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Self-Recognition: A Constraint on the Formation of Electrical Coupling in Neurons

Peter B. Guthrie, Robert E. Lee, Vincent Rehder, [a]Marc F. Schmidt, and [b]Stanley B. Kater

Program in Neuronal Growth and Development, Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, Colorado 80523

Electrical coupling between specific neurons is important for proper function of many neuronal circuits. Identified cultured neurons from the snail *Helisoma* show a strong correlation between electrical coupling and presence of gap junction plaques in freeze-fracture replicas. Gap junction plaques, however, were never seen between overlapping neurites from a single neuron, even though those same neurites formed gap junctions with neurites from another essentially identical identified neuron. This observation suggests that a form of self-recognition inhibits reflexive gap junction formation between sibling neurites. When one or both of those growth cones had been physically isolated from the neuronal cell body, both electrical coupling and gap junction plaques, between growth cones from the same neuron, were observed to form rapidly (within 30 min). Thus, inhibition of electrical coupling between sibling neurites apparently depends on cytoplasmic continuity between neurites, and not the molecular composition of neurite membrane. The formation of gap junctions is not likely due to the isolation process; rather, the physical isolation appears to release an inhibition of reflexive gap junction formation. These data demonstrate the existence of a previously unknown constraint on the formation of electrical synapses.

[Key words: gap junctions,* Helisoma*, autapse, electrical coupling, synapse specificity, freeze fracture, fura-2]

Gap junctions are widespread throughout the animal kingdom and have been proposed to serve a number of functions, including metabolically linking cells, controlling morphogenesis, and serving as an integral part of neuron circuits (Larsen, 1983; Bennett et al., 1991). A number of studies have examined the development of coupling (Liu and Nicholls, 1989; Swenson et al., 1989; Barrio et al., 1991), regulation of coupling (Murphy and Kater, 1980; Hadley et al., 1982; Bulloch, 1985; Janse et al., 1986; Pelletier, 1988; Carrow and Levitan, 1989; Matsumoto et al., 1991), and the regulation of gap junction activity (Liu and Nicholls, 1989; Swenson et al., 1989; Barrio et al., 1991). Gap junctions between cells have been found to form across species (Epstein and Gilula, 1977), and even from heterologous connexon proteins (Swenson et al., 1989; Traub et al., 1989; Barrio et al., 1991). Gap junctions can also be found between different processes of a single cell (called reflexive gap junctions) (Iwyama, 1971; Herr, 1976; Majack and Larsen, 1980). These observations suggest that processes whose membranes have connexons present (or nearby) might normally be expected to couple with other, equally competent, processes. Relatively few studies have investigated the mechanisms regulating the specificity of gap junction formation. What is not clear is what the mechanisms are that might prevent inappropriate gap junction formation between competent processes.

Studies of the specificity of electrical synapse formation in the pond snail *Helisoma* have shown that synapse specificity can be different *in vivo* than *in vitro. In vivo,* regenerating buccal neurons will reconnect to their appropriate targets and will also form novel electrical connections (Hadley et al., 1982). Not every neuron will be connected, however, and many of the electrical connections are transient (Cohan et al., 1987). In contrast, regulation of electrical coupling *in vitro* appears to depend solely on the growth state of the neurons (Hadley and Kater, 1983; Hadley et al., 1983, 1985). Electrical coupling is observed between *every pair* of neurons that overlap when both are in an active growth state, suggesting that every neuron, in culture, is competent to form gap junctions. Coupling is almost completely absent when one of the neuron ceases active outgrowth before overlap of neurites occurs.

We report here that a specific inhibition of gap junction formation occurs between sibling neurites from a single neuron. These neurites, which are quite competent to form gap junctions with neurites from other growing neurons, fail to establish gap junctions with equally competent sibling neurites from the same neuron. This form of self-recognition is unlikely to occur because of molecular recognition of sibling membrane, since ultrastructurally and functionally defined gap junctions can be formed between sibling neurites after they have been physically isolated from the parent neuron. These results point to a novel mechanism regulating the formation of gap junctions within neurons.

Methods and Materials

Cell culture. Individual identified neurons were isolated from adult *Helisoma* nervous system and cultured as previously described (Mattson and Kater, 1987). For the ultrastructural and optical experiments, neurons were plated onto glass coverslips on the bottom of 35 mm culture dishes. B5 and B19 neurons were used. Different cultures were used for ultrastructural, electrophysiological, and optical experiments to avoid ultrastructural damage that might occur from the electrophysiological or optical recording procedures.

Growth cone isolation. Growth cones were isolated from the rest of the neuron by severing the neurite immediately proximal to the growth

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Correspondence should be addressed to Dr. Peter B. Guthrie, Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, CO 80523.

[a] Present address: Department of Biology, Georgia State University, P.O. Box 4010, Atlanta, GA 30302-4010.

[b] Present address: Division of Biology 216-76, California Institute of Technology, 1201 East California, Pasadena, CA 91125.

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cone, using a glass electrode as a microknife (Rehder et al., 1991). Such isolated growth cones have been observed to resume normal elongation within 30 min of isolation, and to exhibit normal branching and turning behaviors, electrophysiological properties, and responsiveness to neurotransmitters for several days following isolation (Davis et al., 1992b). Growth cones that showed unusual swelling or other signs of trauma were not used in these experiments.

Freeze-fracture procedures. Freeze-fracture procedures were as described previously (Guthrie et al., 1989). Briefly, neurons were grown on glass coverslips and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hr at 4°C. The cultures were then photographed to permit identification of individual neurites in the freeze-fracture replicas. The coverslips were washed twice in 0.1 M phosphate buffer. The material was cryoprotected by infiltration of 25% glycerol in 0.1 M phosphate buffer at 4°C for 30 min. Pieces of glass containing the neurons were obtained by scoring and breaking the coverslip. The neurons were frozen according to the method of Pauli et al. (1977), which consists of placing a drop of a solution containing polyvinyl alcohol, phosphate-buffered saline, glycerol, and dimethyl sulfoxide onto a 3-mm-diameter gold specimen support with a central 1 mm hole. The glycerol solution was drained from the piece of glass coverslip with the attached neuron, and the glass was placed neuron-side down onto the polyvinyl alcohol solution drop on the specimen support. The specimen was then frozen by plunging the gold support into partially liquid Freon-12 cooled in liquid nitrogen.

