

Synaptic Plasticity *in vitro*: Cell Culture of Identified *Aplysia* Neurons Mediating Short-Term Habituation and Sensitization

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The gill withdrawal reflex of the marine mollusk, *Aplysia californica*, shows habituation and sensitization, two simple forms of learning. In order to extend the cellular studies on synaptic plasticity underlying the changes in the reflex behavior, and to explore further the development of synaptic plasticity during synapse formation, we have sought to establish the neural circuit of the gill withdrawal reflex *in vitro*. We report here the reconstruction of the elementary gill withdrawal circuit in cell culture and find that the cells show short-term homosynaptic depression and heterosynaptic facilitation, the cellular mechanisms of habituation and sensitization, respectively.

The gill withdrawal reflex of the marine snail, *Aplysia californica*, is a simple defensive reflex mediated by a population of about 24 sensory cells, six motor cells, and several interneurons (Kandel, 1979). The reflex undergoes two forms of nonassociative learning, habituation and sensitization, as well as classical conditioning (Carew et al., 1983; Kandel and Schwartz, 1982). The elementary cellular substrate underlying these forms of learning consists of sensory neurons that monosynaptically connect with a motor cell and a facilitatory neuron that synapses onto the presynaptic terminals of the sensory neurons (Bailey et al., 1979, 1981). Analysis of these forms of learning at the cellular level indicates that stimulus experience modulates transmitter release from the terminals of the sensory neurons (Hawkins et al., 1983; Klein et al., 1980). Habituation of the reflex is mediated by homosynaptic depression, a decrease in transmitter release from the sensory neuron presynaptic terminals with repeated stimulation (Castellucci and Kandel, 1974). Sensitization is based on heterosynaptic (presynaptic) facilitation, an increase in transmitter release from sensory neurons due in part to a reduction in a K^+ current, and a consequent increased Ca^{2+} influx (Castellucci and Kandel, 1976; Klein and Kandel, 1978; Klein et al., 1982). Sensitization in the intact nervous system is mediated either by activity in a number of facilitator neurons, some of which are identified, or by the exogenous facilitatory neurotransmitters, namely, serotonin (5-HT) or the small mol-

luscan cardiac peptides (Abrams et al., 1984; Brunelli et al., 1976; Hawkins et al., 1981b).

To gain direct access to the synapses mediating the learning exhibited by this reflex, and to enable us to explore the developmental processes involved in the acquisition of plastic properties by newly formed synapses, we have sought to establish the elementary gill withdrawal circuit *in vitro*. Here we describe the cell culture of dissociated sensory and motor neurons that mediate the reflex and the culture of a modulatory cell, the serotonergic metacerebral cell (MCC), which simulates the *in vivo* facilitating pathway. The MCC was used as a facilitator cell for two reasons. First, it can be identified and easily isolated from the cerebral ganglion (Schacher, 1985). Second, the MCC produces a 5-HT-mediated modulation of muscle activity via an increase in cAMP (Weiss et al., 1978, 1979) that is comparable to facilitation at the LE-L7 connections (Kandel and Schwartz, 1982). The three cells form the characteristic synaptic connections after 5 d in culture, and show homosynaptic depression and heterosynaptic facilitation. Bath application of 5-HT also produces facilitation of the sensorimotor connection.

Materials and Methods

Cell culture preparations

The isolation and maintenance of *Aplysia* neurons in cell culture have been described (Schacher, 1985; Schacher and Proshansky, 1983). Abdominal and cerebral ganglia from 1-3 gm animals raised in the laboratory (Kriegstein, 1977; Kriegstein et al., 1974) were bathed in proteolytic enzyme for 2 hr, and adult abdominal ganglia excised from 100-150 gm animals (Sea Life Supply, Sand City, CA) were exposed to the same proteolytic digest for 2.5 hr. The ganglia were pinned in Sylgard- (Dow Corning, Midland, MI) coated dishes and desheathed. The gill motor cell L7 was isolated from the juvenile abdominal ganglion, the MCC was isolated from the juvenile cerebral ganglion, and the LE mechanosensory cells were isolated from the adult abdominal ganglion. Cells were identified by size, position, and pigmentation, and axon distribution during removal from the ganglion. The isolated cells were transferred to polylysine-coated culture dishes with medium containing *Aplysia* hemolymph and L15 (Flow Laboratories, McLean, VA), with salts added to adjust the medium to marine saline conditions. The cultures were maintained for 5 d at 18°C with medium changes on alternate days.

