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Growth and Morphogenesis of an Autonomic Ganglion. II. Establishment of Neuron Position

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The developmental events affecting the positioning of neurons were examined in the frog cardiac ganglion. Use of a neuron-specific marker enabled the position of all neurons in the ganglion to be quantified at different developmental stages. Subsets of neurons born at specific times were labeled with ^{3}H-thymidine, and their positions were mapped at different developmental stages. This technique identifies a subset of cells within a seemingly homogeneous pool of neurons and provides an opportunity for studying the position of individual neurons during ganglion morphogenesis.

Comparison of identified neurons in different animals has revealed several unexpected results. First, during a period of dramatic ganglion and cellular morphogenesis there is little or no cell death since the number of identified neurons does not change during this time. Second, the distinctive clusters that are characteristic of parasympathetic ganglia have been shown to be ephemeral because identified cells that were neighbors early in development become separated during ganglion morphogenesis. Third, individual postmitotic neurons do not actively migrate to produce the observed changes in neuron distribution, as evidenced by the fact that their relative position in the ganglion is maintained. Fourth, both ganglion and target undergo intercalary growth since the absolute distance of identified neurons from one another increases while the relative distance remains the same. Finally, the differentiation of neurons is analogous to the inside-out pattern seen in many parts of the CNS. Thus the ability to identify cells within a large ensemble of seemingly equivalent neurons has made it possible to investigate ganglion morphogenesis at the level of individual cells.

A cell's position in the nervous system is generally fixed early in its differentiation. The positioning of a neuron is significant because it establishes a set of neighbors with whom it may compete and interact. The result is an orderly array of neurons that function in a cohesive manner. One factor that influences the position of neurons is their time of origin. In many areas of the nervous system, cells originating at later times migrate past earlier-arising cells to produce a characteristic laminar structure. The position that a neuron assumes can be associated with its function. Thus, not only are neurons with similar functions grouped together, but within the group, cells are often arranged in a topographic order (e.g., the somatosensory homunculus; Penfield and Rasmussen, 1950; Powell and Mountcastle, 1959). The rules that govern the initial placement of neurons may provide clues concerning the formation of these arrays of cells within the nervous system.

This paper examines the position of individual neurons in a rather simple autonomic ganglion. The cardiac ganglion of the frog consists of a single type of neuron (a pool of motorneurons) whose cells derive from the neural crest. They become postmitotic over an extended period of time and continue to accumulate in the ganglion even after metamorphosis (Heathcote and Sargent, 1984, 1987). Since neurons are added to the ganglion over a long period of development, there are opportunities to manipulate the developing system and investigate how the various levels of morphological organization are established. This study shows that the first neurons present occupy a distinct position in the ganglion. As gangliogenesis proceeds there is a shift in the distribution of neurons, which is the result of new neurons taking up positions distal to the older ones in a manner reminiscent of inside-out development in the CNS. During morphogenesis, an apparently homogeneous pool of neurons follows a set of developmental rules that are responsible for establishing a rough ordering of cell position.

Materials and Methods

Embryos and larvae of the frog *Xenopus laevis* were obtained from a laboratory breeding colony. Hearts were removed and fixed, and the neurons were stained for AChE (Heathcote and Sargent, 1984, 1987). The position of neurons was traced using a camera lucida attachment to a compound microscope. The *relative* position of neurons was determined after dividing each atrium into 10 equal segments from the top (the sinus venosus, defined by the border of pigmented cells that line the atrium) to the bottom (the site where the ventricle attaches to the atrium), as illustrated in Figure 1. The *absolute* position of neurons was assessed by measuring the distance from the center of the neuron to the top of the atrium.

Neurons originating at a specific stage of development were labeled with ^{3}H-thymidine. A neuronal precursor undergoing its final cell division at the time of exposure to ^{3}H-thymidine will permanently incorporate the labeled base into its DNA. The amount of incorporated label will be halved following each episode of DNA replication. When injected into embryos or young larvae, ^{3}H-thymidine is present as a short pulse and is incorporated into only those cells undergoing DNA synthesis within 2 hr following injection (Heathcote and Sargent, 1984). In order to increase the number of labeled cells, animals were immersed for longer periods in 1 mCi/ml methyl-^{3}H-thymidine (50–80 Ci/mmol)

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in 52.5% L-15 medium. [This is the approximate osmolarity of 75% Ringer; salt is required for the uptake of thymidine in amphibian embryos (Loeffler and Johnston, 1964), yet too much salt results in deformed embryos.] Immersion for 6 hr resulted in labeling at least one cardiac ganglion neuron undergoing its final cell division and several precursors destined to undergo further divisions. After rearing animals for appropriate periods of time, the hearts were removed and embedded in a plastic wafer after staining neurons for AChE. The position of all neurons was traced with a camera lucida attachment, ganglia were serially sectioned *en face* at 1 μm, and the sections were exposed to emulsion, developed, and examined (Heathcote and Sargent, 1984). Each neuron was located and the number of grains over its nucleus was counted in the section containing the nucleolus. The number of grains over each labeled neuronal nucleus was divided by the number of grains over the nucleus of the most heavily labeled cells in the atrium to assess the number of divisions undergone since labeling (Heathcote and Sargent, 1984). The position of the labeled cells within the atrium was then mapped on the tracing prepared before sectioning.

