Calcium Wave Fronts that Cross Gap Junctions May Signal Neuronal Death during Development

Laura R. Wolszon,¹ Vincent Rehder,^{2,a} Stanley B. Kater,² and Eduardo R. Macagno¹

¹Department of Biological Sciences, Columbia University, New York, New York 10027 and ²Program in Neuronal Growth and Development, Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, Colorado 80523

Embryonic anterior pagoda (AP) neurons in the leech interact with their segmental homologs in adjacent ganglia through transient axons that overlap during a critical period of development and then retract. However, when an AP neuron is ablated mechanically or by irradiation during this period, an adjacent homolog responds by reinitiating growth of its overlapped axon and thereby taking over vacated territory (Gao and Macagno, 1987b; Gao, 1989). The death of an AP cell is therefore communicated to its homolog, but the mechanism underlying this signaling is presently unknown.

Since it was recently found that AP homologs are electrically and dye coupled through their transient axons (Wolszon et al., 1994), we investigated the possibility that gap junctions may mediate the cell death signal that could occur between developing neurons. Among several candidate intercellular signals, we began by studying calcium dynamics in embryonic AP cells, *in situ*, since calcium is known to cross gap junctions and is implicated in cell death in many systems. We found that elements that usually increase $[Ca^{2+}]$, in adult neurons, such as releasable internal stores or voltage-dependent calcium channels, were not present at the critical period. Instead, mechanisms that *reduce* free calcium, such as buffering and pumping, were the most robust. When a large, focal calcium rise was produced in an AP axon by making a lesion with a UV microbeam (leading to eventual death of these neurons), calcium did not rise quickly throughout the cell, but rather moved in a slow (0.05–0.25 $\mu\text{m}/\text{sec}$) wave front away from the lesion site, into other processes of the damaged cell. Furthermore, when a calcium wave front reached the growth cone of a transient axon, it *crossed at the gap junctions into the coupled axon of the neighboring AP neuron*, but went no further. Since it is known that an AP responds to a neighbor's death by reinitiating growth only in that axon that contacts the dying cell (Gao and Macagno, 1987b; Gao, 1989), these observations are consistent with

calcium playing a role in the signaling of cell death to homologs that are coupled to a dying cell.

[Key words: *Hirudo medicinalis*, growth cone, homeostasis, gap junctions, cell death]

Although much is now known about cell death during the development of the nervous system (reviewed in Oppenheim et al., 1992), one of the most important but least understood aspects is how death of a neuron affects the cells in contact with it. Whether neuronal death occurs naturally or by experimental means, adjacent cells can change their differentiation or fate radically in response to loss of a nearby neighbor. A key step in understanding this process is to discover how a dying neuron communicates its death to the cells around it, a necessary prelude to any cellular response. We address that issue here, using the developing, intact leech as a model system.

Recent work in our laboratory has focused on the development of segmentally iterated leech neurons called the “anterior pagoda” (AP) cells, found as bilateral pairs in 20 of the 21 segmental ganglia of the CNS. Their function is unknown, but their distributed innervation of the body wall suggests a role in modulation of sensory input (W. B. Gan, H. Wang, and E. R. Macagno, unpublished observations). These neurons use a developmental strategy that is commonly employed in many vertebrate and invertebrate systems to ensure complete target innervation, in that they initially extend more axons than they will retain in the adult. Therefore, embryonic APs that lie on the same side of the animal, but in adjacent ganglia, extend transient longitudinal axons toward each other that overlap extensively (Fig. 1A, left). These transient axons interact for a critical period of 5–8 d, inhibiting each other's further outgrowth by an as yet unknown mechanism, until they finally retract (Fig. 1A, center; Gao and Macagno, 1987b). Other developing leech neurons exhibit a similar behavior (Gao and Macagno, 1987a; McGlade-McCulloh and Muller, 1989).

Interestingly, the function of the transient AP projections seems to be to seek out neighboring AP cells, in order to determine how much territory to innervate. Gao and Macagno found that killing one AP cell in an intact embryo, either by manual decapitation or by photoablation (Miller and Selverston, 1979), results in a coupled (previously inhibited) axon reinitiating growth. Only the particular axon that contacts the dying cell actually responds to the “distress” signal sent by the AP. The axon begins to take over vacated territory within 10–12 hr (Fig. 1A, right) and is maintained through adulthood (Gao and Macagno, 1987b; Gao, 1989). How does this coupled AP axon learn of its neighbor's death?

Received Sept. 14, 1993; revised Nov. 4, 1993; accepted Nov. 24, 1993.

We are grateful to Dr. William Roberts (University of Oregon, Institute of Neuroscience) for enlightening discussions and comments on an earlier version of the manuscript, and Nik Necles for assistance with the photography. This work was supported by NIH Grant NS20336 and NSF Grant BNS-8819970 to E.R.M. and an NRSA fellowship to L.R.W.

Address correspondence to Dr. Laura R. Wolszon, Columbia University, Department of Biological Sciences, 1003 Sherman-Fairchild Center, New York, NY 10027.

^aPresent address: Department of Biology, Georgia State University, P.O. Box 4010, Atlanta, GA 30302-4010.

Copyright © 1994 Society for Neuroscience 0270-6474/94/143437-12\$05.00/0

Figure 1. *A*, Schematic diagrams of AP cells in the leech *Hirudo medicinalis* under normal and experimental conditions. *Left*, Two adjacent AP cells at E10–E14. The APs are found as bilateral pairs in 20 of the 21 midbody ganglia (MG) of the CNS. Each AP's primary neurite crosses the midline within the ganglion and branches, sending two axons out the contralateral roots, and one projection partway into each of the anterior and posterior contralateral connective nerves (a mirror-image AP homolog resides on the opposite side of each ganglion but only its soma is illustrated,

Since it was recently found that transient AP axons communicate with each other via gap junctions (Wolszon et al., 1994), we can ask whether a dying neuron signals its death to its neighbor via the gap junctions that connect them. Such a mechanism would have the advantage of ensuring that only particular cells (or parts of a cell) would respond to that death. There are several developing systems in which it is thought that gap junctions allow the passage of molecular signals between cells, including chick (Allen et al., 1990), *Hydra* (Fraser et al., 1987), *Xenopus* (Warner et al., 1984), and mouse (Lee et al., 1987; Bevilacqua et al., 1989) embryos (reviewed in Warner, 1992). In the *Daphnia* visual system, pioneer axons form transient gap junctions with undifferentiated neural precursors near the midplane (LoPresti et al., 1974), and it was suggested that a molecular signal might cross the junctions to initiate neuronal differentiation. Gap junctional signaling was also postulated for developing grasshopper pioneer neurons studied *in situ*, which dye-couple to "guidepost" neurons in the CNS and in the periphery (Taghert et al., 1982); studies using the calcium indicator fura-2 suggest that calcium levels in these axons can change after coupling to guidepost cells (Bentley et al., 1991). However, in no case is the identity of any molecular or ionic signal actually known, and further, the question of whether gap junctions can mediate the communication of cell death during development has not been addressed, to our knowledge.

