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Growth and Morphogenesis of an Autonomic Ganglion. I. Matching Neurons with Target

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Regulation of the number and size of neurons presumably plays a role in the matching of a group of neurons to their target. In this paper the relationship of the cardiac ganglion neurons of the frog to their target is examined. Neurons in this ganglion first appear in the embryo and continue to accumulate for several months, even after the animal has completed metamorphosis, and eventually reach a fixed number of cells in the adult. This prolonged period of neuron production has provided an opportunity to manipulate development and test various mechanisms of neuronal regulation. Manipulation of animal culture conditions and hormone levels has shown that the addition of neurons to the ganglion continues up to the characteristic adult number and depends upon neither the chronological age nor the developmental stage of the animal. The size of neurons also changes markedly during development. The *average* cell body size initially decreases due to the addition of many smaller cells to the ganglion. After metamorphosis neuron size increases dramatically. The changes in size and number complement one another such that the total volume of neuronal cell bodies increases in proportion with the size of both the target and the entire body. The relationship holds for changes in animal size that extend over 4 orders of magnitude and follows a power function of the form $y = bx^m$.

Regulation of cardiac ganglion size can be divided into 3 overlapping phases: (1) the arrival of neurons and precursors from the neural crest, (2) an increase in neuron number, and (3) an increase in neuron size. A common denominator for all phases is that the size of the ganglion is, in a coherent way, precisely matched to the size of its target.

The number of cells in a particular developmental pathway is often genetically determined. One method by which this is attained is the rigid production of a set number of cells (Lawrence, 1966; Chalfie et al., 1981). An alternative method is overproduction followed by the death of excess numbers of homologous cells (Shankland, 1984; Zackson, 1984). Both of these mechanisms can be mediated by cell–cell interactions, and both could be important in the formation of structures that are invariant, such as the number of metameric segments in an animal or the number of cells in a sensory receptor. The regulation of neuron cell number in structures that continually increase in size might have quite different requirements. One possibility is that an internal clock times the sequential addition of cells (Temple and Raff, 1986). This could be a chronological clock, such that the number of cells is determined by the absolute age of the animal. Alternatively, the clock could be developmental, with the number of cells determined by the animal's developmental stage. The ultimate result of either clock is that there should be a predictable number of cells at any point in the life of the animal.

The regulation of neuron number could be more dynamic than that provided by a rigid clock and might involve cell–cell interaction. Thus, homotypic cell–cell interactions (between 2 identical cell types) or heterotypic interactions might be important in regulating the size of a growing structure. Within the nervous system such heterotypic interactions could take the form of communication between neurons and their targets. In fact, the "matching" of populations of neurons with their targets is thought to occur throughout the nervous system (see reviews by Hollyday, 1980; Hamburger and Oppenheim, 1982). In the present study the regulation of cell size and number in a simple autonomic ganglion has been analyzed. The cardiac ganglion of the frog contains only a single class of neuron, whose size and number increase well into adult life. Although the final number of neurons in the mature adult is apparently predetermined, there is considerable plasticity in neuronal number throughout development. This variation is the result of a mechanism that "matches" the total volume of the neuronal synthetic machinery (cell bodies) with the size of the developing heart, which is the target of the cardiac ganglion. Since there is no detectable neuronal cell death in the ganglion (Heathcote and Sargent, 1985a, 1987), these results raise the possibility that the innervation of the heart may be regulated by a direct action of the target on neuronal growth and proliferation. [These results have been presented in abstract form (Heathcote and Sargent, 1985b).]

Materials and Methods

Larvae of *Xenopus laevis* were obtained from a laboratory breeding colony and staged according to the criteria of Nieuwkoop and Faber (1967). Lab-raised frogs with a snout–rump length of 5.7 cm are approximately a year of age and were the adults used in these experiments (see Fig. 2*B*).

The size of developing animals and their ganglia was manipulated and measured in the following ways. Animals were maintained for indefinite periods at a given size and stage (usually stage 48) by over-

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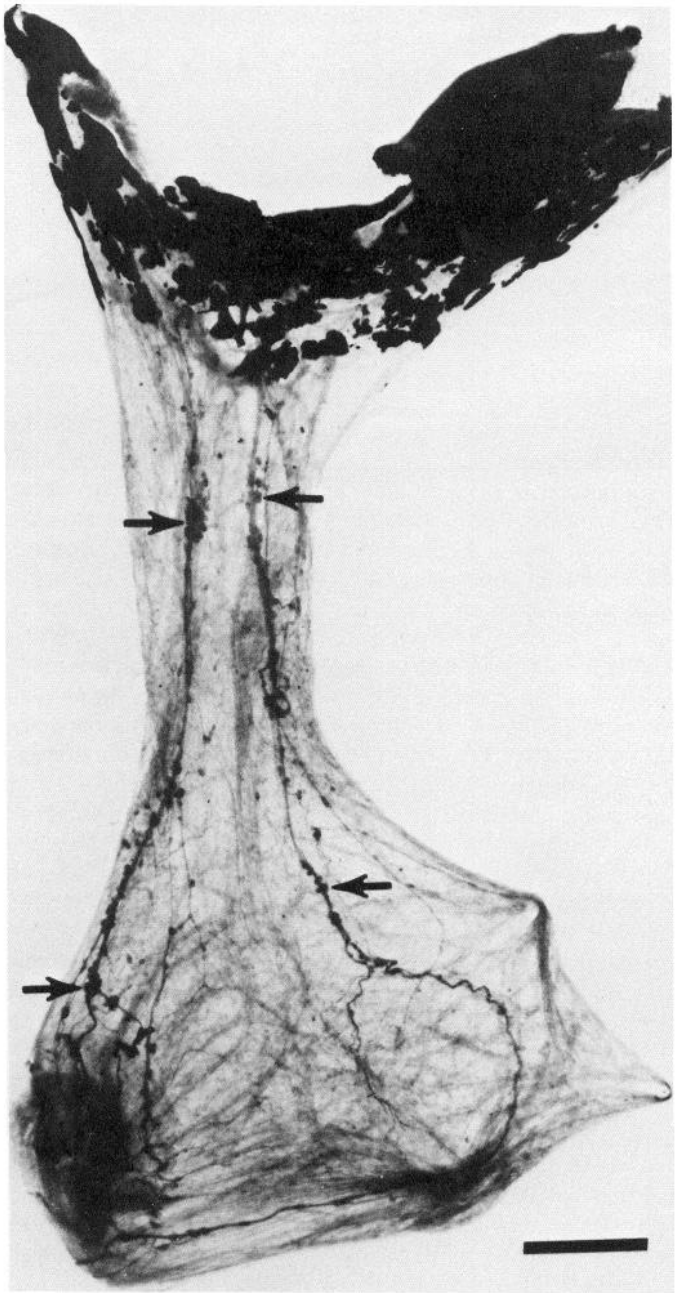