The frozen neurons on glass were fractured in a Balzers 301 at −100°C and 10^{-6} millibar by placing the cold knife under the glass and raising the knife. The glass popped off with the fracture plane typically passing through the neuron. Platinum–carbon was immediately deposited on the fractured surface at an angle of 40°. Carbon was evaporated from above to strengthen the replicas. The specimen was dissolved in full-strength bleach for 4 hr, and the replicas washed in water and picked up on grids. The replicas were viewed in Philips 400T and JEOL-2000 transmission microscopes.

Regions of the replicas were identified by comparison of the low-power electron micrographs and the phase photomicrographs. Overlapping regions were examined where the plane of fracture passed between the two neurites. Overlapping regions were counted for statistical purposes only if both neurites could be traced unambiguously to a neuronal cell body. An overlap region was counted as lacking a gap junction only if the entire region of overlap was visible on the replica.

Electrophysiological methods. General electrophysiological procedures were similar to those described in Guthrie et al. (1989). Standard patch-electrode recording procedures (whole-cell configuration) were used to characterize electrical coupling between isolated parts of the same neuron and between neuron pairs. Current flow between the two recording electrodes was directly measured in voltage-clamp mode. Electrodes were fabricated on a Narishigi PP-83 Patch Pipette Puller from borosilicate glass (1.5 mm o.d.; F. Haer and Co.). The intracellular patch solution contained 55 mM K-aspartate, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES; pH 7.4, 125 mmol/kg osmolality. Electrode resistances for cell body recording were 3–5 MΩ; for isolated growth cones, 15–20 MΩ electrodes were used. Two Dagan 8900 patch amplifiers with a 0.1 GΩ headstage were controlled by an Indec LSI 11/23-based computer system, which was also used to collect and analyze the data.

Coupling between an isolated growth cone region and the parent neuronal cell body was estimated with a double voltage-clamp system. Both electrodes were clamped at a holding potential of −60 mV. One electrode was stepped ±10 mV; the steady state current generated within the stepped electrode was divided by the current required to keep the other electrode at the holding potential (−60 mV) to estimate the current transfer function. The other electrode was then stepped ±10 mV. All four values (stepping each electrode ±10 mV) were averaged to a single current transfer function estimate. Six preparations in which there was no overlap of processes served as controls for interelectrode coupling (current transfer function = 0.015 ± 0.009, mean ± SD; range = 0.005 − 0.03). Coupling was accepted as significant if the current transfer function exceeded the maximum values observed in the controls.

For stimulation of neuronal cell bodies in optical experiments, the cell body was penetrated with a standard glass microelectrode filled with 0.7 M K_2SO_4. A Getting-style microelectrode amplifier was used for current-clamp stimulation and recording.

Potassium depolarization. Cultures were depolarized for 30 min by replacement of normal defined medium with a defined salt solution containing 34 mM K^+ (Rehder and Kater, 1992). Four neurons were immediately fixed after the 30 min depolarization; four additional neurons were washed with normal defined medium, and cultured for an additional 24 hr before fixation. The cultures were fixed and processed for freeze-fracture examination as described above.

Calcium measurements. Free cytoplasmic calcium concentrations were measured as described (Rehder et al., 1991). Briefly, the cell body was penetrated with a microelectrode containing fura-2 (pentapotassium salt, 10 mM in distilled H_2O; Molecular Probes, Eugene, OR). The fura-2 was injected with hyperpolarizing current for a maximum of 5 min, yielding intracellular fura-2 concentrations estimated at less than 60 μM. The preparation was allowed to recover for 15 min before manipulations began. Fura-2 fluorescence was measured on a Zeiss ICM microscope using a quartz collector on the HBO 50 mercury lamp and a 40× oil Nikon Fluorite objective. The excitation light was directed through a computer-controlled shutter/filter wheel system for the two fura-2 excitation wavelengths (350 ± 10 nm and 380 ± 10 nm). Neutral density filters were inserted in the excitation path as necessary to reduce bleaching and saturation of the fluorescence. The image was directed to an intensified CCD camera (Quantex Corp, Sunnyvale, CA). The output of the camera was fed to a Quantex QX7 Image Acquisition/Analysis System (Quantex Corp, Sunnyvale, CA). Calcium concentrations were estimated from the ratio of emission intensities (495 nm long-pass) at the two excitation wavelengths according to Grynkiewicz et al. (1985).

Results

Gap junctions are found only between neurites from neurons known to be electrically coupled

Previous work demonstrated that electrical coupling between cultured neurons occurs only when both neurons are in an active growth state during the time of neurite overlap; virtually no electrical coupling is seen if one of the neurons is not in an active growth state when neurites from the second neuron reach it (Hadley and Kater, 1983; Hadley et al., 1983, 1985). When both neurons are in an active growth state during neurite overlap, freeze-fracture replicas revealed gap junction plaques at virtually every site of neurite overlap (Fig. 1) (20 of 20 overlap regions from nine cell pairs). Plaques were seen between identical neuron pairs (i.e., two B19 neurons) and between mixed neuron pairs (e.g., one B19 neuron and one B5 neuron). Plaques such as these have been classically identified as the morphological substrate for electrical coupling in many systems (Larsen, 1983; Bennett et al., 1991), including *Helisoma* nervous system *in vivo* (Berdan et al., 1987).