There are several potential signaling molecules that can cross gap junctions. These include calcium, nucleotides, cAMP, and Ins[1,4,5]P₃ (discussed in Saez et al., 1989). While our eventual goal is to test several of these candidates, we began by investigating the passage of calcium between developing AP neurons, using the same ablation conditions as in previous developmental studies (Gao and Macagno, 1987b; Gao, 1989). Calcium has the practical advantage of there being reliable calcium indicators available for its measurement. More importantly, calcium has been implicated in cell death in several neuronal and non-neuronal systems (reviewed in Choi, 1992).

Materials and Methods

Leech embryos were obtained from our laboratory colony of *Hirudo medicinalis*. Their maintenance and staging were as described in Gao and Macagno (1987a), except that they were kept at a constant temperature of 23°C. Embryogenesis lasts 30 d at this temperature. Although many neurons begin extending axons on or before E7, we found that

E11–E12 were the youngest ages at which neurons could be clearly identified and impaled without significant damage.

General dissection techniques. Embryos were anesthetized by immersing them in cold (4°C), sterile, dilute Instant Ocean solution (0.5 gm/liter; Menasha Corporation) for 10 min. The cryptolarval membrane (yolk sac) was split along the anterior-posterior axis with fine forceps to remove the yolk, after which the embryo was transferred to sterile Wenning's saline [40 mM DL-malic acid (Sigma; free acid form, neutralized to pH 7.0 with NaOH), 4 mM KCl, 10 mM disodium succinic acid (Sigma), 10 mM Tris-base, 1.8 mM CaCl₂, and pH adjusted to 7.4 with NaOH; Wenning, 1987] on a Sylgard-coated (Dow-Corning) microscope slide. The embryo was pinned to the slide, ventral side up, using 001 tungsten pins (American Wire Industries), stretching the elastic membrane along the longitudinal and lateral axes in order to minimize movement during filling. An incision was then made in the body wall over 4–10 adjacent midbody ganglia using a sharpened tungsten pin, and any remaining blood sinus cleared from the surface of the ganglia (Fig. 1B).

Injection of fura-2. The preparation was transferred to an electrophysiological recording rig into which was incorporated a microscope with a 40× water-immersion lens and epifluorescence optics. To accommodate the 1.7 mm working distance of this objective, electrode tips were bent to a 45° angle, using a heated nichrome wire.

Electrodes were backfilled first with 30 mM fura-2 (pentapotassium salt; Molecular Probes) in dH₂O and then with 0.1 M KCl. Good cell fills were obtained with 1–1.25 nA of negative current (200 msec at 2 Hz) for 1–2 min; higher currents of 1.5–5 nA or longer injection times of 3–6 min did not improve the quality of the fills, and sometimes resulted in damage to the neurons. A new electrode was used for each subsequent injection. Care was taken to ensure that the dye in the electrode and in the cell was not exposed to UV or blue light, which bleaches the dye (Becker and Fay, 1987).

Injected cells were allowed to stabilize for at least 30 min before measuring fura-2 fluorescence. We found that washing the preparation with cold saline every 5–10 min prolonged the health of the neurons.

Preparation of the embryo for imaging. To use an inverted microscope with a 100× short-working-distance objective for imaging growth cones *in situ*, we developed a preparation that allowed us to view the intact nervous system dorsal side down through a thin (thickness 0) coverslip (Fig. 1C). After filling neurons with fura-2, the embryo was unpinned and then repinned dorsal side up in room temperature Wenning's saline with 7% EtOH (colder temperatures produced muscle contractions). The endoderm was then removed from the dorsal surface by teasing gently with fine forceps since the underlying tissue sticks well to coated glass. The three most anterior and posterior segments (of 21) were then removed to prevent excessive contractions when mounting and imaging the embryo. The pins were again removed and the preparation transferred to a plastic dish whose bottom had been replaced with an affixed polylysine-coated coverslip, and which contained room-temperature Wenning's saline with 7% EtOH. The embryo was stretched slightly and then placed ventral side up on the surface of the glass (Fig. 1C). The nervous system was particularly adherent and did not move once it

for clarity). The longitudinal projections from adjacent AP cells therefore overlap and interact with each other within the connective nerve, beginning on or before E10. It should be noted here that although we sometimes refer to a longitudinal projection as an "axon," each projection generally consists of two or three axons that grow along parallel paths in one connective nerve (Gao, 1989). Each of these axons has a growth cone and interacts with one or more axons from the longitudinal projection of the neighboring AP homolog. *Middle*, At E15–E18, the longitudinal projections begin to retract and finally disappear, although the lateral root projections persist into adulthood and innervate the periphery, where they divide up territory with APs from adjacent ganglia. *Right*, When an AP cell is killed (X) during the period that its longitudinal axons are overlapping with those of its neighbors, the neighboring APs respond to its death. Instead of retracting their own longitudinal projections, they reinitiate growth, *only in those axons facing the dying cell*, finally taking over territory vacated by the killed cell (Gao and Macagno, 1987b; Gao, 1989). *B and C*, Dark-field photomicrographs of *Hirudo medicinalis* embryos at E11. The cryptolarval membrane was opened along the dorsal midline to remove the yolk it envelops, and then the embryo was flattened by pinning it to a Sylgard dish (see Materials and Methods). Anterior is toward the top, and the ventral surface faces the viewer. *B*, The cryptolarval membrane (CM, arrow) was stretched with tungsten pins to minimize movement of the germinal plate (GP), the left edge of which is indicated by the arrowhead. The chain of 21 segmental ganglia is visible through the body wall, but a length of the body wall was opened above the CNS to allow better visibility and electrode penetration for the fura-2 dye fills. Seven ganglia were exposed in this preparation. The head (H) and tail (T) suckers are already being formed at this age. Development proceeds in an anterior-to-posterior direction, the germinal plate widening and elongating until it completely surrounds the yolk sac and zippers along the dorsal midline. *C*, A higher-power view of the embryo in *B*, after trimming and transfer to a polylysine-coated coverslip, used for fura-2 imaging (see Materials and Methods). The position of midbody ganglion 10 (MG10) is indicated by the arrowhead. Individual ganglia are connected to each other via a pair of connective nerves (CN, arrow), which contain the axonal projections from the AP cells imaged for these experiments. The ganglia are also connected to the periphery via two pairs of lateral root nerves that are obscured here by the body wall. Note the transparency of the CNS, and that even at this magnification, one can begin to distinguish individual somata. Scale bar: 350 μm for *B*, 75 μm for *C*.

A

B

Fig. 2. Continued.

made contact with the coverslip. The dish was then transferred to a Zeiss ICM405 inverted microscope and individual ganglia of the embryonic CNS were located using Nomarski optics and a 20× Nikon Fluor objective.

Calcium quantification using fura-2. Imaging was carried out at room temperature as described in Bentley et al. (1991), except that we used 30 mM fura-2 and a 100× Zeiss NeoFluar oil-immersion objective. Fluorescence images were acquired with a Quantex imaging system (QX7-210, Quantex, Santa Clara, CA) and a Quantex intensified CCD camera. Measurements were made of emission intensities, using a 495 nm long-pass emission filter, when light of 350 ± 10 nm (fura-2 bound to calcium) or 380 ± 10 nm (fura-2 free of calcium) excited the dye; these values are reported as $A_{350}:A_{380}$ ratios. Using these ratios, intracellular Ca^{2+} concentrations were estimated from the equation

$$[Ca^{2+}] = K_D \cdot [(R - R_{min}) / (R_{max} - R)] \cdot (F_0/F_s)$$

(Gryniewicz et al., 1985) after calibrating the system using known amounts of Ca^{2+} . For our calibration conditions, K_D (the dissociation constant of fura-2 under these conditions) = 224 nM, R_{max} (the $A_{350}:A_{380}$ ratio at saturating $[Ca^{2+}]_i$) = 13.87, R_{min} (the ratio at zero Ca) = 0.7, and (F_0/F_s) (the A_{380} value of zero Ca divided by that of a saturating $[Ca^{2+}]_i$) = 15.79.

