# Changes in the Number of Chick Ciliary Ganglion Neuron Processes with Time in Cell Culture

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*Abstract.* The purpose of this study was to describe the shape of chick ciliary ganglion neurons dissociated from embryonic day 8 or 9 ganglia and maintained in vitro. Most of the neurons were multipolar during the first three days after plating, with an average of 6.0 processes extending directly from the cell body. The neurons became unipolar with time. The remaining primary process accounted for >90% of the total neuritic arbor. This striking change in morphology was not due to the selective loss of multipolar cells, or to an obvious decline in the health of apparently intact cells. The retraction of processes was neither prevented nor promoted by the presence of embryonic muscle cells. Process pruning occurred to the same extent and over the same time course whether the cells were plated on a monolayer of embryonic myotubes or on a layer of lysed fibroblasts. Process retraction is not an inevitable consequence of our culture conditions. Motoneurons dissociated from embryonic spinal cords remained multipolar over the same period of time. We conclude that ciliary ganglion neurons breed true in dissociated cell culture in that the multipolar–unipolar transition reflects their normal, in vivo, developmental program.

THE development of embryonic chick ciliary ganglia has been described in great detail (Pilar and Tuttle, 1982). This preparation has also proved useful for studies of neuronal development and nerve–muscle synapse formation in vitro. Conditions have been defined that permit the dissociation and survival of virtually 100% of neurons in sparse cell culture (Nishi and Berg, 1977, 1979; Varon et al., 1979; Tuttle et al., 1980). During the course of our own studies (Role et al., 1982, 1983, 1985, 1987) we confirmed earlier observations that most of the neurons extend several processes during the first few days in culture.

Ciliary ganglia contain two types of neurons. Ciliary neurons innervate striated muscle in the iris and ciliary body; choroid neurons innervate smooth muscle in the choroid coat of the eye. Both ciliary and choroid neurons are unipolar in adult ganglia, so it might be supposed that the multipolar form of cultured cells is an artifact, perhaps reflecting altered adhesion to an artificial, two-dimensional surface. In the initial study of dissociated ciliary ganglion neurons (Helfand et al., 1976), the cells were plated on polylysine and multipolar cells were found after 1 wk in vitro. On the other hand, Landmesser and Pilar (1974*a*, 1976) have shown that ciliary and choroid neurons pass through a multipolar phase during the course of development in vivo. On embryonic day 7 (E7), which corresponds to Hamburger–Hamilton stages 30–33, the cells extend extensive dendritic processes. By E11 (stage 37), most of the dendrites have retracted, and only a few spine-like pseudodendrites remain. In addition, E7 ciliary neurons possess more than one axon. The axon/soma ratio is maximum on E11 and then declines steadily until hatching on E20–E21. (Landmesser and Pilar, 1976). Thus, it is possible that the multipolar form in vitro is an accurate reflection of the normal, in vivo developmental program. Ciliary ganglion neurons are not unique in their initial overproduction and subsequent retraction of neurites (Cowan et al., 1984). Two examples are quite similar to the pattern of change observed in ciliary ganglia. Neurons in the nucleus magnocellularis of the chick (Jahveri and Morest, 1982) and in the cardiac ganglion of the frog heart (Heathcote and Sargent, 1985) prune elaborate neuritic arbors to achieve an essentially unipolar shape. In the former case the extra processes are undoubtedly dendrites. In the latter, they are probably axons.

We have investigated the fate of ciliary ganglion cell neurites in vitro by injecting cell bodies with Lucifer Yellow and measuring the number and length of dye-filled processes at various times between 6 h and 3 wk after plating. Our population study strongly suggests that each neuron undergoes a multipolar–unipolar transition, and that this transition occurs over approximately the same time course as that observed in vivo.

## Materials and Methods

### Cell Culture

Nerve and muscle cells were grown on collagen-coated glass coverslips that were glued with medical-grade adhesive (Dow Corning Corp., Midland, MI) over an 18-mm hole cut in a 60-mm tissue culture plate. The glass-bottomed well contained ~200 μl of medium.

Portions of this work have appeared in abstract form (1982. *Soc. Neurosci.* 8:129 and 1983. *Soc. Neurosci.* 9:1179).

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Muscle cultures were prepared as previously described (Fischbach, 1972; Frank and Fischbach, 1979). Briefly, pectoral muscle dissected from 11-d-old chick embryos (E11) were minced, incubated in a $Ca^{++}/Mg^{++}$-free, phosphate-buffered saline (Puck's $D_1G$) for 30 min, and then mechanically disrupted by repeated passage through a fire-polished pasteur pipette. Approximately $4 \times 10^5$ mononucleated cells were added to each dish in 3 ml of Eagles minimum essential medium (MEM) supplemented with horse serum (10% vol/vol), chick embryo extract (5% vol/vol), glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). After 3–4 d, fibroblasts were eliminated by adding cytosine arabinoside ($10^{-5}$ M) to the medium for 24 h.