Gap junction plaques usually consisted of an average of 170 particles, at a density of 50 particles/0.1 μm^2. The particles were 10–14 nm in diameter. The gap junction plaques could be readily distinguished from the other two types of particle clusters seen in cultured neuronal *Helisoma* membranes. Adhesion plaques, which consisted of regular arrays of large intramembranous particles (IMPs) (14–16 nm diameter) in the protoplasmic face of the plasma membrane, were observed infrequently where growth cones contacted the glass coverslip surface. Partial septate junctions, consisting of 5–10 relatively short rows of IMPs, were seen where a growth cone was growing along a neurite from the same neuron. These junctions were seen only in the membrane of the growth cone, and never in the membrane of the neurite along which the growth cone was advancing.

In neuron pairs where one of the neurons was no longer in an active growth state during neurite overlap, plaques were never seen (0 of 12 overlap regions in eight cell pairs). These observations demonstrate that the plaques of IMPs seen between neurites from different neurons represent the ultrastructural correlate of gap junctions and are responsible for the electrical coupling seen by previous workers.

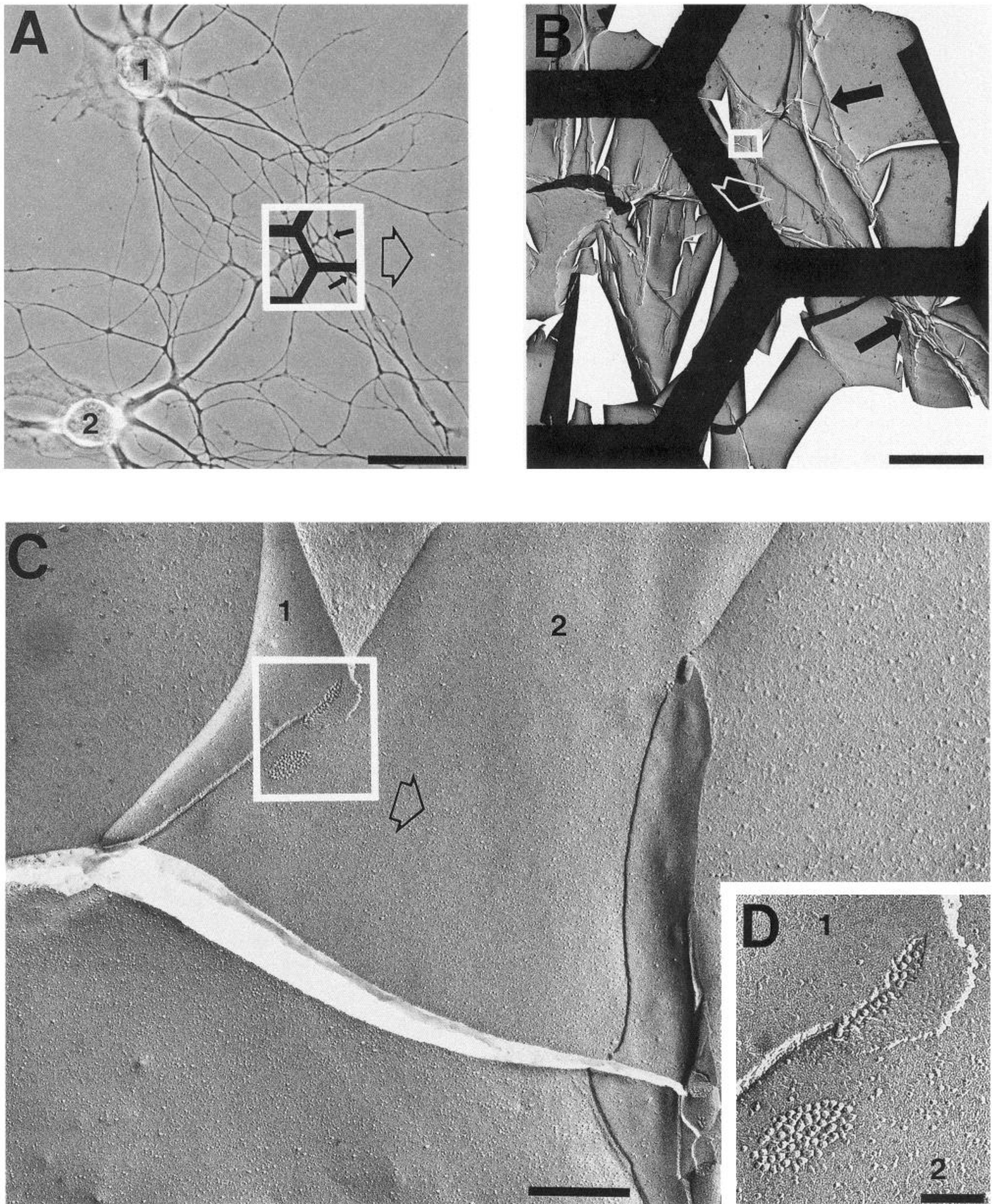


Figure 1. Identification of gap junction plaques between neurites from two growing B19 neurons in culture. *A,* A low-power phase microphotograph showing the two neurons with extensive overlapping neurites. This culture was fixed, frozen, and fractured. The freeze-fracture replica of the region indicated by the *boxed area* was examined. The *two arrows* indicate distinctive neurites in the phase image. The *gridbars in B* were added to aid in identification. *B,* A low-power electron micrograph of the *boxed area in A.* The *arrows* indicate the same neurites marked in *A.* The *boxed area in B* is presented in *C. C,* A high-power electron micrograph of a region of overlapping neurites from the two neurons. These neurites can be unequivocally traced back to the two neurons in *A* with neurite *1* (external membrane leaflet: E-face) coming from neuron 1 and neurite *2* (protoplasmic membrane leaflet: P-face) coming from neuron 2. There are two gap junction plaques between the two neurites. *D,* An enlargement of the gap junction plaques from *C.* Scale bars: *A,* 100 μm; *B,* 10 μm; *C,* 1.0 μm; *D,* 0.25 μm.

