Concanavalin A Alters Synaptic Specificity Between Cultured *Aplysia* Neurons

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Factors that regulate synaptic specificity were investigated with *Aplysia* buccal and bag cell neurons in primary cell culture. In the presence of fetal calf serum electrical synapses are formed between buccal-buccal or bag-bag cell pairs, but not between buccal-bag cell pairs. Instead, buccal neurons make inhibitory chemical synapses on bag cells. However, in the presence of nanomolar concentrations of the lectin concanavalin A this pattern changes, such that more than 75 percent of buccal-bag pairs exhibit electrical synapses and the frequency of occurrence of buccal-bag chemical synapses is reduced. Such changes in synaptic specificity may be important in determining the types of synapses formed during neuronal development and neurite regeneration.

THE FORMATION OF SPECIFIC SYNAPSES between neurons early in development is critical for the proper functioning of neural circuits. Although they are specific, many synapses are also plastic and can be changed by injury to, or learning in, the nervous system (*1, 2*). To understand better how synapses are formed and modified, investigators have used tissue culture techniques to study synaptogenesis between isolated neurons (*3–9*). Identified invertebrate neurons retain many of their in situ properties in culture, including the formation of specific synapses (*4–8*). For example, bag cell neurons from the abdominal ganglion of the sea hare *Aplysia californica* will make electrical synapses with each other when dissociated in culture (*5*), just as they do in the intact ganglion. Likewise, neurons from the buccal ganglion will form electrical synapses with each other in culture (*6, 8*), although the extent to which this reflects the situation in vivo is less clear. These studies (*5, 6, 8*) indicate clearly that cultured buccal and bag cell neurons are both competent to form electrical synapses. Yet in spite of this, buccal neurons do not form electrical synapses with bag cell neurons when growing adjacent to them in tissue culture dishes. They will, however, make inhibitory chemical synapses on bag cell neurons (*8*). Thus these neurons exhibit selectivity in their choice of synaptic partners, and specificity in the kinds of synapses that they form.

One advantage of tissue culture is the ability to change the conditions under which the neurons grow and ask whether this changes their properties. For example, factors in conditioned medium from heart cells can change the transmitter phenotype of cultured sympathetic neurons from adrenergic to cholinergic (*10*). We now show that adding nanomolar concentrations of the lectin concanavalin A (Con A) to the culture medium changes the pattern of synaptogenesis in *Aplysia* neuron cultures by inducing the formation of electrical synapses between buccal and bag cell neurons.

Buccal and bag cell neurons were dissociated and cultured in sterile 35-mm dishes filled with modified L15 medium containing fetal calf serum (*6, 8*). After 3 to 10 days in culture, microelectrodes were inserted into pairs of cells with overlapping neurites to determine the type of synapses formed between the cells (*11*). As reported previously (*6*), buccal neurons grown in medium without Con A will form electrical synapses with one another, as measured by the passage of hyperpolarizing current between cell pairs (Fig. 1A). Similarly, a bag cell will form an electrical synapse with another bag cell in culture (Fig. 1B) (*5*). These responses are still seen in saline containing 10 m*M* cobalt to block chemical synaptic transmission, confirming that they are indeed mediated by electrical synapses. However, there are no buccal-bag cell electrical synapses under these culture conditions, even though the neurites of the cells overlap extensively and a buccal-to-bag cell inhibitory chemical synapse is often seen (Fig. 1C) (*8*).