Figure 1. Neurons in a postmetamorphic cardiac ganglion. The atrium of the heart of a stage 66 animal has been stained for AChE. The hundreds of cholinesterase-positive neurons are present in large clumps (*arrows*) along stained intracardiac extensions of the vagus nerves. The number of neurons at this stage of development greatly exceeds the approximately 30 cells present at stage 48 or 1 week of development (Heathcote and Sargent, 1984). Calibration, 200 μm.

crowding (Lamborghini, 1981). Varying the density of animals alters the rate of development and can produce a series of animals at different stages of development that are all the same chronological age. Oversized larvae were produced by blocking the production of thyroxine with 0.01% phenylthiourea (PTU) in 10% Holtfreter's solution (Hoskins and Grobstein, 1984). Animals immersed continually in this solution at stage 48 of development do not progress beyond stage 53 but continue to grow in size (Hoskins and Grobstein, 1984; Sperry and Grobstein, 1985). Dry weight was used as a measure of animal size. Total heart protein was assayed (Bradford, 1976) and used as a measure of heart size. Hearts were homogenized in 0.1 *N* NaOH, and extractable protein was estimated in triplicate assays using BSA as a standard. Animal dry weight and the total heart protein at the same stage are directly correlated (see Results).

Cardiac ganglion neurons can be visualized with Nomarski optics, and a method has been devised in which all such neurons can be stained for AChE (Heathcote and Sargent, 1984). Hearts were removed from animals anesthetized in 1 mM tricaine. The atria were pinned to a dish, fixed, and stained for AChE as described earlier (Heathcote and Sargent, 1984). Access of the reaction substrate to AChE is a critical problem in older tissue and can be facilitated by pinning out atria as flat as possible and by continually agitating the reaction mixture (e.g., on a rotary shaker). The progress of the reaction was monitored with a compound microscope.

The number and volume of neurons were determined in ganglia at different stages of development. Counts of neuronal number were performed a minimum of 2 times for each whole-mounted ganglion. Since the ganglion is essentially a flat sheet of cells, counts were readily reproducible, and in cases where neurons clustered together a camera lucida was used to aid counting. Cell body volume was determined after analyzing approximately 50 cells from each of 3 animals at every developmental stage examined. Cell body size is related to both axon size and the size of the field of innervation (Henneman et al., 1965). Since the cell body is the sole site of protein synthesis (these cells have no dendrites), its size is probably the parameter that best reflects neuron growth. The neurons measured were the first 50 cells encountered in 3 different randomly chosen areas of the ganglion. The outline of the cells was traced with a camera lucida attachment to a compound microscope. The shape of the neurons is that of a prolate ellipsoid (Sargent, 1983), and their volume was calculated from the major ($2a$) and minor ($2b$) axes (where $V = 4\pi ab^2/3$). The accuracy of the volumes obtained was tested in an experiment in which the volume of 11 neuronal cell bodies was calculated from their major and minor axes as above. The volume was then measured more directly after serially sectioning them at 1 μm intervals and adding together the volumes (area $\times$ 1 μm) of each slab. Measurements made by the 2 methods are directly related ($r = 0.99$, $P < 0.001$).

Results

The cardiac ganglion is a parasympathetic ganglion located within the heart. The neurons are cholinergic, and when activated by the vagus nerve, release ACh onto cardiac muscle and inhibit the rate and amplitude of contraction. Differentiated cardiac ganglion neurons are present in the heart starting at approximately 3 d of development and accumulate at a constant rate for some time (Heathcote and Sargent, 1984). Neurogenesis in the ganglion is prolonged and is the likely source of future neurons (Heathcote and Sargent, 1984). The animal (and its heart) develops and grows both before and after metamorphosis (see Fig. 2*B*). Adult or postmetamorphic *Xenopus* can continue to grow for at least 1–2 years (Gurdon, 1967). Therefore, the cardiac ganglion, like many other neural structures, is confronted with the problem of effectively innervating a target that continually increases in size.

Neuron number

Effective innervation of a target presumably depends upon the appropriate number of neurons as well as the appropriate type. At the time the first cardiac neurons are seen in *Xenopus* (3.3 d; stage 42), the average number present is 3.7. This increases steadily, and at one week of development (stage 48) approximately 30 neurons are present (Heathcote and Sargent, 1984). A ganglion from an animal that has just completed metamorphosis (approximately 3 months; stage 66) is shown in Figure 1. The hundreds of neurons present show that the increase in cell number seen early in development continues for quite some time. Direct counts of neurons at various stages, including 3 weeks (stage 52; the start of metamorphosis) and the adult, illustrate that the number increases more than 2 orders of magnitude during the developmental stages studied (Fig. 2). The increase in number is not confined to larval life but continues even after metamorphosis. The observed increase in number of

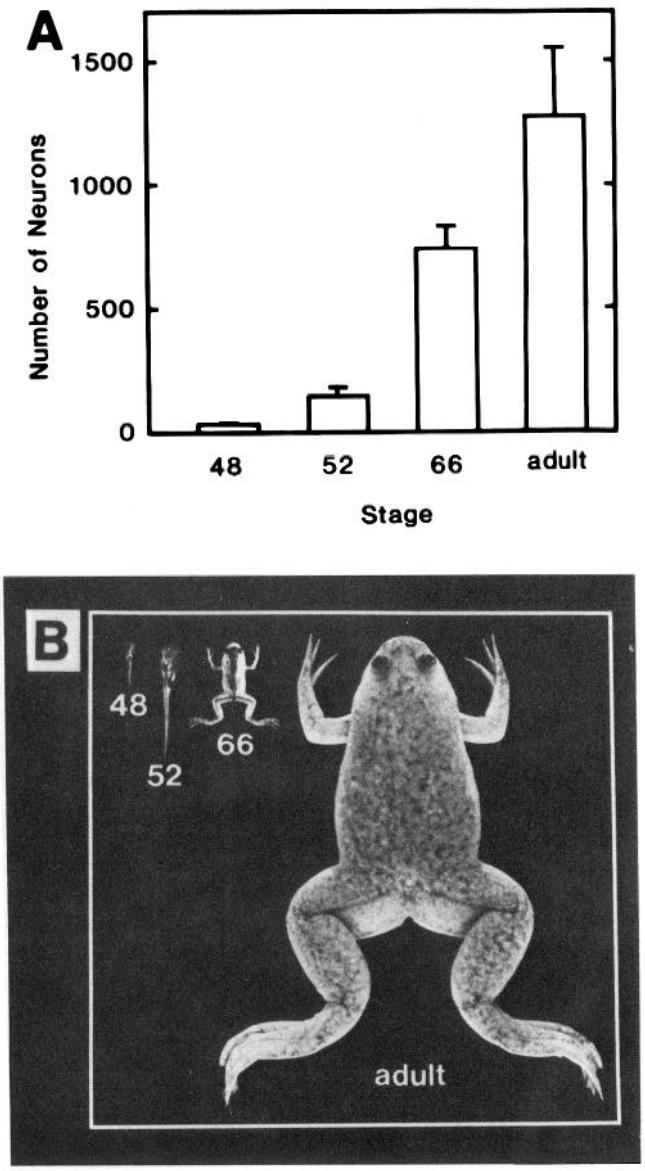

Figure 2. Developmental increase in neuron number and animal size. *A*, Average number of neurons at 4 stages of development (bars represent SD). All stages are postembryonic. *B*, Size of animals at the same 4 stages of development. All animals are depicted at the same scale. The snout–rump length of the adult is 5.7 cm, the size of all the adults used in *A*. Neuron number increases along with animal size even after metamorphosis.