Ciliary ganglia were dissected from E8 or E9 embryos, and dissociated as described by Nishi and Berg (1977). The medium was withdrawn from the 60-mm plates, and neurons were added directly to the culture wells at a density of 25,000/cm² (1,500/coverslip). The neurons were plated on myotubes 5–7 d after the cultures were seeded with myoblasts, or they were plated on a substrate of lysed fibroblasts. Fibroblasts were dissociated from the skin of E10 embryos. After they formed a confluent monolayer, they were lysed by exposure to distilled water for 2 h (Nishi and Berg, 1979).

Spinal cord motoneurons were labeled in vivo by retrograde transport of Lucifer Yellow–wheat germ agglutinin conjugates, as described by O'Brien and Fischbach (1986). Briefly, the conjugates were injected into hindlimbs of E5 embryos. The eggs were returned to the incubator, and 24 h later lumbar spinal cord segments were excised and dissociated. Motoneurons were distinguished from other spinal cord neurons in vitro by the presence of bright fluorescent speckles within their cell bodies and proximal processes.

### *Electrophysiology*

Electrophysiological recordings were performed at room temperature on the stage of an inverted microscope (Leitz, Diavert) equipped with phase contrast, interference contrast, and epifluorescence optics. The culture medium was replaced with recording medium containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM $NaH_2PO_4$, 0.9 mM Mg $SO_4$, 3.6 mM $CaCl_2$, 6 mM glucose, and 12 mM Hepes, pH 7.4.

Intracellular recordings were obtained from ciliary neurons with patch electrodes (Hamill et al., 1981). Borosilicate glass capillaries (World Precision Instruments, New Haven, CT) were drawn in two stages on a vertical electrode puller (David Kopf Instruments, Tujunga, CA). The resistance of the electrodes ranged between 8 and 15 MΩ when filled with an intracellular solution containing 140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA-K, 1 mM $CaCl_2$ and 10 mM Hepes, pH 7.3. Thin-walled glass (0.25 mm) pulled in the same manner was used for local application of drugs by pressure ejection (Choi and Fischbach, 1981). Lucifer Yellow CH was injected into individual neurons through fine-tipped microelectrodes that measured 50–100 MΩ resistance when filled with a 4% solution of the dye in 0.25 M LiCl. We routinely used 0.5-s pulses of inward current repeated at 0.5 Hz.

In some experiments, neurons were stimulated through cell-attached patch electrodes filled with KCl (140 mM), $MgCl_2$ (2.0 mM), EGTA KOH (11.0 mM), $CaCl_2$ (1.0 mM), Hepes (10.0 mM); pH, 7.4. The efficacy of stimulation was documented by simultaneously recording the soma spike through the same electrode connected in the active bridge circuit of an amplifier (701; WPI).

### *Video Microscopy*

Lucifer Yellow-filled cells were illuminated with a 100 W mercury source and viewed through appropriate filters (excitation λ:390–490, barrier λ:515) and either a 40× (NA, 1.0) or a 63× (NA, 1.3) phase contrast objective lens. Images obtained with an SIT camera (series 65; Dage-MTI, Inc., Wabash, MI) were simultaneously displayed on a high resolution monitor (Audiotronix, Spring Lake Park, MN) and recorded with a video tape recorder equipped with single frame viewing capability (NV 8030; Panasonic). The SIT camera/video tape recorder system allowed us to rapidly scan the culture surface at several planes of focus before significant fading of the fluorescent signal occurred. Several frames were traced to reconstruct the image of the entire cell. Measurements of neurite length and branching pattern were made with the aid of a GITCO digitizing pad and a MINC computer (Digital Equipment Corp., Maynard, MA). Programs for the data analysis were written by Viken Matossian (Washington University).

## *Results*

Many dissociated ciliary ganglion neurons settled directly on myotubes, but many others settled on the collagen surface between the muscle cells. In either case, neurites emerged rapidly from the cell bodies. Although some variation in soma diameter was evident throughout the period of observation, we did not attempt to distinguish between ciliary and choroid neurons. Therefore, the following description of ciliary ganglion neurons probably applies to both populations. The initial outgrowth could be followed with interference or phase contrast optics. However, as the neuritic arbor became more complex, individual processes were lost as they passed along the edge of refractile myotubes or formed fasicles with other neurites. Therefore, to follow the course of the neurites, the cells were injected with Lucifer Yellow. Neurites of the youngest cells, including the finest growth cone filopodia, were filled after only 1–2 min of repetitive injection pulses (Fig. 1). Longer times (5–10 min) were required to fill distal branches of older cells.