Electrophysiology

Using standard electrophysiological technique, sensory, motor, and metacerebral cells were impaled simultaneously with single-barreled electrodes filled with 2.5 M KCl. Motor cells were hyperpolarized 50 mV below their resting potential by intracellular current injection, in order to block spontaneous and stimulus-evoked activity. Sensory neurons were then stimulated with short depolarizing pulses every 20 sec. After five stimuli, either the MCC was fired at 3-5 Hz for 15 sec, using a long

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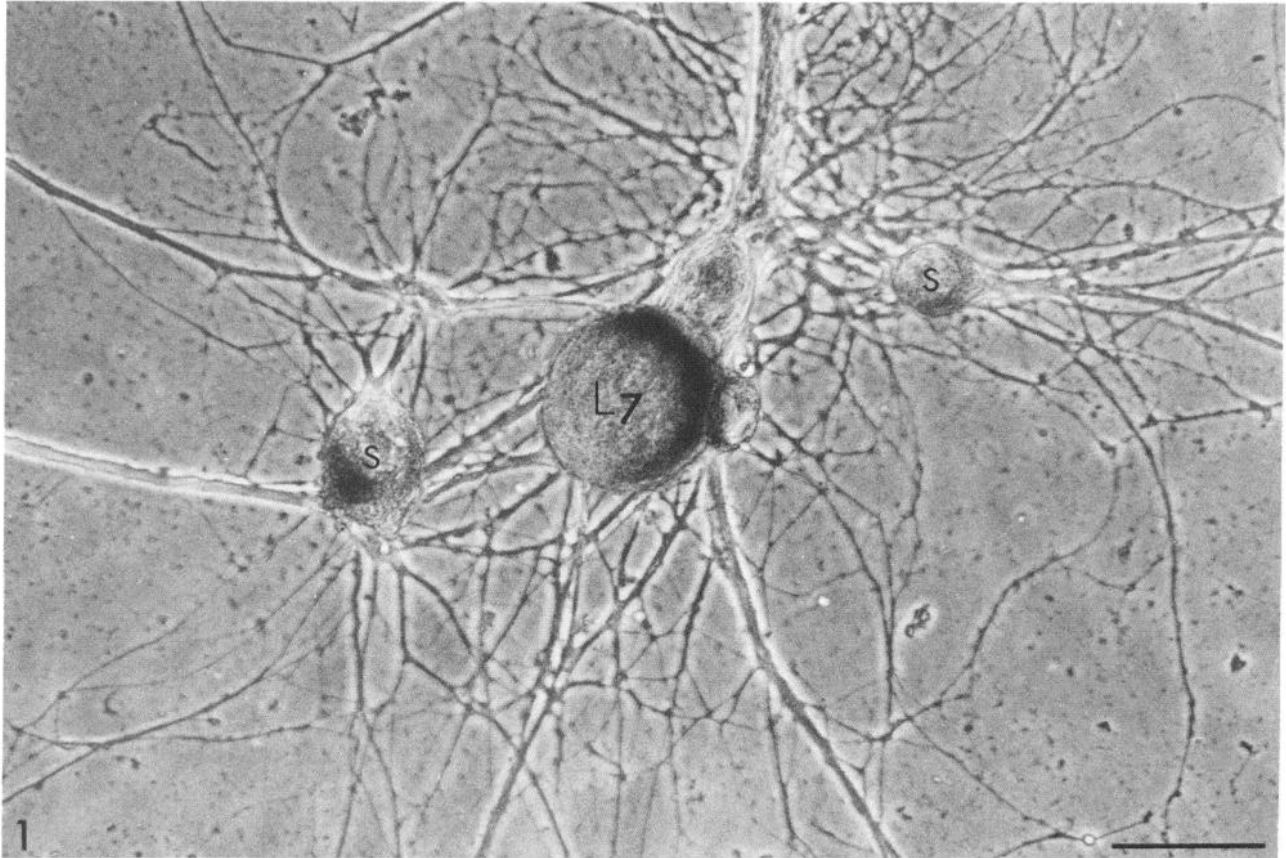