cardiac ganglion neurons concomitant with animal size is likely to help maintain effective innervation of the growing target.

Among the possible mechanisms that could provide an increasing number of neurons to the developing ganglion is one in which new neurons are added at regular intervals of time according to a chronological clock. This possibility could be tested if siblings born at the same time were forced to develop at different rates. Such treatment would produce a series of animals at different stages and sizes but with the same chronological age. Lamborghini (1981) has shown that if larvae are raised at high density, their rate of development can be retarded or stopped. Sibling larvae were raised at different densities to produce a series of animals whose cardiac ganglia contained as few as 27 and as many as 105 neurons (Fig. 3*A*). This 4-fold

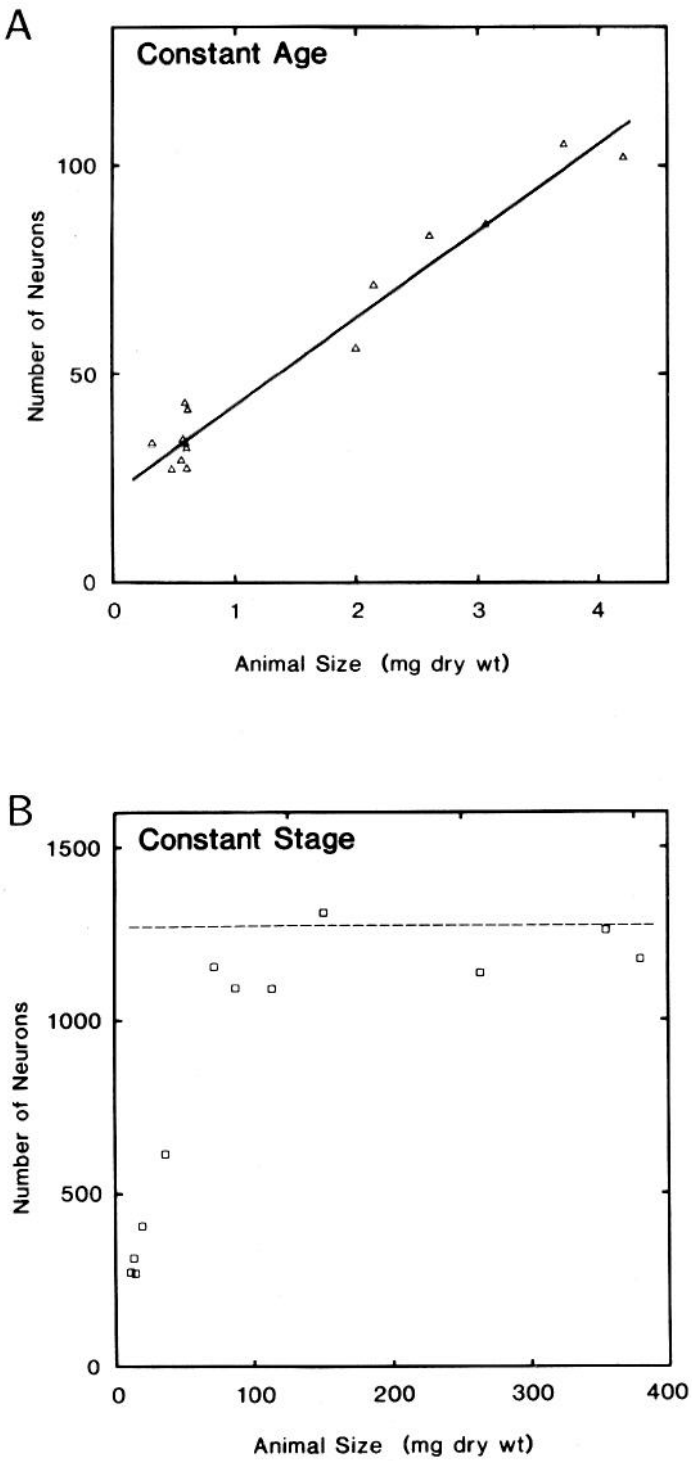

Figure 3. Neuron number is independent of chronological age and developmental stage. *A*, Sibling animals who were identical in age but raised under different conditions produced ganglia whose number of neurons varied over a wide range. Although the number of neurons is not related to age, there is a significant correlation between the number of neurons and the size of the animal measured as dry weight. The relationship is $y = 21x + 21$ with $r = 0.98$, $p < 0.001$. *B*, Animals were kept at stage 53 by treatment with the thyroxine analog PTU, yet the number of neurons increased in parallel with animal size to the level normally found in adults (*dotted line*). This relationship (for the first 6 points) is $y = 14x + 120$ ($r = 0.99$, $p < 0.001$). The number of neurons is correlated, over a certain range, with the size of the animal and not with its age or its developmental stage.

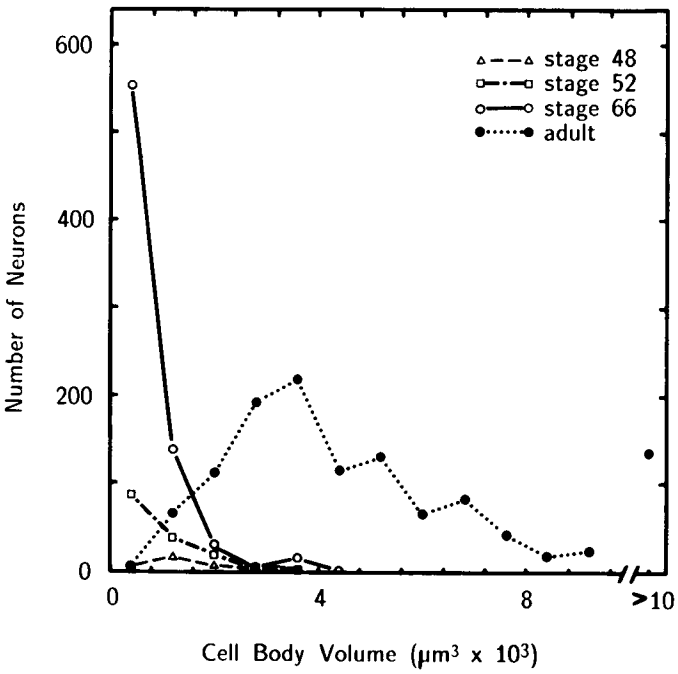

Figure 4. Distribution of cell body sizes at 4 stages of development. Development through larval stages and metamorphosis is characterized by a prominent increase in the number of small cells, which is responsible for the decrease in the *average* size of neuronal cell bodies from stage 48 to 52 to 66 (Table 1). In the adult, very few small neurons are present, indicating that there has been dramatic growth in the size of the neurons during adult life.