Neurons born at the same time (cells that we will call "birthmates") are often found in common clusters of neurons. In order to assess whether this is the result of a tendency for birthmates to cluster, an analysis was made of the extent to which such clustering would be expected simply from random occurrence. If neurons from a specific age class are distinguished from all others and randomly assigned to positions within the ganglion, then the probability of finding k neurons of the same age class in a cluster of n neurons within a ganglion having M neurons, M_w of which are of the specified age class, is determined by the following expression (Parzen, 1960):

$$P[k] = \binom{M_w}{k}\binom{M - M_w}{n - k}\left[1 \Big/ \binom{M}{n}\right]$$

where the binomial coefficient

$$\binom{M_w}{k} = \frac{M_w!}{k!\,(M_w - k)!}.$$

For example, the probability of finding 3 neurons (k) out of a total of 9 of a specific age class (M_w) within a cluster of 7 cells (n) in a ganglion having a total of 34 cells (M) will be 0.198. Thus, the occurrence of such a cluster is not unexpected if birthmates were randomly assigned to their positions within the ganglion. If a confidence level of 0.05 is used, then one ought to detect "unlikely" events about 5% of the time, assuming that the process by which birthmates are assigned positions within the ganglion is random. If the individual probability values consistently fall below 0.05, then birthmates tend to stay together. On the other hand, if only a small number of probability values fall below 0.05, then the observed clustering is likely to be the result of a random process.

Results

The morphology of the cardiac ganglion suggests that several factors are important in the positioning of neurons within the heart. The first is that differentiated neurons are restricted to the atrium of the heart. Neurons are not present in the ventricle, and only during the first days of development are a few neurons seen outside the pericardium at the venous end of the heart (Heathcote and Sargent, 1984). Second, most neurons are situated along extensions of the bilateral branches of the vagus nerve (Fig. 1), with only a few isolated cells located off the major nerve trunks. Another organizational parameter is that neurons tend to be clustered, forming distinctive aggregates within the ganglion (Fig. 1). Finally, whereas neurons are evenly distributed along the length of the atrium in mature ganglia, the first neurons present are concentrated at the venous end of the heart (Heathcote and Sargent, 1984). Thus, neuronal position within the cardiac ganglion depends on the age of the animal and perhaps also on the age of the neurons themselves.

Pattern of neuron condensation

During the period that neurons accumulate in the cardiac ganglion, their distribution within the heart changes. Initially, neurons cluster at the venous end of the heart, adjacent to the sinus venosus (Heathcote and Sargent, 1984). This asymmetric distribution is not seen at later times. A quantitative map of relative neuronal position at different developmental stages is shown in Figure 2*A*. Each atrium has been divided into 10 equal sections along the axis from the sinus venosus to the ventricle (Fig. 1). The average percentage of total neurons in each of the 10 sections is plotted in Figure 2*A*. At one week of development (stage 48), more than half of the neurons present are in the 10% of the ganglion closest to the sinus venosus. By 3 weeks of development (stage 52), the distribution is more even, and by the end of metamorphosis (stage 66), the original gradient of neurons has disappeared. One explanation for the loss of asymmetry would be that the first neurons present near the sinus venosus become redistributed during subsequent development. Alternatively, new

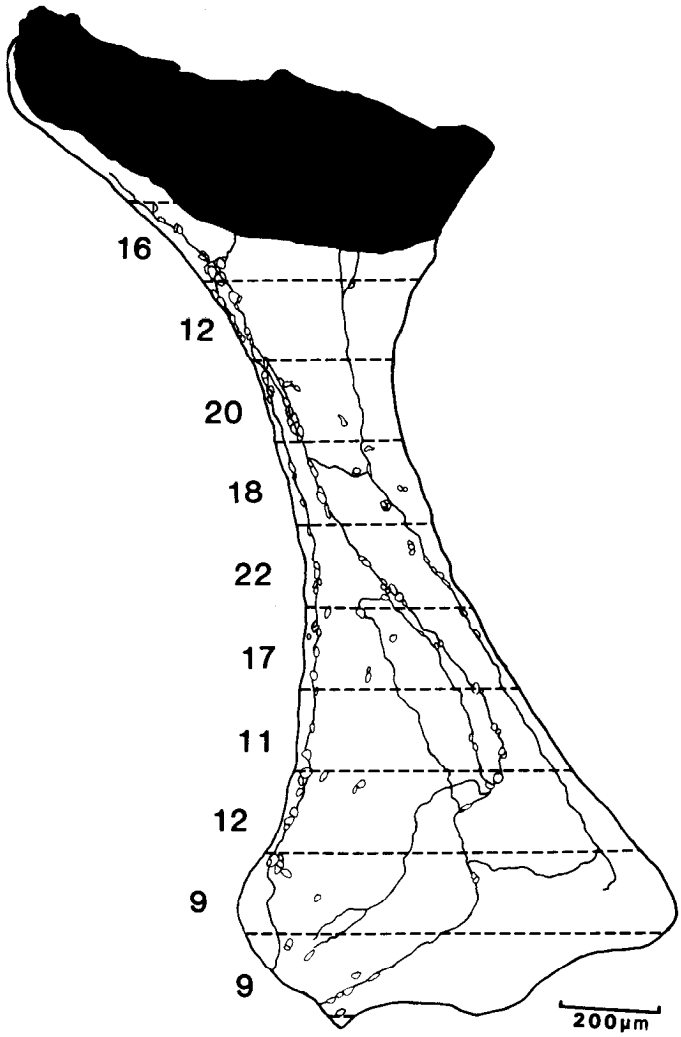

Figure 1. Neuron position in the cardiac ganglion. The atrium of a stage 52 animal was fixed and stained for AChE, and the cell bodies and nerve trunks of the ganglion were traced with a camera lucida. Most of the neurons are located along nerve trunks that are extensions of the vagus nerve. Neurons also form distinctive clusters within the ganglion. To determine the relative position of neurons, the ganglion has been divided into 10 equal segments (*dashed lines*). The number of neurons is indicated for each segment from the black pigmented pericardium (the venous end) to the ventricular end of the atrium. At this stage, there is a slight tendency for more cells to be found at the venous end of the heart.