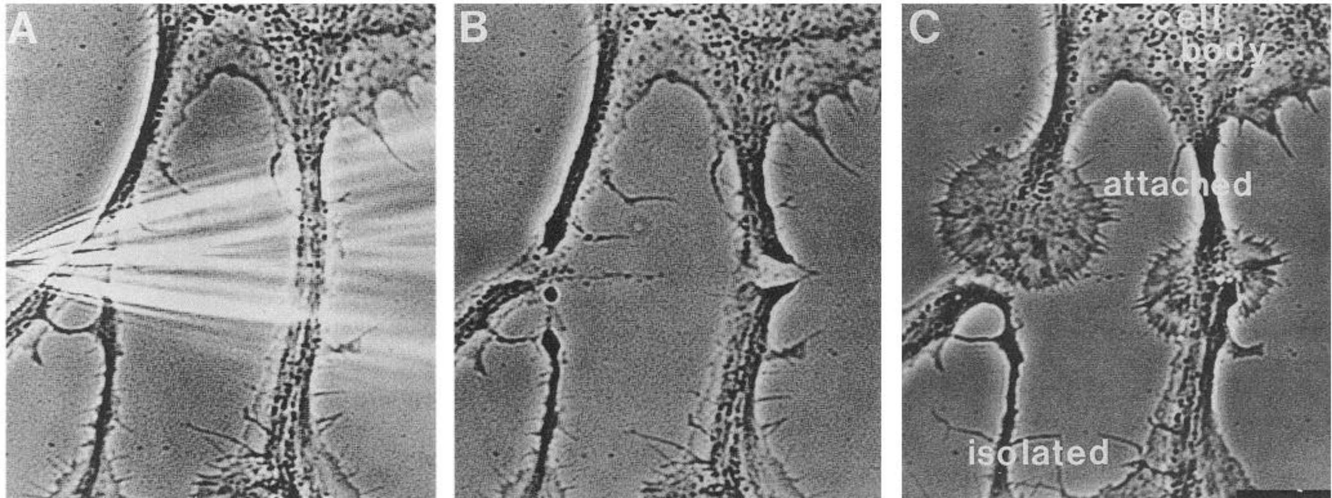


Figure 2. Isolation of growth cones. *A,* A glass microknife is in position over two growth cones to be isolated. *B,* The glass microknife is rapidly drawn across the neurites to sever them (image taken 10 sec following isolation). *C,* New growth cones are generated at the proximal ends of the neurites, which immediately begin elongating. The newly generated growth cones often overlap the isolated growth cones within 30 min (image taken 45 min following isolation). Scale bar, 20 μm.

These ultrastructural observations suggest that every neurite is competent to form a gap junction plaque. The animals used for these experiments are from a highly inbred line maintained within the laboratory for more than 10 years. We would therefore expect that specific identified neurons would have virtually identical genotypes. Electrical coupling was observed between essentially identical identified neurons, and even between bilateral pairs of neurons removed from the same animal. These observations would suggest that every neurite should be capable of forming a gap junction with any other competent neurite, even one from the same neuron.

Reflexive gap junctions are not found in Helisoma *neurons*

Reflexive gap junctions between sibling neurites from the same neuron were never seen (total observations: 0 of 27 overlap regions from 20 neurons). In experiments where neurites from two growing neurons overlapped (and hence formed gap junctions with each other), sibling neurites did not develop reflexive gap junctions (0 of 9 overlap regions). In experiments where a growing (and hence competent) neuron overlapped with a nongrowing (and noncompetent) neuron, sibling neurites did not form reflexive gap junctions (0 of 8 overlap regions). In experiments where single neurons were cultured in isolation, reflexive gap junctions were not found (0 of 10 overlap regions). An even stronger case can be made for the absence of reflexive gap junctions in *Helisoma*: gap junction plaques were not seen in any of the freeze-fracture replicas made from 50 individual neurons.

Some form of self-recognition must exist to prevent the formation of reflexive gap junctions and electrical coupling. This observation was surprising since essentially identical neurons will couple with each other in culture. The inhibition of gap junction formation is therefore not likely due to specific recognition of neuronal differences (i.e., membrane markers), but might rather be due to the cytoplasmic connection between the sibling neurites.

Physically isolated sibling neurites do become electrically coupled

Growth cones and associated neurites can be mechanically isolated from the parent cell body by severing the connecting neurite with a glass microknife (Rehder et al., 1991). The isolated growth cone/neurite survives for days, and retains all the active membrane properties and motility of an attached growth cone (Guthrie et al., 1989; Davis et al., 1992b). This preparation was used to demonstrate, using three separate techniques, that sibling neurites can form gap junctions *if at least one of the sibling neurites is physically isolated from the parent cell body.*

Individual B5 neurons were plated in isolation into culture. Several growth cones were isolated from each neuron; newly generated outgrowth from the neurite stumps was allowed to overlap the isolated growth cones (Fig. 2). These preparations were examined by freeze-fracture techniques. Figure 3 shows that plaques of gap junction particles were found at overlapping regions of isolated and attached neurite. In all, six gap junction plaques were found in three such neurons examined; each neuron had at least one gap junction plaque. Since only one neuron was present, the gap junctions must have formed between sibling processes. These data show that reflexive gap junctions can form if the processes are physically isolated from each other.

To test for functional coupling between isolated sibling processes, we developed a combined optical/electrophysiological assay using the calcium indicator fura-2. Fura-2 was injected into the cell body of isolated B5 neurons and allowed to diffuse into all regions of the cell. Several of the growth cones were then isolated from the cell body. After 30–60 min, newly formed growth cones from the neurite stumps had reached and overgrown the isolated growth cones. The cell body was then stimulated with a microelectrode to fire action potentials, and the resulting changes in intracellular free calcium levels evoked by the electrical activity were monitored in both the attached and in the isolated growth cones. Figure 4 shows that as the cell body is stimulated, calcium levels rise simultaneously in the growth cones still attached to the cell body as well as in the isolated growth cones.

