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Interactions during a Critical Period Inhibit Bilateral Projections in Embryonic Neurons

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The anterior pagoda (AP) neurons in the CNS of the medicinal leech are found as homologous pairs in 20 of the 21 midbody ganglia. Each AP is the mirror image of its mate, extending its main axon across the midline of the CNS and eventually into the contralateral body wall, thereby attaining a unilateral pattern of innervation. Certain features of the adult AP morphology are known to arise through interactions among homologs early in development (Gao and Macagno, 1987b), but it is not known whether the contralateral nature of the projection pattern is due to intrinsic "one-sidedness" or rather to cell–cell interactions that inhibit the formation of a second, ipsilateral projection. In the experiments described in this report, we tested the possibility that an AP's contralateral homolog itself inhibits the formation of bilateral projections. One AP was photoablated in the intact embryo early in development and then the response of the remaining AP was examined. We found that an AP can extend bilaterally symmetrical projections when its homolog is missing, but only during a critical period that, interestingly, begins when an AP's interactions with *other* specific neurons come to an end. To determine whether synaptic communication between AP homologs could be responsible for the timing of this critical period, we recorded electrophysiologically from pairs of embryonic AP neurons. Although no detectable chemical signaling was observed, AP cells were electrically coupled throughout the entire critical period. Further, the junctions between these neurons were permeated by 5-HT, whereas larger molecules such as carboxyfluorescein were impermeant. This dye coupling decreased with age even while electrical coupling persisted, suggesting but not proving that the properties of the gap junctions between AP neurons may also change with time.

We conclude that unilateral AP cells possess the intrinsic ability to project bilaterally, but are inhibited from doing so by age-dependent interactions with homologous neurons, possibly mediated by gap-junctional communication.

[Key words: CNS, inhibition, 5-HT, commissure, axon outgrowth, growth cones, gap junctions, electrical coupling, leech,* Hirudo medicinalis*]

In bilaterally symmetrical nervous systems, a neuron that projects contralaterally has typically a mirror image homolog. The axons of such paired homologs cross the midplane in opposite directions, sharing the same commissure, and then make a turn to travel some distance along parallel but separate longitudinal tracts. These lateral tracts appear to be defined by one or more molecular cues expressed on cell surfaces (Macagno et al., 1983; McKay et al., 1983; Bastiani et al., 1987; Dodd et al., 1988). A neuron therefore can recognize and follow the same commissural cues as its homolog, but will only follow the lateral cues that are on the opposite side of the nervous system, even though presumably identical cues must be present on its own side to guide that homolog. An interesting developmental question arises from this behavior: by what mechanism(s) do these growing neurons avoid responding to the nearer ipsilateral cues, either before or after crossing the commissure?

There are several possible answers to this question. One is that there are intrinsic identifiers of "leftness" and "rightness" (Brown and Wolpert, 1990; Wood, 1991), much as there are molecular cues that distinguish anterior from posterior regions and dorsal from ventral (reviewed in McGinnis and Krumlauf, 1992, and Puelles and Rubenstein, 1993). Contralaterally projecting cells would then seek opposite-side cues whereas neurons that project ipsilaterally would recognize same-side signals. Another possibility is that cues are simply available at different times, the commissural ones appearing earlier than the lateral ones. While this possibility is not unlikely *a priori,* perhaps only those neurons growing very early would find this temporal order available, and those that differentiate later would find both types of cues present simultaneously.

An interesting alternative hypothesis is that the ability of axons to *respond* to lateral cues may require a prior interaction with commissural substrates (e.g., Dodd et al., 1988). If this were the case, once the commissures were traversed, either side would be equally attractive to growing processes. The choice of lateral tract would then depend only upon an axon's proximity to lateral cues. Such a scheme would result in the primary axon continuing to project contralaterally, but if a collateral branch happened to extend some distance into *ipsilateral* territory, it might there encounter lateral cues and thus become a second efferent projection. Since branching can be quite profuse within the neuropil on both sides of the CNS, one might expect then that unilateral morphologies would be rare. The fact that they are not implies that there may be a specific inhibitory influence that prevents the expression of a bilateral phenotype.

We have begun to explore whether direct inhibition between

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homologs is important in maintaining a unilateral morphology, by examining the circumstances under which a unilateral neuron can be made to project in a bilaterally symmetrical manner. We studied the anterior pagoda (AP) cells of the medicinal leech, which are unipolar and are found as bilateral pairs in the first 20 of the 21 midbody ganglia (MG). By ablating one AP cell *in vivo,* we could ask whether the remaining AP would follow ipsilateral cues when the putative inhibition by the killed cell was removed. We found that the contralateral homolog of a deleted AP could indeed generate bilateral projections, and did so in an age-dependent manner. Since these paired cells were also found to be electrically- and dye-coupled to each other throughout this period of plasticity, we suggest that electrical communication, via gap junctions, might mediate the normal inhibitory signal between these cells early in development.

Materials and Methods

Hirudo medicinalis embryos were generated in our laboratory and staged as described in Gao and Macagno (1987a), except that they were maintained at 23°C. Embryogenesis is considered to end with hatching, which occurs at 30 d (E30) at this temperature, but the animals continue to develop to sexual maturity for approximately 1 year. Leeches from E9 to P5 (postembryonic day 5), as well as adult leeches, were used for this study.

Cell ablations. Leeches from E9 to P5 were anesthetized in 7–10% ethanol in 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 $CaCl_2$, and the pH adjusted to 7.4 with NaOH; Wenning, 1987] for 3–5 min. The intact animals were then placed ventral-side-up into a groove cut in a Sylgard-coated slide. Embryos older than E14 had one tungsten pin (002; American Wire Industries) placed in each sucker to prevent gross movements. The CNS was then visualized through the body wall using a high-intensity fiberoptic illuminator and a small incision was made over the 8th midbody ganglion (MG8), taking care not to disturb the cocoon fluid. For embryos at E17 or older, a small window in the blood sinus surrounding the ganglion was opened.

The preparation was transferred to the electrophysiological recording rig described below and a 40× water-immersion objective was used to identify the neuron to be photoablated. The cell was filled iontophoretically (−1 to −5 nA, 500 msec at 1 Hz) with Lucifer yellow for 2–4 min while continuously irradiating the ganglion with 480 nm light, after which the electrode was withdrawn. The illumination continued for 2–10 min, until one or more obvious signs of death were observed: dark spots in the cytoplasm, cytoplasm leaking from the soma, and beading of the axons and dendrites. The pins were then removed and the animal placed in sterile artificial spring water overnight at 18°C to facilitate recovery. The following day they were moved to a 23°C incubator and the sterile spring water was changed daily. Within 24 hr there was no sign of damage to the body wall, and development proceeded normally.

