an increase in both the amplitude of CS-elicted generator potentials and the number of action potentials elicited by the CS, may be a major contributor to changes in the duration and amplitude of CS-elicted complex postsynaptic potentials (PSPs) and enhanced CS-elicted spike activity observed in type I interneurons (Crow and Tian 2002b). However, facilitation of the monosynaptic IPSP between identified type B photoreceptors and type A photoreceptors may be due to both pre- and postsynaptic mechanisms (Frysztak and Crow 1994). In addition, facilitation of the amplitude of the monosynaptic IPSP between type B photoreceptors and type I interneurons and the monosynaptic EPSP between type B photoreceptors and type I interneurons of conditioned animals may also involve pre- and postsynaptic mechanisms (see Fig. 5).

*Figure 4* Semi-intact partial split foot preparation used to measure ciliary locomotion elicited by depolarization of VP1 and VP3 motor neurons and foot contraction evoked by depolarization of type Ib interneurons (red) and VCMNs (blue). Depolarization of type I interneurons (green) can inhibit VP1 ciliary activating motor neurons (brown) and ciliary locomotion.
Modifications in Components of the CS Pathway Contributing to Generation of the CR

An examination of CS-elicited changes in excitability and PSPs in the neural circuit generating ciliary locomotion has provided an explanation for the light-elicited suppression of the locomotor CR produced by Pavlovian conditioning. As summarized in the circuit diagram shown in Figure 5, studies of conditioned animals have shown that light inhibits the tonic spike activity of VP1 ciliary activating pedal motor neurons (Fig. 5, inset F at top) below their prelight baseline activity (Crow and Tian 2003b). In contrast, recordings from pseudorandom controls exhibited a significant increase in light-elicited tonic firing of VP1 neurons (Fig. 5, inset F at bottom). An analysis of changes in other components of the CS pathway of conditioned animals revealed that type Ie interneurons exhibited an intrinsic enhanced excitability with conditioning in contrast to pseudorandom controls (Fig. 5, inset E). Therefore, a combination of synaptic facilitation and intrinsic enhanced cellular excitability can account for light-elicited inhibition of locomotion. Facilitation of the synaptic connection between type B-photoreceptors and type Ie (Fig. 5, inset C) interneurons in conjunction with intrinsic enhanced excitability in type B-photoreceptors (Fig. 5, insets A and B) and type Ie interneurons of the CS pathway would result in an increase in spike activity of type IIIi inhibitory interneurons and inhibition of VP1 and VP3 ciliary motor neurons of conditioned animals (see Fig. 5, inset F). The changes in terminal branch morphology were detected within an hour after in vitro conditioning. Interestingly, in double-labeling experiments of the B photoreceptors and hair cells, terminal changes were characterized by a reduction of dendritic boundary volumes enclosing labeled medial-type-B photoreceptor arborizations (Kawai et al. 2002). The changes in terminal branch morphology were detected within an hour after in vitro conditioning. Using confocal microscopy, it was shown that five conditioning trials produced a contraction of terminal branches of fluorescently labeled type B photoreceptors along a contralateral axis as compared with unpaired controls (Kawai et al. 2002). The changes in terminal branch morphology were detected within an hour after in vitro conditioning.