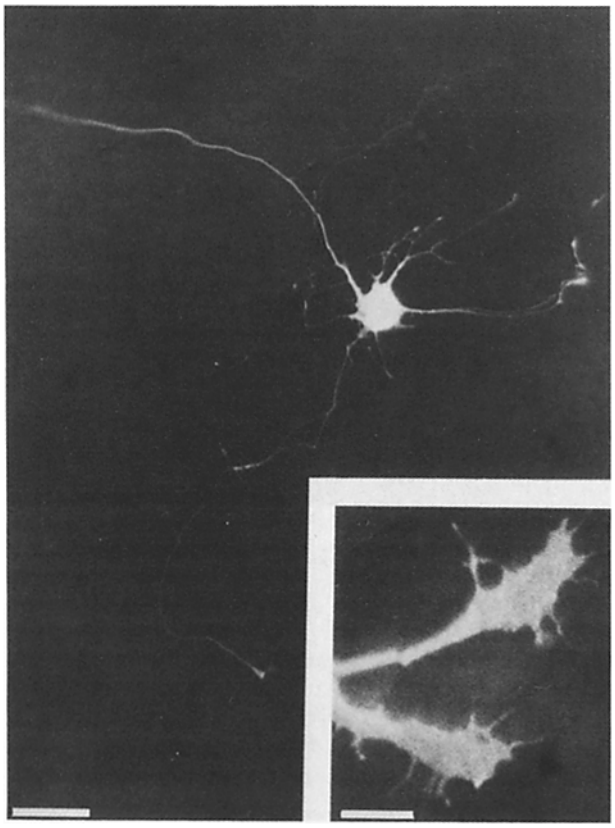

*Figure 1.* A ciliary ganglion neuron injected with Lucifer Yellow CH on the second day after plating. Injection pulses repeated at 0.5 Hz for 2 min hyperpolarized the membrane by 20 mV. Note that dye fills the finest processes. (*Inset*) Dye-filled growth cone filopodia from another cell. Cells were visualized without fixation under epifluorescence illumination (excitation λ:390–490, barrier λ:515). Bars: 20 μm; (*inset*) 5 μm.

Within the first 12 h after plating most of the neurons were multipolar, and prominent growth cones were evident at the ends of the major processes (Fig. 2 *A*). All of the neurons tested during this interval were electrically excitable (Fig. 2 *B*), some displayed spontaneous action potentials (Fig. 2 *C*), and they were all sensitive to acetylcholine (Fig. 2 *D*). The neurons could be stimulated through fire-polished micropipettes that were sealed by gentle suction to the neuron soma (Fig. 3). This technique, which has proved extremely useful (Role et al., 1987) merits some discussion. With seal resistances of 0.05–0.1 GΩ, spikes were reliably evoked by cur-

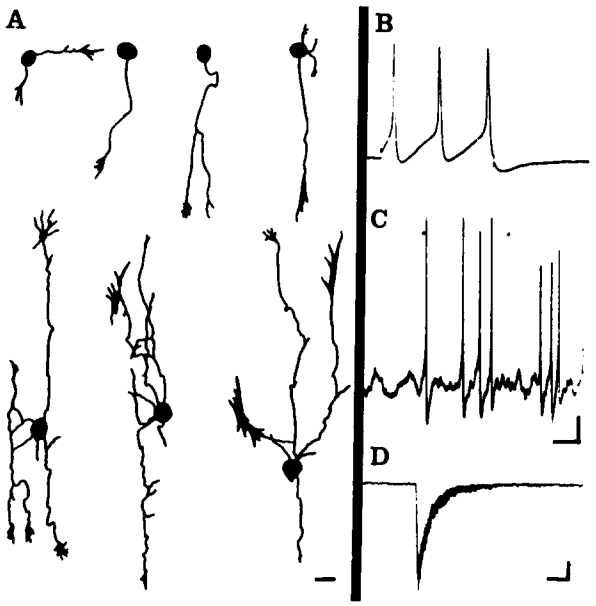


*Figure 2.* Shapes and electrical properties of ciliary ganglion neurons within the first 12 h after plating. The neurons shown in *A* were injected (*top row, left to right*) at ~4, 6, 6, and 8 h and (*bottom row, left to right*) 10, 12, and 12 h after plating. Bar, 10 μm. (*B*) Repetitive spikes evoked in a neuron 2 h after the cell settled on the culture surface. (*C*) A recording from another cell at the same time in vitro that exhibited spontaneous activity. Some neurons remained active for up to an hour of continuous recording. Calibration, 10 mV × 50 ms. (*D*) An inward current evoked in a neuron (1 h after plating) after application of 100 μm acetylcholine. The cell was voltage-clamped at −50 mV and acetylcholine was applied by pressure ejection. Calibration; 50 pA × 1 s.