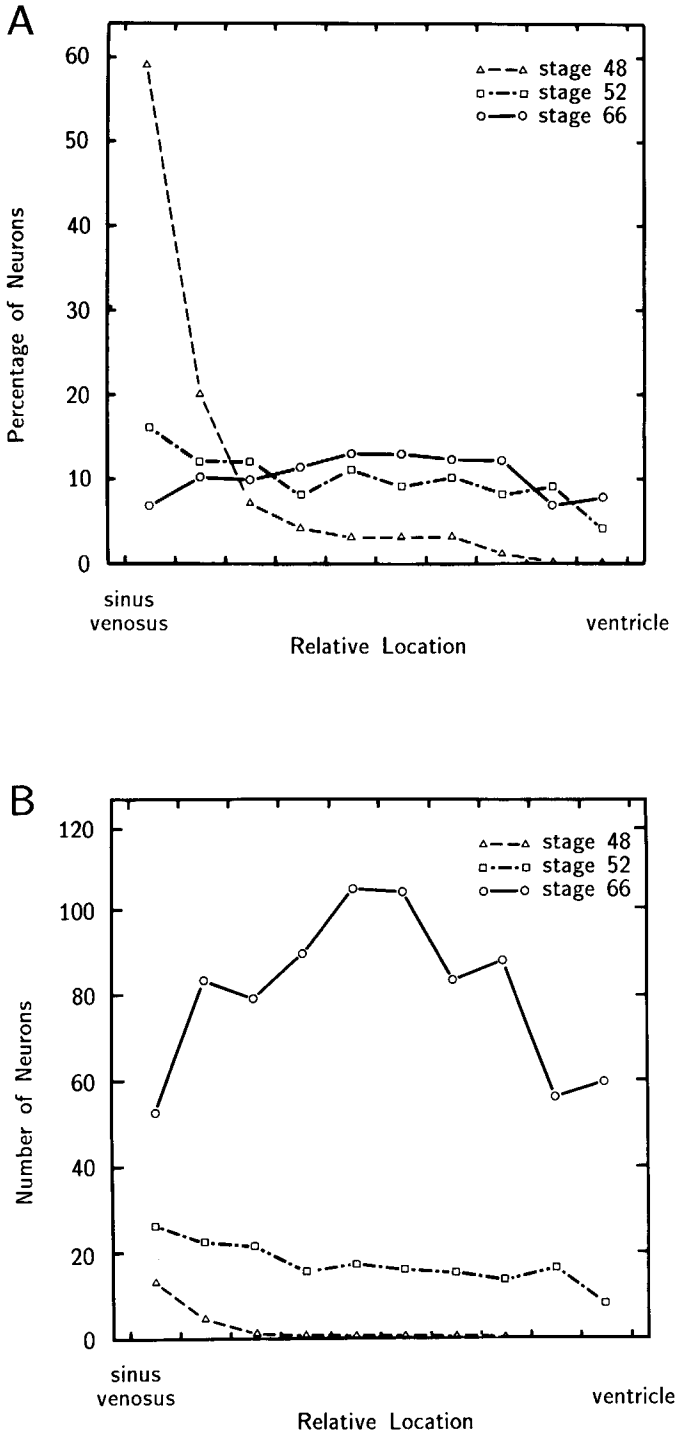

Figure 2. Development of neuron distribution in the cardiac ganglion. The percentage (*A*) and number (*B*) of neurons occupying each relative position is shown for animals at stages 48, 52, and 66. *A* shows that at stage 48 most of the cells are located near the venous end of the atrium, but by stage 66 (the end of metamorphosis) the distribution is fairly uniform, with slightly more cells located in the middle of the atrium than at either end. *B* shows that the absolute number of neurons increases in all parts of the ganglion during development. The loss of asymmetry occurs because most of the younger neurons are added to more distal parts of the ganglion.

neurons could be preferentially added to unoccupied areas of the ganglion. Measurement of the absolute number of cells at different positions within the atrium reveals that there is a substantial increase in cell number during the interval when asymmetry is lost (Fig. 2*B*). The distribution of neurons at stage 48 is asymmetric; however, the total number of cells at this stage is quite small. By stage 52 neurons are more or less equally distributed in all parts of the ganglion. At stage 66 more neurons are present in the central part of the ganglion than at either end. Although these results do not exclude the possibility that existing cells are redistributed within the ganglion, they indicate that after the initial deposition of cells at the venous end of the heart, neurons are added to all parts of the ganglion. This subsequent addition of neurons would not need to be restricted to a particular zone of the ganglion; however, since fewer neurons are present at the venous and ventricular ends of the heart, it is possible that the preferential deposition of neurons continues to occur but shifts its focus to the central part of the ganglion.