Since simultaneous patch-clamp recordings from an isolated growth cone and a cell body are technically very difficult, we instead chose a paradigm employing B19 neurons, which offer the following advantage: B19 neurons can be plated in culture with long, electrophysiologically accessible, axonal remnants. After 24 hr in culture (during which time new outgrowth has

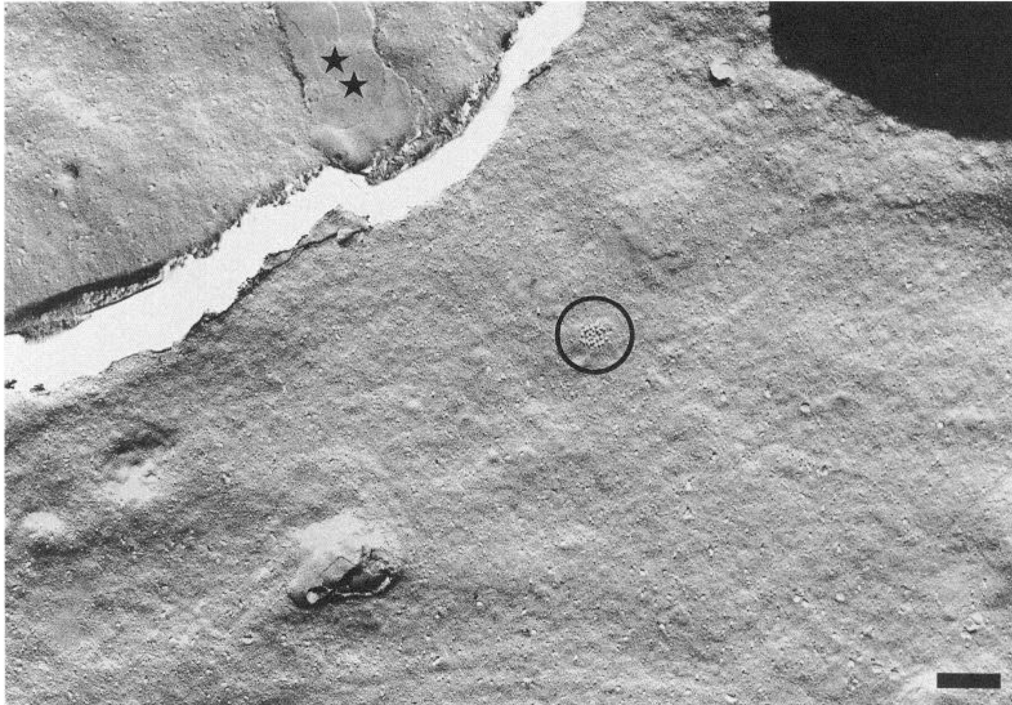


Figure 3. Ultrastructural identification of reflexive gap junctions between isolated and attached sibling neurites. A single B5 neuron was plated into a culture dish. One day after plating, neurites on one side of the neuron were severed with a glass microknife. New outgrowth (*double stars*; E-face) initiated from the severed ends of neurites that subsequently overlapped the isolated growth cones (P-face). Plaques (*circle*) were observed in all three experimental preparations. Scale bar, 0.2 μm.

initiated from the cell body and from the ends of the axonal remnants), the axons were severed with a glass microknife. New outgrowth, initiated from the cut end of the proximal axonal remnant, overlapped the original outgrowth from the now isolated distal axonal segment. Patch electrodes were used to establish whole-"cell" recording configurations from both the cell body and from the distal axonal remnant (Fig. 5). Clear electrical coupling was seen in 12 of 21 cases.

The process of isolation was not responsible for gap junction formation

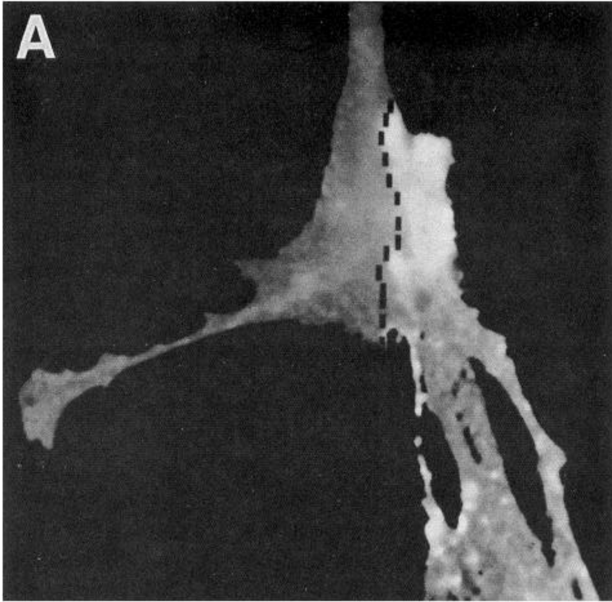
The isolation procedure has immediate effects on both membrane potential and calcium concentrations in growth cones (Rehder et al., 1991). The ability of isolated processes to form reflexive gap junctions could have occurred as a consequence of the trauma associated with the isolation procedure. This is unlikely, since gap junction plaques were not seen on the control side (uncut side) of the neuron. Additional experiments also rule this possibility out. K^+ depolarization has been shown to produce large elevations in calcium concentrations similar to those observed after growth cones isolation (Rehder and Kater, 1992). Four cells were examined immediately after 30 min of depolarization; no reflexive gap junctions were seen in these cultures (0 of 12 overlap regions). An additional four cells were depolarized for 30 min, returned to normal defined medium, and cultured for an additional 24 hr before fixation; again, no reflexive gap junctions were seen (0 of 11 overlap regions). It is clear that the sibling neurites must be physically isolated from one another in order for gap junctions to form.