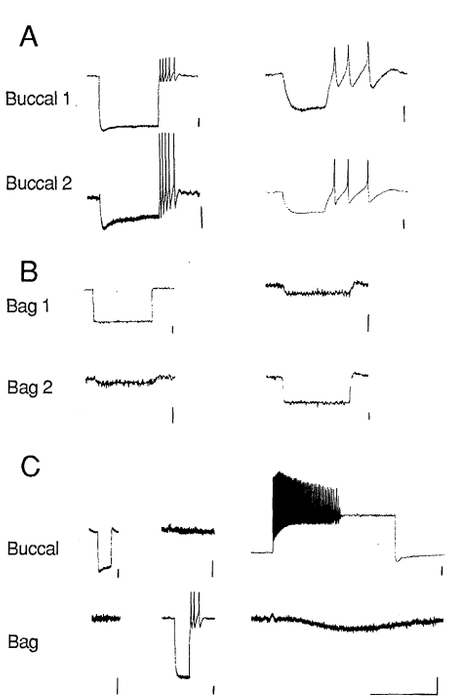

Fig. 1. Electrical synapses between buccal-buccal (**A**) and bag-bag (**B**) cell pairs, but not buccal and bag cell pairs (**C**). Only cell pairs with extensively overlapping neurites were examined. Hyperpolarizing current was injected into the cell in the top trace in the left columns and in the bottom cell in the right columns. Hyperpolarizing responses in the paired cells in (A) and (B) demonstrate the presence of an electrical synapse. In (C) buccal and bag cells do not make electrical synapses with each other (left and middle traces). However, a depolarizing pulse given to the buccal neuron, causing it to fire a train of action potentials, reveals an inhibitory chemical synapse on the bag cell (right traces). These results confirm previous reports (*5, 6, 8*) and are shown here for comparison with those in Fig. 2. Vertical scales, 10 mV; horizontal scales, 5 seconds, except (A) (right column), 1 second.

Addition of Con A to the culture medium induces electrical synapses between buccal and bag cell neurons in more than 75% of the cell pairs we examined (Fig. 2). The Con A–induced electrical synapse can occur in the absence (Fig. 2A) or the presence (Fig. 2B) of the inhibitory chemical synapse, and is seen at Con A concentrations ranging from 10 n*M* to 1 μ*M*. With increasing Con A concentrations, it appears that the proportion of buccal-bag pairs with electrical synapses increases, whereas the frequency of chemical synapses decreases (Fig. 3). Although we cannot entirely rule out the possibility that this apparent decrease in the proportion of chemical synapses is due to their being masked by the extensive electrical coupling, it seems unlikely because of the very different time courses of the two types of synapses and the fact that we can readily observe examples of dual synaptic responses (Fig. 2B). Other lectins tested, such as wheat germ agglutinin and lima bean lectin, do not alter the pattern of synapse formation between buccal and bag cell neurons.

In addition to changing the type of synapses formed between buccal and bag cell neurons, Con A enhances formation of lamellipodia and neurite outgrowth from cultured *Aplysia* neurons (Fig. 4, A and B). Similar effects of Con A are also seen in cultured chick dorsal root ganglion (*12*) and leech (*13*) neurons. Furthermore, large identified *Aplysia* neurons greater than 100 μm in diameter, which rarely attach and sprout neurites in normal culture medium, do so readily in the presence of Con A (Fig. 4C).

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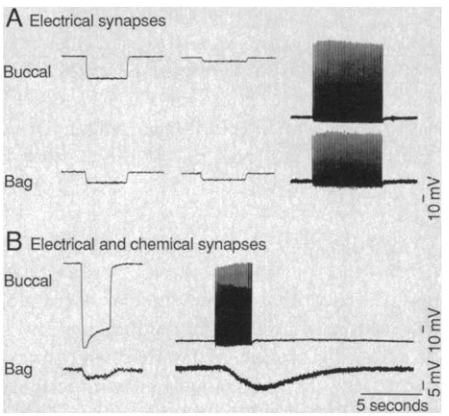


Fig. 2. Buccal and bag cell neurons will form electrical synapses in the presence of 100 n*M* Con A. (**A**) Hyperpolarizing current injected into buccal (left traces) and bag cells (middle traces) and depolarizing current injected into the buccal cell (right traces) show strong coupling between the two cells. The buccal-bag pair in (**B**) is coupled somewhat less strongly (left traces) and also displays a chemical synapse (right traces). We have seen no electrical synapses between buccal and bag cell neurons grown in fetal calf serum in the absence of Con A, but it is possible that the cells are coupled to some very small extent that is below our level of detection and that the addition of Con A increases the degree of coupling to a higher level.