Movement of differentiated neurons

The distribution of neurons shown in Figure 2 suggests that cells are added to all parts of the ganglion; however, these results do not rule out the possibility that individual neurons can move within the ganglion. Postmitotic migration is characteristic of several types of neurons and probably does occur in the cardiac ganglion during the first week of development (Heathcote and Sargent, 1984). In order to determine whether movement of neurons occurs during the period when polarity is lost within the ganglion, individual cells were marked with an age-specific label. Neurons born at 2 d of development (stages 40–42) were labeled by immersing animals in ^{3}H-thymidine. The animals were allowed to survive to either 1 week (stage 48) or 3 weeks (stage 52/53) of development, at which time their hearts were examined and the position of all cardiac ganglion neurons was noted. Following serial sectioning and autoradiography, the cells born around the time of immersion (i.e., those with labeled nuclei) were mapped (Fig. 3). The positions of all neurons, both labeled and unlabeled, are shown for representative stage 48 (Fig. 3*A*) and stage 52/53 ganglia (Fig. 3*C*). Both ganglia are displayed at the same magnification, which emphasizes the dramatic increase in both heart size and neuron number between these 2 stages (Heathcote and Sargent, 1987). In the older ganglion, labeled neurons are not restricted to a small area near the venous end of the heart; rather, they extend over a distance more than 3 times greater than in stage 48 ganglia (Fig. 3). Thus it is clear that cardiac neurons undergo a shift in their absolute position (as measured by the distance from the venous pericardium) as the heart and animal grows.

The shift in the absolute position of neurons shown in Figure 3 could be caused by either an active or a passive movement of labeled neurons. The first situation would describe a true postmitotic migration, while the second is what would be expected if growth of the atrium were occurring isomorphically, such that increases in ganglion size occur equally at all places. The position of labeled cells in ganglia from both stages appears to be concentrated at the venous end of the heart, suggesting that the relative position is unchanged (Fig. 3). Quantitative mapping of the relative position of labeled neurons in several ganglia at both time points is shown in Figure 4. This figure demonstrates that the relative position of cells born during a specific period of developmental time does not change between the 2 stages studied. The "movement" of labeled cells during development does not involve the independent motion of individual cells; rather, all cells remain approximately in place as the ganglion undergoes an isomorphic or intercalary form of growth. Once an immature cell differentiates and condenses in

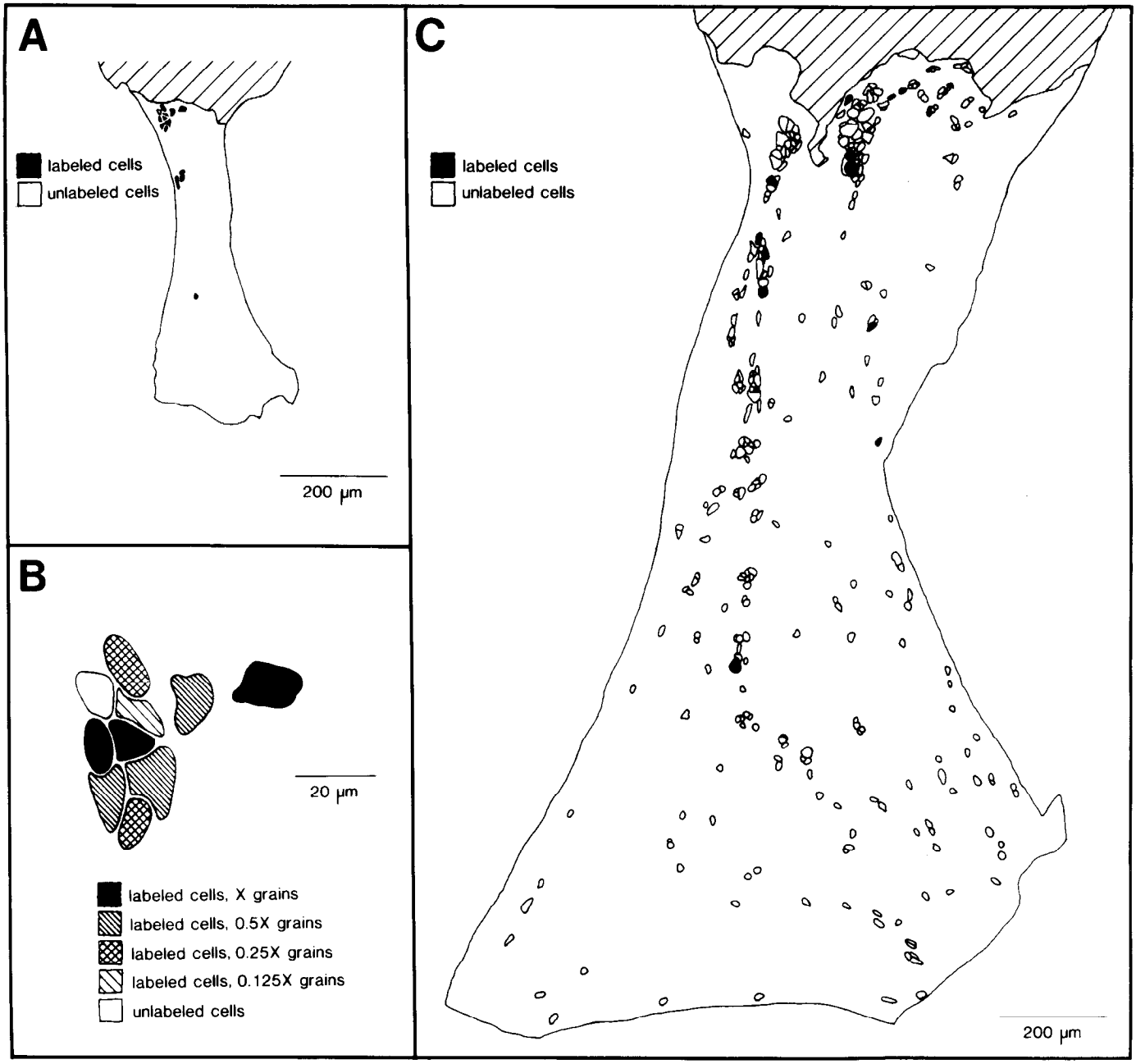

Figure 3. Movement of labeled cells. Neurons born early in development were labeled by exposing a group of siblings to ^{3}H-thymidine (see Materials and Methods). Labeled neurons were identified by serial section autoradiography, and their position within the ganglion was mapped at 2 different developmental stages. *A* shows a ganglion at stage 48. Most of the neurons are labeled and are located at the venous end of the ganglion in a small number of clusters. *C* shows a sibling ganglion at stage 53. The number of cells and cell clusters has increased markedly with labeled cells now being outnumbered by younger unlabeled cells. The absolute distance spanned by the labeled cells has increased between the 2 stages, but labeled cells remain in the venous portion of the ganglion. *B* is an enlargement of the top group of neurons in *A* and shows the age class of individual labeled cells. The number of grains over the nucleus indicates whether the cell has undergone 0, 1, 2, or 3 rounds of DNA synthesis since labeling (Heathcote and Sargent, 1984).