After the animal recovered for 7 or more days, it was opened along the dorsal midline and any remaining cocoon fluid was removed. The experimental ganglia and a square of the surrounding body wall (about 5 mm per side) were pinned ventral-side-up on a Sylgard-coated microscope slide. The AP cell contralateral to the photoablated cell was then filled with Lucifer yellow as described below, but only enough 480 nm illumination was used to confirm the cell's identity, thereby minimizing damage and avoiding bleaching of the dye. After filling, the dye was allowed to diffuse for 5 min. The preparation was then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 45 min. It was rinsed in 0.01 M PBS, pH 7.4, and then cleared with a series of 25%, 50% and 100% glycerol (in 0.01 M PBS), 5 min each. The preparation was subsequently mounted in glycerol and photographed.

Adult APs were ablated after anesthetizing a young adult leech (1.5–2.5 gm) with 10 mM chlorobutanol in sterile saline. The animal was pinned through the suckers, ventral side up, in a large Sylgard-coated holder. A small hole was opened in the ventral body wall over MG8, and the blood sinus removed over the anterior packets of the ganglion, in which the two AP cells reside. Two Minutien pins were bent into the shape of staples and were placed firmly across the leech's body, anterior and posterior to the opening, and pushed into the Sylgard so that the ganglion bulged out slightly from the body wall. This was done in order to improve stability and facilitated visualization of the cells. The ganglion was then illuminated from the side with a fiberoptic cable. Adult APs were impaled with thick-walled electrodes that were filled with 0.5% protease type VIII (Sigma) with 0.2% fast green and 0.15 M KCl (Muller et al., 1981), beveled, and then backfilled with 0.2 M KCl. These electrodes had resistances of 30–60 MΩ The solution was pressure injected until the cell turned green. The animal was kept overnight in sterile artificial spring water at 18°C, and then transferred to a 23°C incubator for 13 or 28 more days. After this period, the animal was opened and MG8 removed and pinned ventral-side-up in a Sylgard-coated dish. The remaining AP was filled with 4–8% Lucifer yellow in 0.2 M LiCl using the pressure-injection technique described below. The ganglia were then fixed, cleared in glycerol, mounted and photographed.

Dissection of embryos for physiological recording. Embryos were partially immobilized in cold (2°C) sterile spring water (0.5 gm/liter Instant Ocean, Menasha Corporation) for 10 min. For animals up to E13, the cryptolarval membrane that surrounds the cocoon fluid was then torn along the longitudinal axis to remove the fluid. Older embryos (E14–17) had more viscous fluid, requiring them to be pinned (with 001 tungsten wire), dorsal-side-up, on a Sylgard (Dow-Corning)-coated microscope slide, and then cut along the dorsal midline and washed with a stream of saline. The embryo was then anesthetized by transferring to 6% ethanol in sterile Wenning's saline, after which it was repinned, ventral-side-up, to the Sylgard slide. The elastic cryptolarval membrane was stretched in all directions during pinning in order to minimize movement of the germinal plate during recording, since the leech CNS and surrounding body wall contain many muscle cells that contract strongly even in ethanol. Longitudinal muscle contractions were also reduced by pinning both sides of the body wall near the ganglia from which the recordings were to be made, and then making short, lateral cuts in the skin and muscle between segments, on each side. If these treatments did not stop movement, the embryo was immersed for 1 min in 0.1% paraformaldehyde in sterile saline and then immediately rinsed; this procedure did not affect physiological responses of the neurons. Finally, to expose the CNS, a longitudinal incision was made in the ventral body wall over 4–10 adjacent midbody ganglia, using an etched tungsten pin, and any remaining blood sinus was removed.

For embryos older than E18, individual ganglia could be removed entirely from the animal and pinned to a Sylgard-coated dish for subsequent recording, avoiding treatments to immobilize the preparation.

Electrophysiological recording. The electrophysiology rig used for embryos had a microscope equipped with epifluorescence optics and a 40× water-immersion objective to allow identification of embryonic neurons. The working distance of this objective necessitated bending the fine electrode tips to a 60° angle using a heated nichrome wire. Every electrode contained Lucifer yellow dye (3–8% in 0.2 M LiCl, microfiltered; Sigma) in order to confirm the identity of the neurons at the end of each experiment. They therefore had high resistances (150–300 MΩ), causing frequent blockage and significant AC interference.

Measurements of electrical coupling. To measure electrical coupling between pairs of AP cells within the same ganglion, we used dual recordings to measure voltage responses to current pulses of ±1–7 nA (300–600 msec duration). Positive pulses in embryonic cells usually produced blockages of the electrode tips so that the data were not further analyzed; all quantification of coupling coefficients (for example, in Fig. 5) is therefore based upon negative current pulses. However, in the larger, postembryonic neurons blocked electrodes could be cleared, and in adults, two intracellular electrodes could be used within the same neuron, so the values for positive currents in these experiments are reliable and are used in the analysis of rectification at these ages.

After recording from each cell pair, we tested for electrode crosstalk and nonspecific electrical coupling by sequentially withdrawing the electrodes from the cells and applying the same current pulses in the saline.

To stabilize the preparations for recording, the microscope stage was fixed in the vertical dimension. The fragility of embryonic neurons made these cells susceptible to damage, however, so only a few current pulses could be applied before resting potentials deteriorated. Reliable dual recordings could be obtained from E10 to E12 embryos for up to 1.5 min and from E13 to E15 embryos for up to 3 min, after which the electrical coupling diminished. Data were digitized at 600–2100 Hz for subsequent analysis, but the time limitations imposed by the fragile preparations precluded digital averaging of repeated measurements.

Therefore, numbers for coupling coefficients were calculated for the first few traces of a series, and then averaged if and only if deterioration did not occur.

Since fine electrode tips were necessary to minimize neuronal damage, electrode resistances ranged from 150–300 MΩ. This often caused a transient blockage of the tips, making bridge-balance using the rising phase of the pulse nearly impossible. We therefore bridge-balanced using the falling phase, as described in Wolszon et al. (1994a), and did so during the analysis of the digitized traces, using the same criteria as are traditionally applied during live recordings. The time constant of decay of the electrode voltage was sufficiently fast, relative to that of the cell, that it was possible to determine the intersection of the corresponding exponential curves, providing the electrodes were capacity compensated. However, when there was uncertainty in the measurements, the numbers were verified by fitting the falling phases of the voltage responses to two exponentials and extrapolating the slow (neuronal) response to time zero. These measurements of presynaptic voltages were equivalent to those done by eye, both in embryos and adults, providing we restricted our measurements to the linear region of the current–voltage relationship.

Coupling coefficients in cells in which there was much spontaneous or anode-break activity were sometimes difficult to measure; when there was any ambiguity in determining the baseline, these data were omitted.