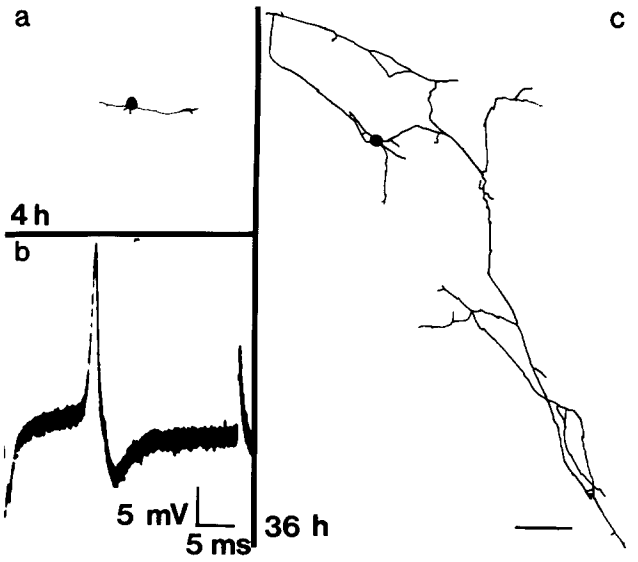


*Figure 3.* Extracellular stimulation of a cell (*a*) through a sealed-on microelectrode. The soma spike (*b*) was evoked during a 30-ms, 0.5-nA current pulse. After 1 h of stimulation at 1 Hz, the culture was returned to the incubator. The cell was relocated 30 h later and injected with Lucifer Yellow. Note the extensive growth of the neuritic arbor (*c*).

rents of <1 nA. Slightly larger currents were required when the stimulating electrodes were filled with a solution containing 140 mM NaCl instead of 140 mM KCl, presumably because the conductance of the sealed-off membrane patch was lower in this solution. More conventional extracellular stimulation with the electrode simply placed adjacent to the soma required much larger currents, on the order of several microamps, and in this situation the youngest neurons often degenerated after a few pulses. In comparison, stimulation through sealed-on electrodes was relatively innocuous; cells stimulated for 1 h at 0.5–1.0 Hz and then relocated 1 d later had grown to an extent comparable to unstimulated controls (Fig. 3, *a* and *c*). Moreover, the seals could be broken and reformed several times without obvious deterioration of the response.

A summary of total neurite length, based on 75 neurons examined at various times between 4 h and 3 wk after plating, is shown in Fig. 4. An initial phase of rapid outgrowth was maintained for ~2 d. During this interval the total process length increased at a rate of ~40 μm/h. The total process length reached a plateau after 3 d, and did not increase significantly over the next 18 d. This plateau does not imply that all neurites stopped growing. Indeed, the longest process of each cell continued to grow, albeit at a slightly reduced rate (~20 μm/h), for at least 6 d after plating (Fig. 5). Thus, some neurites must retract during the same interval.

In fact, we found that the number of processes that issued directly from the soma for a distance of at least 20 μm declined with time. Between 12 and 24 h after plating, the neurons exhibited 6.0 ± 0.5 (mean ± SEM, $n$ = 12) primary processes, whereas cells examined between 2 and 3 d after plating had only 3.0 ± 0.4 ($n$ = 12) primary neurites. After 1 wk, all of the 23 neurons examined were essentially unipolar, with 90% of the total neurite length accounted for by the single dominant process. Representative tracings of neurons studied between 12–24 h and 6–9 d after plating are shown in Fig. 6. The remaining primary process branched extensively (beginning at 150–200 μm from the soma), but the decrease in the number of neurites that extended directly from the cell body is clearly evident.

To determine if the appearance of a unipolar geometry depended on the presence of target myotubes, we plated dissociated ciliary ganglion cells on a layer of lysed fibroblasts. The neurons attached to this substrate as well as they did to myotube containing collagen-coated coverslips, and the ini-

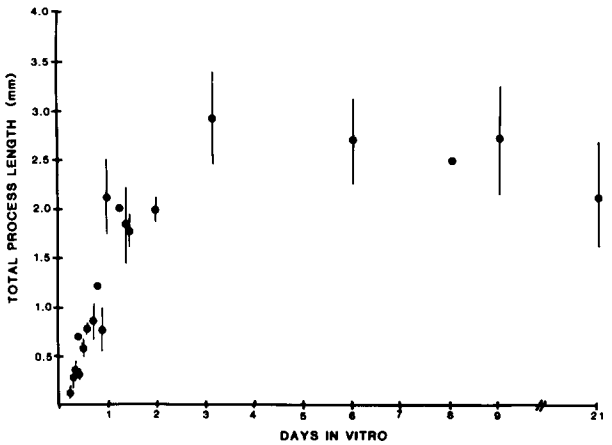


*Figure 4.* Total process length as a function of time in vitro. The total length of all neurites was determined with a digitizing tablet for 77 neurons injected with Lucifer Yellow between 4 h and 21 d of co-culture. Data are expressed as mean ± SEM.

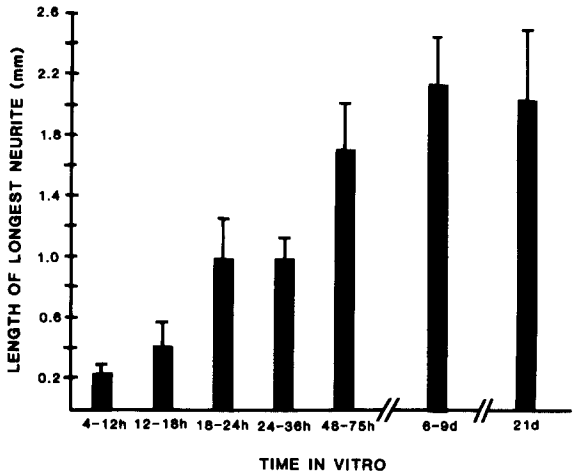


*Figure 5.* Length of the longest neurite as a function of time in vitro. The longest neurite continues to grow until 6–9 d in vitro.

tial outgrowth of neurites was at least as luxuriant (Fig. 7; top). The neurons did not remain multipolar. 1 wk after plating, they were essentially unipolar (Fig. 7; bottom), with the longest process accounting for >90% of the total neuritic length. At this time, the length of the dominant process (2.6 mm ± 0.3; $n$ = 15) was not significantly different from the length of the dominant process of neurons grown with muscle (2.2 mm ± 0.3; $n$ = 14). A summary of the relative length of the dominant processes in the two types of culture at various intervals after plating is presented in Table I.