the ganglion, its relative position appears to remain fixed. Thus, the movement of cells within the ganglion is primarily passive and is the result of interstitial growth.

Younger neurons occupy positions further from the venous end of the heart than older neurons (Figs. 2, 4). Are neurons differing by only a single generation found in slightly different parts of the atrium? The "age class" of neurons labeled at early times was determined at both stage 48 and stage 52/53 in sibling animals (by counting the amount of label in each cell; see Fig. 3*B*), and their positions were plotted as a function of the absolute distance from the venous end of the heart (Fig. 5). At stage 52/53 the heart has increased in size, and intercalary growth has resulted in the spreading out of labeled cells. The difference between the positions of the oldest 3 classes and the youngest 2 classes of cells, which is suggested by the stage 48 data, is readily apparent at this later time. This shows that neurons belonging to the same age class take up similar positions within the ganglion and that slightly younger cells are deposited further from the venous border of the ganglion than their predecessors.

Neurons that share birthdays take up similar positions along

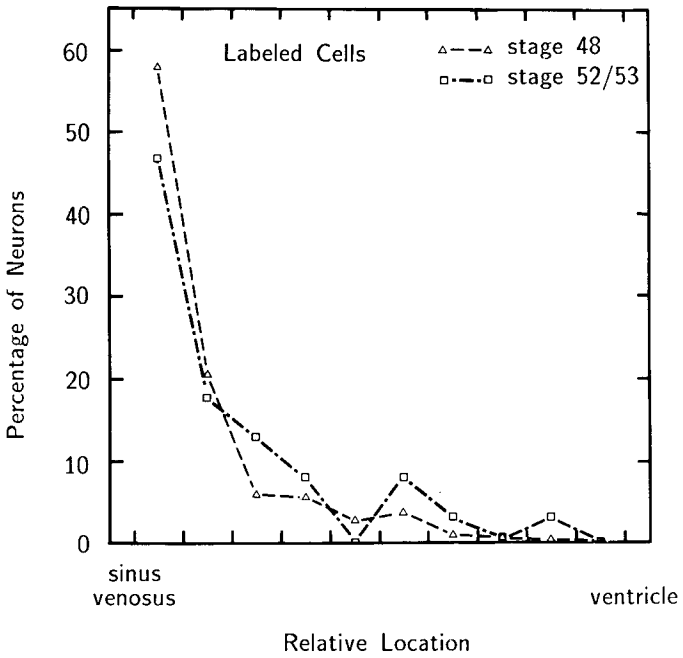

Figure 4. The relative location of identified neurons is constant. The relative postion of ^{3}H-thymidine-labeled neurons was examined in siblings at stages 48 and 52/53 (see Fig. 3). During this interval the asymmetric distribution of the entire population of cells is largely lost (Fig. 2) but is retained by the labeled cells.

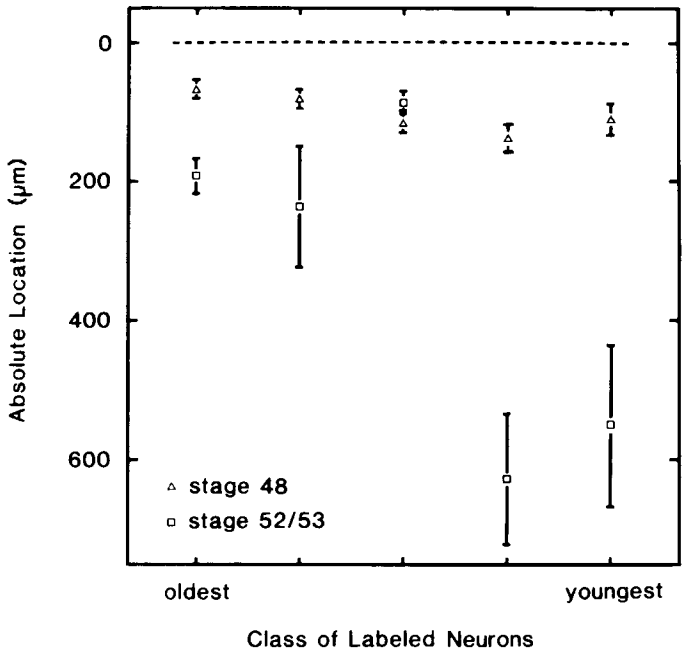

Figure 5. Neuron position is related to both birth order and animal age. The absolute position of neurons (±SEM) is indicated relative to the venous end of the heart (*dashed line*). The birth order of neurons born within a period of several days following ^{3}H-thymidine labeling was determined for sibling animals at 2 stages of development (see Materials and Methods). Older neurons tend to be located closer to the venous end of the heart. In addition, the absolute distance from the venous end of the heart of 4 of the 5 age classes increased between stages 48 and 52/53. Thus the absolute position of cells born at a particular time changes during development, and slightly younger neurons tend to be deposited further from the venous end of the heart.