Tests for dye-coupling using 5-HT. The injection of 5-HT into embryonic AP cells and its visualization using anti–5-HT monoclonal antibodies is described in detail in Wolszon et al. (1994a). Briefly, a solution of 5% (130 mM) serotonin (Sigma) in 0.2 M KCl and 0.05 M Tris (pH 7.4) was iontophoresed intracellularly using 200 msec pulses of positive current for 1–2 min (1–2 nA, 2 Hz). After allowing 10 min for dye diffusion, the embryos were fixed and then incubated at 4°C overnight in anti–5-HT antibodies (made and provided by Drs. Kathy French and Bill Kristan, UCSD) which were later visualized using the ABC Vector Kit (Vector Laboratories). The preparation was then cleared and mounted in glycerol and photographed. Using these techniques, we were able to see 5-HT immunoreactivity only until E20.

Results

Background. A brief description of the normal development of AP cells (Gao and Macagno, 1987b) will be useful to the reader. Early in embryogenesis, around E9–E10, the AP's primary neurite, already having crossed the midline to the contralateral side, branches there to produce four extraganglionic projections (Fig. 1, left). Two lateral branches exit the CNS through the contralateral root nerves, but the two longitudinal branches remain within the CNS, projecting anteriorly and posteriorly within the connective nerves that link adjacent ganglia. The longitudinal axons interact with those of the adjacent AP homologs for 5–8 d, mutually inhibiting each other until they finally retract, so that they are completely absent in the adult (Fig. 1, center).

In previous work from this laboratory, the interactions between AP neurons and their *ipsilateral* segmental homologs in adjacent ganglia were studied. It was found that if an AP cell is deleted on or before E14, during the 5–8 d period that longitudinal AP projections interact with each other, then the previously inhibited axon of the adjacent homolog starts growing again (Gao and Macagno, 1987b), finally taking over vacated territory (Fig. 1, right). From E15 on, the incidence of this response decreases dramatically and finally becomes zero, perhaps because the synaptic communication between adjacent AP cells disappears (Wolszon et al., 1994a). However, the *contralateral* AP homolog, the subject of this article, does not respond to cell ablation in embryos up to E14 (2 out of 45 cases; Gao and Macagno, unpublished observations), even though its soma and secondary branches are much closer physically to the damaged cell. Thus, there appears to be a competition between the contralateral and ipsilateral homologs for territory vacated by a dying cell, and up to E14, the ipsilateral homolog "wins."

Unilateral cells become bilateral after early cell ablation. To examine the outcome of the competition between contralateral and ipsilateral AP homologs at later ages, after the ipsilateral homolog begins to retract its transient projections, we ablated AP cells in intact embryos at E15 or later. We found that at these ages, the competition was instead biased in favor of the contralateral AP, as shown in Figure 2. When the AP was ablated at E15 (Fig. 2*A,B*) and its contralateral homolog filled with Lucifer yellow 7 d later, in 9 out of 10 experiments the contralateral AP cell produced one or two additional axons exiting the ganglion from the ipsilateral roots, something never observed in unoperated embryos of any age (see below). The pattern of axon growth was somewhat variable: in 20% of the cases, the axons sprouted from the soma, in 50%, from the main axon, often near the original site of bifurcation, and in 30%, from secondary processes; these ratios were maintained even at older ages (see below). In addition, there were variations in the directness of the axonal paths out of the ganglion (see the meandering process in Fig. 2*A*, for example). When there were two novel axons generated, there was always one axon per ipsilateral root, with two exceptions (not shown): one was a case in which there were four extra ipsilateral axons, two exiting from each root, and in an-

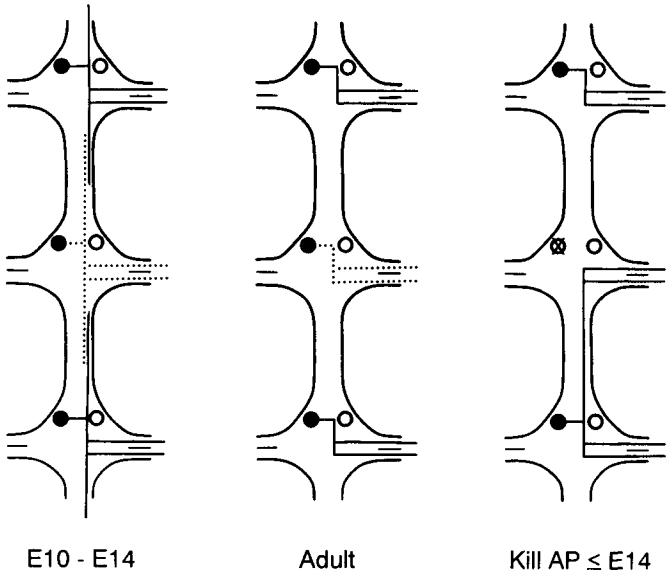

Figure 1. Schematic drawings of AP morphology in three consecutive ganglia under normal and experimental conditions. *Left,* From E10–E14, AP cells extend four axons, two of which grow into the connective nerves that join adjacent ganglia. These longitudinal projections overlap with those of ipsilateral, adjacent AP homologs (indicated with *solid circles*) for 5–8 d. (Successive AP homologs are indicated with alternating *solid* and *dotted lines* to distinguish the axons of adjacent neurons.) The other two projections exit the CNS via two contralateral nerve roots. On the opposite side of the ganglion lie the mirror-image contralateral homologs, the subjects of this report; these cells are symbolized with *open circles* but their projections are not illustrated, for simplicity.

Center, In the adult, AP cells possess only the contralateral projections, as the longitudinal axons are retracted during development. Retraction begins at E15–E18 and is nearly complete by the end of embryogenesis (E30).

Right, Adult AP morphologies after ablating one AP (marked with an *X*) during the period in which longitudinal axons overlap in the connective nerves (E10–E14). In 72% of 54 photoablation experiments, the posterior ipsilateral homolog takes over territory vacated by the killed cell (Gao and Macagno, 1987b), instead of retracting its longitudinal axon. In another 19%, the anterior homolog fills this role, and in the remaining 5% of the experiments, both homologs do, each sending one branch to the periphery via the operated ganglion.