Due to differences occurring when calibrating in a test tube compared with calibrating *in situ*, we used the $A_{350}:A_{380}$ ratios in order to quantify changes in $[Ca^{2+}]_i$ instead of reporting absolute calcium concentrations.

In order to provide the reader with a general idea of the $[Ca^{2+}]_i$ reflected in these ratios, absolute calcium levels, derived from the above equation, are occasionally provided with the ratioed values.

To compare identical experiments, changes in ratios are expressed as *percentage change* compared to pretreatment values, with starting values being provided in the figure legends. It should be noted here that the *percentage change* in a ratio will often appear much smaller than the equivalent *percentage change* in $[Ca^{2+}]_i$, as there is a nonlinear relationship between these parameters.

Manipulation of intracellular calcium levels. In some neurons we damaged the cell membrane to allow calcium influx from the bath (Guthrie et al., 1991). For these “zaps” we removed all input filters and focused the UV beam to a 10-μm-diameter spot that was then directed at the process. This procedure did not compromise the sensitivity of fura-2 in the areas not directly exposed to the UV beam, since the raw (non-ratioed) fluorescence of the dye was unchanged. The best zaps were those in which the local $[Ca^{2+}]_i$ immediately rose to high values (several times above baseline levels) while other parts of the cell remained normal. This usually occurred after irradiating for 30 sec, although it could sometimes be achieved with zaps of 10 sec. Zaps of longer than 1 min usually irreversibly damaged the neurons.

For those experiments in which we observed small or no increases in $[Ca^{2+}]_i$, we verified the integrity of the fura-2 dye at the end of each experiment by either zapping the cells or by applying ionophore. Large increases in the $A_{350}:A_{380}$ ratios in response to these treatments indicated that the fura-2 was functioning properly. As controls for $[Ca^{2+}]_i$ increases caused by experimental manipulations, we also monitored untreated, fura-2-filled cells in the same embryo to ensure that increases in ratios were not due to a general deterioration of the preparation.

Results

Calcium homeostasis in developing AP neurons—a highly stabilized system

Resting calcium distribution. Calcium measurements were made on those parts of AP cells that could be easily and repeatedly identified: the soma, the primary neurite, the neuropilar fork (near the sites of synaptic contact within the ganglion), the lateral roots, and the anterior and posterior growth cones. We found that calcium was distributed homogeneously within these neurons under resting conditions, as illustrated in Table 1. The only part of the cell that was statistically different from the others was the soma (significant values indicated by an asterisk; $P < 0.01$, ANOVA), the site of dye injection, which may have been damaged slightly in some experiments by the fura-2 injections. This was not the case for the cell of Figure 2A, however, in which the resting levels of $[Ca^{2+}]_i$ in various parts of one AP neuron are illustrated. Although these images were taken in the intact CNS within the embryo, most features of the neuron were clearly discernible and well focused (a Lucifer yellow fill of an AP in a dissected and cleared embryonic ganglion is shown in the inset for comparison).

Embryonic AP neurons do not have detectable voltage-dependent calcium signals at E11–E12. One way by which neurons

Figure 2. The production of a calcium wave. *A*, Resting levels of $[Ca^{2+}]_i$ are indicated by levels of fura-2 fluorescence in an E11 AP neuron. The figure is a composite of several images taken of the soma, primary neurite, neuropilar fork, lateral roots, and anterior and posterior growth cones. Resting calcium levels are about 80 nM and are equivalent for various parts of the cell (see text). The inset is a Lucifer yellow fill of part of an E11 neuron to allow comparison with the fura-2 images. The arrow indicates the location of the zap that produced the calcium wave in the panel below. Anterior is to the right. *B*, Images of the neuropilar fork taken before (0) and at various times after zapping the posterior lateral root at the site indicated in *A*. Note how the calcium wave front gradually moved into the neuropil from the periphery: at 23' it progressed so far that the frame had to be shifted to the right in order to follow it. The wave front continued to move until 35', at which time the solution was changed to one containing no added calcium but 1.8 mM magnesium. Within 5', the wave front receded until at 21' the neuropil returned to nearly baseline values. The color scale refers to pseudocolor images and gives calculated calcium nanomolar concentrations. Scale bar, 10 μm for the fura-2 images. *C*, A plot of the changes in the fluorescence ratio (as percentage) versus time for the experiment illustrated in *B*. Ratios were fairly constant until after the zap, which produced larger increases in those parts of the cell closer to the lesion. Starting ratios: 0.91 (equivalent to 57 nM Ca^{2+} ; position *A*), 0.90 (55 nM Ca^{2+} ; *B*), and 0.86 (44 nM Ca^{2+} ; *C*). *D*, Decapitation also produced a calcium gradient by allowing a transient influx from the bath, followed by resealing of the membrane. Note again that sites closer to the (missing) soma had the largest increases. Starting ratios: 1.16 (equivalent to 128 nM Ca^{2+} ; *A*), 1.23 (149 nM Ca^{2+} ; *B*), 1.17 (132 nM Ca^{2+} ; *C*), 1.17 (132 nM Ca^{2+} ; *D*), and 1.20 (139 nM Ca^{2+} ; *E*).

Table 1. Resting calcium levels within AP cells

Location	<i>n</i>	$A_{350} : A_{380}$ (mean $\pm$ SD)	[Ca ²⁺] _i (nM) (mean $\pm$ SD)
Soma	68	1.241* $\pm$ 0.465	156.9* $\pm$ 147.3
Prim. neurite	13	0.982 $\pm$ 0.142	77.9 $\pm$ 40.1
Fork	71	1.032 $\pm$ 0.237	92.8 $\pm$ 73.8
Ant. root	13	0.942 $\pm$ 0.154	66.6 $\pm$ 42.8
Post. root	22	0.987 $\pm$ 0.158	79.2 $\pm$ 44.9
Ant. GC	61	0.941 $\pm$ 0.139	66.3 $\pm$ 39.2
Post. GC	57	0.956 $\pm$ 0.167	70.8 $\pm$ 48.4

Values are given as both ratios and absolute calcium concentrations, the former being more reliable due to calibration considerations (see Materials and Methods). Ant., anterior; post., posterior; GC, growth cone.