To rule out the possibility that all neurons maintained in vitro under our culture conditions pare down to a simple unipolar geometry, we investigated the shape of dissociated spinal cord motoneurons. Fig. 8 shows representative tracings of motoneurons identified 6–7 d after plating and then injected with Lucifer Yellow CH. It is evident that several major processes were maintained; the longest process accounted for only 46 ± 7% ($n$ = 12) of the total process length at this time.

The predominance of unipolar ciliary ganglion neurons after 1 wk was not due to the loss of multipolar cells. About 1,500 neurons were added to each culture well and cell counts in randomly selected fields showed that ~1,300 (90%) were present in myotube and in lysed fibroblast cultures 1–2 wk after plating. The retraction of processes was not associated with a general decline in the health of apparently intact cells. Changes in passive and active membrane properties were noted during the period of process pruning but they were quite small (Table II). The membrane potential and action potential amplitude increased slightly, and as expected, the input resistance decreased by ~50% during the interval of rapid process outgrowth. The neurites of multipolar neurons were also healthy in that they conducted impulses. This was demonstrated by recording spikes with focal extracellular electrodes positioned along individual processes at different distances from the cell bodies (Fig. 9), and also by the fact that soma stimulation reliably evoked synaptic potentials in innervated myotubes (Role et al., 1985, 1987). From the delay in stimulus–spike or stimulus–epp intervals, the conduction velocity of ciliary neurites was ~0.1 m/s.

## Discussion

The great majority (34/41) of neurons dissociated from E8–E9 ciliary ganglia are multipolar during the first 2–3 d after plating, whereas the great majority (31/38) of neurons examined between 1 and 3 wk after plating were unipolar. We did not follow individual neurons over time, but it is unlikely that the change in the population comes about by the selective loss of multipolar cells. Cell death does occur in intact ganglia (Landmesser and Pilar, 1974*b*); ~50% of the neurons are lost between E8 and E14, an interval that corresponds to the period of morphological transformation in our cultures. However, we confirmed previous reports that doomed

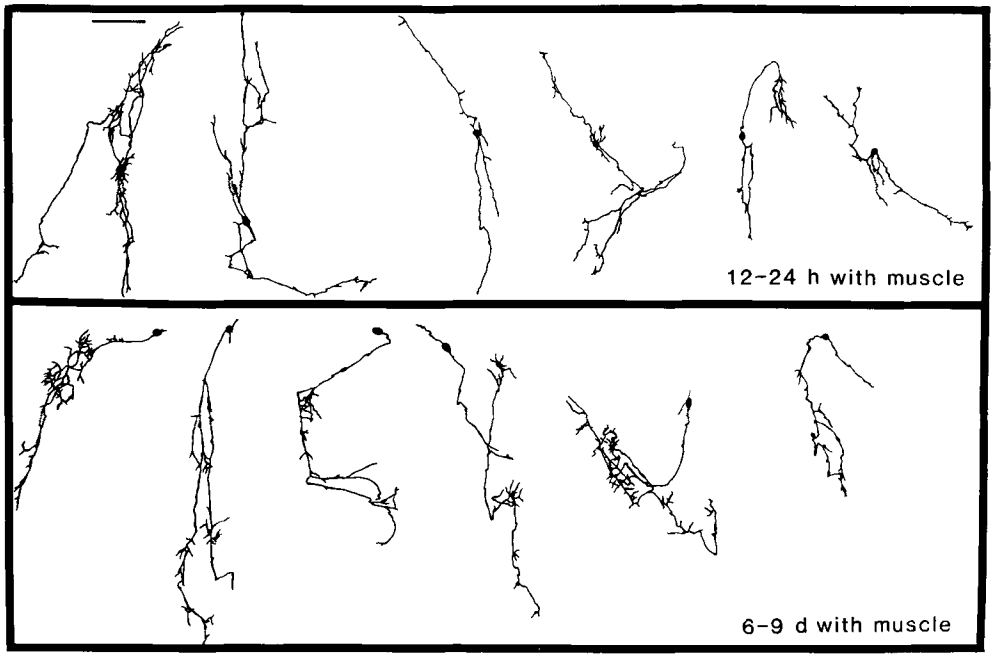


*Figure 6.* The shape of ciliary ganglion neurons after 1–2 d and after 6–9 d of co-culture with myotubes. The arbors of Lucifer Yellow–injected neurons were reconstructed from video images as described in the text. Neurons were initially multipolar, but after 1 wk in vitro they were essentially unipolar with one major, branching process. The examples shown here were selected at random from the 38 neurons studied at these times. Bar, 100 μm.