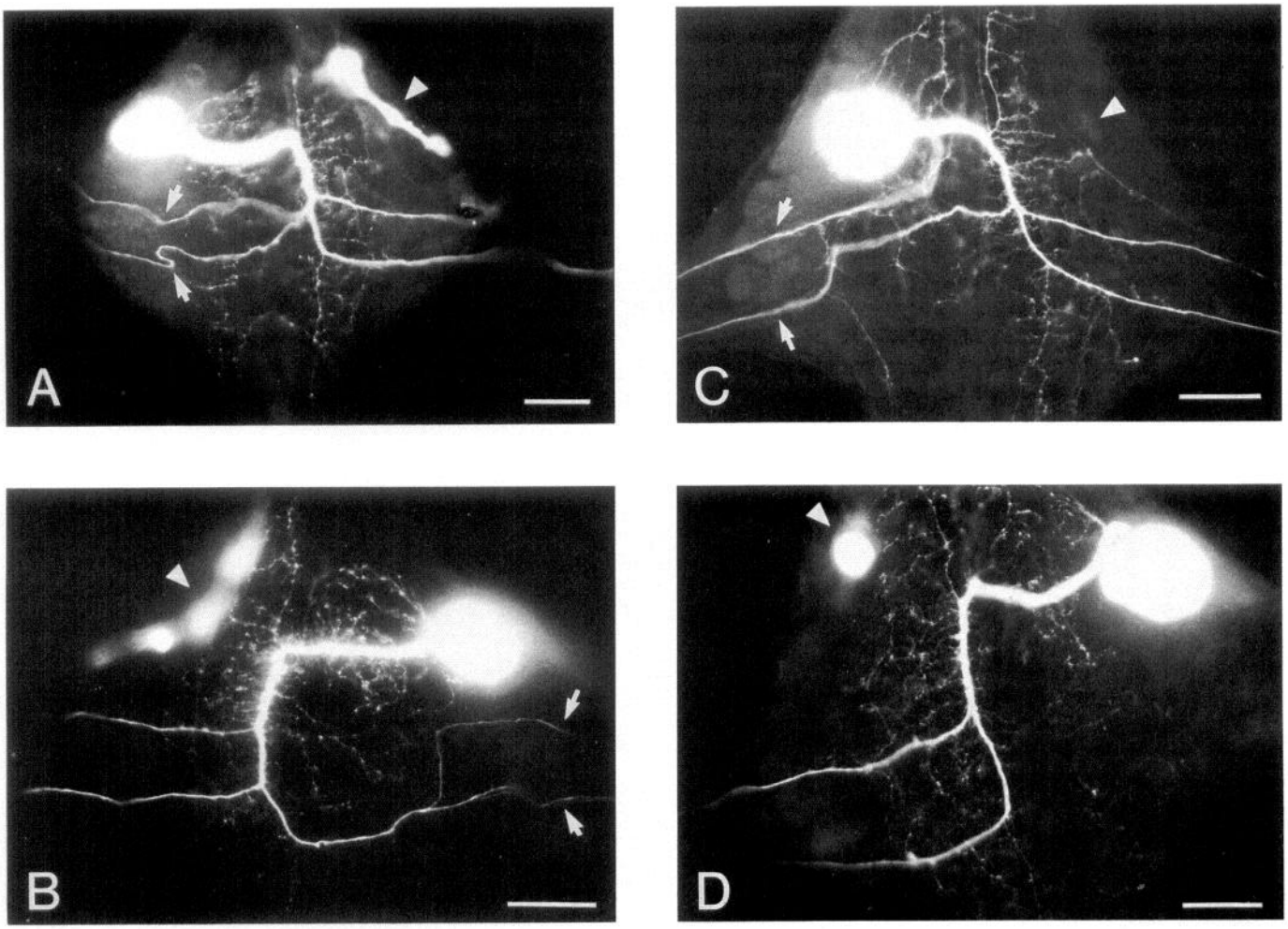

Figure 2. AP cell morphology 7 d after photoablation of its contralateral homolog. In *A–C*, two novel projections (*arrows*) exit the ganglion via the ipsilateral nerve roots. Photoablations were done at E15 in *A* and *B*, at E16 in *C* and at E18 in *D*. Note the lack of ipsilateral processes in *D*. *Arrowhead* indicates the fluorescent debris of the killed cell, sometimes visible for up to 2 weeks after photoablation (though it has been absorbed almost completely in the experiment of *C*). Scale bars, 20 μm.

other, two axons projected out the anterior root. Similar growth patterns were observed when APs were deleted at later ages (e.g., E16, Fig. 2*C*), although the frequency of occurrence declined with age (see Fig. 2*D*), as described below. Finally, in one case, the remaining AP cell also generated two extra *contralateral* axons (not illustrated).

Several days were required for the generation of these novel projections, as bilaterality was never detected fewer than 5 d after photoablation ($n = 4$ additional experiments), even when ablation occurred at E15. However, once generated, the extra axons remained well into adulthood, as determined by experiments in which APs were ablated at E15 and the contralateral AP cells filled 60 d later ($n = 4$ out of 5 additional experiments).

To rule out nonspecific effects of the photoablations, two types of control experiments were done. First, we photoablated cells whose somata or primary axons are adjacent to the APs, such as T, P, and N sensory cells (on both sides of the ganglion) and Retzius cells, at E15 ($n = 17$) or E20 ($n = 10$). None of the APs generated ipsilateral projections in these controls. Second, we filled E15 APs with Lucifer yellow but did not irradiate them, the cells therefore remaining viable, and there was no response in any of the contralateral APs ($n = 4$). The dye injection per se was therefore not responsible for the observed effects.

The bilateral response is age dependent. To determine whether the ability of an AP cell to generate ipsilateral axons was a property that changes with age, photoablations were carried out at various embryonic stages and in the 1 year old adult. The results are shown in Figure 3. Interestingly, there was a dramatic change in the ability to become bilateral at E15 versus E16, when the incidence of occurrence fell to less than 50% (see the Fig. 3 caption for the statistical analysis). This rate was maintained at a relatively constant level until the end of embryogenesis (E30), when another sudden change occurred, the incidence dropping to zero. Since adult AP cells never recovered their ability to generate the bilateral projections, we conclude that there is a critical period for this behavior, defined by either or both of the following properties: their ability to *detect* the death of their homologs, or their ability to *respond* to that death by extending a novel ipsilateral projection.