Morphological Modifications in the CS Pathway

Ultrastructural and electrophysiological analyses have shown that synaptic interactions between photoreceptors, other sensory neurons, and interneurons is in the neuropil of the cerebropleural ganglion (Crow et al. 1979). Recent studies of labeled photoreceptors have focused on changes in the morphology of secondary and terminal photoreceptor processes in the neuropil. Structural changes characterized by a reduction of dendritic boundary volumes enclosing labeled medial-type-B photoreceptor arborizations were observed in conditioned animals as compared with unpaired controls (Alkon et al. 1990). The structural changes in type B photoreceptors associated with conditioning have been examined further using an in vitro conditioning procedure. Using confocal microscopy, it was shown that five conditioning trials produced a contraction of terminal branches of fluorescently labeled type B photoreceptors along a contralateral axis as compared with unpaired controls (Kawai et al. 2002). The changes in terminal branch morphology were detected within an hour after in vitro conditioning. Interestingly, in double-labeling experiments of the B photoreceptors and hair cells, terminal con-
traction was not observed at the synaptic connection between the hair cell and photoreceptor (Kawai et al. 2002). The structural remodeling of the photoreceptor terminal branches following in vitro conditioning can be blocked with anisomycin pretreatment (Kawai et al. 2003). The further analysis of this type of structural remodeling is of interest, as Pavlovian conditioning produces synaptic facilitation of the monosynaptic connection between type B photoreceptors and type A photoreceptors (Frysztak and Crow 1994; Gandhi and Matzel 2000) and between type B photoreceptors and type I, Ii interneurons (Crow and Tian 2002b). In addition to changes in dendritic volume, changes in the morphology of photoreceptor somas have also been reported to occur following activation of PKC, a signaling molecule implicated in learning (Lederhendler et al. 1990). Phorbol-induced changes involved outgrowth from the cell surface similar to blebs or ruffling that altered the soma volume. The functional significance of the morphological changes in both dendritic and soma volume has not been established.

Second-Messenger Systems

Studies of the signal transduction pathways responsible for the modification of diverse K+ currents of type B photoreceptors of conditioned animals have identified several second messenger systems. Both protein kinase C (PKC; Farley and Auerbach 1986; Neary et al. 1986; Matzel et al. 1990a; Crow et al. 1991; Farley and Schuman 1991) and extracellular signal-regulated protein kinase (ERK; Crow et al. 1998) have been reported to contribute to modifications of excitability and synaptic efficacy of conditioned Hermissenda. Light and rotation have spatially separated physiological consequences on type B photoreceptors. However, both the CS and US increase cytosolic Ca2+ levels (Sakakibara et al. 1993; Blackwell 2000, 2002a; Muzzio et al. 2001). Both GABA and 5-HT have been proposed to mediate the effects of activation of the US pathway during conditioning (see section on anatomy of the US pathway during conditioning (see section on anatomy of the US and CS pathways). Light, the CS, activates phospholipase C (PLC) to produce inositol trisphosphate (IP3) and diacylglycerol (DAG) (Sakakibara et al. 1986, 1994). Inositol trisphosphate opens rhabdomeric Na+ and Ca2+ channels, which result in a depolarizing generator potential and Ca2+ influx (Blackwell 2000). Two distinct Ca2+ currents have been identified in the soma of photoreceptors (Yamoah and Crow 1994). Inositol trisphosphate can also bind to its receptor (IP3R), which triggers Ca2+ release from the smooth endoplasmic reticulum (Blackwell and Alkon 1999). The Ca2+ influx from the rhabdomere and the IP3R-gated storage compartment can cause Ca2+ release from the ryanodine receptor-gated (RyR) compartment (Blackwell and Alkon 1999).

Rotation, the US, produces a depolarizing generator potential in identified statocyst hair cells and elicits a monosynaptic GABAergic IPSP in the photoreceptors (Alkon et al. 1993; Sakakibara et al. 1993; Rogers et al. 1994; Blackwell 2002a). The US is also proposed to activate a serotonergic polysynaptic pathway that projects to type B photoreceptors (Land and Crow 1985; Crow and Forrester 1986, 1991). The primary focus of 5-HT release has been on the modulation of membrane conductances (e.g., Farley and Wu 1989; Acosta-Urquidi and Crow 1993; Yamoah and Crow 1996). In addition, the induction of 5-HT-dependent enhanced excitability of type B photoreceptors is Ca2+-dependent (Falk-Vairant and Crow 1992). However, the precise role of 5-HT in the activation of second-messenger systems is poorly understood. It has been proposed that GABAergic IPSPs in photoreceptors activate phospholipase A2 (PLA2) to liberate arachidonic acid (AA; Muzzio et al. 2001) and create a back-propagating wave of Ca2+ released from intracellular stores (Ito et al. 1994; Blackwell 2002a). When the CS and US are repeatedly paired, the Ca2+ influx, due to light IP3R stores, RyR stores, and voltage-gated Ca2+ channels sums together (Blackwell and Alkon 1999). The large increase in cytosolic Ca2+ combined with DAG and AA act to synergistically activate PKC by translocation of PKC to the membrane (Lester et al. 1991). Each pairing of the CS and US has been proposed to incrementally increase the proportion of PKC translocated to the membrane (Muzzio et al. 1997). Both 5-HT (Rogers and Matzel 1995; Yamoah and Crow 1996) and GABA (Yamoah and Crow 1996) are linked to a per mensus-toxin sensitive G-protein. These proteins can activate multiple second messenger systems, several of which are involved in one-trial and/or multitraining Pavlovian conditioning.