range suggests that neuronal number is not controlled by a chronological clock. Although all the data plotted in Figure 3*A* were taken from animals of the same age, neuronal number varied with both the developmental stage and the size of the animal. Animals with the smallest number of neurons had not progressed beyond stage 48, while the ones with the largest number of neurons were at stage 51. The number of neurons varied with the size of the animals (Fig. 3*A*). This relationship is very strong ($r = 0.98$, $p < 0.001$), and the slope indicates that approximately 20 additional neurons are present for each milligram increase in animal dry weight. The initial accumulation of differentiated neurons in the ganglion is due to the migration of neural crest cells (Kirby and Stewart, 1983), and its timing may or may not be adequately explained by a chronological clock. The accumulation of neurons at subsequent stages is clearly not regulated by a chronological clock.

Steady increases in neuronal number could be regulated by mechanisms that depend on developmental stage or size of the animal. In the case of developmental stage, the mechanism is essentially another type of clock, one that provides for the addition of new neurons at appropriate stages. A way to distinguish between stage- and size-dependent mechanisms would be to examine different-sized animals at the same stage of development. Normal differences in animal size at specific stages are not very marked. However, dramatic differences can be generated at a specific stage of *Xenopus* development by hormonal manipulation (Hoskins and Grobstein, 1984; Sperry and Grobstein, 1985). Oversized larvae are generated by raising animals in PTU, a derivative of thyroxine that halts development at stage 53. Giant stage 53 larvae continued to increase in size and were sacrificed at various sizes, with the largest animal achieving a dry weight almost 400 times that of normal stage 53 larvae

Table 1. Average neuronal cell body size

Stage	Diameter[a] (μm)	Volume[b] (μm³)
48	15.5	1534
52	13.1	934
66	11.5	671
Adult	22.8	5738

[a] Diameter is the average of the major ($2a$) and minor ($2b$) axes.
[b] Volume was calculated using the formula $V = 4\pi ab^2/3$.

(Fig. 3*B*). For animal sizes between 10 and 100 mg (dry weight), the number of cardiac ganglion neurons increased monotonically from approximately 250 to more than 1000 neurons (Fig. 3*B*). The rate of increase of neurons with size in this initial range (14 neurons/mg dry weight) is roughly similar to that for animals raised at different densities (21 neurons/mg dry weight; Fig. 3*A*) and may represent the maximal rate of neuron addition to the ganglion. Since all the data displayed in Figure 3*B* were taken from stage 53 animals, developmental stage is clearly not an absolute determinant of neuronal number. The relationship between neuronal number and animal size for stage 53 larvae eventually reaches a plateau at 1200–1300 neurons (Fig. 3*B*). This is the total number of neurons present in ganglia from 5.7 cm adults (Fig. 2*A*), as well as much larger animals (data not shown). Thus, the plateau in neuronal number achieved by stage 53 larvae (Fig. 3*B*) likely reflects an absolute limit to the number of neurons that can be generated in the ganglion. Before this limit is reached, neuronal number is regulated by neither a chronological nor a developmental clock; rather, it appears to be regulated together with animal size.

Neuron size

Effective innervation of a target could depend upon the size of neurons as well as their number. One might expect that as animals get larger, their neurons also increase in size. Curiously, the average size of the neuronal cell body actually *decreases* during larval development and metamorphosis (Table 1). It is only during postmetamorphic growth that the average cell body size increases (Table 1). The initial decrease in size indicates that the first neurons present in the ganglion are relatively large. These large neurons may die, shrink, or become outnumbered by small neurons, thereby resulting in a decrease in average cell size. The number of neurons with different sized cell bodies was determined for each of the stages studied by normalizing data collected from a sample (see Materials and Methods) to the actual number of cells present at that stage (Fig. 2*A*). The distribution of neuronal cell body sizes show that no fewer large cells are present later in development and that the reduction in *average* size between stages 48 and 66 is explained by the addition of many small cells to the ganglion (Fig. 4). The absolute number of large cells (with a somatic volume >2400 μm³) does increase slightly during this time, but its contribution is minor compared to the increase in number of smaller cells (with a somatic volume <800 μm³; Fig. 4). During postmetamorphic life there is a dramatic increase in animal size that accompanies equally dramatic increases in the size of virtually all cardiac ganglion neurons as reflected in the size of their cell bodies (Table 1, Fig. 4).

During larval life, animal growth is accompanied primarily by an increase in neuronal *number,* whereas during adult life,

animal growth is accompanied primarily by an increase in neuronal *size.*

Total volume of neuronal cell bodies

The effective innervation of an organ like the heart almost certainly depends on a process that scales or matches neurons with their growing target. In the cardiac ganglion an increase in both size and number of neurons characterizes the development of the ganglion. Although the number of neurons often appears to track increases in animal size (Fig. 3*A*), there appears to be a finite limit beyond which numbers will no longer increase (Fig. 3*B*). Thus, animal size cannot be used to predict either neuronal number or cell body size over the entire range of development. Perhaps the important parameter for target innervation is a combination of both size and number. The product of the number of cardiac ganglion neurons and the average cell body volume gives the total neuronal cell body volume. Figure 5*A* shows that the total volume of neuronal cell bodies is strongly correlated with animal size over the entire range of development studied ($r = 0.995$, $p < 0.01$). The relationship is a power function of the form $y = bx^m$, where m is the slope of the log–log plot and equals 0.55. Power functions of this form have been called relative growth or allometric functions and have been used to describe the increase in size of various organs or parts of the brain during development (Huxley, 1932; Jacobson, 1978). In the cardiac ganglion this relationship allows one to predict the total volume of neuronal cell bodies in an animal whose size is known. If the average size of the neurons is known, the growth function can be used to estimate their number.