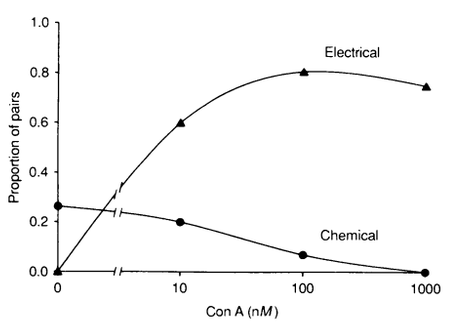
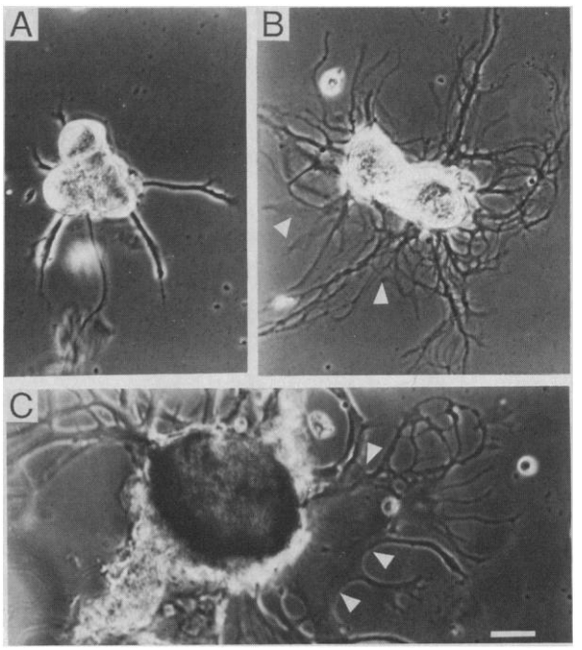


Fig. 3. The percent of buccal and bag cell pairs examined exhibiting electrical synapses (triangles) and chemical synapses (circles) as a function of the Con A concentration in the culture medium. The percent of chemical synapses formed in the absence of Con A is somewhat lower than that reported previously (*8*), possibly because recordings from the buccal-bag cell pairs in the present experiments were made after a shorter time in culture. Actual number of pairs examined for each concentration are: 0 Con A, 19 pairs; 10 n*M* Con A, 5 pairs; 100 n*M* Con A, 57 pairs; 1 μ*M* Con A, 8 pairs. The five cell pairs grown in 10 n*M* Con A were all from the same culture dish. For all other concentrations the data were obtained from at least three separate dishes.

The mechanism by which buccal and bag cell neurons in culture recognize one another in order to form electrical synapses specifically with their own cell type and not the other is unknown. Cell-cell recognition between these neurons may involve glycoproteins such as the neural cell adhesion molecule in vertebrate nervous systems (*14*), or some other uncharacterized membrane proteins. Concanavalin A has been shown to bind to *Aplysia* neurons and to rapidly modify their receptor-mediated electrical response to iontophoretic application of glutamate (*15*), and it is possible that cell recognition or gap junction proteins (or both) on the plasma membrane are also modified by Con A so that they alter their specificity. The effect of Con A could be mediated by adenosine 3′,5′-monophosphate (cAMP), which modulates electrical synapses in several systems [(*16–19*); for a review, see (*20*)], presumably via cAMP-dependent phosphorylation of gap junctions (*16*). However, the adenylate cyclase activator forskolin, added to the culture medium together with a phosphodiesterase inhibitor, does not affect the strength of electrical coupling between buccal-bag pairs grown in the presence of Con A.

Alternatively, Con A might be acting as a growth factor as evidenced by the increased neurite outgrowth and formation of extensive lamellipodia (Fig. 4). Other growth factors, such as insulin, can increase the strength of electrical synapses between cultured sympathetic neurons (*21, 22*). In *Helisoma*, the formation of electrical synapses between isolated cultured neurons occurs only between growing neurites (*9*). Although in *Aplysia* neurons there are factors other than, or in addition to, growth state that regulate electrical synapse formation (*8*), it is possible that the induction by Con A of electrical synapses between buccal and bag cell neurons is related in part to enhanced neurite outgrowth. The precise mechanism by which Con A induces changes in *Aplysia* neurons is not yet known, but by investigating how such changes occur we may better understand how neurons make specific synapses with one another and how these synapses can be modified.

Fig. 4. Effects of Con A on neurite outgrowth and lamellipodium formation. (**A**) Buccal neurons grown in the absence of Con A after 7 days in culture. (**B**) Buccal neurons grown in the presence of 100 n*M* Con A after 4 days in culture. Note the more numerous neurites and flat lamellar processes (arrowheads) extending from the cell bodies. These features are more evident in (**C**), which shows a large identified medial cell from the pleural ganglion, about 200 μm in diameter, cultured in the presence of 100 n*M* Con A. Note the very broad lamellipodia (arrowheads) from which neurites and growth cones extend. Neurons this large only rarely regenerate neurites in the absence of Con A. Scale bar, 60 μm.