Activation of PKC is necessary for the induction of cellular plasticity in Hermissenda (Crow et al. 1991; Crow and Forrester 1993a,b). Down-regulation of PKC and pretreatment with kinase inhibitors block the induction of short-term excitability, but not long-term excitability (Crow and Forrester 1993b). This indicates that short- and long-term memory in this system may involve parallel processes. PKC may phosphorylate two K+ currents, Ik,A and Ik,Ca, decreasing their maximum conductance and producing increased input resistance and evoked spike frequency (Farley and Auerbach 1986; Frysztak and Crow 1997). Conditioning also induces the activation of ERK (Crow et al. 1998). Serotonin activates ERK through a Ca2+-dependent PKC pathway and a PKC-independent pathway (Crow et al. 2001).

Proteins Regulated by Pavlovian Conditioning

The regulation of several proteins has been examined following conditioning. Calexcitin (CE) is a GTP- and Ca2+-binding protein found in Hermissenda photoreceptors (Neary et al. 1991; Alkon et al. 1998; Kuzirian et al. 2001). CE is activated by Ca2+ influx, can decrease K+ currents, and may bind to the RyR to increase cytosolic Ca2+ concentrations (Nelson et al. 1996, 1999; Ascoli et al. 1997). CE is proposed to be phosphorylated by PKC, which produces translocation to the membrane. Phosphorylation of CE also causes it to bind to the Ca2+-ATPase transporter to increase the rate of Ca2+ removal from the cytosol (Alkon et al. 1998). Behavioral conditioning has been reported to increase CE in B photoreceptors, specifically in Ca2+ sequestering organelles such as endoplasmic reticulum and within mitochondria and photopigments (Kuzirian et al. 2001). It has been proposed that increased CE levels in B photoreceptors of conditioned animals causes increased excitability via K+-channel inactivation and internal Ca2+ release from ER due to increased CE binding to ryanodine receptors.

One-trial conditioning regulates proteins found in the CS pathway (Crow et al. 1996, 1997, 1999; Crow and Siddiqi 1997). A protein whose phosphorylation is regulated by Pavlovian conditioning is cytoskeleton-related protein 24 (Csp24), a member of the family of β-thymosin repeat proteins (Crow and Xue-Bian 2000, 2002; Crow et al. 2003). Csp24 is phosphorylated with Csp24, and is colocalized with Csp24 in the cytosol of B-photoreceptor cell bodies (Crow and Xue-Bian 2002). Csp24 is phosphorylated by procedures that produce enhanced intermediate-term and long-term excitability, but not after procedures that result in short-term excitability of photoreceptors (Crow and Xue-Bian 2000). Incubation of isolated Hermissenda nervous systems with Csp antisense oligonucleotides decrease Csp24 expression. Treatment with antisense oligonucleotides before one-trial conditioning blocked intermediate-term enhanced excitability, without affecting the induction of short-term immediate enhanced excitability (Crow et al. 2003). Because Csp24 is associated with the actin cytoskeleton, its regulation by conditioning may influence K+ channel activity by the spatial and temporal control of actin dynamics.