Figure 1. Neurons mediating gill withdrawal *in vitro*. The circuit consists of two LE sensory neurons and one gill motor cell L7 after 5 d in culture. S = LE sensory cell; L7 = motor cell. Calibration, 100 μm .

depolarizing step, or 5-HT (Sigma, St. Louis, MO) was added to the bath to a final concentration of 2×10^{-6} M. The five subsequent stimuli were recorded to measure facilitation. The cultures were perfused continuously with buffered artificial seawater (pH 7.6) throughout the recordings, except during the addition of 5-HT. In some cultures, seawater containing 50 mM tetraethylammonium (TEA) was used to enhance measurements of widths of action potentials evoked in sensory neurons before and after MCC stimulation or 5-HT application.

Results

Elementary gill withdrawal circuit shows homosynaptic depression and 5-HT-induced facilitation

The elementary circuit established in cell culture consisted of one or more LE sensory cells, the major gill motor cell L7, and, in some cultures, the serotonergic MCC, which we used as a facilitator neuron. After 5 d in culture, the cells had formed an extensive neuritic network (Figs. 1 and 4A), and in 80% of the cultures (11 of 14), there were robust connections between LE and L7, with an average postsynaptic potential (PSP) amplitude of 8.0 ± 2.5 mV ($n = 11$), latency of 3–5 msec, and a configuration characteristic of the *in vivo* connection. The LE–L7 connection was unidirectional, and there was no detectable electrical coupling between the cells.

As in studies in both the intact animal and the isolated nervous system, a single spike elicited in an LE sensory cell resulted in an excitatory PSP in L7 (Figs. 2A₁ and 4B₁). Repeated stimuli every 20 sec—a rate that produces habituation in the intact animal—led to a gradual decrease in the PSP to 30% of the initial value, paralleling homosynaptic depression *in vivo* (Figs. 2 and 4B). If allowed to rest for 10 min, the strength of the

connection returned to 80% of its initial level (not shown). Rapid facilitation occurred when, after the fifth stimulus, 5-HT was added to the bath to a final concentration of 2×10^{-6} M. As in *in vivo*, 5-HT produced a small (1–5 mV) decreased conductance depolarization in the sensory neurons [see also Belardetti et al. (1985) on 5-HT effects on sensory neurons *in vitro*] and slow depolarization (2–6 mV) in L7 (Fig. 2A₁). By the seventh stimulus, the PSP size increased by nearly 300% (Fig. 2B). To rule out the effects of depolarization alone in enhancing transmitter release, current was injected into sensory neurons to produce a 1–5 mV depolarization. This had little or no effect on PSP amplitude (data not shown). Serotonin also produced a change in the time course of the PSP, an increase in rise time, and slower decay (compare stimuli 1 and 5 with stimulus 7 in Fig. 2A₂). These 5-HT effects are similar to those observed *in vivo* (Klein et al., 1980, Figs. 25 and 26) and are consistent with the 5-HT-induced effects on the shape of the sensory neuron action potential. *In vitro* 5-HT application led to spike broadening of 10% in normal seawater (Fig. 3, $n = 3$) and 50% in seawater containing 50 mM TEA ($n = 3$). Such changes in the action potential duration are capable of producing significant increases in transmitter release from the sensory neurons both *in vivo* and *in vitro* (Hochner et al., 1985; Klein et al., 1980).