Discussion

This report brings together ultrastructural, optical, and electrophysiological techniques to examine the rules governing the establishment and distribution of electrical synapses made by *Helisoma* neurons in culture. This report makes a strong case for the presence of gap junction plaques to be necessary for electrical coupling, and also demonstrates that sibling neurites can form identifiable gap junction plaques under specific circumstances. The simplest conclusion to be drawn is that sibling neurites are inhibited from forming electrical connections between each other under normal circumstances. The inhibition of electrical coupling between sibling neurites is not likely to lie at the level of the molecular composition of the membrane, but might lie instead in some interaction through the cytoplasm. These results suggest a novel mechanism that might play a role in the regulation of electrical synapse formation.

Correlation of gap junction plaques with electrical coupling

Several manipulations were used to correlate the presence of gap junction plaques with electrical coupling. Previous studies have shown that only neurons that are in an active growth state during neurite overlap will form electrical connections (Hadley and Kater, 1983; Hadley et al., 1983, 1985). Freeze-fracture replicas revealed abundant gap junction plaques between neurons that were in an active growth state during overlap. Conversely, electrical coupling was never seen between an actively growing neuron and a neuron that was no longer actively growing during the time of overlap. Likewise, no gap junction plaques were seen in freeze-fracture replicas from similar preparations. Electrical coupling in the absence of ultrastructurally defined gap junctions has been described in one instance (Williams and DeHann, 1981). Nonetheless, almost every fracture plane that passed between growing neurites from different neurons provided a replica with evidence of gap junction plaques. We conclude that, in this preparation, the presence of a gap junction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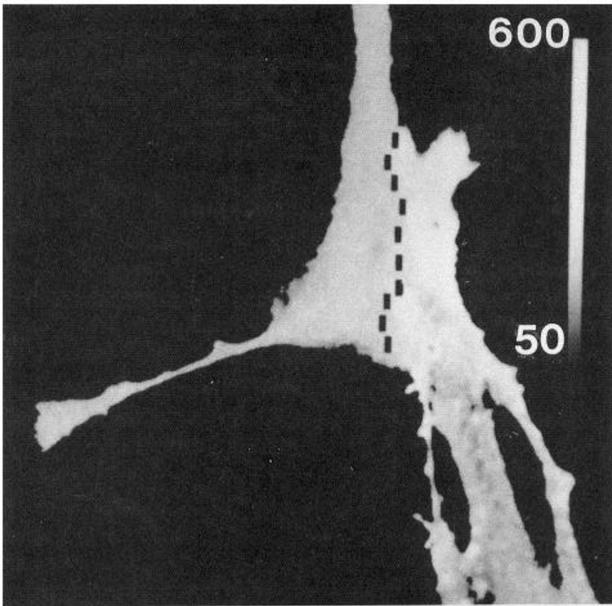
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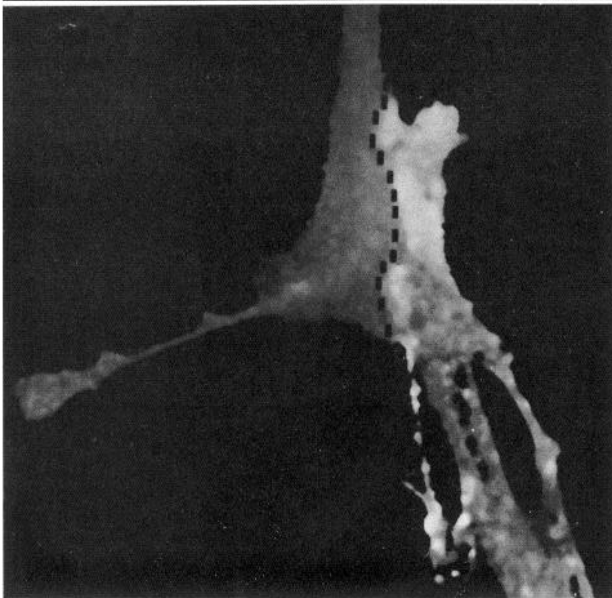