Contralateral homologs are electrically coupled. One way that cells might detect the death of a neighbor is via synaptic signaling. We therefore recorded electrophysiologically from two embryonic AP neurons within the same ganglion to assess

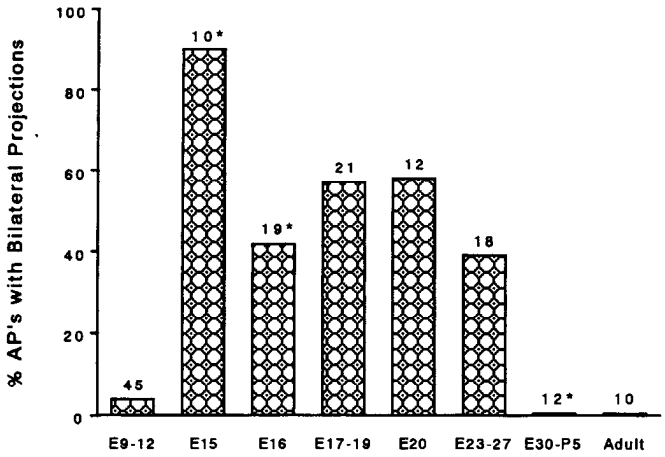

Figure 3. Percentage of AP cells exhibiting bilateral branching after photoablation of contralateral homolog, as a function of age. The number of cells tested at each age is given above the *bars*. When photoablation occurred *before* E15, bilateral projections were rare, but when done at E15, such projections were seen 90% of the time. Only 1 d later, this value dropped to approximately half, where it remained until the end of embryogenesis. To determine statistical significance of temporal changes, a χ^2 statistical analysis ($\alpha = 0.01$) was done at each time point, comparing values just before and just after. Values for embryos from E16 through E27 were not significantly different from each other, but significant ($p < 0.01$) changes occurred from E9–12 to E15, from E15 to E16, and from E23–27 to E30–P5. The values for E9–12 are taken from Gao and Macagno (1987b) and Gao (1989).

whether they communicate in this fashion. Whereas stimulating one AP cell produced no detectable chemical postsynaptic potential in the second cell, significant electrical coupling was observed, probably mediated by gap junctional synapses. An example is shown in Figure 4, in which dual recordings were obtained from contralateral AP homologs at E11. Although coupling was consistently observed, it was not strong, and action potentials in one AP produced no detectable voltage changes in the second cell.

The improved impalements obtained in postembryonic day 3 (P3) and adult AP neurons (see Materials and Methods) permitted more quantitative comparisons of ±1 nA pulses to determine whether these junctions rectify. At P3, the electrical synapses between AP cells were partially rectifying (traces not shown), with coupling coefficients averaging 0.340 ± 0.069 (mean ± SD) for negative pulses and 0.185 ± 0.082 for positive ones ($p < 0.005$, Student's two-tailed t test; $n = 4$). In contrast, for adults (1–3 years) these junctions did not rectify, negative pulses producing coupling coefficients of 0.035 ± 0.016 and positive ones producing coefficients of 0.047 ± 0.021 ($p > 0.2$; $n = 5$).

Coupling coefficients, and other electrical properties, vary with age. Since the ability of APs to generate bilateral projections varied with age, and since we suspected that gap junctions might be involved in this process, we tested whether electrical communication between these neurons persisted throughout the critical period. We recorded from pairs of contralateral AP homologs at various ages and measured their coupling coefficients. As shown in Figure 5, the coupling coefficients increased from E11 to E15, and then stayed constant through P3 until they dropped to very low values in adults. The electrical signaling between these neurons was therefore present throughout the critical period (compare this graph to that of Fig. 3) and even outlasted it. This finding is consistent with, but does not prove, the

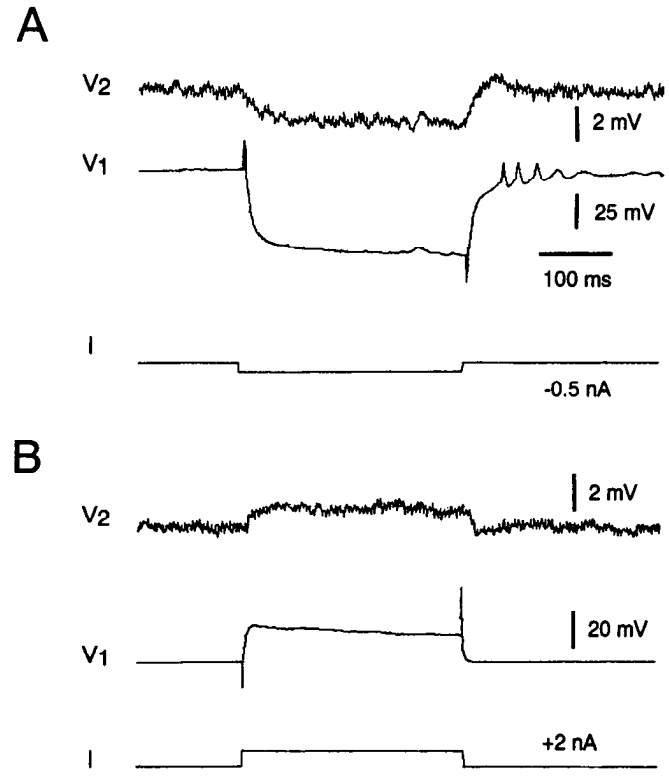

Figure 4. AP cell pairs within the same ganglion are electrically coupled throughout embryogenesis. Simultaneous recordings from AP neurons of an E11 embryo reveal strong electrical coupling with negative (*A*) and positive (*B*) pulses of current (*I*) injected into the presynaptic (V_1) cell. Presynaptic traces are not stimulus canceled (see Materials and Methods).

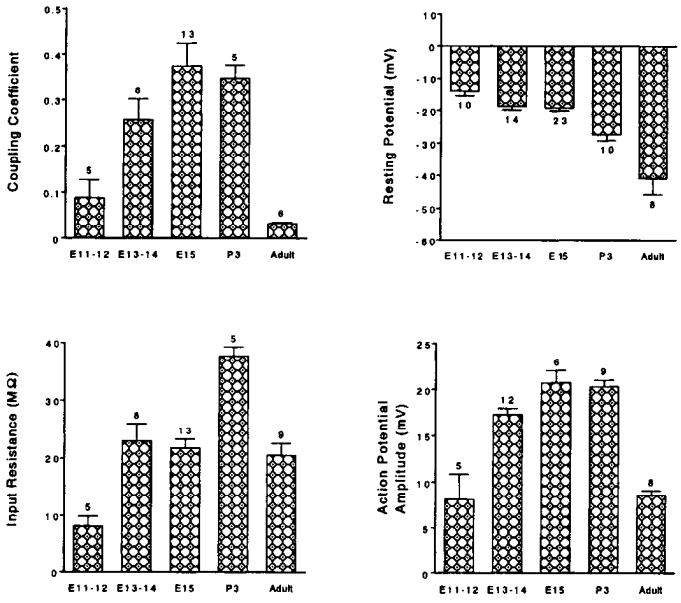

Figure 5. Measurements of coupling coefficients, input resistances, resting potentials, and action potential amplitudes of AP neurons of different ages. The coupling coefficient initially rose and then remained relatively constant throughout embryogenesis, but fell to low values in the adult. The time course of electrical coupling appears not to be directly related to changes in passive membrane properties. The numbers of experiments are indicated above the *bars*.