* Statistical significance ($p < 0.01$, ANOVA).

can elevate [Ca²⁺]_i is by generating currents through voltage-sensitive calcium channels. To determine whether AP cells can respond to depolarization with an increase in [Ca²⁺]_i, we applied medium with elevated KCl to the preparation while monitoring ratioed images of fura-2 fluorescence. A 10-fold increase in potassium (20 mM KCl) did not cause detectable increases in [Ca²⁺]_i, and even 50 mM KCl had little or no effect: in all 10 experiments in which calcium levels were measured from 20 sec to tens of minutes after application of 50 mM KCl, fura-2 ratios increased by 6% or less and remained at those levels for the duration of the experiment. Similarly, prehyperpolarization of the cells with zero-KCl solution ($n = 2$) did not result in an increase in [Ca²⁺]_i upon switching to 50 mM KCl. Calcium distributions remained uniform throughout the neurons and did not change significantly upon returning to normal saline.

To verify that AP cells can be depolarized by 50 mM KCl, electrophysiological recordings were made under identical conditions ($n = 3$) and in all cases the cells were rapidly and reversibly depolarized to -2.7 ± 5.1 mV from their normal resting level of -20 ± 4.7 mV (mean $\pm$ SD).

The lack of a detectable voltage-dependent calcium signal was not a characteristic unique to the AP cells, as three sensory ("N") neurons were also tested for their responsiveness to depolarization and their calcium levels were found to be insensitive to 50 mM KCl as well.

Embryonic AP cells contain negligible releasable pools of calcium. Calcium signals in many systems can be propagated or enhanced by a regenerative release of calcium from internal stores (see Discussion). To determine whether such stores exist in these embryonic neurons, we tested whether caffeine, an activator of one of the most prominent stores (e.g., Bandtlow et al., 1993) and 4-Br-A23187, an ionophore that causes calcium flux across all types of cell membranes (Deber et al., 1985), could elevate [Ca²⁺]_i in AP neurons. In three experiments in which caffeine was applied at 25 mM, 37.5 mM, and 50 mM concentrations while monitoring [Ca²⁺]_i in AP cells, no increase in [Ca²⁺]_i was seen. To test for the generality of this finding, caffeine was applied to another neuron, the HAL cell (Stewart et al., 1991), and this neuron was also unaffected. We conclude that there are no detectable caffeine-sensitive calcium stores in these neurons at this stage of development. The results employing the calcium ionophore 4-Br-A23187, which is incorporated into the plasma membrane (permitting influx from the extracellular medium) as well as into intracellular organelles (Deber et al., 1985), depended on which part of the cell was

measured. When we tested high concentrations (1.5–3 μ M) of this agent in normal (1.8 mM Ca²⁺) saline ($n = 3$), the ratios taken from the soma rose an average of 134 ± 146 ($\pm$ SD) percentage within about 12 min, whereas those in the processes of the cell, such as the neuropil and growth cones, rose much more ($526 \pm 212\%$). Therefore, it appeared at first glance that the soma had a greater ability to buffer large rises in [Ca²⁺]_i, than did the small processes. However, since processes have higher surface:volume ratios than the somata, more ionophore per unit volume would be inserted into their membranes, causing higher calcium concentrations. Indeed, when the same tests were done after applying zero-Ca saline to the preparation ($n = 2$), thereby nearly eliminating calcium influx from the bath, the increases in the somata were comparable (112% and 16%), whereas there was no increase (zero and 3%) in the processes. Taken together, these results suggest that there are no significant calcium stores in the AP axons and growth cones at this stage of development (E11), and those that exist in the soma are very weak.

Generation of a slow calcium wave front within embryonic neurons. Since we wished to determine whether calcium could act as a signal of cell death, the next series of experiments was designed to test whether cell ablation conditions (of either mechanical or photoablation) could generate such a signal *in situ*. To this end, we adjusted a UV microbeam to a diameter of approximately 10 μ m and focused it on a particular region of a fura-2-filled cell. We then irradiated ("zapped") the neuron with this narrow beam for 30 sec, allowing a calcium influx through the damaged membrane; the movement of calcium from that site was then monitored. In spite of the strong intracellular calcium buffering, this influx initiated an internal calcium wave front that progressively invaded other parts of the cell ($n = 31$ of 37 experiments). Figure 2 illustrates one such experiment, in which the posterior root was zapped at the point indicated by the arrow in *A*. Images taken of the neuropilar fork (Fig. 2*B*) show the entry of calcium into this region and the progression of the calcium wave front. When the preparation was then changed into medium containing no calcium and 1.8 mM magnesium, the elevated [Ca²⁺]_i dissipated quickly. A graph of these data is shown in Figure 2*C*, in which fura-2 ratios are plotted as a function of time; in parts of the neuropil the ratio rose as high as 225% of control within 25 min of the zap.

We next tested whether a calcium wave front could be generated by mechanically severing the soma from the rest of the cell, the first method of cell ablation used by Gao and Macagno (1987a,b) to demonstrate the reinitiation of growth by a stopped axon. As seen in Figure 2*D*, within minutes of decapitation, a calcium wave front from the "neck" of the axon moved into the primary neurite and progressed into the neuropilar fork. The stump of the axon resealed relatively quickly, preventing protracted calcium entry, since [Ca²⁺]_i peaked and then began to decline. Notice the progression of the wave front, in that the anterior root (position E) rose slightly sooner than did the second bifurcation of the fork (position C). The results of these experiments are consistent with the idea that a calcium transient resulting from decapitation of an AP neuron might be involved in signaling its death to neighboring APs.

Wave fronts typically advanced at a rate of 0.05–0.25 μ m/sec, much slower than can be accounted for by unhindered diffusion, suggesting that there is considerable calcium buffering and/or pumping in these cells (see Discussion). Indeed, in the remaining 6 of the 37 experiments, we observed a standing wave front with no movement of the calcium gradient through the

cell. We interpret this finding as reflecting a balance between the amount of calcium that entered before membrane resealing, and the amount buffered or extruded by the cell.

Initiation of the wave front requires extracellular calcium. Since there were no significant internal calcium stores or detectable voltage-dependent calcium signals, we next tested whether the generation of the calcium wave front was dependent on calcium influx from the extracellular medium. To this end, AP cells were zapped in medium in which calcium was replaced with magnesium and to which 1 mM EGTA was added. The zaps produced no significant change in the ratio until the preparations were put back into normal medium ($n = 3$ of 3), indicating that the initiation of the wave front is indeed dependent on calcium influx.

Bathing the preparation in medium containing no calcium caused a uniform decline in $[Ca^{2+}]_i$ throughout the cells (as in the experiment of Fig. 2A–C), even when the neurons were not zapped. In six experiments, the ratios decreased $15.2 \pm 4.2\%$ (mean $\pm$ SD) within 9–21 min, and in five more experiments, ratios dropped $17.4 \pm 9.9\%$ within 35 min. An example is shown in Figure 3, in which an AP cell was placed in zero-Ca medium while fura-2 fluorescence was monitored. Notice the gradual decrease in the ratio with time, until the preparation was returned to normal medium. Subsequent addition of $3 \mu M$ 4-Br-A23187 to the medium (as in Fig. 3) indicated clearly that the decrease in fura-2 ratios in zero Ca was not due to compromise of the indicator.

Calcium signals pass between transiently coupled neurons

The next series of experiments focused on the pivotal issue of whether a calcium wave front initiated by photoablation, which is known to alter AP cell morphology (Gao, 1989), could pass from the damaged AP to its neighbor. To address this question, we filled two adjacent AP cells with fura-2 ($n = 6$), or, instead, filled one with fura-2 and one with rhodamine dextran-amine (RDA; $n = 1$). One of the two cells was then zapped in order to generate a calcium wave front, and the progress of this wave front was monitored (except in the case of the RDA-filled cell), paying particular attention to the $[Ca^{2+}]_i$ in the growth cone of the neighboring AP. We found that in six of seven cases, the calcium wave front did, indeed, cross into the neighboring cell.