REFERENCES AND NOTES

1. E. Kandel, *Cellular Basis of Behavior* (Freeman, San Francisco, 1976).
2. A. G. M. Bulloch and S. B. Kater, *Science* **212**, 79 (1981).
3. P. H. O'Lague, K. Obata, P. Claude, E. J. Furshpan, D. D. Potter, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3602 (1974).
4. D. F. Ready and J. Nicholls, *Nature (London)* **281**, 67 (1979).
5. L. K. Kaczmarek, M. Finbow, J. P. Revel, F. Strumwasser, *J. Neurobiol.* **10**, 535 (1979).
6. D. Dagan and I. B. Levitan, *J. Neurosci.* **1**, 736 (1981).
7. J. Camardo, E. Proshansky, S. Schacher, *ibid.* **3**, 2614 (1983).
8. R. Bodmer, D. Dagan, I. B. Levitan, *ibid.* **4**, 228 (1984).
9. R. D. Hadley, D. A. Bodnar, S. B. Kater, *ibid.* **5**, 3145 (1985).
10. P. H. Patterson, *Annu. Rev. Neurosci.* **1**, 1 (1978).
11. Buccal ganglia and bag cell clusters were treated overnight in 1% dispase (Boehringer Mannheim) in modified L-15 medium (Gibco) (*8*). The ganglia were desheathed and individual neurons were dissociated and plated near each other on a glass coverslip in 35-mm dishes. Fetal calf serum (4%) was present in the culture medium (*8*). In some dishes, Con A was added to the medium soon after cells were plated. Microelectrodes (10 to 20 Mohm) filled with 3*M* KCl were used to record voltage from and inject current into buccal and bag cell neurons whose neurites overlapped in culture. Buccal and bag cell neurons in culture were identified by their differences in pigmentation and electrophysiological properties (*8*). Hyperpolarizing currents (1 to 5 nA) were injected into each cell, and the coupling coefficient was determined by the change in voltage in the follower cell as compared to that of the injected cell. The voltage change due to electrode resistance was balanced by a bridge amplifier or subtracted before determining the coupling coefficient. Only cell pairs with coupling coefficients greater than 0.01 were considered to be coupled by electrical synapses.
12. J. J. DeGeorge, N. Slepecky, S. Carbonetto, *Dev. Biol.* **111**, 335 (1985).
13. M. Chiquet and S. E. Acklin, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6188 (1986).
14. D. R. Buskirk, J.-P. Thiery, U. Rutishauser, G. M. Edelman, *Nature (London)* **285**, 488 (1980).

15. J. Kehoe, *ibid.* **274**, 866 (1978).
16. J. C. Saez *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2473 (1986).
17. E. M. Lasater and J. E. Dowling, *ibid.* **82**, 3025 (1985).
18. M. Piccolino, H. M. Gerschenfeld, J. Neyton, *J. Neurosci.* **4**, 2477 (1984).
19. E. C. Wiener and W. R. Loewenstein, *Nature (London)* **305**, 433 (1983).
20. D. C. Spray and M. V. L. Bennett, *Annu. Rev. Physiol.* **47**, 281 (1985).
21. E. J. Wolinsky, P. H. Patterson, A. L. Willard, *J. Neurosci.* **5**, 1675 (1985).
22. J. A. Kessler, D. C. Spray, J. C. Saez, M. V. L. Bennett, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6235 (1984).
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Pheromone Components and Active Spaces: What Do Moths Smell and Where Do They Smell It?

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The pheromone-mediated flight behavior of male Oriental fruit moths was observed in the field to test the hypothesis that male activation far downwind of a female is initiated by the major, or most abundant, component of the pheromone blend. Males responded at significantly greater distances to the three-component pheromone blend produced by females than to the major component alone or to either binary mixture containing the major component and one minor component. These results support the alternative hypothesis that the active space of a multicomponent pheromone is a function of male perception of the female-released blend of components, rather than of the major component alone, and that so-called minor components have a greater impact on male behavior farther downwind of a female than previously thought.