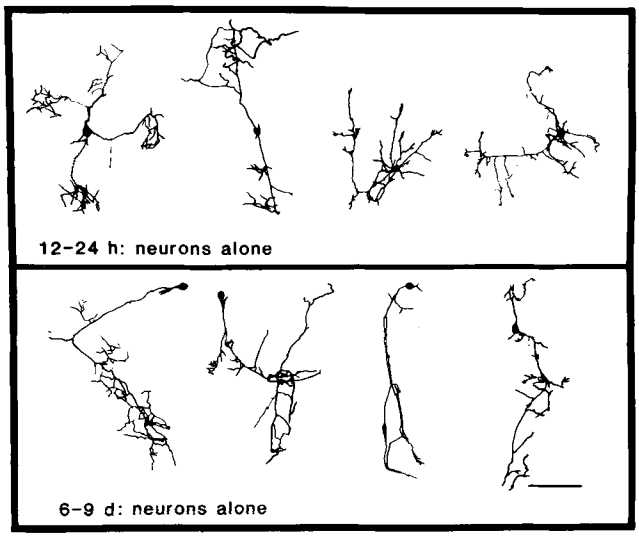


*Figure 7.* The shape of ciliary ganglion neurons after 1–2 d and after 6–9 d in vitro on a layer of lysed fibroblasts. The neurons changed from a multipolar to a unipolar shape when grown without myotubes (cf. Fig. 6). The examples shown were selected at random from the population of 40 neurons studied. Bar, 100 μm.

*Table I. Change in Neuritic Arbor of Ciliary Ganglion Neurons in Vitro*

Length of longest process per length of total neuritic arbor

| | Ciliary ganglion neurons | |
|---|---|---|
| Time in vitro | With muscle | Grown alone |
| 18–36 h | 0.62 ± 0.05 (22) | 0.53 ± 0.09 (8) |
| 2–3 d | 0.73 ± 0.07 (11) | – |
| 6–9 d | 0.90 ± 0.13 (14) | 0.89 ± 0.10 (15) |
| 21 d | 0.90 ± 0.19 (9) | – |

Ciliary ganglion neurons were plated on a monolayer of myotubes or grown alone on a layer of lysed fibroblasts. The length of Lucifer Yellow-filled neurites was determined as described in the text. Entries are mean ± SEM. The number of cells examined is indicated in the parenthesis.

ciliary ganglion neurons can be rescued when they are dissociated and plated on muscle or lysed fibroblasts (Nishi and Berg, 1977, 1979). To account for the observed multipolar–unipolar transition between 2 d and 1 wk after plating, cell death would have to exceed 60%, whereas, under our conditions, no more than 10% of the neurons were lost during this interval. Therefore, the change in morphology with time in vitro most likely reflects retraction of processes by otherwise healthy neurons, rather than selective loss of multipolar cells. Since ciliary and choroid neurons are present in about equal numbers in embryonic ganglia, it seems likely that both types of neuron prune their processes in vitro.

We do not know if the neurites extended by ciliary ganglion neurons in culture are axons or dendrites. Ciliary and choroid neurons in E8 ganglia possess several dendrites, and they have already extended axons that arborize within the iris, ciliary muscle, and choroid coat of the eye (Landmesser and Pilar, 1972; 1974*a*, *b*; 1976). Several arguments suggest that the majority of neurites are axons. All of the processes were thin at their point of exit from the cell body and they did not taper rapidly with distance from the soma. Moreover, all of the processes tested conducted impulses and they formed synapses on contacted myotubes (see Role et al., 1987). The number of processes per neuron and the time course of their elimination in vitro is consistent with their identity as axons. Landmesser and Pilar (1976) found that the number of axons, counted in electron micrographs of E11 (stage 37) ciliary nerves, was about five and a half times the number of ciliary cell bodies in the ganglion at the same stage. This corresponds to our finding of 6.0 neurites per cell body 2–3 d after plating cells from E8–E9 ganglia. As expected, there was a dramatic loss of axons in vivo between E9 and E12, coincident with the rapid phase of neuron degeneration. However, Landmesser and Pilar (1976) also provided evidence for axon elimination by healthy cells in that the axon/soma ratio decreased from 5.5 to ~1.5 by ~E20.