the venous/ventricular axis of the ganglion. However, it is not known whether birthmates tend to coalesce in common clusters of neurons and become neighbors. The distinctive clusters of neurons that characterize intramural parasympathetic ganglia are, in the cardiac ganglion, present during all stages of development and are arranged along the length of the vagal nerve trunks (Fig. 6). The relationship among neurons in clusters was analyzed by examining maps of neuronal age class (Fig. 3*B*) for all the clusters in stage 48 (1 week) ganglia. Within 12 ganglia, a total of 226 labeled neurons were located in 37 clusters and an additional 40 labeled neurons were present as individual cells. In 50 instances a cluster having labeled neurons was found to contain at least 2 neurons of the same age class. A statistical analysis was performed to determine if the occurrence of birthmates in common clusters was higher than would be expected from a random assignment of birthmates to the positions occupied within their ganglion (see Materials and Methods). The probability of obtaining the observed clustering varied between 0.01 and 0.67 for all 50 occurrences. Five out of the 50 cases had a probability of less than 0.05, an incidence that is not itself unexpected ($p > 0.05$). Thus at one week of development, neurons sharing birthdays are just as likely to be in the same cluster of neurons as in separate clusters of neurons.

An analysis of the tendency for birthmates to cluster produces different results when performed on stage 52/53 larvae. At stage 48 (1 week) most of the cells are labeled after immersion of embryos in ^{3}H-thymidine and belong to one of the age classes analyzed. By stage 52/53, however, these labeled cells have been joined by a large number of unlabeled cells, and labeled cells are found in only a relatively small fraction of all the clusters in the ganglion (Fig. 3*C*). Since the labeled neurons are restricted to only a part of the ganglion and to only a few of its neuronal clusters, birthmates do tend to be found in common clusters. Recall that for neurons examined at stage 48, soon after they have differentiated, birthmates showed little or no tendency to cluster (see above). This difference is a result of the dynamics of ganglion growth. Cells, which at the time of their birth must have been adjacent, may become separated from one another primarily by interstitial growth, which does not alter their relative position along the venous/ventricular axis of the ganglion. Although neurons in the ganglion are randomly distributed following their differentiation, the pattern of growth and accumulation of new neurons results in a distinctly nonrandom arrangement of neurons in older animals.

During development the interstitial growth of the ganglion causes neighboring cells to become separated. The extent of this passive movement of neurons can be seen by comparing Figure 3, *A* and *C* (see also Fig. 5). Another consequence of this pattern of growth is that the constituents of clusters change continually during development. This arises from the addition of new cells to established clusters, as well as the passive movement of existing cells away from one another. At stage 48, labeled cells are found within an average of 2.2 clusters of neurons, whereas at stage 52/53, these "same" identified cells are dispersed among an average of 7.8 clusters of neurons (Table 1). One possible explanation for this phenomenon is that labeled neurons which are individuals at stage 48 become incorporated into clusters by stage 52/53. However, the number of individual cells that are labeled is similar at the 2 stages (Table 1). Thus, labeled cells that were originally neighbors within the same cluster of neurons tend to disperse and subsequently to reside in different clusters. This suggests that the cluster associations of neurons

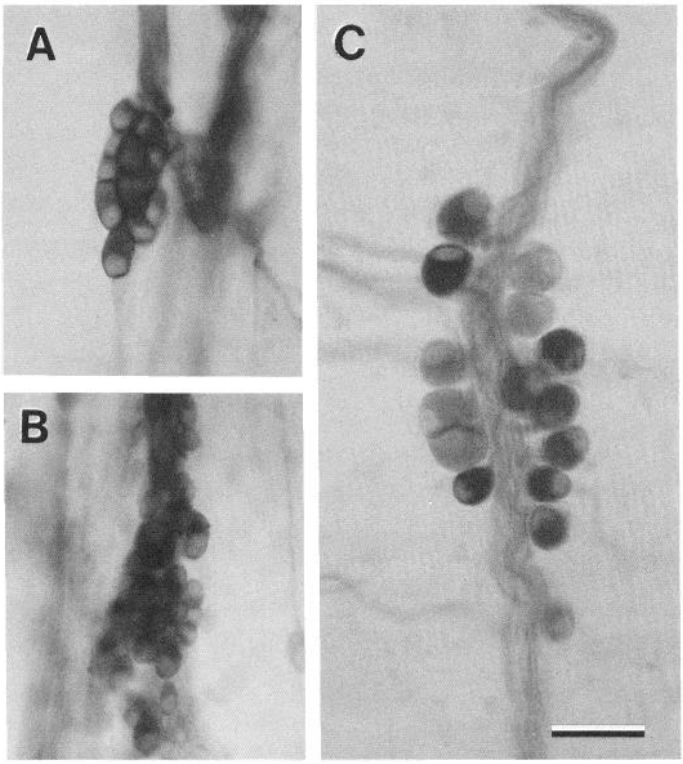

Figure 6. Neuron clustering persists throughout development. Clusters are shown from stage 48 (*A*), 66 (*B*), and adult (*C*) ganglia. All are shown at the same magnification. Although clusters of neurons persist throughout development, the association among neurons in clusters is ephemeral (see text). Calibration, 50 μm.

are ephemeral during development. Although the relative position of a cell within the ganglion as a whole is maintained during development and growth, a cell's immediate neighbors can change. This effect is likely to be produced by the deposition of new neurons around old ones as interstitial growth increases the distance between existing cells.