ONE OF THE MAJOR QUESTIONS IN the study of lepidopteran multicomponent sex pheromones concerns the role of individual chemicals in influencing the dimensions of the active space, defined here as the area downwind of a calling female over which males are able to detect and respond to the pheromone (*1, 2*). Two conflicting hypotheses have been presented to explain this problem. The first, or component, hypothesis states that the maximum distance at which a male orients to the odor plume and initiates upwind flight is a function of the male's ability to detect the major, or most abundant, component in the blend (*3–7*). Accordingly, minor components do not participate in long-range attraction; rather, they initiate short-range approach, landing, and courtship behaviors. The second, or blend, hypothesis states that the female-released blend of components acts as a unit to effect optimal sensitivity in males over the entire response range (*8, 9*). Even though minor components may be present in very small amounts (usually <10% of the major component), these components are necessary for optimal levels of activation and upwind flight.

To test these two hypotheses, we recently conducted a series of flight tunnel tests with three moth species, the Oriental fruit moth (OFM) [*Grapholita molesta* (Busck)], the red-banded leaf roller (RBLR) [*Argyrotaenia velutinana* (Walker)], and the cabbage looper (CL) [*Trichoplusia ni* (Hübner)] (*10*). Our results showed (i) that over the dose series used the female-released blends significantly enhanced the number of males activating and completing upwind flights to the source over that observed with the major component alone, and (ii) that male response to lower doses of the major component (at which no upwind flights were recorded) was significantly enhanced by addition of the complement of minor components (*11*). From these results we hypothesized that downwind of a female, represented in the flight tunnel by lower doses of pheromone, males are most responsive to the complete blend of components and that the active space of the pheromone is a function of male perception of the female-released blend and not simply the major component. Here we present additional evidence for the blend hypothesis from studies with the OFM in which the active space of the pheromone was measured under field conditions. We suggest that this hypothesis represents an important general principle in insect chemical communication systems and is most consistent with the existing paradigm stating that multicomponent pheromones in the Lepidoptera function as species-specific mating signals.

The behavior of male OFM to synthetic pheromone [(*Z*)-8-dodecenyl acetate (Z8-12:OAc), with 6% (*E*)-8-dodecenyl acetate (E8-12:OAc) and 3% (*Z*)-8-dodecenol (Z8-12:OH) (*12*)] was observed in a large open field (*13*). The pheromone plume was localized by a parallel stream of bubbles released at a point 1 m from the pheromone source (*14*). In the first test, males were exposed to the major component, Z8-12:OAc, alone and to the three-component mixture at three doses (1, 10, and 100 μg) and over two temperature ranges (19° to 21°C and 25° to 28°C) representing the extremes that occur during the activity period of this insect. Males exhibited vigorous wing-fanning and walking responses in significantly greater numbers and at greater distances at all three doses of the three-component blend than to the major component (Fig. 1) (*15, 16*). The distance at which males responded was approximately doubled at the higher temperature range. In the second test males were

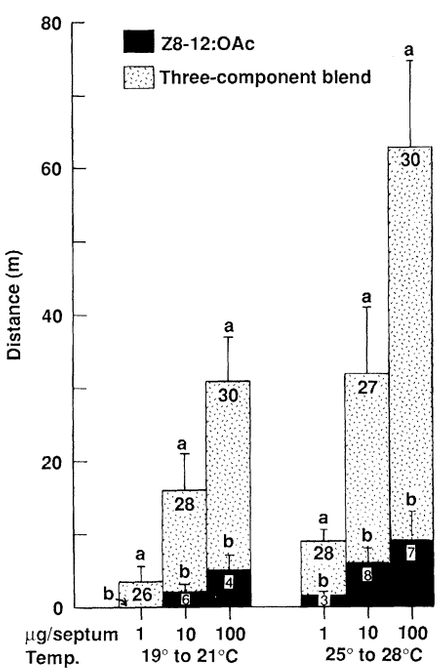

Fig. 1. Distance downwind (in meters) of a pheromone source that prompted male OFM wing-fanning and walking responses. Three doses of the major component (Z8-12:OAc) or the three-component blend (Z8-12:OAc with 6% E8-12:OAc and 3% Z8-12:OH) were used over two temperature ranges. Response values are means ± SD, $n = 30$, for each treatment. Values inside each bar are the number of responders. Comparisons of mean response values were made between the pair of treatments at each dose within each temperature range by analysis of variance. Means within each pair with different letters indicate a significant difference ($P < 0.05$).

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