Nature and Nurture in Development of the Autonomic Neuron

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Summary. Arguments are presented for the hypothesis that during an early stage of development the cells which become principal neurons of the autonomic nervous system possess information regarding the positions they will occupy within the body. A second stage of development, during which a decision is made regarding which neurotransmitter to employ, is delayed until each neuron has assumed its permanent position in the body and has sampled, presumably via its growing axons, the peripheral field which it will innervate. The development of cholinergic mechanisms takes precedence; adrenergic neurons may develop only when cholinergic sites have been occupied. An extended period during which the differentiation of transmitter mechanisms may be modulated permits the neuron to adequately sample the periphery prior to commitment to a specific transmitter economy.

Several recent observations related to the embryology of the autonomic nervous system (ANS) provide new insights into the factors that may influence the autonomic neuron during development. These observations derive, in part, from the fact that peripheral portions of the ANS are relatively well characterized and are particularly amenable to experimentation. It seems reasonable to anticipate that mechanisms elucidated in this system may also apply to neuronal development in other neural centers.

In this article, we review these recent observations on development of the ANS (as well as related older literature) and present the framework and substantial support for a hypothesis concerning the development of the autonomic neuron which involves two major, and quite distinct, steps or stages. We draw on studies of the "principal," cholinergic or adrenergic, neurons of the system. Accumulating evidence, however, indicates that the traditional concept of two types of neurons is being replaced by that of a complex system of cell types with a variety of neurohumoral substances, including, in the gastrointestinal tract, for example, 5-hydroxytryptamine, somatostatin, substance P, and enkephalin (*1*). It seems reasonable to anticipate that concepts derived from information concerning the development of the "principal" neurons of the ANS may be applicable to those more recently discovered subpopulations where similar information is not yet available.

The hypothesis then can be summarized as follows. During the first stage of development, the primary aspect of the neuron's behavior is its assumption (by somal migration and axonal growth) of position within the body tissues. During the second stage, differentiation of certain properties of the neuron (specifically the final selection of the neurotransmitter to be employed) is substantially influenced by interactions with these tissues. This scheme is presented below as a hypothesis, some aspects of which can be supported; other aspects await future testing.

Two observations prompted this reevaluation of developmental mechanisms. The first, from experiments in which the techniques of experimental embryology were used, demonstrated that the tissue of origin of the several parts of the ANS (the neural crest) exhibits an impressive pluripotentiality (*2, 3*). Those portions of neural crest that normally provide sympathetic neurons may, under certain experimental conditions, provide parasympathetic neurons instead. The second observation was direct in its simplicity but offered a considerable challenge in interpretation; neurons from a predominantly adrenergic sympathetic ganglion grown under certain tissue culture conditions form cholinergic synapses among themselves and on several types of target tissue in coculture (*4–9*).

Several possible explanations of this latter result have now been ruled out. The cultured neurons providing cholinergic synapses are not derived from the small population of cholinergic neurons thought to be present in the sympathetic ganglia under study. Selection of a specific neuronal type as cells proliferate or die (or both) with time did not occur; thus the change from adrenergic to cholinergic function did not occur by selection of subpopulations of neurons (*10*). The shift was not idiosyncratic of the culture system, in that it did not occur under certain culture conditions or with adrenergic neurons taken from more mature animals (*11*). The unexpected conclusion appears to be that neurons clearly expressing adrenergic properties are capable of developing fully functional cholinergic mechanisms.

Observations from Experimental Embryology

Although the question of the sources of autonomic neurons within the embryo has engendered considerable dispute, it now appears generally accepted that neurons for all parts of this system derive from neural crest cells (*12*). Certain of the neural crest cells that migrate ventrally between the developing neural tube and somite take up permanent residence in this position and form the sensory ganglia. Other cells continue to migrate ventrally to provide autonomic neurons in widely scattered locations including (i) sites lateral to the developing vertebral bodies (the paravertebral ganglia), (ii) sites lateral and anterior to the aorta (the prevertebral ganglia), and (iii) sites along the gut wall, by extensive migrations from the "vagal" and sacral regions forming the intrinsic enteric plexuses of Auerbach and Meissner. Several other specific migratory routes are known, including the route taken by cells which colonize the adrenal medulla.