In the experiment illustrated in Figure 4A, both AP cells were filled with fura-2. Their resting $[Ca^{2+}]_i$ was normal, though not identical, before the zap (time = T_0). In order to generate a wave front, the posterior AP neuron was zapped at the primary neurite within the neuropil, as indicated in the schematic diagram on the left. Fura-2 fluorescence images on the right show how the calcium wave front resulting from this zap moved anteriorly, toward the neighboring AP cell. By 130 min after the zap, the wave front not only crossed into the neighbor's projection (referred to here as the "receiver"), but had advanced almost to the margin of the next ganglion, where it stopped. Note how the rightmost of the receiver's two processes near the ganglionic margin had more calcium transferred to it than the other, suggesting that the signaling may have been stronger at this junction.

Another example of a wave front transfer between adjacent AP homologs is shown graphically in Figure 4B, in which the increases in fura-2 ratios are plotted as a function of time for different locations within the connective nerve; this is our most robust example of calcium transfer. The wave front gradually

Figure 3. The effect of zero Ca plus 1 mM EGTA on fluorescence ratios. Zero Ca caused a gradual decrease in ratios until the preparation was returned to normal saline. Before complete recovery, the integrity of the fura-2 was measured by adding $3 \mu M$ 4-Br-A23187 to the solution. Values went so high as to be off the scale for these axes. The final percentage increases in the ratios are indicated next to each plot, with the corresponding time in parentheses; for example, the posterior growth cone (diamonds) rose 733% at the 71 min time point (8 min after A23187 application); this corresponds to a percentage increase in $[Ca^{2+}]_i$ of over 5300%. Starting values: 0.96 (equivalent to 71 nM Ca^{2+} ; soma), 1.03 (91 nM Ca^{2+} ; neuropil), 0.89 (52 nM Ca^{2+} ; anterior growth cone), and 0.91 (57 nM Ca^{2+} ; posterior growth cone).

progressed from one cell to the other, again stopping before it reached the next ganglion.

Transfer of the wave front between neurons could occur in either direction, from anterior to posterior or *vice versa*, depending on which homolog was zapped, and could also occur when the zapped cell was filled with RDA.

In all six positive cases, the increases in $[Ca^{2+}]_i$ in the receiver were confined to the processes that contacted the dying cells, never entering even the neuropil or lateral roots, for the duration of the experiments (about 3 hr). This observation may be important in that Gao and Macagno (1987b; Gao, 1989) found that only one of the neighboring cell's growth cones resumed growth when an AP was killed, namely, the one touching the dying cell, and is in agreement with other observations reported here that calcium homeostasis is strong in these neurons.

The extent and time course of calcium transfer varied significantly for some experiments. This did not appear to be correlated with the amount of calcium that entered after the zap, but could have been due to differing abilities of individual cells to control $[Ca^{2+}]_i$, or to differences in the extent of coupling between neighboring cells. Such variability makes it inappropriate to average the responses over all experiments in order to quantitate the extent of transfer. However, some general properties can be extracted from the data: (1) wave front transfer required 15–20 min after the zap before being detectable in the receiving cell, (2) the rate of increase in A_{350}/A_{380} in the receiving growth cone ranged from 0.23% to 5.4% per minute (the corresponding increases in $[Ca^{2+}]_i$ were from 0.51% to 26% per minute), and (3) the maximum increase in the receiving growth

A

T_0

T_{130}

Fig. 4. Continued.

cones ranged from 9% to 321% increase in fura-2 ratios (equivalent to a 35–3700% increase in $[Ca^{2+}]_i$).

In one experiment, we monitored the receiving growth cone for 6 min before changing to zero-Ca saline. Interestingly, calcium continued to be transferred after this solution change, peaking 6 min later; the rate of increase in A_{350}/A_{380} during this 12 min period was 1.33% per minute. As observed in the zero-Ca experiments described earlier, $[Ca^{2+}]_i$ eventually decreased all over this cell, the receiving growth cone reaching baseline values 17 min after the solution change and continuing to drop. This finding suggests that the *crossing* of the wave front is itself not dependent on calcium influx from the bath.

Discussion

We have found that calcium wave fronts were generated in embryonic AP cells, *in situ*, when using the same conditions as are required to kill an AP neuron and cause growth of an adjacent AP's axon (Gao and Macagno, 1987b; Gao, 1989). We propose that these wave fronts have two major effects: they damage irreversibly the irradiated cell, and they communicate a resultant "death signal" to a coupled homolog. It is important to point out at this time that we are not proposing that the calcium rise is *sufficient* to reinitiate growth; rather, that it signals the death of a coupled neuron, and the remaining axon may or may not respond to that signal depending on other factors.

The basis of the mutual inhibition seen between AP axons in normal embryos is unknown, and was not investigated in these experiments. It is therefore impossible to distinguish at this time between two possible causes for reinitiation of axon growth: first, that the death of the AP resulted in the removal of an inhibitory influence, and second, that a novel signal was produced that caused the coupled axon to overcome inhibition and restart growth. There is evidence in the literature for both mechanisms. In the embryonic grasshopper nervous system, an ablated neuroblast is replaced by a nearby neuroectodermal cell that is usually not fated to become a neuroblast (Doe and Goodman, 1985). It is thought that these neuroblasts normally inhibit adjacent neuroectodermal cells from becoming neuroblasts, an interaction which in *Drosophila* is thought to involve neurogenic genes (reviewed in Campos-Ortega, 1991; Greenspan, 1992). Similar conclusions were drawn after laser-ablation of somatic cells in the gonad of the nematode *Caenorhabditis elegans* (Kimble, 1981; Seydoux and Greenwald, 1989). In contrast, the generation of a novel signal by a dying cell has been shown to occur in lymphocytes, where a phosphatidylserine becomes exposed on the surface membranes of dying cells that triggers their removal by macrophages (Fadok et al., 1992).

Two experimental results in the embryonic leech argue in favor of a novel signal being generated in response to damage of an AP cell. First, laser-cutting experiments reveal that when a transient AP axon is severed from the soma, the adjacent AP's axon begins to grow again, even though the distal stump of the severed axon remains alive for at least 2 d, and presumably retains its ability to inhibit growth (W. B. Gan and E. R. Macagno, unpublished observations). Second, Gao and Macagno (1988) found that when they cut the peripheral roots of one ganglion, thereby preventing target contact by one AP but leaving all AP somata and longitudinal projections intact, both transient axons of the AP cell whose root axons were cut began to grow again, *even though the overlapping axons of neighboring AP cells were still present and still, themselves, inhibited from growing further*. Thus, the inhibitory influence of one AP axon on another can be overcome when a novel distress signal indicates that one cell is damaged.