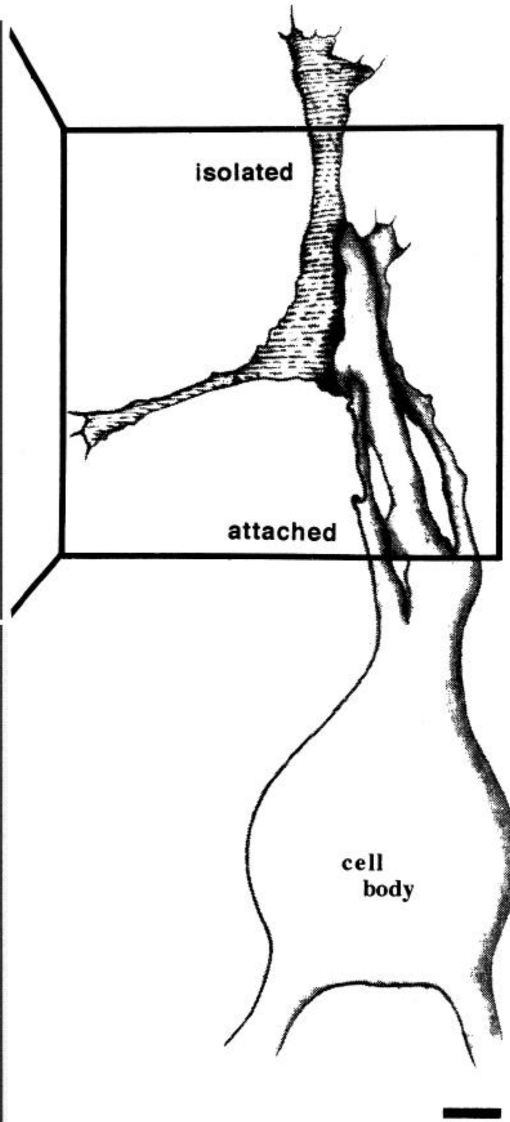
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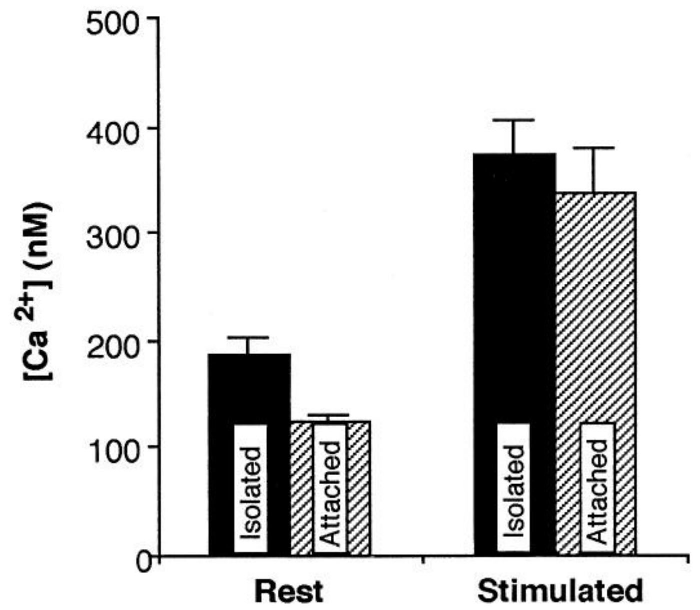
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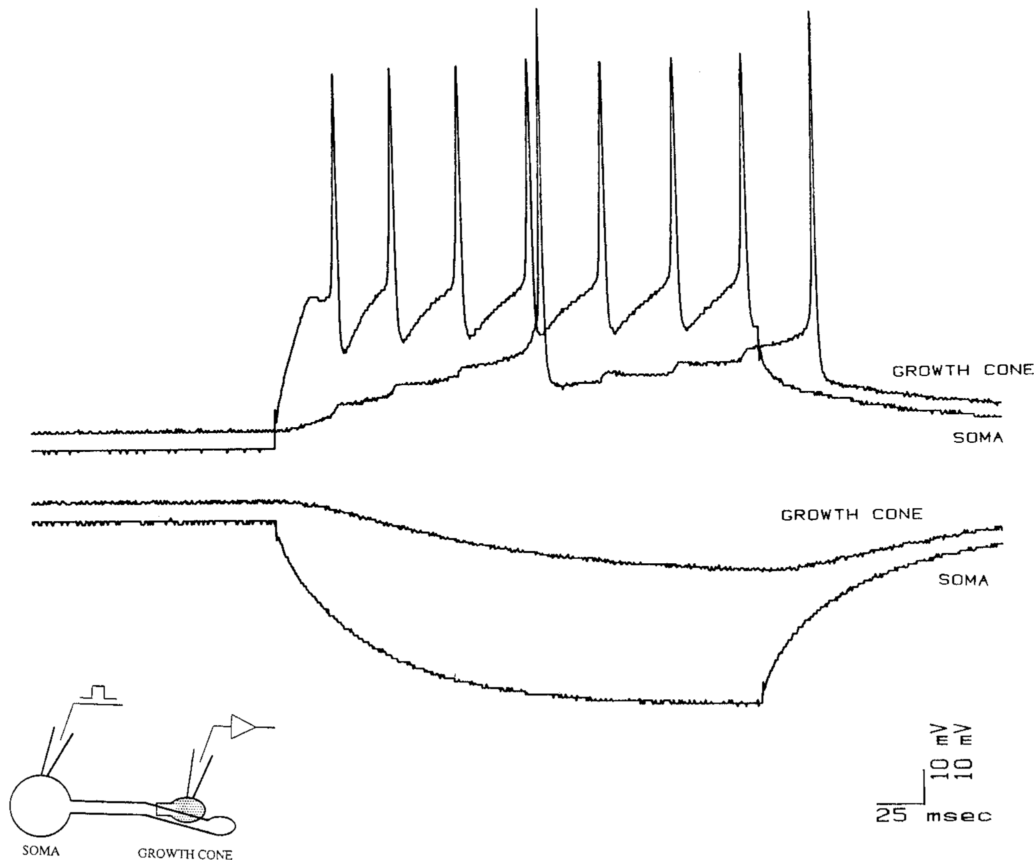

Figure 5. Two-electrode demonstration of electrical coupling between the cell body and isolated growth cones/axonal regions of the same neuron. Passage of current into the cell body (*SOMA*) resulted in a change in the membrane potential of the isolated growth cone/axonal remnant (*GROWTH CONE*). The coupling was electrical, since both depolarizing and hyperpolarizing currents passed into the isolated growth cone/axonal remnant. No evidence of rectification was seen. For quantitative data, both electrodes were voltage clamped, and current passing from one electrode to the other was measured. The *inset* at the *lower left* shows the experimental setup.

plaque is necessary for electrical coupling, and that virtually every growing neurite is competent to form a gap junction.

This point is further strengthened by our results showing that every overlapping neurite contributes to the overall electrical coupling between two neurons. When a fraction of the overlapping neurites between two neurons was cut, a similar fraction of the electrical coupling disappeared (data not shown). Both the ultrastructural and the electrophysiological evidence point to an equal competence of each neurite to form an electrical synapse.

Can sibling neurites form reflexive gap junctions?

The data presented above strongly suggest that reflexive gap junctions are not normally found between sibling neurites. Given the capricious nature of the fracture plane in freeze fracture, it is possible that a rare reflexive gap junction might have been missed. However, none of the replicas from 50 different individual isolated neurons showed evidence for any gap junction plaques. In addition, none of the neurons participating in electrical coupling via neurites from one side of the cell body showed evidence for any gap junction plaques between sibling neurites from the other side of the cell body. These observations make it unlikely that reflexive gap junctions are present to any functionally significant degree.