These arguments in favor of axons are not entirely conclusive. In the absence of their normal synaptic input, dendrites may conduct impulses, and they may also innervate myotubes. Dendrites of cultured chick anterior horn motoneu-

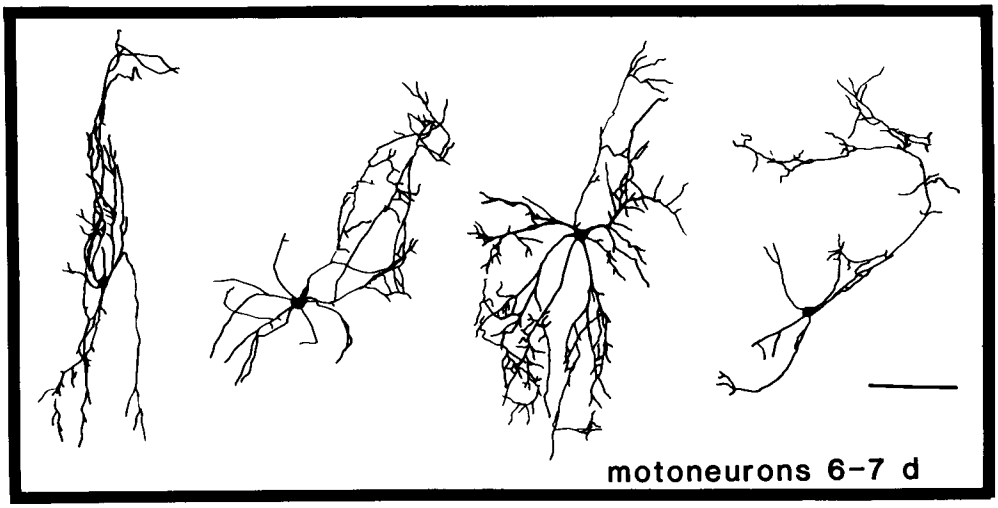


*Figure 8.* The shape of identified spinal cord motoneurons after 6–7 d in vitro. In this experiment, the neurons were plated on a layer of myotubes. Motoneurons in the lateral motor column were labeled in vivo before dissociation by retrograde transport of Lucifer Yellow–wheat germ agglutinin conjugates. Motoneurons identified by the presence of intrasomatic fluorescent speckles were injected with Lucifer Yellow CH and reconstructed from video images. They remain multipolar for at least 1 wk after plating. Bar, 100 μm.

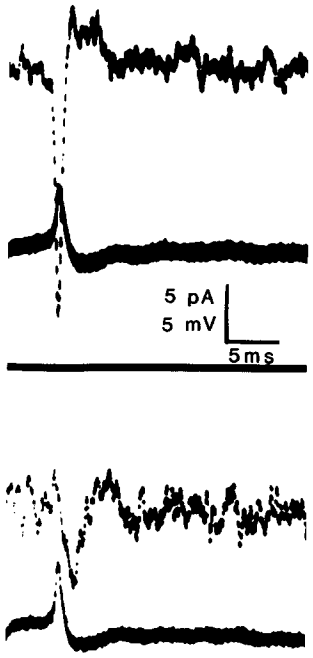


*Figure 9.* Conducted action potentials. The top traces of each pair show neurite spikes recorded with an extracellular electrode and the bottom traces show soma spikes recorded through the stimulating electrode. In the top pair, the electrode was positioned near the soma and in the bottom pair it was located some distance away along the same neurite.

*Table II. Electrical Properties of Ciliary Ganglion Neurons In Vitro*

| | Time after plating | | |
|---|---|---|---|
| | 1–12 h | 24–36 h | 1–2 wk |
| $V_m$ (*mV*)* | 48.4 ± 1.1 (23) | 55.6 ± 1.0 (33) | 54.0 ± 1.4 (17) |
| $R_{in}$ (GΩ) | 2.2 ± 0.2 (21) | 0.93 ± 0.08 (27) | 1.02 ± 0.28 (12) |
| Spike amplitude (*mV*) | 77 ± 3 (19) | 81 ± 2 (28) | 86 ± 3 (12) |
| Repetitive spikes (%) | 77 (18) | 81 (27) | 84 (13) |
| Acetylcholine current (*pA*) | 316 ± 54 (19) | 254 ± 21 (22) | 247 ± 42 (11) |

Ciliary ganglion neurons were dissociated and plated on a monolayer of chick myotubes for the indicated times. Input resistance ($R_{in}$) was estimated from the steady state electronic potential achieved during 300 ms, pulses that hyperpolarized the membrane by 5–10 mV. Repetitive spiking was assessed by injecting depolarizing current for ~100 ms. The response to 100 μM acetylcholine was examined in neurons voltage-clamped at −50 mV. Numerals in parentheses indicate the number of cells tested.
* $V_m$, resting membrane potential.

rons apparently behave in this manner (Role et al., 1985). Dendrites of ciliary neurons (and probably of choroid neurons as well) are retracted between E11 and E14 (stage 37–40), so the time course of pruning in culture is also consistent with their identity as dendrites. Axons have been distinguished from dendrites in other types of cultured neurons based on the electron density of the cytoplasm (Landis, 1976) and on the distribution of synaptic vesicles, polyribosomes, and microtubule-associated proteins (Bartlett and Banker, 1984*a*, *b*; Caceres et al., 1984, 1986), and the distribution of certain neurofilament subunits (Hirokawa et al., 1984). These markers might prove useful in future studies of ciliary ganglion neurites.