It might be expected that the increase in size of various organs would be scaled in proportion to body size. In particular, does the target of the cardiac ganglion, the heart, increase isomorphically with the rest of the body? Animal size, measured as dry weight, increases over 4 orders of magnitude from larval stage 48 to 5.7 cm adults (see abscissa, Fig. 5*A*). The dry weight of the youngest larval hearts could not be easily measured. Instead, the total protein content of hearts was taken as a measure of heart size. Total protein increases over several orders of magnitude during the developmental period studied (see abscissa, Fig. 5*B*) and is directly related to body size over the entire range ($r = 0.996$, $p < 0.01$; graph not shown). Since there is a correlation between heart size and animal size, one should expect total neuronal cell body volume to be correlated with heart size as well as animal size. Figure 5*B* shows that the total volume of neuronal cell bodies is also correlated with heart size over several orders of magnitude ($r = 0.992$, $p < 0.01$). Since the *density* of cardiac ganglion neuron cytoplasm is likely to remain constant, this implies that the weight of the target is proportional to the weight (volume × density) of the neurons innervating it. The matched growth of both total neuronal cell body volume and target size over a prolonged length of developmental time could insure the effective innervation of a continually expanding target.

Discussion

The size of the cardiac ganglion increases in 3 distinct stages. First, during embryonic development, a dowry of neurons is provided to the ganglion by cells migrating from the neural crest. Second, after the dissipation of the neural crest, new neurons continue to be added to the ganglion. The addition of these cells is related to the size of the animal and the size of the neuronal target and corresponds neither to chronological nor develop-

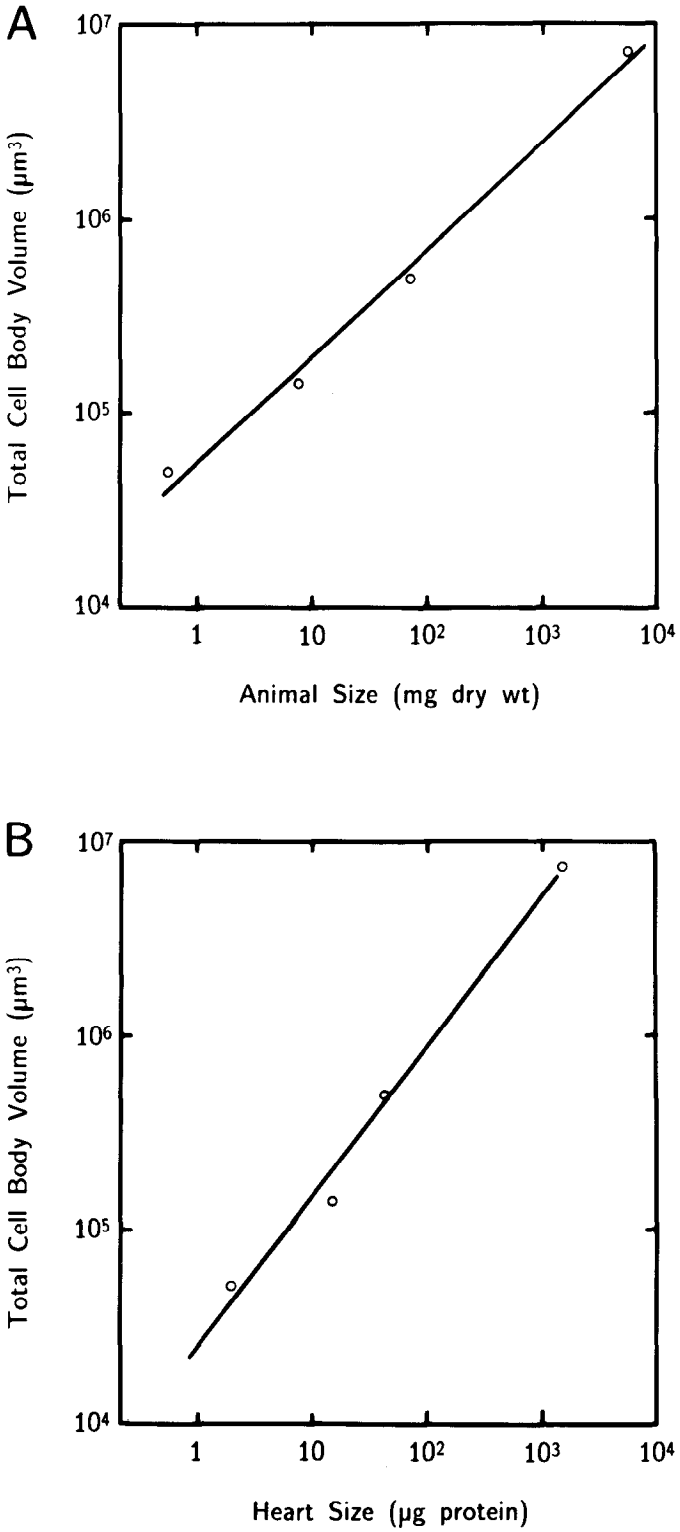

Figure 5. Total volume of neuronal cell bodies is matched with animal and target size. *A*, From 1 week of development to the adult, total cell body volume (average number × average cell body size) maintains a constant relationship with the size of the animal as size increases over almost 4 orders of magnitude. The log–log relationship is a power function with $y = 5.5(10^4)x^{0.55}$ ($r = 0.995$, $p < 0.01$). *B*, Over the same range of development, the total volume of neuronal cell bodies is also related to the size of its target, the heart. Heart size was taken to be proportional to total heart protein. The relationship between total cell body volume and heart size is also a power function, with $y = 2.9x^{0.71}$ ($r = 0.992$, $p < 0.01$). Thus, the size of the heart is quantitatively matched to the total size of the neurons innervating it.

mental age. Third, as the adult number of neurons is approached, the existing cells begin to increase in size. During all 3 overlapping stages, the relationship between the total volume of neuronal cell bodies and target or animal size is maintained. The maintenance of this relationship throughout the entire span of development suggests that there is a common denominator for the initial colonization, proliferation, and growth of neurons in the cardiac ganglion, namely, they all are regulated in concert with target or animal size.

Establishment of neuronal number

Cell generation and cell death are 2 developmental processes responsible for establishing the number of cells present in any structure. In the cardiac ganglion, proliferation is the major determinant of neuronal number, and cell death is either absent or plays a very minor role in ganglion morphogenesis (Heathcote and Sargent, 1985a, 1987). The proliferation of neurons in the ganglion could be regulated by endogenous clocks, which have often been implicated in triggering developmental events. For example, a developmental clock apparently counts cell divisions of the clonal descendents of oligodendrocyte progenitors (Temple and Raff, 1986). In the cardiac ganglion the constant increase in neurons during early development is consistent with the idea that a clock might be involved in the generation of neurons arriving from the neural crest. After the dissipation of the neural crest, the production of neurons can be retarded or even stopped by changing the rate of growth. A clock based solely on elapsed time would not behave in this way. Another possibility is that instead of elapsed time, a mechanism based on a developmental clock could be responsible for generating new neurons. Thus, new neurons would be formed as the animal progressed through each new stage of development. The number of neurons, however, continues to increase in animals that have been arrested at a specific developmental stage. This shows that a developmental clock does not direct the increase in neuronal number. On the other hand, during all developmental manipulations described here, the number of neurons present in the ganglion was always correlated with target or animal size. These data are consistent with a mechanism in which the size of the target (or the animal itself) regulates the proliferation of neurons in the cardiac ganglion.