It is also possible that contact with a nonsibling neurite could induce a general competence for the formation of gap junctions, including reflexive gap junctions. Our data cannot completely rule out the possibility that reflexive gap junctions could form in the vicinity of gap junctions between nonsibling neurites. However, we have never found gap junction plaques between two neurites that could be unambiguously traced back to the same cell body (with the exception of the isolated growth cone experiments). All overlapping regions that could be traced back to the same cell body were without gap junctions. Finally, gap

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Figure 4. Fura-2 demonstration of communication between isolated and attached growth cones from the same cell (neuron B5). *A,* A drawing of the experimental preparation is shown on the *right.* A B5 neuron was plated in isolated cell culture. The neuron was injected with fura-2 (K^+ salt). One of the growth cones was physically isolated. New outgrowth from the neurite stump overlapped the isolated growth cone within 60 min. Gray-scale calcium maps (*left*) show calcium concentrations (range, 50–600 nM) of the isolated and the attached growth cone before (*top*), during (*middle*), and 5 min after (*bottom*) stimulation. The cell body was penetrated before any of the images were acquired. The stimulation consisted of 60 depolarizing current pulses (1 Hz) sufficient to fire an action potential within the cell body. Scale bar, 20 μm. *B,* Averages from three similar experiments showing that calcium levels rose to similar amounts in attached and in isolated growth cones immediately following stimulation of the cell body. Two to four growth cones of each type were measured in each experiment; the error bars indicate the SEM.

junctions were seen only in the area of overlap between neurites from two different neurons; other regions of the culture (where neurites from only one neuron were to be found) were completely devoid of gap junction plaques. Since ultrastructural techniques can only identify full gap junction plaques, the question of whether sibling neurites normally have some linked, single gap channels must remain unanswered for the present. Nonetheless, these data clearly support the suggestion that sibling neurites do not normally form reflexive gap junctions.

Isolated growth cones can form electrical connections

Newly generated growth cones were allowed to overlap recently isolated growth cones. Fura-2 calcium imaging showed that stimulation of the cell body evoked calcium increases both in the attached growth cones and in the isolated but overlapped growth cones. This optical/electrophysiological procedure was developed to assay coupling between small, fragile, isolated processes. To eliminate the possibility that the connections were chemical, a similar preparation was used with two-electrode patch recording. The results clearly showed that *isolated* parts of a single neuron could form electrical synapses with other parts of the same neuron. Examination of freeze-fracture replicas from similar preparations showed the unequivocal presence of gap junction plaques. Therefore, sibling neurites can form electrical synapses with each other *if they have been physically disconnected from each other.*

What underlies this self-recognition–induced inhibition of electrical synapse formation?

Our results give some suggestions about the mechanism(s) that inhibits electrical coupling between sibling neurites. Each neurite is inherently competent to form electrical synapses. In addition, electrical coupling between isolated sibling neurites developed rapidly after overlap. Since overlap occurred as early as 15 min or as late as 24 hr after isolation, the mechanism that underlies the negation of the normal self-recognition must be both rapid in onset and long lasting in duration.

The rapid onset of electrical coupling is not unusual since many investigations have shown that electrical coupling can be a rapid event, with onset of detectable coupling occurring within 2–20 min after membrane contact (Sheridan, 1971; Rash and Fambrough, 1973; DeHaan and Hirakow, 1982). The rapid onset of coupling does suggest that *de novo* synthesis of new connexin proteins is unlikely to be involved. Although isolated *Helisoma* growth cones are capable of synthesizing proteins (Davis et al., 1992a), the turnover rate for connexins, at least in mammalian systems, is 1.5–2 hr (Traub et al., 1989). In addition, immunohistochemical evidence suggests that a pool of precursor hemichannels can exist in the plasma membrane (Dahl et al., 1992). These data imply that connexins are present in the membrane of all neurites. Therefore, some interaction between sibling neurites must be preventing the linking of hemijunctions in the respective neurite membranes and/or the consolidation of linked junctions into full gap junction plaques.

It is unlikely that the process of isolation disables the self-recognition mechanism, and allows sibling neurites to form electrical connections. Electrical connections were seen between sibling neurites that overlapped at very different times after isolation (ranging from 15 min to 12 hr). Also, no reflexive gap junction plaques were seen on the control (uncut) side of neurons. The direct depolarization of single neurons with 34 mM K^+, a treatment that results in substantial increases in cytoplasmic calcium concentrations and mimics some of the effects seen upon neurite transection, did not result in gap junction formation.

It is most likely that cytoplasmic continuity is responsible for the self-recognition–induced inhibition of electrical synapse formation between sibling neurites. Coincident electrical activity in sibling neurites might prevent consolidation of gap junction plaques. It is possible that a difference in transjunctional potential must exist between the internal faces of the two hemijunction proteins for them to link and form a functional channel. Some connexins can sense the transmembrane potential (Barrio et al., 1991); a difference in transmembrane potential for the two hemichannels might be needed to stabilize an intact gap junction channel.

Functional rationale for the inhibition of reflexive gap junction formation

Reflexive gap junctions would be likely to serve roles similar to those served by gap junctions between different neurons: communication. If two sibling neurites were present in the same region of the neuropil, they are likely to be electrically close, so reflexive gap junctions would have little effect on the electrical communication between the neurites. If the two neurites were electrically distant from each other, the neurites would represent separate information processing areas; in this case, reflexive gap junctions would prevent the separate processing of inputs, and might interfere with proper function of the neuron. Therefore, there is little reason to need, and several reasons to avoid, establishing communication between sibling neurites. The self-recognition–dependent inhibition of reflexive gap junction formation provides another tool to restrict the formation of connections within the nervous system.

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