Irradiation or decapitation of AP neurons produces calcium wave fronts

As wave fronts traveled throughout the damaged cells, very high levels of $[Ca^{2+}]_i$ were often attained, in the micromolar range or higher. A role for high levels of intracellular calcium in nerve

Figure 4. Calcium wave fronts pass between coupled neurons. *A*, The schematic diagram on the left illustrates the zap site of the posterior AP cell (curved arrow), as well as four of the 12 loci that were monitored throughout the experiment. Anterior is up. Note in the fura-2 images on the right that although the longitudinal projections of each cell consist of two parallel axons (see comment in Fig. 1*A* caption), in this preparation we were able to find a focal plane such that only one growth cone from each cell was visible, in the panel that corresponds to the region of overlap (the right growth cone belongs to the posterior cell and the left to the anterior cell). The prezap values (T_0) indicate that the sender (posterior cell) and receiver (anterior cell) were at similar resting calcium levels. After the zap (T_{130}), the sender's axon and growth cone were extremely high in $[Ca^{2+}]_i$, and the calcium wave moved into the adjacent axon via the gap junctions. Note that in the receiver, the wave reached the margin of the ganglion and then stopped (subsequent data not illustrated), suggesting that calcium levels can be controlled locally. The color scale refers to pseudocolor images and gives calculated calcium concentrations in nM. Scale bar, 10 μ m. *B*, Fura-2 ratios in another wave-crossing experiment, showing graphically the movement of calcium between cells. The schematic diagram at the top indicates the site of the zap (X) in the sender (dashed line) and the direction of movement of the calcium wave front (dotted arrow) into the receiver (solid line). Anterior is to the left. Note the temporal delays between $[Ca^{2+}]_i$ rises in adjacent segments of each projection, for example, that section B rises before C, and so on. Further, the receiving growth cone rose even higher than the sending axon, but we reserve our interpretation of this phenomenon as we did not observe it often. At most locations $[Ca^{2+}]_i$ peaked and then declined temporarily, to then continue rising for the duration of the experiment (3 hr). As in the previous example, the postsynaptic wave front was localized to that part of the process that was adjacent to the zapped cell; position F rose only slightly and G changed almost not at all. Starting values: 1.15 (equivalent to 125 nM Ca^{2+} ; A), 1.03 (91 nM Ca^{2+} ; B), 1.14 (122 nM Ca^{2+} ; C), 0.92 (60 nM Ca^{2+} ; D), 0.96 (71 nM Ca^{2+} ; E), 0.96 (71 nM Ca^{2+} ; F), and 1.03 (91 nM Ca^{2+} ; G). GC, growth cone.

cell death, possibly through the activation of calcium-dependent endonucleases, proteases, and phospholipases, is well supported by experimental evidence (see Siesjö, 1988; Choi, 1992). For example, neuronal death resulting from abnormally high levels of excitation is inhibited by agents or treatments that prevent increases in intracellular calcium (e.g., Mattson et al., 1988; Abele et al., 1990; Randall and Thayer, 1992; Tymianske et al., 1993; Wahlestedt et al., 1993), a phenomenon also observed with adult leech neurons *in vitro* (Grumbacher-Reinert and Nicholls, 1992; Neely, 1993). Since most of this evidence stems from experiments on cultured neurons, observations that show an involvement of calcium in neuronal death *in situ* are generally lacking.

Why do calcium wave fronts propagate slowly in these neurons?

Calcium wave fronts in embryonic AP cells moved at 0.05–0.25 $\mu\text{m}/\text{sec}$. These values are comparable to those observed in hippocampal pyramidal cells (0.1 $\mu\text{m}/\text{sec}$) after zapping under very similar conditions (Guthrie et al., 1991), but are much slower than the 5–200 $\mu\text{m}/\text{sec}$ calcium waves observed in virtually every other cell type measured *in vitro* (e.g., Charles et al., 1991; Finkbeiner, 1992). It is possible that this difference in rate is due, at least in part, to the fact that we were working with neurons *in situ*, which may have calcium dynamics that differ from those observed in cultured cells; indeed, even cultured leech neurons manifest different calcium responses, depending upon the substrate upon which they are plated (Ross et al., 1988).

It was surprising that 50 mM KCl did not cause a detectable calcium influx in these neurons even though the physiological recordings proved that this concentration does depolarize them, since elevated KCl causes sustained rises in $[\text{Ca}^{2+}]_i$ in a variety of other neurons (Lipscombe et al., 1988; Collins et al., 1991; Rehder and Kater, 1992). Adult AP cells in culture have significant inward calcium currents at the membrane potentials reported here (–20 to 0 mV; Stewart et al., 1989), and embryonic Retzius cells *in situ* do have a voltage-dependent calcium current at comparable ages (Schirrmacher and Deitmer, 1989). A possible explanation is that these embryonic AP cells may have a rapidly desensitizing voltage-dependent calcium conductance that ends too quickly to be seen with our methods, the temporal resolution of this calcium imaging system being limited to detecting changes in ratios on the order of seconds instead of milliseconds. However, embryonic (Schirrmacher and Deitmer, 1991) and adult (Stewart et al., 1989; Bookman and Liu, 1990) leech neurons *in vitro* have voltage-dependent calcium currents that only partially inactivate. Furthermore, considering the effects of KCl depolarization on other neurons *in vitro*, even a transient rise in $[\text{Ca}^{2+}]_i$ should be detectable in a fura-2-filled cell for several minutes (Lipscombe et al., 1988; Collins et al., 1991; Rehder and Kater, 1992). Therefore, the most plausible explanation is that AP cells do not express voltage-dependent calcium channels at this stage of development, a property that may contribute to the slowness of the wave front in these neurons.

It is also possible that the mechanism by which calcium wave fronts were generated may have influenced their speed, since the calcium distribution at the source necessarily affects the rate of diffusion. The wave fronts described in this report were initiated by allowing calcium to enter the cell at a discrete site, through a hole in the membrane produced by either spot irradiation of an AP axon or manual decapitation. Most calcium

waves studied in other systems have been generated using stimuli such as depolarization or transmitters to elicit delocalized calcium entry from the bath, or by applying caffeine or $\text{Ins}[1,4,5]\text{P}_3$ -generating agents to release calcium from internal stores (reviewed in Berridge, 1990; Rink and Merritt, 1990; Cheek, 1991; Henzi and MacDermott, 1992; Lytton and Nigam, 1992). However, Sanderson and colleagues initiated waves in epithelial cells (Sanderson et al., 1990), glial cells (Charles et al., 1991), and glioma cells transfected with connexin 43 cDNA (Charles et al., 1992) using mechanical deformation of the cell membrane, leading to the production of calcium waves in neighboring cells. These waves had propagation rates that were very similar to those produced through other means (20–30 $\mu\text{m}/\text{sec}$). The method of wave generation, therefore, does not appear to be strongly correlated with wave speed.

We conclude, therefore, that wave fronts in developing APs move slowly because these cells have strong buffering and extrusion systems, and perhaps more importantly, almost no calcium-enhancing mechanisms such as voltage-dependent calcium channels or releasable internal stores. Since the latter can act as feed-forward calcium sources and thereby increase the rate of wave front propagation, their absence results in calcium wave fronts traveling slowly in these neurons when compared with rates in other cell types.