Table 1. Statistical significance of changes in electrophysiological properties with age

Comparison: Age_1 vs Age_2	Coupling coefficient	Input resistance	Resting potential	Spike amplitude
E11–12/E13–14	—	*	—	*
E11–12/E15	*	*	—	*
E11–12/P3	*	*	*	*
E11–12/Adult	—	*	*	—
E13–14/E15	—	—	—	—
E13–14/P3	—	*	*	—
E13–14/Adult	*	—	*	*
E15/P3	—	*	*	—
E15/Adult	*	—	*	*
P3/Adult	*	*	*	*
F test	15.204	9.601	24.876	30.488
p	0.0001	0.0001	0.0001	0.0001

This table shows the results of ANOVA (Fisher PLSD) test for significant differences in physiological properties among leeches of different ages. Asterisk indicates that the two specified groups are significantly different from each other (at 1% level). Results comparing E11–12 embryos with other groups should be interpreted with caution, since cell damage sometimes occurs with electrode impalement at these ages.

idea that AP cells may use gap junctional communication early in development to assess whether their homologs are present to innervate territory.

Since we measured electrical coupling between cells that synapse in the neuropil, which is electrotonically distant from the somatic recording sites, we could not determine gap junctional conductance and thus measured coupling coefficients. Because the latter parameter is influenced by a cell's input resistance and, sometimes, resting potential, we measured these properties as well. The results are shown in Figure 5, and the corresponding statistical analyses are given in Table 1.

No correlation was found between resting potentials and the degree of electrical coupling at any age. Similarly, changes in input resistance were not responsible for changes in coupling except possibly at E11–12. Indeed, at P3, although the coupling coefficient was unchanged from the previous time point, the input resistance nearly doubled. This change in resistance probably reflects that at this age, the improvement in impalement increases the measured input resistance more than the larger cell surface area decreases it. In contrast, the coupling coefficients of (the even larger) adult AP cells decreased to almost zero, although input resistances returned to average values. Taken together, these data suggest that the differences in electrical coupling with age probably reflected changes in the degree of gap junctional communication rather than changes in passive membrane properties, with the possible exception of E11–12 embryos.

The amplitude of action potentials also varied with age, in parallel with values for coupling coefficients but independent of changes in input resistance (e.g., compare *E15* with *adults* in Fig. 5). This was surprising in that one might expect spike amplitudes to be indirect indicators of input resistance in AP cells, since they have no spike-initiating zones on the soma or primary neurite (Gu et al., 1991).

Contralateral AP cells are also dye-coupled. To confirm the existence of gap junctions between AP cells, we tested for dye passage between them. Since AP cells resemble many other leech neurons (reviewed in Muller et al., 1981) in that their gap junctions do not pass traditional tracers such as carboxyfluorescein, Lucifer yellow, biocytin, or neurobiotin, we used a smaller marker (Wolszon et al., 1994a). The 5-HT ion has a molecular weight of 212 Da, and can be readily visualized in embryos up to E20 with anti-5-HT antibodies.

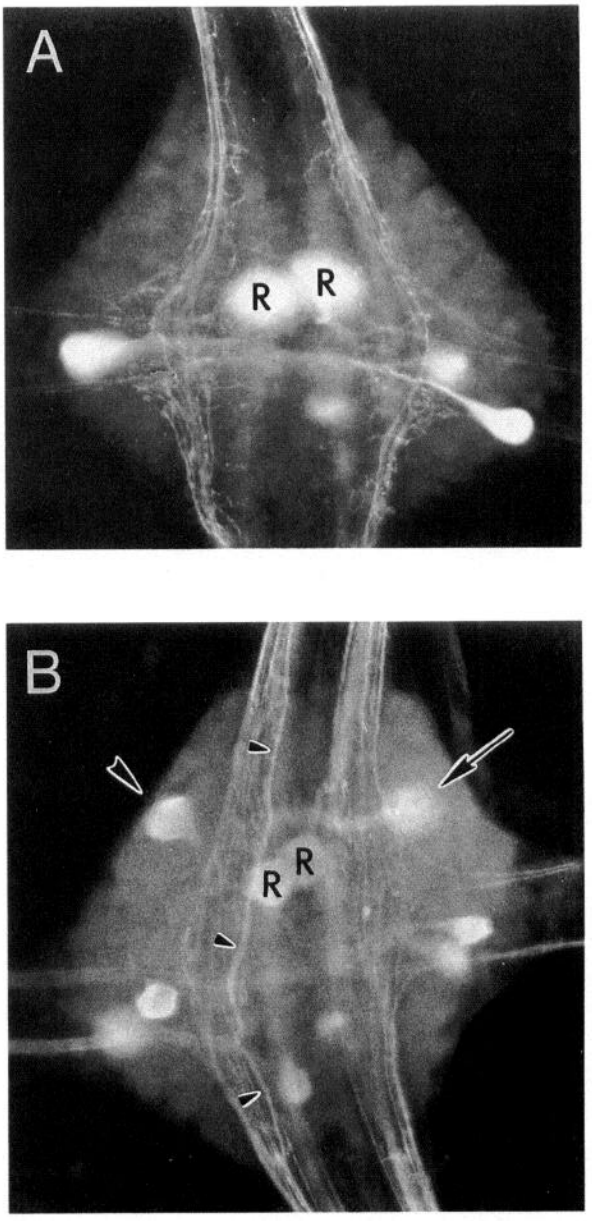

Figure 6. Contralateral AP neurons are "dye"-coupled. 5-HT was injected into AP cells and later visualized with anti–5-HT monoclonal antibodies. *A,* A control (uninjected) ganglion of a late E12 embryo, showing four pairs of 5-HT–containing neurons, including the large Retzius (*R*) cells. Note the absence of endogenous AP staining. *B,* 5-HT was injected into the right AP neuron (*arrow*; out of the plane of focus), and allowed to diffuse long enough to fill the contralateral processes of the injected cell (*small arrowheads*) and also cross specifically into the contralateral AP homolog (*large arrowhead*) via gap junctions.