The time course of neuronal proliferation in the cardiac ganglion is quite long, extending well into postnatal life. Generally it is thought that most populations of neurons are generated and start to differentiate during embryogenesis (Jacobson, 1978). This is the case for many of the peripheral ganglia that are derived from the neural crest (D'Amico-Martel, 1982). However, cardiac ganglion neurons continue to be generated after hatching (Heathcote and Sargent, 1984), and it is likely that the entire postnatal addition of neurons reflects concomitant activity of the neuronal precursors. In addition, it has recently been shown that neuronal precursors in the mammalian enteric ganglia continue dividing postnatally (Benjamin et al., 1985). In all animals there are non-neural derivatives of the neural crest that continue to divide throughout the life span of the animal (e.g., Schwann and pigment cells). Other neurons that do not derive from the neural crest but continue to divide postnatally include the ectodermally derived olfactory neurons (Graziadei and Monti-Graziadei, 1978) and the granule cell populations in the mammalian brain (see Jacobson, 1978), including the hippocampus (Bayer et al., 1982), as well as many regions of the brain of lower vertebrates (Kirsche, 1983). With the exception of the olfactory neurons, the neuronal precursors eventually become postmitotic in mature adults. Among derivatives of the neural crest, the time that cells become postmitotic forms a continuum, with some cells ceasing mitotic activity during embryogenesis, while others continue dividing throughout the life of the animal. Some neurons derived from the neural crest, including those in the cardiac ganglion, lie in the middle of this spectrum of proliferative activity and continue to be formed postnatally and even during adult life.

Proliferation in the cardiac ganglion eventually stops, resulting in a characteristic number of neurons in the mature adult. Fixity of neuronal number is seen in many parts of the vertebrate nervous system. However, what regulates the mitotic activity and thus the number of neurons in any given area is not well understood (Cowan, 1978). Invertebrates tend to have a fixed number of cells, many of which have a unique identity (Goldschmidt, 1908; Nicholls and Baylor, 1968). Apparently, this results from the programmed cell lineages of the neuronal precursors (Goodman and Spitzer, 1979; Sulston et al., 1983). Uniquely identifiable cells are less common in vertebrates, and in their place are large numbers of cells that appear to be functionally equivalent. The genesis of these large ensembles or pools of neurons could be similar to that of a set of uniquely identifiable cells in invertebrates. Thus each organ or group of neurons could be endowed with a fixed number of precursors that undergo characteristic patterns of division that result in the appropriate number of cells. Experiments on chimeras of mutant and wild-type mice have shown that the cerebellar Purkinje cells are generated by a small, set number of precursors that become committed during neurulation (Wetts and Herrup, 1982). In the cardiac ganglion the number of neurons present can be decreased by ablating various portions of the neural crest during embryogenesis (Yntema and Hammond, 1954; Kirby and Stewart, 1983). In these studies the ganglia were examined before maturity, and it is unclear whether the mature ganglia would have contained fewer neurons than normal. Following ablation of some of the precursors, one might expect to see the discrete quantal steps in number of neurons present in the adult, as seen in the experiments on cerebellar Purkinje cells.

Modulation of cardiac neuron production can be affected by altering the conditions of growth. Although increasing target size could stimulate proliferation of innervating neurons, there is a point at which increased size no longer stimulates production of additional neurons. Postmetamorphic animals and large, developmentally arrested tadpoles continue to grow after they attain the adult number of neurons. These results suggest that once a certain "critical size" is reached, the number of neurons no longer changes. In addition, after the cardiac ganglion precursor pool is determined early in development, increasing size cannot be dissociated from the proliferation of neurons. Whether the proliferation of neurons is indeed gated by size, the end result is a roughly fixed number of neurons that is characteristic of the adult ganglion.

Increase in neuronal size

Although the initial size of a class of neurons often depends on the time and place of origin, it is not clear whether such a relationship exists among a population of equivalent cells. Consequently, groups of similar neurons can have different functions based on individual cell size (Henneman et al., 1965). A general

rule within the nervous system is that the largest classes of neurons tend to be generated first (Jacobson, 1978). For example, in the cerebellum the largest neurons (Purkinje cells) are generated first and the smallest cells (granule cells) last (Miale and Sidman, 1961). In the PNS, several sensory and autonomic ganglia are composed of 2 populations of different-sized neurons, and the large cells are generated first (Lawson et al., 1974; D'Amico-Martel, 1982; Ayer-LeLievre and LeDouarin, 1982; D'Amico-Martel and Noden, 1983). Although the cardiac ganglion of the frog consists of only a single neuronal cell type, the correlation of size and time of origin appears to hold to a limited degree. The cells that differentiate during the first week of development have a larger cell body, on average, than those that appear later in larval development. After metamorphosis the cell bodies of all cardiac neurons become much larger. It is not known whether the largest neurons in juveniles are the largest ones in the adult; however, in the trigeminal ganglion embryonic neuron size is not related to adult neuron size (Noden, 1980). Whether the differences in size (and perhaps function) between individual cardiac ganglion cells are maintained during development has not yet been determined.

Developmental increases in cell size are often characteristic of cells that become postmitotic before growth has stopped. D'Arcy Thompson (1942) noted that continuously dividing cells will tend to attain a constant size, whereas those that stop dividing (particularly neuronal ganglion cells) continue to grow, with their increase in size being a function of the length of the animal's life. Interestingly, both of these principles are illustrated during the prolonged period of development of the cardiac ganglion. During the proliferative stage of ganglion development, the overwhelming majority of cells tend to be in the smallest category of size (see Fig. 4). Later, and perhaps only after the adult number of neurons has been reached, there is a dramatic increase in cell size. Thus within this ganglion increases in neuronal size accompany animal growth in the adult.

During development cardiac ganglion neurons gradually switch from proliferation to growth. This switch normally starts after metamorphosis, a period in which drastic changes occur within the animal. It is possible that the metamorphic hormones could act directly on the neuronal precursors or that one of the many metamorphic changes in the animal is responsible for eventually halting precursor proliferation. However, neuronal proliferation stops before metamorphosis in PTU-treated animals. Thus, events associated with metamorphosis, including the action of thyroid hormone, are not necessary for the switch from proliferation to growth.