The slow passage of calcium signals from a dying AP neuron through gap junctions provides a spatially restricted distress signal

The characteristic homeostatic properties of AP neurons at this age—in particular, the lack of voltage-dependent calcium channels and cytoplasmic calcium stores—are very well suited to confine death to a particular cell and to provide specificity in the response of neighboring neurons. First, the slow and controlled movement of the wave front in the damaged cell probably prevents the immediate uncoupling of gap junctions by abnormally high calcium that is observed in some *in vitro* systems (reviewed in Bennett and Verselis, 1992); this allows signals to cross the gap junctions into the neighboring AP before uncoupling can occur. Second, the lack of releasable calcium stores ensures that the distress signal is confined to the *coupled axon* of the receiving cell, consistent with the observation by Gao and Macagno (1987b) that only this projection reinitiates growth, all others being unaffected. A third way these homeostatic properties provide specificity in the response is by ensuring that the receiving cell does not respond to calcium signals under the wrong circumstances, for example, after repetitive activation. [The latter idea depends upon calcium signals actually being *sufficient* to reinitiate the growth of the coupled AP axon (for review, see Kater and Mills, 1991; Franklin and Johnson, 1992), which was not specifically addressed in the experiments described here.] It is possible that at later stages of development, when we know that AP axons become incapable of responding to ablation of their homologs (Gao and Macagno, 1987b), releasable pools and voltage-dependent channels could then become functional without danger of initiating unwanted growth.

Although calcium waves are known to cross gap junctions in many cell types (Brehm et al., 1989; Saez et al., 1989; Bentley et al., 1991; Boitano et al., 1992; Christ et al., 1992; Finkbeiner, 1992; Yuste et al., 1992), there is some possibility that the postsynaptic AP cell's calcium rise could have occurred via other mechanisms. For example, there could have been depolarization-dependent release of a presynaptic molecule that caused

calcium entry postsynaptically. This is unlikely for the AP neurons, however, not only because these cells don't possess a detectable voltage-dependent calcium signal, but also because the time course of calcium's appearance in the receiving growth cone was extremely slow (15–20 min). Furthermore, when a preparation was bathed in zero-calcium medium after wave front initiation, the gradient continued to move into the adjacent cell, even though no calcium ions were available for pre- or postsynaptic entry. We also rule out the possibility that a molecule such as $\text{Ins}[1,4,5]\text{P}_3$ crossed the junctions and released calcium from postsynaptic releasable pools, since we report here that there are no releasable stores in AP neurons at this age. The simplest explanation is therefore that calcium ions moved directly between AP neurons via the gap junctions that connect them.

How might the distress signal be interpreted by a coupled growth cone?

The fact that calcium signals can cross the AP's gap junctions into a neighboring axon is particularly intriguing because no information is as yet available regarding the effects of calcium on growth cones *in vivo*. There is a wide range of possible calcium actions (reviewed in Henzi and MacDermott, 1992), including increased synthesis of growth-related proteins (e.g., Brostrom et al., 1989), cytoskeletal rearrangements via regulation of actin-binding proteins (Lankford and Letourneau, 1989; reviewed in Forscher, 1989), and the induction of immediate-early genes such as *fos* and *jun* (Morgan and Curran, 1986, 1988; Bading et al., 1993). If very large and rapid increases in $[\text{Ca}^{2+}]$, were to occur under certain conditions, the presumed uncoupling of the gap junctions between these neurons could itself be interpreted as a signal. Indeed, a similar model has been proposed for regenerating leech neurons (S cells) that overgrow when gap junctional communication is interrupted (Scott and Muller, 1980). When good molecular or pharmacological tools become available to block gap junctions in the leech, as there are already in other systems, it will be feasible to explore some of these possibilities in developing leech neurons *in situ*.

References

- Abele AE, Scholz KP, Scholz WK, Miller RJ (1990) Excitotoxicity induced by enhanced excitatory neurotransmission in cultured hippocampal neurons. *Neuron* 2:413–419.
- Allen F, Tickle C, Warner A (1990) The role of gap junctions in patterning of the chick limb bud. *Development* 108:623–634.
- Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* 260:181–186.
- Bandtlow CE, Schmidt MF, Hassinger TD, Schwab ME, Kater SB (1993) Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. *Science* 259:80–83.
- Becker PL, Fay FS (1987) Photobleaching of fura-2 and its effect on the determination of calcium concentration. *Am J Physiol* 253:C613–C618.
- Bennett MVL, Verselis VK (1992) Biophysics of gap junctions. *Semin Cell Biol* 3:29–47.
- Bentley D, Guthrie PB, Kater SB (1991) Calcium ion distribution in nascent pioneer axons and coupled preaxonogenesis neurons *in situ*. *J Neurosci* 11:1300–1308.
- Berridge MJ (1990) Calcium oscillations. *J Biol Chem* 265:9583–9586.
- Bevilacqua A, Loch-Caruso R, Erickson RP (1989) Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos. *Proc Natl Acad Sci USA* 86:5444–5448.
- Boitano S, Dirksen ER, Sanderson MJ (1992) Intercellular propagation of calcium waves mediated by inositol trisphosphate. *Science* 258:292–295.
- Bookman RJ, Liu Y (1990) Analysis of calcium channel properties in cultured leech Retzius cells by internal perfusion, voltage-clamp and single-channel recording. *J Exp Biol* 149:223–237.
- Brehm P, Lechleiter J, Smith S, Dunlap K (1989) Intercellular signaling as visualized by endogenous calcium-dependent bioluminescence. *Neuron* 3:191–198.
- Brostrom CO, Chin KV, Wong WL, Cade C, Brostrom MA (1989) Inhibition of translational initiation in eukaryotic cells by calcium ionophore. *J Biol Chem* 264:1644–1649.
- Campos-Ortega JA (1991) Genetic and molecular bases of neurogenesis in *Drosophila melanogaster*. *Annu Rev Neurosci* 14:399–420.
- Charles AC, Merrill JE, Dirksen ER, Sanderson MJ (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* 6:983–992.
- Charles AC, Naus CCG, Zhu D, Kidder GM, Dirksen ER, Sanderson MJ (1992) Intercellular calcium signaling via gap junctions in glioma cells. *J Cell Biol* 118:195–201.
- Cheek TR (1991) Calcium regulation and homeostasis. *Curr Opin Cell Biol* 3:199–205.
- Choi DW (1992) Excitotoxic cell death. *J Neurobiol* 23:1261–1276.
- Christ GJ, Moreno AP, Melman A, Spray DC (1992) Gap junction-mediated intercellular diffusion of Ca^{2+} in cultured human corporal smooth muscle cells. *Am J Physiol [Cell Physiol]* 263:C373–C383.
- Collins F, Schmidt MF, Guthrie PB, Kater SB (1991) Sustained increase in intracellular calcium promotes neuronal survival. *J Neurosci* 11:2582–2587.
- Deber CM, Tom-Kun J, Mack E, Grinstein S (1985) Bromo-A23187: a nonfluorescent calcium ionophore for use with fluorescent probes. *Anal Biochem* 146:349–352.
- Doe CQ, Goodman CS (1985) Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Dev Biol* 111:206–219.
- Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148:2207–2216.
- Finkbeiner S (1992) Calcium waves in astrocytes—filling in the gaps. *Neuron* 8:1101–1108.
- Forscher P (1989) Calcium and phosphoinositide control of cytoskeletal dynamics. *Trends Neurosci* 12:468–474.
- Franklin JL, Johnson EM Jr (1992) Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. *Trends Neurosci* 15:501–508.
- Fraser SE, Green CR, Bode H, Gilula NB (1987) Selective disruption of gap junctional communication interferes with a patterning process in *Hydra*. *Science* 237:49–55.
- Gao W-Q (1989) Axonal extension and retraction by developing neurons in the leech central nervous system. PhD thesis, Columbia University.
- Gao W-Q, Macagno ER (1987a) Extension and retraction of axonal projections by some developing neurons in the leech depends upon the existence of neighboring homologues. I. The HA cells. *J Neurobiol* 18:43–59.
- Gao W-Q, Macagno ER (1987b) Extension and retraction of axonal projections by some developing neurons in the leech depends upon the existence of neighboring homologues. II. The AP and AE cells. *J Neurobiol* 18:295–313.
- Gao W-Q, Macagno ER (1988) Axon extension and retraction by leech neurons: severing early projections to peripheral targets prevents normal retraction of other projections. *Neuron* 1:269–277.
- Greenspan RJ (1992) Initial determination of the neuroectoderm in *Drosophila*. In: *Determinants of neuronal identity* (Shankland M, Macagno ER, eds), pp 155–188. New York: Academic.
- Grumbacher-Reinert S, Nicholls J (1992) Influence of substrate on retraction of neurites following electrical activity of leech Retzius cells in culture. *J Exp Biol* 167:1–14.
- Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450.
- Guthrie PB, Segal M, Kater SB (1991) Independent regulation of calcium revealed by imaging dendritic spines. *Nature* 354:76–80.
- Henzi V, MacDermott AB (1992) Characteristics and function of Ca^{2+} and inositol 1,4,5-trisphosphate-releasable stores of Ca^{2+} in neurons. *Neuroscience* 46:251–273.