It is noteworthy that ciliary ganglion neurons pruned their neurites in the absence of their normal presynaptic input, supporting glial cells and target myotubes. Intrinsic factors must play a major role in determining the overall shape of these neurons in dissociated cell culture. Intrinsic determinants of cell shape have also been emphasized in studies of dissociated embryonic rat hippocampal neurons (Banker and Cowan, 1977, 1979; Bartlett and Banker, 1984*a*, *b*). Pyramidal shaped neurons with a prominant apical dendrite, a few basal dendrites, and one or two long axons develop in relatively sparse cultures in which few contacts are established with other cells. Similar observations have been made in cultures of cortical (Kriegstein and Dichter, 1983) and spinal cord neurons (Neal et al., 1978). The powerful influence of endogenous determinants of neuronal form is underscored by Solomon's (1979, 1981) remarkable finding that mitotically related (sibling) neuroblastoma cells assume shapes that are mirror images of one another. In these cells, the distribution of microtubule organizing centers and microtubule-associated proteins may determine soma shape and the sites of neurite outgrowth (Solomon, 1981; Spiegelman et al., 1979).

In view of the likely possibility that many of the neurites of multipolar ganglion cells are axons, it is significant that co-cultured myotubes did not prevent their retraction. Process retraction in vitro may be related to the elimination of axon branches from multiply innervated endplates in embryonic and neonatal muscle, and also to the phenomenon of motor nerve terminal sprouting in partially denervated adult muscle. In both cases, it is widely believed that motor axons compete, in some way, for muscle-derived axon growth factors (Betz, 1980; Pilar et al., 1980). (In some muscles, synapse elimination proceeds in the absence of competing motor axons [Brown et al., 1976]. Thus, intrinsic determinants may play a role here as well as in vitro.) Either the same factors are not produced in sufficient quantity by the embryonic myotubes, or different factors are required. Soluble and substrate-attached factors derived from presumptive target tissues promote the initial outgrowth of ciliary ganglion neurites (Collins, 1978; Collins and Dawson, 1982; Collins and Lee, 1984) and of spinal cord cell neurites (Henderson et al., 1981, 1984; Gurney, 1984; Berg, 1984). Such factors were probably present in our complex medium, which contained embryo extract. Indeed, our estimate of the initial rate of neurite outgrowth, ~40 μm/h, is comparable to rates observed in previous studies of cultured ciliary and sympathetic ganglion neurons (Collins and Dawson, 1982; Bray, 1973; Argiro and Johnson, 1982; Argiro et al., 1984). Unfortunately, nothing is known about factors that might regulate the long term maintenance of neurites in vitro.

It is also clear that myotubes are not directly responsible for the retraction of processes in vitro, as retraction proceeded to the same extent and over the same time course when the neurons were grown in the absence of myotubes on a layer of lysed fibroblasts. Here again our findings parallel results obtained in vivo. Landmesser and Pilar (1976) found that ciliary ganglion neurons differentiated to a remarkable degree in embryos from which the eyecup was removed at an early stage of development. In this situation, nearly all of the neurons within the ganglion degenerate. However, before the period of cell death, neurons in peripherally deprived ganglia extend and retract dendrites to the same degree as in control ganglia. Axons reach the periphery, but the great majority are lost coincident with the exaggerated phase of neuronal degeneration. Our results show that at least one process survives in the absence of the target when cell death is prevented.

The surviving ciliary ganglion nerve process did not continue to grow throughout the 3-wk period examined. Between 2 and 6 d after plating, the net rate of advance was about half the initial rate of outgrowth. 1 wk after plating, the longest neurites measured ~2.5 mm, and they did not elongate further during the next 2 wk. Growth cones often pause when they contact a myotube (our unpublished observations), but it is unlikely that such contacts, or the formation of synapses, stunt the growth of ciliary ganglion cells because neurites cultured on lysed fibroblasts stabilize at the same length. Interestingly, this is about the same length achieved by most of the longest processes of hippocampal pyramidal neurons (Banker and Cowan, 1979), spinal cord neurons (Neal et al., 1978), and sympathetic ganglion neurons (Wakshull et al., 1979) in long term cell cultures. Argiro et al. (1984) have shown that the net growth of sympathetic ganglion neurites reflects a balance between the maximum rate of growth cone advance and frequent, short retractions. Thus, the cessation of growth of the longest process may reflect the same mechanisms that lead to the complete retraction of the others. We have emphasized intrinsic determinants of cell form, but extrinsic factors, such as the directed migration of nonneuronal cells and the rapid growth of the postsynaptic target, must play a significant role in regulating neurite length in vivo.

In sum, we have shown that the multipolar form of ciliary ganglion neurons in short term cultures is a transient phenomenon. Dissociated neurons appear to follow their in vivo program to assume a unipolar shape between 3 and 7 d after plating. In this regard, our results compliment those of Nishi and Berg (1979) who found that the activity of choline acetyltransferase in dissociated ciliary ganglion neurons increases according to the in vivo schedule. Skeletal myotubes do not play a major role in the phenomenon of process elimination by cultured neurons or in the regulation of neurite length. The possibility that myotubes influence the choice of which process survives is discussed in the following paper (Role et al., 1987).

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