Three-cell gill withdrawal circuit in vitro shows homosynaptic depression and heterosynaptic facilitation

To further compare the culture system to the *in vivo* circuit, we cultured sensory and motor cells with the serotonergic MCC ($n = 5$) and examined the effects of MCC spike activity on LE–L7 connections (Fig. 4). Repeated LE cell action potentials every

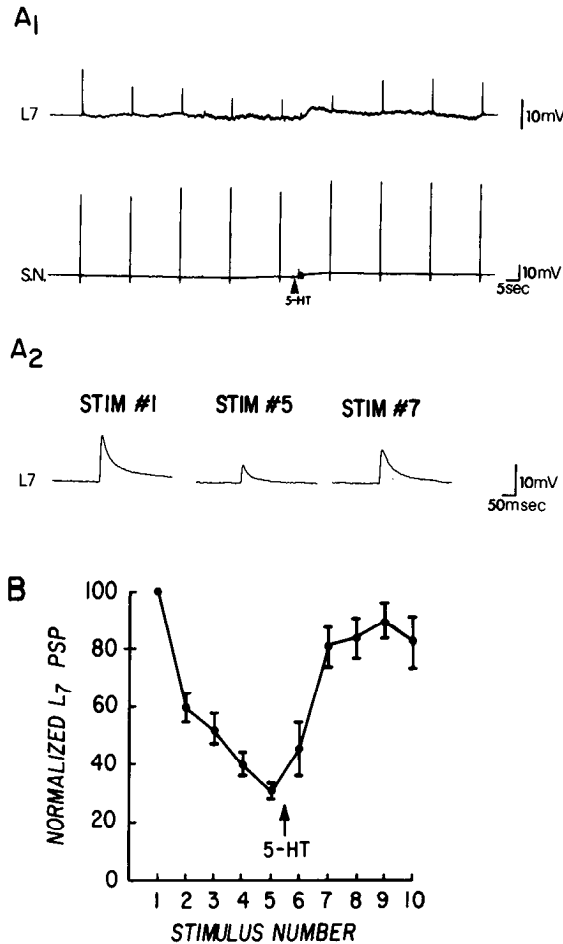


Figure 2. Sensorimotor circuit shows homosynaptic depression and 5-HT-induced facilitation. *A*₁, In a slow sweep record, sensory and motor cells were simultaneously impaled with single-barreled electrodes. The motor cell (L7) was hyperpolarized 50 mV below rest. The sensory neuron was fired with a brief depolarizing pulse every 20 sec (*lower trace*). By the fifth stimulus, the PSP amplitude in L7 (*upper trace*) was depressed. Between the fourth and fifth stimuli, the perfusion was halted, which occasionally produced a small (0.5–1 mV) depolarization. After the fifth stimulus, 5-HT was applied (*arrowhead*) to a final concentration of 2×10^{-6} M. Within a few seconds, both the sensory neuron and motor cell showed a small, slow depolarization. The initial depolarization in the motor cell typically was followed by a small repolarization, which then plateaued and remained at that level for the duration of the 5-HT exposure. By the seventh stimulus, the PSP amplitude was increased about 3-fold from the depressed level. *A*₂, Fast sweep record of the EPSP in L7 for the first, fifth, and seventh stimuli from the series shown in *A*₁. *B*, Summary of homosynaptic depression and 5-HT facilitation from five experiments. The LE–L7 PSPs were normalized relative to the initial PSP in each experiment. Points are means and SEM.

20 sec evoked PSPs in L7 and produced synaptic depression (Fig. 4*B*) with kinetics similar to that observed for the two-cell circuit (Fig. 2*B*). Interposing repeated MCC spikes at 3–5 Hz for 15 sec between the fifth and sixth stimuli produced a 200% enhancement of the PSP (Fig. 4*B*), replicating heterosynaptic facilitation *in vivo*. MCC stimulation also produced a small depolarization (1–2 mV) in the sensory and motor cells, the typical responses observed with exogenous 5-HT application (see Fig. 2*A*) and facilitator neuron activity in the intact nervous system (Hawkins et al., 1981a).