Of particular interest are the studies of LeDouarin and Teillet and their associates (*2, 3*), who, using the technique of implanting neural crest anlage from quail into chick embryos, have made important new observations on very early autonomic development. Structural dif-

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ferences in the interphase nucleus in these two species allow recognition of cells of quail origin within the developing chick embryo. This experimental approach circumvented the chief disadvantage of thymidine marking of neural crest cells—that is, the dilution of label as the cells continue to divide during migration. The quail-chick chimera technique makes it possible to follow the migration of neural crest cells through the whole of embryonic development, and thus to the point where these cells have reached their definitive locations. A series of studies based on this approach has provided evidence that the portion of the neural crest which normally provides cells for the adrenal medulla may, if transplanted to the rostral region of the embryo, provide cells that migrate into the intestinal wall and form neurons located within the intrinsic gut plexuses. These neurons do not exhibit the fluorescence characterizing adrenergic neurons; presumably they are expressing cholinergic function. Conversely, neural crest cells taken from upper trunk somite levels, which normally complete the long migration to colonize the gut autonomic plexuses will, if placed in the mid-trunk region, provide neurons for the sympathetic chain ganglia and cells for the adrenal medulla. By this technique, these cells can be shown to fluoresce as do intrinsic adrenergic cells in these regions. These workers have also placed quail tissue containing neural crest cells in direct contact with chick embryo gut, thus eliminating the migratory pathway normally traversed. Under these conditions, quail cells were able to populate intrinsic plexuses of the gut wall with nonfluorescent neurons. This experiment demonstrated that cells which are normally progenitors of adrenergic neurons are capable of providing gut plexus neurons, some of which are demonstrably cholinergic (*3*, *13*), even when bypassing their normal migratory route through embryonic tissues. From these and other experiments, Le Douarin and Teillet concluded that differentiation of autonomic neuroblasts is controlled by the environment that these neural crest cells occupy at the end of their migration. Because these neurons are in the process of cell division even as they migrate to their final locations, and because this route is not as critical as the site the neurons finally occupy, Le Douarin and Teillet conclude that the differentiation of the postmitotic neuron is influenced by its immediate environment. They noted no neurons in abnormal locations; in this experimental situation the neuron retains the clear sense of the position it is to assume. However, functional expression, in terms of the type of neurotransmitter synthesized, appears to be labile.