- Kater SB, Mills LR (1991) Regulation of growth cone behavior by calcium. *J Neurosci* 11:891–899.
- Kimble J (1981) Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev Biol* 87:286–300.
- Lankford KL, Letourneau PC (1989) Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. *J Cell Biol* 109:1229–1243.
- Lee S, Gilula NB, Warner AE (1987) Gap junctional communication and compaction during preimplantation stages of mouse development. *Cell* 51:851–860.
- Lipscombe D, Madison DV, Poenie M, Reuter H, Tsien RY, Tsien RW (1988) Spatial distribution of calcium channels and cytosolic calcium transients in growth cones and cell bodies of sympathetic neurons. *Proc Natl Acad Sci USA* 85:2398–2402.
- LoPresti V, Macagno ER, Levinthal C (1974) Structure and development of neuronal connections in isogenic organisms: transient gap junctions between growing optic axons and lamina neuroblasts. *Proc Natl Acad Sci USA* 71:1098–1102.
- Lytton J, Nigam SK (1992) Intracellular calcium: molecules and pools. *Curr Opin Cell Biol* 4:220–226.
- Mattson MP, Dou P, Kater SB (1988) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J Neurosci* 8:2087–2100.
- McGlade-McCulloh E, Muller KJ (1989) Developing axons continue to grow at their tip after synapsing with their appropriate target. *Neuron* 2:1063–1068.
- Miller JP, Selverston AI (1979) Rapid killing of single neurons by irradiation of intracellularly injected dye. *Science* 206:702–704.
- Morgan JJ, Curran T (1986) Role of ion flux in the control of *c-fos* expression. *Nature* 322:552–555.
- Morgan JJ, Curran T (1988) Calcium as a modulator of the immediate-early gene cascade in neurons. *Cell Calcium* 9:303–311.
- Neely MD (1993) Role of substrate and calcium in neurite retraction of leech neurons following depolarization. *J Neurosci* 13:1292–1301.
- Oppenheim RW, Schwartz LM, Shatz CJ (1992) Neuronal death, a tradition of dying. *J Neurobiol* 23:1111–1115.
- Randall RD, Thayer SA (1992) Glutamate-induced calcium transient triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. *J Neurosci* 12:1882–1895.
- Rehder V, Kater SB (1992) Regulation of neuronal growth cone filopodia by intracellular calcium. *J Neurosci* 12:3175–3186.
- Rink TJ, Merritt JE (1990) Calcium signaling. *Curr Opin Cell Biol* 2:198–205.
- Ross WN, Arechiga H, Nicholls JG (1988) Influence of substrate on the distribution of calcium channels in identified leech neurons in culture. *Proc Natl Acad Sci USA* 85:4075–4078.
- Saez JC, Connor JA, Spray DC, Bennett MVL (1989) Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. *Proc Natl Acad Sci USA* 86:2708–2712.
- Sanderson MJ, Charles AC, Dirksen ER (1990) Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. *Cell Regul* 1:585–596.
- Schirrmacher K, Deitmer JW (1989) Membrane properties of identified embryonic nerve and glial cells of the leech central nervous system. *J Comp Physiol [A]* 164:645–653.
- Schirrmacher K, Deitmer JW (1991) Sodium- and calcium-dependent excitability of embryonic leech ganglion cells in culture. *J Exp Biol* 155:435–453.
- Scott SA, Muller KJ (1980) Synapse regeneration and signals for directed axonal growth in the CNS of the leech. *Dev Biol* 80:345–363.
- Seydoux G, Greenwald I (1989) Cell autonomy of lin-12 function in a cell fate decision in *C. elegans*. *Cell* 57:1237–1245.
- Siesjo BK (1988) Historical overview: calcium, ischemia and death of nerve cells. *Ann NY Acad Sci* 522:638–661.
- Stewart RR, Nicholls JG, Adams WB (1989) Na^+ , K^+ and Ca^{2+} currents in identified leech neurones in culture. *J Exp Biol* 141:1–20.
- Stewart RR, Gao W-Q, Macagno ER (1991) Segmental differentiation in the leech central nervous system: proposed segmental homologs of the heart accessory neurons. *J Comp Neurol* 313:431–440.
- Taghert PH, Bastiani MJ, Ho RK, Goodman CS (1982) Guidance of pioneer growth cones: filopodial contacts and coupling revealed with an antibody to Lucifer yellow. *Dev Biol* 94:391–399.
- Tymianski M, Wallace MC, Spigelman I, Uno M, Carlen PL, Tator CH, Charlton MP (1993) Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury *in vitro* and *in vivo*. *Neuron* 11:221–235.
- Wahlestedt C, Golanov E, Yamamoto S, Yee F, Ericson H, Yoo H, Inturrisi CE, Reis DJ (1993) Antisense oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. *Nature* 363:260–263.
- Warner A (1992) Gap junctions in development—a perspective. *Semin Cell Biol* 3:81–91.
- Warner AE, Guthrie SC, Gilula NB (1984) Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* 313:127–131.
- Wenning A (1987) Salt and water regulation in *Macrobrachium decorata* (Hirudinea: Gnathobdelliformes) under osmotic stress. *J Exp Biol* 131:337–349.
- Wolszon LR, Gao W-Q, Passani MB, Macagno ER (1994) Growth cone collapse *in vivo*: are inhibitory interactions mediated by gap junctions? *J Neurosci* 14:999–1010.
- Yuste R, Peinado A, Katz LC (1992) Neuronal domains in developing neocortex. *Science* 257:665–669.