We found that 5-HT passed freely between contralateral AP homologs (Fig. 6), and that the degree of dye-coupling changed with age (Fig. 7). Dye passage increased significantly after E10 (χ^2 test, $p < 0.01$), remained constant for 6 d and then dropped significantly at E17–19. A comparison of Figures 5 and 7 reveals an interesting difference in the time courses of the incidence of

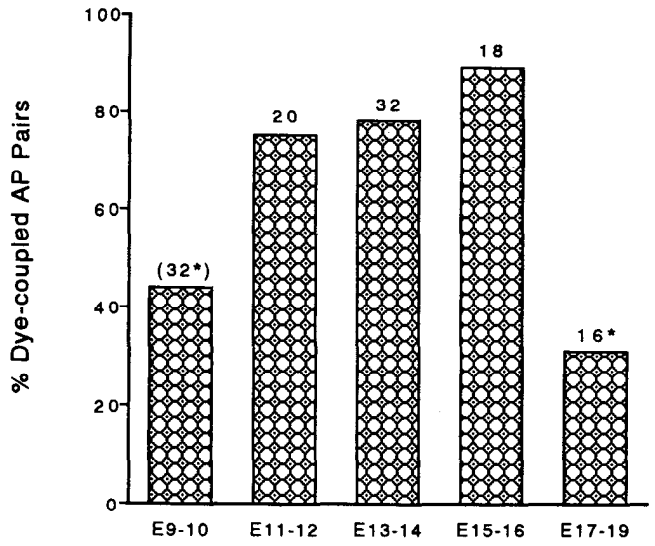

Figure 7. Dye coupling with 5-HT varies with age. The incidence of 5-HT passage between contralateral homologs was measured at all ages from E9 to E19. The results for different ages were binned into groups after determining that they were not significantly different from each other, using the χ^2 test with $\alpha = 0.01$. For example, passage at E15 ($n = 8$) was not significantly different from that at E16 ($n = 10$) so these results were combined into one "E15–16" category ($n = 18$). The numbers of experiments at each time point are indicated above the *bars*. Only at E9–10 and E17–19 was the incidence of dye coupling statistically different (indicated with an asterisk *) from values at intervening ages. The low incidence at E9–10 should be interpreted with caution, however, since dye injection at these ages can compromise the integrity of cells, potentially giving a false-negative result. 5-HT immunoreactivity could not be visualized with our staining techniques in leeches older than E19 (see Materials and Methods).

dye passage and electrical coupling: the ability of 5-HT to cross the gap junctions was markedly reduced at E17–19, whereas electrical coupling remained constant until adulthood. Although there are many examples of electrical coupling occurring in the absence of detectable dye passage (Muller et al., 1981; Audesirk et al., 1982; Bodmer et al., 1988; Wadepuhl, 1989; Ransom and Kettenmann, 1990), the difference in time courses is consistent with the idea that the properties of these gap junctions may change during development, perhaps by gradually restricting the pore size or number. That such properties could change is supported by the fact that the rectification observed at P3 disappeared in the adult.

We conclude from these data that contralateral AP homologs do communicate via gap junctions during development, and that this communication persists throughout the period in which AP cells can become bilateral after homolog deletion.

Discussion

Labeled paths might guide AP axons across the commissure. It is well established that many developing axons grow in paths specified by particular molecular markers (the "labeled pathways" hypothesis; Ghysen and Janson, 1980; Raper et al., 1983). Because AP cells project contralaterally before growing posteriorly, one would assume that there are markers specific for the commissure and others specific for the lateral, longitudinal paths on each side of the CNS. The symmetry of the CNS would suggest that the lateral cues are identical to each other, but AP cells initially ignore the nearby cues, apparently preferring the commissural markers that promote growth across the midline. Once across, they must change their affinity as they move onto the lateral tracts. However, our demonstration here that an embryonic AP neuron can generate an ipsilateral projection after its contralateral homolog is ablated strongly suggests that, at certain stages, AP cells must be capable of recognizing the same guidance cues on both sides of the midline.

The fact that an AP neuron grows initially along a commissural path could be explained by there being transient expression of an attractive substrate, first within the commissures and later within the lateral tracts that guide axons into the periphery. Time- and/or space-dependent expression of surface markers within axon tracts has been described in several developing CNSs (e.g., Kolodkin et al., 1993; reviewed in Whitington, 1993), including that of the leech (Macagno et al., 1983; Peinado et al., 1987; McGlade-McCulloh et al., 1990; Zipser et al., 1994). In the *Drosophila* CNS, for example, members of the fasciclin family are expressed by subsets of tracts (Bastiani et al., 1987; Patel et al., 1987), and the axons that traverse them generally express these proteins only in those sections that are contained within the appropriate bundle (Bastiani et al., 1987; Zinn et al., 1988). In fact, certain projection interneurons express fasciclin I in the commissure but fasciclin II in longitudinal axon tracts (Harrelson and Goodman, 1988). The important role of proteins like these in neuronal development is illustrated with single and double mutants in which commissural or longitudinal axon pathways are missing. In these cases, some axons that normally cross the midline instead remain ipsilateral, and vice versa (Seeger et al., 1993). Interestingly, axons that are forced in this manner to take ipsilateral tracts do not generally follow them as well as in normal animals, suggesting that it is necessary for them first to cross the commissure before they are endowed with the ability to read ipsilateral cues in an error-free fashion.

The above experiments of Seeger et al. (1993) suggest that the ability of axons to recognize cues may vary with "experience." This idea was proposed by Dodd et al. (1988) to account for the behavior of early commissural axons in the developing spinal cord of the rat. As such axons project toward the ventral midline, they express TAG-1, an axonal surface glycoprotein, and continue to do so until they emerge from the floorplate. Within the floorplate, they begin to express L-1, another surface glycoprotein. Once they make the turn to project longitudinally, TAG-1 expression is completely absent, and expression of L-1 increases, although only on the distal (contralateral) segments of the axons. Since the commissural axons ignore lateral cues on their own side of the cord but respond to them on the opposite side, these authors suggest that the crossing of the midline modifies cell surface receptors, making axons responsive to rostrocaudal guidance cues.

There is evidence to support this idea in the developing leech. Kuwada (1984) studied the behavior of the L motor neuron in mutant embryos in which the germinal plate failed to fuse at the midline. In these animals, L motor neurons were physically blocked from their usual crossing to the opposite side and were forced to grow ipsilaterally. However, they failed to either consistently follow or avoid the lateral cues that their contralateral homologs would have used to exit the CNS, thus appearing generally incapable of responding to the ipsilateral markers appropriately.

The above finding suggests that L motor neurons must first cross the midline to acquire the ability to read lateral cues, a requirement that may also apply to the AP cells. However, this mechanism alone is not sufficient to explain why AP neurons

are not normally bilateral. After establishing their primary axonal projections, they exhibit profuse secondary branching within the neuropil, with many fine branches actually crossing back over the midline into ipsilateral space. Presumably, once an AP is endowed with the ability to recognize lateral cues, it would do so on both sides, and follow those cues in two directions. Yet we never see bilateral AP neurons unless we first ablate a homolog on the opposite side of the ganglion. In most of those cases, the extra axon originated on the far side of the ganglion (see Fig. 2*A,B*), though sometimes an axon was found to grow from the primary neurite (or both: Fig. 2*C*).