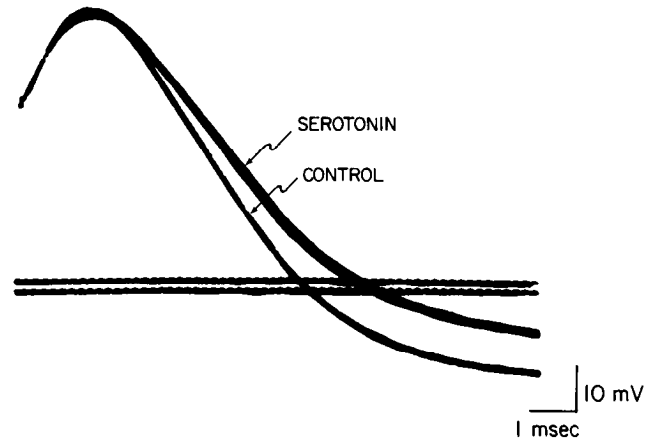


Figure 3. Spike broadening with 5-HT application in normal seawater. 5-HT, 2×10^{-6} M, led to a notable increase in spike width as well as a small depolarization from baseline.

Discussion

For the first time, a neural circuit with a known behavioral role has been established in cell culture. Although modulation of the gill withdrawal reflex in the intact animal involves other interneurons (Hawkins et al., 1981a), an elementary three-cell circuit consisting of a sensory neuron, a motor neuron, and a modulatory neuron, is both necessary and sufficient to show short-term homosynaptic depression and heterosynaptic facilitation, the cellular mechanisms of habituation and sensitization. These plastic properties, especially homosynaptic depression, are specific to the LE–L7 circuit. Other *Aplysia* cells that form chemical connections *in vitro* show stable PSP amplitudes, with stimulation rates that depress the LE–L7 connection (Camardo et al., 1983; Schacher et al., 1985). Heterosynaptic facilitation of LE–L7 connections can also be produced by other cells. Preliminary experiments with cultures in which the serotonergic MCC was replaced with the identified abdominal ganglion facilitator L29 (Hawkins et al., 1981b) also showed heterosynaptic facilitation (Hawkins and Schacher, unpublished observations). Further, the circuit may be capable of long-term facilitation. Synaptic transmission between LE and L7 can be enhanced in cultures with spontaneously active MCCs or following repeated applications of exogenous 5-HT (Montarolo et al., 1985; Rayport and Schacher, 1984). The elementary circuit may, in future work, be explored for the capacity to show activity-dependent facilitation, the cellular mechanism mediating classical conditioning of this reflex (Hawkins et al., 1983). For studying both nonassociative and associative mechanisms, the exposed processes and visualizable terminals of the circuit *in vitro* offer the possibility of examining subcellular mechanisms of synaptic plasticity in the previously inaccessible synaptic region itself (Belardetti et al., 1985).

Although the MCC does not interact with abdominal ganglion cells *in vivo*, activity in the MCC evokes small 5-HT-like depolarizing responses of long latency and duration in both the sensory and motor cells and facilitates sensorimotor connections. We do not know as yet whether these MCC effects are the result of the formation of synapses by MCC with sensory or motor cells or are caused by an activity-dependent release of 5-HT that diffuses to neighboring sensory and motor structures. The release of transmitter from neurons grown in culture, in the absence of target cells, has been recently demonstrated for spinal cord and ciliary ganglion cells (Hume et al., 1983; Young and Poo, 1983) and individual neuropeptidergic buccal cells of *Aplysia* (Weiss et al., 1985). Thus, local transmitter release from