Cell death

Neuronal morphogenesis is often accompanied by massive amounts of cell death (Cowan et al., 1984). In vertebrates, cell death can occur uniformly throughout a population of neurons without reference to the time of origin of a cell (Clarke et al., 1976). By following an identified population of cells at 2 different stages of development, it is possible to examine the extent of cell death during that period. Neurons were labeled at 2 d of development by immersion in ^{3}H-thymidine and examined at both 1 week (stage 48) and 3 weeks (stage 52/53) of development. There was no significant difference in the number of labeled neurons in each proliferative class at these 2 stages (data not shown) nor in the total number of labeled neurons (15.25 and 15.50; Table 1), suggesting that all of the labeled cells present at stage 48 are also present at stage 52/53. These data imply that cell death has, at most, a minor role in the morphogenesis of this ganglion, since it cannot be detected during the time that the distribution of neurons within the ganglion undergoes a dramatic change.

Discussion

Among an apparently homogeneous group of autonomic neurons, small numbers of cells were identified with respect to their time of origin. Comparison of identified neurons in different animals has revealed several unexpected results. First, during a period of dramatic ganglion and cellular morphogenesis, there is little or no cell death in the cardiac ganglion. Second, the clusters that are characteristic of parasympathetic ganglia are ephemeral associations of neurons. Third, individual postmitotic neurons do not undergo active migration to produce the observed changes in the overall distribution of neurons. Fourth, both ganglion and target undergo intercalary growth during morphogenesis. Finally, the pattern of differentiation of cardiac ganglion neurons is analogous to the inside-out pattern seen in many parts of the CNS. Thus, the ability to identify cells within a large ensemble of seemingly equivalent neurons has made it possible to investigate ganglion morphogenesis at the level of individual cells.

Table 1. An identified set of neurons occupies an increasing number of clusters

Stage	Labeled cells	Clusters with labeled cells	Individual cells labeled
48	15.2 ± 2.6	2.2 ± 1.0	3.0 ± 2.2
52/53	15.5 ± 6.4	7.8 ± 4.1[a]	2.5 ± 1.3

^{3}H-thymidine-labeled neurons from sibling animals were examined at 2 different stages. The number of labeled cells was the same, indicating that cell death does not occur between these 2 stages. The number of clusters with labeled cells increased during the interval examined, while the number of individual labeled cells remained constant. Thus the original members of neuronal clusters do not remain neighbors but become separated from one another and form or join new clusters.

[a] Significantly different from stage 48 value ($p < 0.05$).

Morphogenesis

The asymmetric distribution of neurons that is apparent early in the formation of the cardiac ganglion disappears because younger cells migrate past older cells before differentiating. The establishment of the polarity and its disappearance has been quantified; however, knowledge about the overall distribution of neurons in the ganglion provides no insight concerning the cellular basis of the rearrangement. Consequently, a small group of identifiable cells was labeled and their position charted at different developmental times. These experiments showed that early in development younger neurons take up positions that are, on average, distal to older neurons. The difference in position is not as apparent at the time of neuron condensation as it is after ganglion growth has increased the absolute distances between cells (see Fig. 5). There is a distinctive proximodistal order to the differentiation of the neurons in the ganglion that is superficially similar to the wave of the differentiation in enteric ganglia (LeDouarin and Teillet, 1973; Allan and Newgreen, 1980; Rothman and Gershon, 1982). However, in the cardiac ganglion, the distal neurons are not in place awaiting differentiation (Heathcote and Sargent, 1984). The pattern of cardiac ganglion differentiation appears to be analogous to the generation of the different laminae of some cortical structures in the mammalian brain, where younger neurons generated in a germinal zone migrate past older neurons before differentiating (Angevine and Sidman, 1961). These neurons are often said to differentiate in an inside-out manner. Neuron condensation in the cardiac ganglion also occurs from the inside out. Thus, at least this pattern of morphogenesis can be shared by ensembles of neurons in both CNS and PNS.

During development of the cardiac ganglion, differentiated

neurons are located primarily along branches of the vagal nerve trunks. Branches of the presynaptic nerve could serve as a migratory substrate for neurons and/or precursors. Early in development AChE-positive neurons are often associated with branches of the vagus nerve in the extrapericardial region outside the heart (Heathcote and Sargent, 1984, and unpublished observations). Axonal pathways have often been hypothesized as substrates for guiding other axons (Harrison, 1910; Weiss, 1941; Bate, 1976; Keshishian, 1980; Heathcote, 1981; Bastiani et al., 1984). In the cardiac ganglion, the migrating neuroblasts are thought to accompany the growing vagal axons (Kirby et al., 1980). Recently, bundles of axons have been proposed as a substrate for the migrating neuroblasts of cerebellar granule cells (Hynes et al., 1986). Although it is not known precisely when the preganglionic axons first reach the heart, they are present very early in development, and at least some are in place before virtually all of the cardiac ganglion neurons are either born or differentiate. Thus it is possible that most cardiac neurons migrate along a portion of the vagus nerve before they differentiate.

Another morphogenetic pattern characteristic of the condensation of neurons in the cardiac ganglion is their aggregation into clusters. Clusters are formed by the first neurons in the heart and continue to be formed as neurons are added to the ganglion. Whatever mechanism promotes clustering of the first neurons may also affect cells that differentiate even after metamorphosis. A possible mechanism is that migrating neurons or their precursors could coalesce and differentiate around existing neurons, which would have a function analogous to that of "seed pearls." Also, passive movements of the growing ganglion could serve to fractionate existing clusters, effectively separating cells that were at one time neighbors and forming new clusters. Clusters are also present in mature adults and indicate that the mechanism that promotes clustering persists throughout development.