The matched increase in the total volume of neuronal cell bodies and heart size during development may be directed by at least 3 different mechanisms. One possibility is that increases in ganglion size elicit corresponding increases in the size of the target. This orthograde regulation appears to occur between CNS neurons and their target neurons in the superior cervical ganglion, which cease proliferating when they are decentralized (Black et al., 1971). Other examples of orthograde regulation include the mitogenic influence of axons upon glial cells (Wood and Bunge, 1986) and the dependence of secondary myotube production upon innervation (Betz et al., 1980; Harris, 1981). A second mechanism that can explain matched growth of the cardiac ganglion and its target is that increases in target cell size are responsible for inducing increases in the size of the ganglion. The best known examples of retrograde regulation are those demonstrating an effect of target size upon the size of neuronal populations (reviewed by Hamburger and Oppenheim, 1982). A third possible mechanism is one in which both ganglion and target respond in a parallel fashion to some external growth stimulus. Among possible external growth stimuli are nutrients, growth factors, trophic factors, and mitogens. Animal growth is highly dependent on appropriate nutrition, and it is possible that many organs grow as a direct result of the amount of nutrients present. The present experiments do not distinguish among these possibilities. Elucidation of the mechanism responsible for matched growth of the cardiac ganglion and the heart will require identification of the signal(s) involved, as well as their source and target.

Matching of neurons to target

There is a constant relationship between the total size of cardiac ganglion neuronal cell bodies and target size that is maintained throughout development. This relationship is described by the allometric or relative growth function, which is a power function of the form $y = bx^m$ (Huxley, 1932) and which describes the relationship between a specific body part and the rest of the animal during growth. This function is usually linear when plotted on a log–log scale and illustrates the multiplicative aspect of growth, namely, that for every doubling of body size (x) there is an increase in size of the body part (y) by a constant factor (m). In the cardiac ganglion, this relationship can be thought of as one between the protein synthetic machinery of the neurons and the target they innervate. Thus the weight of the cell bodies (volume × density) is proportional to the weight of either the tissue they innervate or the entire animal. It is possible that a similar relationship exists for other ganglia in both CNS and PNS, and in instances where the target size of a specific neuronal population is unknown or diffuse, it might be possible to estimate ganglion size by measuring the size of the entire animal. In this study we have been able to distinguish the neural components of the ganglion from the non-neural structures, something difficult to do in, for example, the brain, where the relationship between the whole organ and body size is the only parameter that has been investigated. Since the slope (m) is <1, the largest ratio of neuronal cell bodies to either body or target mass occurs in the youngest animals. This may represent the maximum possible density of neurons in the target. Limits to the number of neurons that can populate a young ganglion will require subsequent proliferation in order to meet the needs of the growing heart. The allometric relationship holds under all conditions examined so far, and in these situations the independent variable (either body or target size) can be used to predict ganglion size.

The constant relationship of neuron to target or body size may also exist for animals of various species. Much work has been done on the phylogenetic relationship of brain weight to body weight. This relationship is also $y = bx^m$ and it is generally believed that although the constant (b) can vary for different groups of animals, the slope (m) appears to be relatively invariant, estimates of its magnitude ranging from $\frac{5}{6}$ (Brummelkamp, 1939) to $\frac{2}{3}$ (Jerison, 1969). Recently, the number of sympathetic neurons in the superior cervical ganglion of a variety of adult mammals of different sizes was examined (Purves et al., 1986). Ganglia of larger animals do have more neurons but not in proportion to the increase in animal size. Increases in neuronal size are also seen, and it may be that total volume of neuronal

cell bodies (weight) is proportional to animal size. In general, the uniformity of the relationship between groups of neurons and target or body size across the animal kingdom indicates that certain properties associated with body size (e.g., metabolism) may limit or actually dictate the growth of neural tissue.

It has often been postulated that the regulation of neuronal number by target size is a mechanism for matching a population of neurons with the target they innervate. Experimental manipulations of developing systems have shown that the numbers of somatic and autonomic motoneurons, interneurons, and sensory neurons all depend to some extent on the size of available target. Thus if the target is removed, there is a significant decrease or even complete loss of the neurons that normally innervate it (Hamburger and Levi-Montalcini, 1949). Expansion of the target for somatic motoneurons results in a slight but significant increase in the normal number of neurons (Hollyday and Hamburger, 1976). Hamburger (1934) and Laing (1982) have removed various parts of the target of somatic motoneurons and shown that there is a linear relationship between the number of surviving neurons and the amount of remaining target. Sperry and Grobstein (1985) provided a larger target for somatic motoneurons during a relatively late stage of development and found that the number of innervating neurons remained the same; however, those innervating larger targets were larger in size. Even during normal development, the largest α-motoneurons command the largest motor units (Henneman et al., 1965). Therefore, there is evidence that matching neurons to their targets has incorporated strategies in which either the size or number of neurons is altered to correspond with the target. In the cardiac ganglion, if both variables are taken into account, there is a precise and quantitative matching of the innervating neurons with their target.

References

Ayer-LeLievre, C. S., and N. M. Le Douarin (1982) The early development of cranial sensory ganglia and the potentialities of their component cells studied in quail–chick chimaeras. Dev. Biol. *94:* 291–310.

Bayer, S. A., J. W. Yackel, and P. S. Puri (1982) Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science *216:* 890–892.

Benjamin, S., T. P. Rothman, and M. D. Gershon (1985) Postnatal generation of neurons in the developing enteric nervous system. Soc. Neurosci. Abstr. *11:* 1152.

Betz, W. J., J. H. Caldwell, and R. R. Ribchester (1980) The effects of partial denervation at birth on the development of muscle fibres and motor units in rat lumbrical muscle. J. Physiol. (Lond.) *303:* 265–279.

Black, I. B., I. B. Hendry, and L. L. Iversen (1971) Trans-synaptic regulation of growth and development of adrenergic neurons in a mouse sympathetic ganglion. Brain Res. *34:* 229–240.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. *72:* 248–254.

Brummelkamp, R. (1939) Das Wachstum der Gehirnmasse mit kleinen Cepalisierungssprungen (sog. $\sqrt{2}$-sprungen) bei Amphibien und Fischen. Acta Neerl. Morphol. Norm. Pathol. *2:* 268–271.

Chalfie, M., H. R. Horvitz, and J. E. Sulston (1981) Mutations that lead to reiterations in the cell lineages of *C. elegans.* Cell *24:* 59–69.

Cowan, W. M. (1978) Aspects of neural development. Int. Rev. Physiol. Neurophysiol. *17:* 149–191.

D'Amico-Martel, A. (1982) Temporal patterns of neurogenesis in avian cranial sensory and autonomic ganglia. Am. J. Anat. *163:* 351–372.

D'Amico-Martel, A., and D. M. Noden (1983) Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. Am. J. Anat. *166:* 445–468.