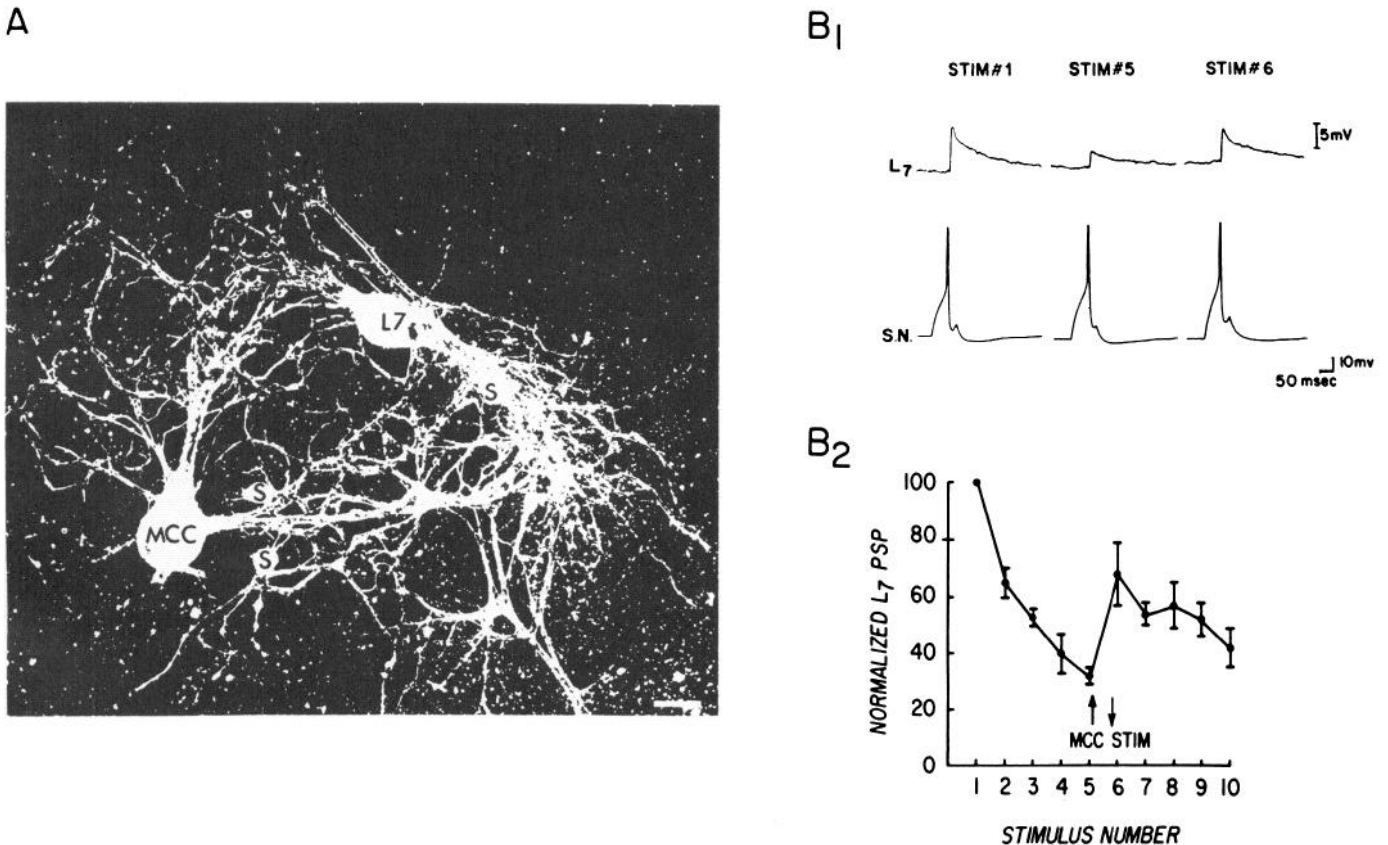


Figure 4. *A*, Elementary three-cell gill withdrawal neural circuit in dissociated cell culture after 5 d. *S*, LE sensory cell; *L7*, motor cell; *MCC*, metacerebral cell. Calibration, 100 μ m. *B*, Electrophysiology of the three-cell circuit. Sensory, motor, and metacerebral cells were simultaneously impaled with single-barreled electrodes. The motor cell was hyperpolarized 50 mV below rest. The sensory neuron was then fired with a brief depolarizing current pulse once every 20 sec. Between the fifth and sixth stimuli, the MCC was fired with a long depolarizing current step, causing it to fire three to five spikes/sec for 15 sec. The records in *B*, show, first, the initial PSP, then the fifth PSP, displaying the magnitude of homosynaptic depression, followed by the PSP after MCC firing, showing maximum facilitation. The graph in *B*₂ summarizes the results of five experiments, normalized by adjusting the initial PSP of each LE-L7 connection to unity. Points are means and SEM.

the MCC may act in a manner equivalent to the exogenous application of 5-HT.