A role for inhibition. We propose that the reason AP neurons do not project bilaterally after elaborating their neuropilar arbors is because they are inhibited from doing so by their contralateral homologs. There are now many examples of cell–cell inhibition governing the outgrowth of neurons *in vitro* (reviewed in Schwab et al., 1993), but thus far this inhibition has been demonstrated between cells of different types, such as retinal and sympathetic neurites (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987a,b), temporal retinal axons and posterior tectal membranes (Walter et al., 1987; Godement and Bonhoeffer, 1989), and neurons and glial cells (Chiquet and Nicholls, 1987; Schwab and Caroni, 1988; Fawcett et al., 1989; Bandtlow et al., 1990). In contrast, axonal growth in several leech neurons, including AP cells, is known to be inhibited by interactions between *homologous* neurons *in vivo* (Gao and Macagno, 1987a,b; Modney and Muller, 1993). The data presented here further show that each AP neuron is inhibited by both of its homologs, the adjacent and the contralateral, and that the *release* of an AP from inhibition depends entirely on when a particular neighbor is killed (Fig. 8). Before E15, an AP can never become bilateral, possibly because it is inhibited from doing so by a newly ingrowing axon from an adjacent homolog (Fig. 8, left). However, when an AP is killed *after* adjacent homologs have begun to retract their longitudinal axons, the contralateral homolog can indeed project bilaterally (Fig. 8, center), as it is then free from inhibition by the ipsilateral AP cell. If this idea is correct, one would expect that if three AP cells in a row were deleted, thus eliminating any renewed growth by adjacent neighbors, the central contralateral AP cell would be free to project bilaterally to innervate vacated territory, even between the ages of E9 and E12. This was found to be the case in an earlier study (Gao and Macagno, 1987b), and is illustrated in Figure 8 (right). Thus, the lack of formation of bilateral projections by normal AP cells is probably not due to the late appearance of same-side cues, as these apparently exist even before E12.

What is the nature of the inhibitory signal? Although there is evidence from *in vitro* studies that neurite retraction can be caused by the release of diffusible molecules (Haydon et al., 1984, 1987; Lankford et al., 1988; McCobb et al., 1988; Luo et al., 1993; Pini, 1993; Pugh and Berg, 1994), it is unlikely that this mechanism pertains in the cases of cells that inhibit their own type, like the AP cells, because they would then inhibit themselves as well. In these cases, inhibition is more likely to arise from direct contact between homologs, perhaps via repellent surface molecules (e.g., Caroni and Schwab, 1988a,b; Cox et al., 1990; Davies et al., 1990; Raper and Kapfhammer, 1990; Bandtlow et al., 1993) or through synaptic signaling. With regard to the latter, it has been shown that electrical activity can cause growth cones to stop growing and even retract (Cohan et al., 1987; Cohan, 1990; Fields et al., 1990), and we recently proposed that formation of transient gap junctions between the overlapping axons of *adjacent* AP homologs might initiate the eventual collapse of their growth cones (Wolszon et al., 1994a). However, since gap junctions are found between cells of many types in developing nervous systems, including cells that are not mutually inhibitory, there must be a mechanism in place for the AP cells that allows cell- or axon-specific signaling.

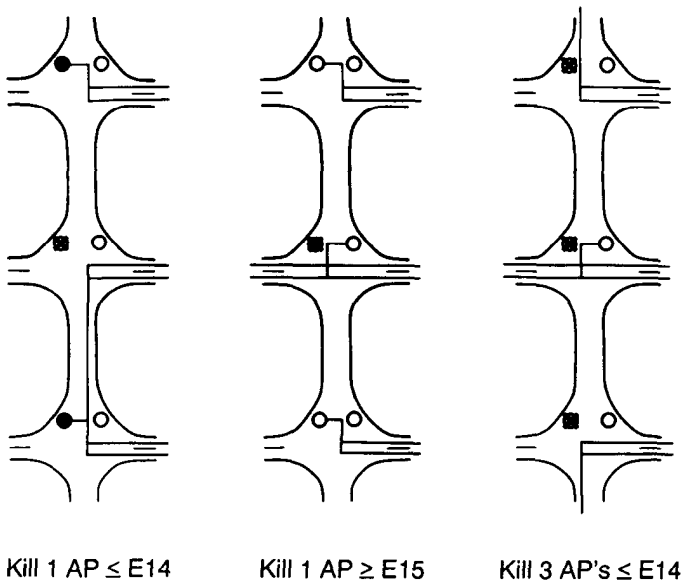

Figure 8. Schematic drawings of the responses of AP cells to deletion of their homologs. *Left,* As illustrated in Figure 1, deletion of an AP during the period of overlap usually causes the posterior homolog to take over territory vacated by the deleted cell (Gao and Macagno, 1987b). *X* indicates the killed cell. *Center,* The conclusion, in this report, that deletion of an AP at E15 or later causes the contralateral homolog to generate bilateral projections. *Right,* An observation, from Gao and Macagno (1987b) that the deletion of three AP cells in a row causes the center AP homolog to generate ipsilateral projections in three out of seven cases. The territory of the anterior and posterior killed cells is taken over by homologous APs in adjacent ganglia, whose somata lie beyond the boundaries of the figure.

Can gap junctional signaling trigger the generation of bilateral projections? The experiments described in this report support the idea that the gap junctional communication between contralateral AP pairs could allow one AP neuron to learn that its neighbor is dying. Molecular signaling via transient gap junctions during development has been proposed in a number of vertebrate and invertebrate systems (Potter et al., 1966; Fischbach, 1972; LoPresti et al., 1973, 1974; Taghert, 1982; Bentley et al., 1991; Yuste et al., 1992; Peinado et al., 1993; reviewed in Warner, 1992). Although no signaling molecule has yet been identified in these systems, likely candidates include calcium and inositol 1,4,5-trisphosphate (Saez et al., 1989; reviewed in Bennett and Verselis, 1992). Indeed, in previous studies examining the response of an AP cell to the deletion of an *ipsilateral* homolog, we have found that calcium waves produced by photoablation can cross the gap junctions that link ipsilateral AP neurons with each other, and remain confined to the nearby longitudinal axon of the receiving cell (Wolszon et al., 1994b). This exchange could act as a signal that tells an inhibited axon to begin growing again, and could serve a function in the generation of bilateral projections described here. Any signals exchanged via gap junctions would have to be specific to particular contacts between cells, or interpreted only locally, since only particular axons of a cell respond to ablation of a neighbor.

The electrical coupling found between AP pairs outlasted their ability to generate bilateral projections. If gap junctions do allow a dying AP cell to communicate with its neighbor, these findings would suggest that while the *detection* of damage is required in order to generate a new projection, the ability to *respond* to the death signal may have its own time course. For example, the lateral pathway cues, or the AP cell's ability to detect or respond to them, may disappear when embryogenesis ends, even though gap junctional signaling is intact. Future experiments will be directed toward looking for surface markers, and for pharmacological or molecular methods to block gap junctional signaling in leech embryos.

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