Cell position and connectivity

Within the nervous system, order is often superimposed upon arrays of cells that are morphologically homologous. In the visual cortex, for example, cells receiving inputs from the thalamus appear to have similar morphologies yet clearly have different sets of inputs and outputs depending upon their position within layer 17 (Gilbert and Wiesel, 1979). A lesser degree of order is apparent in the arrangement of motor neurons in the spinal cord. Although the motor neurons innervating a single muscle in adults tend to be restricted to anatomically distinct clusters, there is a commingling of the neurons that innervate different muscles (Sherrington, 1940; and see Hollyday, 1980a). The lack of precision is less apparent in the embryo, where motor neuron position is correlated to the position of embryonic muscle precursors (Hollyday, 1980b). Early in the development of the cardiac ganglion, neurons occupy a distinct part of the target. Neurons are subsequently deposited throughout the target, and although the relative position of the early neurons does not change, old and new neurons commingle within the ganglion. The distinct location, as well as size (Heathcote and Sargent, 1987) and dramatic sprouting (Heathcote and Sargent, 1985), of early neurons may reflect functional differences from later-arising neurons. Certainly the output of the early cells differs quantitatively from later cells since a relatively small number of neurons project throughout the entire heart (Heathcote and Sargent, 1985). Whether functionally distinct subsets of neurons exist within the cardiac ganglion, a stereotyped pattern of neuron production occurs during development. Similar patterns of neuron generation in other systems have served as templates upon which functionally distinct classes of sensory and motor cells are arranged.

The pattern of innervation of cardiac ganglion neurons may depend upon the timing of neurogenesis in the ganglion. In adult frogs cardiac ganglion cells receive 1 or 2 preganglionic inputs (average, 1.6) from the vagus nerve (Dennis et al., 1971; Dennis and Sargent, 1978; Ko and Roper, 1978). Although the initial development of innervation of the neurons has not yet been studied, it is probably analogous to other parasympathetic ganglia whose neurons are initially polyneuronally innervated (Lichtman, 1977; Johnson and Purves, 1981). The reduction of inputs is thought to be the result of competition among preganglionic axons (Purves, 1983). In the adult submandibular ganglion, Lichtman (1980) has done pairwise recordings from neurons within a cluster and has mapped those that are innervated by common preganglionic axons. He found that ganglion cells that share the same input tend to be dispersed among the target cells of other neurons. This pattern of innervation is similar to that observed in the adult cardiac ganglion (P. B. Sargent and R. D. Heathcote, unpublished observations), as well as adult skeletal muscle (Kugelberg, 1976). Lichtman (1980) noted that "as there is little reason to believe that the submandibular ganglion cells within a cluster differ qualitatively from one another, selectivity is an unlikely basis for the unitary capture of neurons." He proposed that the pattern of innervation results from a competitive mechanism. This is a parsimonious explanation for *both* synapse elimination and the spatial pattern of innervation within clusters. However, we have shown that qualitative differences do exist within a seemingly homogeneous population of cells, namely, neighboring cells are often born at different developmental times. If single preganglionic axons tend to capture target neurons that share birthdays, the time of neurogenesis would be a major factor in determining the spatial pattern or map of synaptic connections. Even though passive movements during growth could separate neighboring cells, the mature motor units would consist of neurons born during a particular window of developmental time and would reflect the spatial order present at that time.

Cell death

Cell death is a regressive phenomenon that is common within the nervous system and can be instrumental in the regulation of neuronal number (Cowan et al., 1984; Clarke, 1985; Oppenheim, 1985). Cell death commonly occurs throughout the nervous system; however, there are some regions, such as the pontine nuclei, in which it has not been detected (Armstrong and Clarke, 1979). The persistence of ^{3}H-thymidine-labeled cardiac neurons during early premetamorphic development suggests that massive cell death does not occur in the ganglion. This conclusion would be incorrect if cell death specifically spared the cells born during the time of labeling. The death of specific neurons does occur among identified cells in invertebrates (Truman and Schwartz, 1982). In pools of vertebrate neurons, cell death can occur uniformly throughout the ganglion, independent of the time of neuron origin (Clarke et al., 1976). Although cell death within the cardiac ganglion does not occur during the period examined, it may occur during other developmental stages. The period examined spans a time when the number of neurons increases from 2 to 20% of the mature adult number and also when individual neurons undergo a dramatic morphological

transformation (Heathcote and Sargent, 1985, 1987). The transformation is a regressive phenomenon in which cells lose supernumerary axons directly from their cell body, as well as numerous secondary and tertiary branches (Heathcote and Sargent, 1985). Neuron number continues to increase during and after this time and eventually reaches a stable number of cells (Heathcote and Sargent, 1987). The cardiac ganglion is distinctly different from the ciliary (Landmesser and Pilar, 1974), sympathetic (Aguayo et al., 1976; Wright et al., 1983), and dorsal root ganglia (Hamburger and Levi-Montalcini, 1949), where cell death is quite pronounced. Unlike the other ganglia mentioned above, the cardiac ganglion is located within its target, and both target and ganglion continually increase in size over a prolonged period of time (Heathcote and Sargent, 1987). The tiny dimensions of the embryonic heart may make the common developmental strategy of overpopulation followed by cell death more difficult to achieve in a ganglion that resides within its target. Cell number in such a ganglion may be controlled by prolonging the proliferation of precursors. It is possible that neuron number in other intramural ganglia are also regulated by prolonging the period of neuron production.

Morphogenesis of the cardiac ganglion is the sum of many different cellular events. These include proliferation, migration, differentiation, growth, and process elimination. The result is a predictable and characteristic arrangement of neurons. Understanding these processes in a relatively simple group of homogeneous cells provides insight into how normal developmental processes arrange groups of neurons in order.

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