During *Aplysia* development, homosynaptic depression is well established before the emergence of heterosynaptic facilitation (Rayport and Camardo, 1984). In culture, cells reestablish connections with mature plastic properties within 5 d. Thus, sequential recordings during the first few days may permit examination of synapse formation, as well as the acquisition of synaptic plasticities mediating habituation and sensitization. Such explorations of the developing gill withdrawal circuit in culture may begin to indicate whether developmental processes underlying synapse formation are carried over to the adult in mediating synaptic plasticity and learning.

References

- Abrams, T. A., V. F. Castellucci, J. S. Camardo, E. R. Kandel, and P. E. Lloyd (1984) Two endogenous neuropeptides modulate the gill and siphon withdrawal reflex in *Aplysia* by means of presynaptic facilitation involving cyclic AMP-dependent closure of a serotonin-sensitive K channel. *Proc. Natl. Acad. Sci. USA* 81: 7956-7960.
- Bailey, C. H., E. B. Thompson, V. F. Castellucci, and E. R. Kandel (1979) Ultrastructure of the synapses of sensory neurons that mediate the gill-withdrawal reflex in *Aplysia*. *J. Neurocytol.* 8: 415-444.
- Bailey, C. H., R. D. Hawkins, M. C. Chen, and E. R. Kandel (1981) Interneurons involved in mediation and modulation of gill-withdrawal reflex in *Aplysia*. IV. Morphological basis of presynaptic facilitation. *J. Neurophysiol.* 45: 340-360.
- Belardetti, F., S. Schacher, E. R. Kandel, and S. A. Siegelbaum (1985) Serotonin produces a decreased conductance EPSP and broadening of the action potential in growth cones of *Aplysia* neurons. *Soc. Neurosci. Abstr.*
- Brunelli, M., V. F. Castellucci, and E. R. Kandel (1976) Synaptic facilitation and behavioral sensitization in *Aplysia*: Possible role of serotonin and cyclic AMP. *Science* 194: 1178-1181.
- Camardo, J., E. Proshansky, and S. Schacher (1983) Identified *Aplysia* neurons form specific chemical synapses in culture. *J. Neurosci.* 3: 2814-2820.
- Carew, T. J., R. D. Hawkins, and E. R. Kandel (1983) Differential classical conditioning of a defensive withdrawal reflex in *Aplysia californica*. *Science* 219: 397-400.
- Castellucci, V. F., and E. R. Kandel (1974) A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in *Aplysia*. *Proc. Natl. Acad. Sci. USA* 71: 5004-5008.
- Castellucci, V. F., and E. R. Kandel (1976) Presynaptic facilitation as a mechanism for behavioral sensitization in *Aplysia*. *Science* 194: 1176-1178.
- Hawkins, R. D., V. F. Castellucci, and E. R. Kandel (1981a) Interneurons involved in mediation and modulation of the gill-withdrawal reflex in *Aplysia*. I. Identification and characterization. *J. Neurophysiol.* 45: 304-314.
- Hawkins, R. D., V. F. Castellucci, and E. R. Kandel (1981b) Interneurons involved in mediation and modulation of the gill-withdrawal reflex in *Aplysia*. II. Identified neurons produce heterosynaptic facilitation contributing to behavioral sensitization. *J. Neurophysiol.* 45: 315-326.
- Hawkins, R. D., T. W. Abrams, T. J. Carew, and E. R. Kandel (1983) A cellular mechanism of classical conditioning in *Aplysia*: Activity-dependent amplification of presynaptic facilitation. *Science* 219: 400-405.
- Hochner, B., S. Schacher, M. Klein, and E. R. Kandel (1985) Pre-