D'Arcy Thompson, W. (1942) *On Growth and Form,* 2nd ed., Cambridge, London.

Goldschmidt, R. (1908) Das Nervensystem von *Ascaris lumbricoides* und *megalocephala.* Z. Wiss. Zool. *90:* 73–136.

Goodman, C. S., and N. C. Spitzer (1979) Embryonic development of identified neurones: Differentiation from neuroblast to neurone. Nature *280:* 208–214.

Graziadei, P. P. C., and G. A. Monti-Graziadei (1978) Continuous nerve cell renewal in the olfactory system. In *Handbook of Sensory Physiology, Vol. 9: Development of Sensory Systems,* M. Jacobson, ed., pp. 55–83, Springer-Verlag, New York.

Gurdon, J. B. (1967) African clawed frogs. In *Methods in Developmental Biology,* F. H. Wilt and N. K. Wessels, eds. pp. 75–84, Crowell, New York.

Hamburger, V. (1934) The effects of wingbud extirpation on the development of the central nervous system in chick embryos. J. Exp. Zool. *68:* 449–494.

Hamburger, V., and R. Levi-Montalcini (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. *111:* 457–501.

Hamburger, V., and R. W. Oppenheim (1982) Naturally occurring neuronal death in vertebrates. Neurosci. Comment. *1:* 39–55.

Harris, A. J. (1981) Embryonic growth and innervation of rat skeletal muscles. I. Neural regulation of muscle fibre numbers. Phil. Trans. R. Soc. London [Biol.] *293:* 257–277.

Heathcote, R. D., and P. B. Sargent (1984) The genesis and differentiation of neurons in a frog parasympathetic ganglion. Dev. Biol. *105:* 102–114.

Heathcote, R. D., and P. B. Sargent (1985a) Loss of supernumerary axons during neuronal morphogenesis. J. Neurosci. *5:* 1940–1946.

Heathcote, R. D., and P. B. Sargent (1985b) Developmental regulation of neuron number in a parasympathetic ganglion. Soc. Neurosci. Abstr. *11:* 1154.

Heathcote, R. D., and P. B. Sargent (1987) Growth and morphogenesis of an autonomic ganglion. II. Establishment of neuron position. J. Neurosci. *7:* 2502–2509.

Henneman, E., G. Somjen, and D. O. Carpenter (1965) Functional significance of cell size in spinal motoneurons. J. Neurophysiol. *28:* 560–580.

Hollyday, M. (1980) Motoneuron histogenesis and the development of limb innervation. Curr. Top. Dev. Biol. Neural Dev. *15:* 181–215.

Hollyday, M., and V. Hamburger (1976) Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. J. Comp. Neurol. *170:* 311–320.

Hoskins, S. G., and P. Grobstein (1984) Induction of the ipsilateral retinothalamic projection in *Xenopus laevis* by thyroxine. Nature *307:* 730–733.

Huxley, J. S. (1932) *Problems of Relative Growth,* Methuen, London.

Jacobson, M. (1978) *Developmental Neurobiology,* 2nd ed., Plenum, New York.

Jerison, H. J. (1969) Brain evolution and dinosaur brains. Am. Nat. *103:* 575–587.

Kirby, M. L., and D. E. Stewart (1983) Neural crest origin of cardiac ganglion cells in the chick embryo: Identification and extirpation. Dev. Biol. *97:* 433–443.

Kirsche, W. (1983) The significance of matrix zones for brain regeneration and brain transplantation with special considerations of lower vertebrates. In *Neural Transplantation Research,* R. B. Wallace and G. D. Das, eds., pp. 65–104, Springer-Verlag, New York.

Laing, N. G. (1982) Motor projection patterns to the hind limb of normal and paralysed chick embryos. J. Embryol. Exp. Morphol. *72:* 269–286.

Lamborghini, J. E. (1981) Kinetics of Rohon-Beard neuron disappearance in *Xenopus laevis.* Soc. Neurosci. Abstr. *7:* 291.

Lawrence, P. A. (1966) Development and determination of hairs and bristles in the milkweed bug, *Oncopeltus fasciatus* (Lygacidae, Hemiptera). J. Cell Sci. *1:* 475–498.

Lawson, S. N., K. W. T. Caddy, and T. J. Biscoe (1974) Development of rat dorsal root ganglion neurones. Studies of cell birthdays and changes in mean cell diameter. Cell Tissue Res. *153:* 399–413.

Miale, I. L., and R. L. Sidman (1961) An autoradiographic analysis of histogenesis in the mouse cerebellum. Exp. Neurol. *4:* 277–296.

Nicholls, J. G., and D. A. Baylor (1968) Specific modalities and re-

ceptive fields of sensory neurons in CNS of the leech. J. Neurophysiol. *31:* 740–756.

Nieuwkoop, P. D., and J. Faber (1967) *Normal Table of* Xenopus laevis *(Daudin),* North-Holland, Amsterdam.

Noden, D. M. (1980) Somatotopic and functional organization of the avian trigeminal ganglion: An HRP analysis in the hatchling chick. J. Comp. Neurol. *190:* 405–428.

Purves, D., E. Rubin, W. D. Snider, and J. Lichtman (1986) Relation of animal size to convergence, divergence, and neuronal number in peripheral sympathetic pathways. J. Neurosci. *6:* 158–163.

Sargent, P. B. (1983) The number of synaptic boutons terminating on *Xenopus* cardiac ganglion cells is directly correlated with cell size. J. Physiol. (Lond.) *343:* 85–104.

Shankland, M. (1984) Positional determination of supernumerary blast cell death in the leech embryo. Nature *307:* 541–542.

Sperry, D. G., and P. Grobstein (1985) Regulation of neuron numbers in *Xenopus laevis*: Effects of hormonal manipulations altering size at metamorphosis. J. Comp. Neurol. *232:* 287–298.

Sulston, J. E., E. Schierenberg, J. G. White, and J. N. Thomson (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans.* Dev. Biol. *100:* 64–119.

Temple, S., and M. C. Raff (1986) Clonal analysis of oligodendrocyte development in culture: Evidence for a developmental clock that counts cell divisions. Cell *44:* 773–779.

Wetts, R., and K. Herrup (1982) Cerebellar Purkinje cells are descended from a small number of progenitors committed during early development: Quantitative analysis of *lurcher* chimeric mice. J. Neurosci. *2:* 1494–1498.

Wood, P. M., and R. P. Bunge (1986) Evidence that axons are mitogenic for oligodendrocytes isolated from adult animals. Nature *320:* 756–758.

Yntema, C. L., and W. S. Hammond (1954) The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J. Comp. Neurol. *101:* 515–534.

Zackson, S. L. (1984) Cell lineage, cell–cell interaction, and segment formation in the ectoderm of a glossiphoniid leech embryo. Dev. Biol. *104:* 143–160.