Conclusions and Discussion

The progress in determining how Pavlovian conditioning is expressed in the generation of behavior in Hermissenda is encour-
aging, and is supported by recent work involving the identification of the neural circuit-controlling locomotion and its modulation by light (CS) and rotation (US). The analysis of Pavlovian conditioning in the neural circuit generating ciliary locomotion showed that both enhanced cellular excitability and synaptic facilitation are expressed in identified circuit components at different loci within the network. The distributed nature of cellular and synaptic plasticity associated with this example of Pavlovian conditioning suggests that an adequate explanation of conditioned behavior requires both an analysis of neural circuits and the identification of mechanisms of CS-US contiguity at convergence sites between the CS and US pathways. Consistent with the view that learning may initially involve changes in pre-existing synaptic connections, conditioned inhibition of phototactic behavior involves modifications of existing synaptic connections between photoreceptors and identified type I interneurons. However, the formation of new connections between neurons in the neural circuit modulating locomotor behavior cannot be dismissed. The acquisition of the foot-shortening CR may involve the establishment of new synaptic connections between photoreceptors, interneurons, and type Ib neurons, involving poly-synaptic pathways. Furthermore, synaptic connections are proposed to operate at this level of the circuit, generating foot-shortening because of the weak and variable influences of light, the CS, on the synaptic activity of type Ib interneurons observed prior to conditioning. Light does not excite VCMNs or posterior foot contraction motor neurons before conditioning, which is consistent with behavioral evidence showing that the CS does not elicit foot-shortening before conditioning.

The analysis of conditioning correlates has revealed that the first site of intrinsic cellular and synaptic plasticity is at the initial site of convergence between the CS and US pathways, the primary sensory neurons of the CS pathway. The mechanisms of temporal contiguity between the CS and US involve both enhanced cellular excitability and enhanced synaptic strength. The changes in excitability produced by conditioning involve reductions in several well-characterized K+ conductances in type B photoreceptors. Moreover, recent modeling studies have indicated that reductions in I_h and I_{K,CS} or an increase in I_K would result in enhanced excitability similar to what is detected experimentally in voltage-clamp and current-clamp studies. The second site of intrinsic enhanced excitability is the type I interneurons of the CS pathway. However, membrane conductances underlying enhanced excitability of type I interneurons have not yet been identified. Taken collectively, the evidence shows that acquisition of Pavlovian conditioning results in the activation of several second-messenger systems. Both one-trial and mult trivial Pavlovian conditioning of Hermissenda involves PKC and the ERK-signaling pathway (ERK). Conditioning is sufficient to activate PKC and ERK, and inhibition of their phosphorylation and activation can block the induction of plasticity.

As in other learning systems, protein synthesis is required to form long-term memory following one-trial and mult trivial conditioning. In addition, an intermediate phase of memory has been identified that is dependent on protein synthesis, but not RNA synthesis. Interestingly, the induction of short-term memory can be blocked without blocking the expression of long-term memory, suggesting that memory may involve parallel processing. Several proteins that are regulated by Pavlovian conditioning have now been identified. CE and Csp2 are two of the proteins that have been examined in some detail. CE is a Ca2+- and GTP-binding protein proposed to enhance excitability via K+-channel inactivation and Ca2+ release from internal stores (ER). Csp24 is a cytoskeletal-related protein whose expression and phosphorylation are required for persistent enhanced excitability. The contribution of Csp24 to synaptic and cellular structural remodeling may be through regulation of the actin cytoskeleton. Excitability could be influenced by alterations in channel density or channel conductances modulated by modification of actin filament dynamics. The cellular and synaptic changes identified following conditioning are distributed at several loci within the network and, therefore, not localized to a single synaptic site or neuron. The distributed nature of learning-dependent changes may account for the complexity of Pavlovian conditioning in Hermissenda, specifically, the emergence of a new response to the CS following conditioning.

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