- synaptic facilitation in *Aplysia* sensory neurons: A process independent of K⁺ current modulation becomes important when transmitter release is depressed. Soc. Neurosci. Abstr.
- Hume, R. I., L. W. Role, and G. D. Fischbach (1983) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. *Nature* 305: 632–634.
- Kandel, E. R. (1979) Cellular insights into behavior and learning. In *The Harvey Lectures*, Ser. 73, D. Marcus, ed., pp. 19–92, Academic, New York.
- Kandel, E. R., and J. H. Schwartz (1982) Molecular biology of an elementary form of learning: Modulation of transmitter release by cyclic AMP. *Science* 218: 433–443.
- Klein, M., and E. R. Kandel (1978) Presynaptic modulation of voltage-dependent Ca²⁺ current: Mechanism for behavioral sensitization in *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* 75: 3512–3516.
- Klein, M., E. Shapiro, and E. R. Kandel (1980) Synaptic plasticity and the modulation of the Ca⁺⁺ current. *J. Exp. Biol.* 89: 117–157.
- Klein, M., J. S. Camardo, and E. R. Kandel (1982) Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in *Aplysia*. *Proc. Natl. Acad. Sci. USA* 79: 5713–5717.
- Kriegstein, A. R. (1977) Development of the nervous system of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* 74: 375–378.
- Kriegstein, A. R., V. F. Castellucci, and E. R. Kandel (1974) Metamorphosis of *Aplysia californica* in laboratory culture. *Proc. Natl. Acad. Sci. USA* 71: 3654–3658.
- Montarolo, P. G., V. F. Castellucci, P. Golet, E. R. Kandel, and S. Schacher (1985) Long-term facilitation of the monosynaptic connection between sensory neurons and motor neurons of the gill withdrawal reflex in *Aplysia* in dissociated cell culture. Soc. Neurosci. Abstr.
- Rayport, S. G., and J. S. Camardo (1984) Development of synaptic plasticity mediating habituation and sensitization in *Aplysia*. *J. Neurosci.* 4: 2528–2532.
- Rayport, S. G., and S. Schacher (1984) Elementary neural circuit of *Aplysia* gill-withdrawal reflex reconstituted in cell culture shows homosynaptic depression and 5HT-induced facilitation. Soc. Neurosci. Abstr. 10: 923.
- Schacher, S. (1985) Differential synapse formation and neurite outgrowth at two branches of the metacerebral cell of *Aplysia* in dissociated cell culture. *J. Neurosci.* 5: 2028–2034.
- Schacher, S., and E. Proshansky (1983) Neurite regeneration by *Aplysia* neurons in dissociated cell culture: Modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* 3: 2403–2413.
- Schacher, S., S. G. Rayport, and R. T. Ambron (1985) Giant *Aplysia* neuron R2 reliably forms chemical connections *in vitro*. *J. Neurosci.* 5: 2851–2856.
- Weiss, K. R., J. L. Cohen, and I. Kupfermann (1978) Modulatory control of buccal musculature by a serotonergic neuron in *Aplysia*. *J. Neurophysiol.* 41: 181–203.
- Weiss, K. R., D. E. Mandelbaum, M. Schonberg, and I. Kupfermann (1979) Modulation of buccal muscle contractility by serotonergic metacerebral cells in *Aplysia*: Evidence for a role in cyclic AMP. *J. Neurophysiol.* 42: 791–803.
- Weiss, K. R., S. Schacher, I. Kupfermann, and P. E. Lloyd (1985) Calcium-dependent release of neuropeptides (the SCPs) evoked by intracellular stimulation of single identified *Aplysia* neurons in cell culture. Soc. Neurosci. Abstr. 11: 482.
- Young, S. H., and M. Poo (1983) Spontaneous release of transmitter from growth cones of embryonic neurons. *Nature* 305: 634–637.