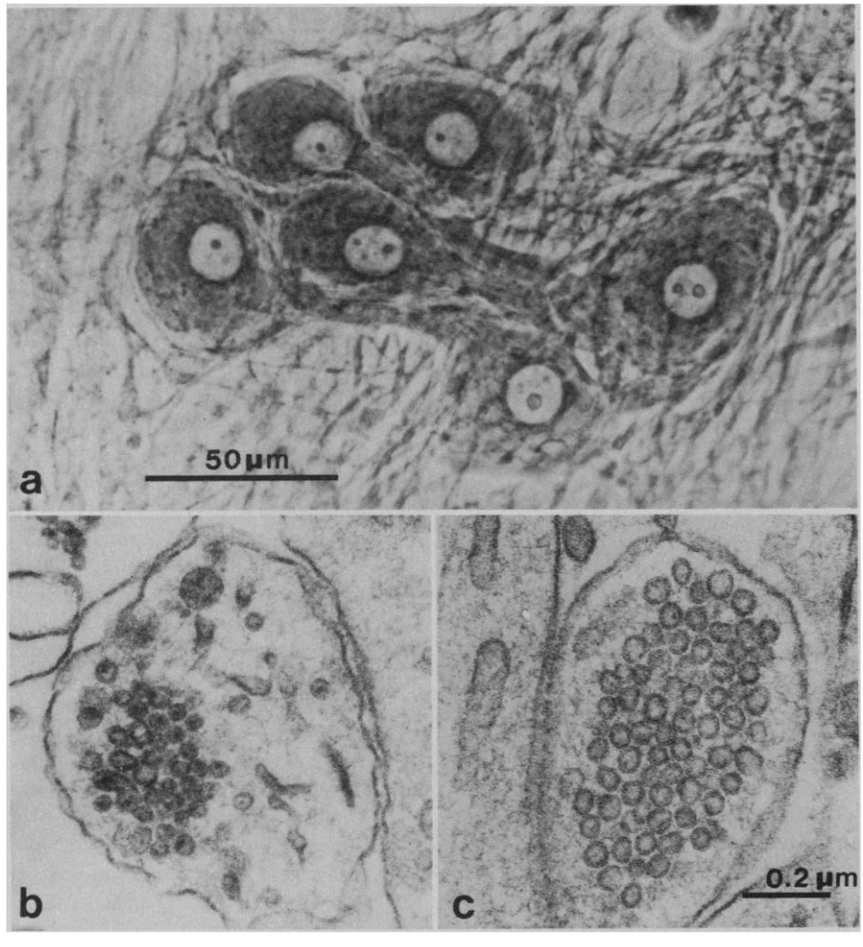

Fig. 1. The "shift" from adrenergic to cholinergic function demonstrated in cultures of neonatal autonomic neurons. The pictures are of neurons from the superior cervical ganglion of newborn rats in dissociated cell cultures with supporting cells. (a) Light micrograph of a cluster of six neurons after 1 month in culture. The neurons are enmeshed in a network of neuronal processes, Schwann cells, and fibroblasts. (b) Synaptic vesicles after 1 week in culture [as in (a)]. This electron micrograph shows an axonal terminal on a neuronal soma after incubation in $10^{-5}M$ norepinephrine and fixation in potassium permanganate. As is characteristic of synaptic vesicle clusters in dissociated perinatal neurons after 1 week in culture, most vesicles contain dense cores, a cytochemical index of the norepinephrine content. Magnification as in (c). (c) Synaptic vesicles after 4 weeks in culture. Preparation as in (b). Characteristically, the synaptic vesicles now show few dense cores. Correlating with this change in vesicle morphology, the cultures show increased levels of choline acetyltransferase and an increasing incidence of cholinergic synaptic interactions between the cultured neurons.

The conclusions of Le Douarin and Teillet are somewhat different from those of Cohen (*14*) and Norr (*15*), who stressed the importance of the migratory pathway of neural crest cells in influencing the differentiation of these cells. Their observations leave no doubt that during migration the neural crest cells may be influenced by their contact with mesoderm of the somite and with the ventral neural tube. This influence imprints a developmental bias on the migrating cell; but it is clear from the tissue cultural experiments discussed in detail below that this is a bias and not a commitment.

The experiments on chick embryos have certain limitations: (i) only embryos during very early phases of development can be manipulated, (ii) cholinergic innervation to chick gut from specific, identified quail neurons has not been directly demonstrated, and (iii) the role of selection is difficult to rule out because the neuronal precursor cells continue to divide as they migrate. Some of these problems can be circumvented if tissue culture techniques are used, as will be discussed below. The observations from experimental embryology do clearly show that the final expression of transmitter function in the neural crest cell which will become an autonomic neuron is influenced by interactions with its environment.

Recent Observations on Autonomic Neurons in Tissue Culture

Long-term cultures of dissociated neurons from autonomic ganglia provide opportunities to study the functional expression of sympathetic neurons in tissue culture (Figs. 1 and 2). For these experiments the starting tissue has been the superior cervical ganglion (SCG) of the perinatal rat. The principal neurons of this ganglion are 95 percent noradrenergic and 5 percent cholinergic (*16*); in addition, there are a small number of small, intensely fluorescent cells, which in some species are known to be dopaminergic (*17*). These small "interneurons" do not survive in the dissociated cell cultures under discussion here. In the SCG of the rat, mitotic activity in neuronal precursor cells is essentially completed by the first postnatal day (*18*). The neonatal neurons are, however, relatively immature, with an eccentric nucleus and a small volume of cytoplasm. Their survival during the first weeks in culture is dependent on the presence of nerve growth factor in the culture medium (*19*); this factor appears to influence their survival but not the course of their differentiation (*20*). The retention of adrenergic characteristics in these dissociated neurons has been described (*4–6*).

The subsequent demonstration that these neurons, taken from a predominantly adrenergic sympathetic ganglion, were capable of making cholinergic synapses with one another (*7, 8*) and with skeletal muscle (*21*) in tissue culture prompted a series of studies aimed at elucidating the mechanism behind this unexpected observation. Several possible explanations have now been excluded. The type of culture used in these experiments is routinely prepared from rat fetuses at the time of birth. As discussed above, it is now known that essentially all neuronal mitoses in this ganglion are completed prior to this time. Mitosis of neuronal precursor cells has not been observed in these cultures, and the total number of neurons does not change appreciably with time (*10, 22, 23*). The accrual of functional neuronal types by selection from subpopulations is thus ruled out.

The proportion of neurons with demonstrable synaptic interactions was often very high (up to 70 percent) (*7, 8*); thus the interacting neurons could not be derived from the small number (5 percent) of cholinergic neurons that may be present in the source tissue. That these cultures do not contain a population of "unexpressed" neurons that elect to express cholinergicity after a period of time in culture may be concluded from cytochemical analysis of synaptic profiles after various periods of time in culture (*10, 22*). The cholinergic neurons in these cultures are not thought to arise from a population of undifferentiated cells, initially unrecognizable as neurons, whose rate of differentiation into cholinergic cells equals the rate of adrenergic neuronal cell death. Neurons in cultures grown in

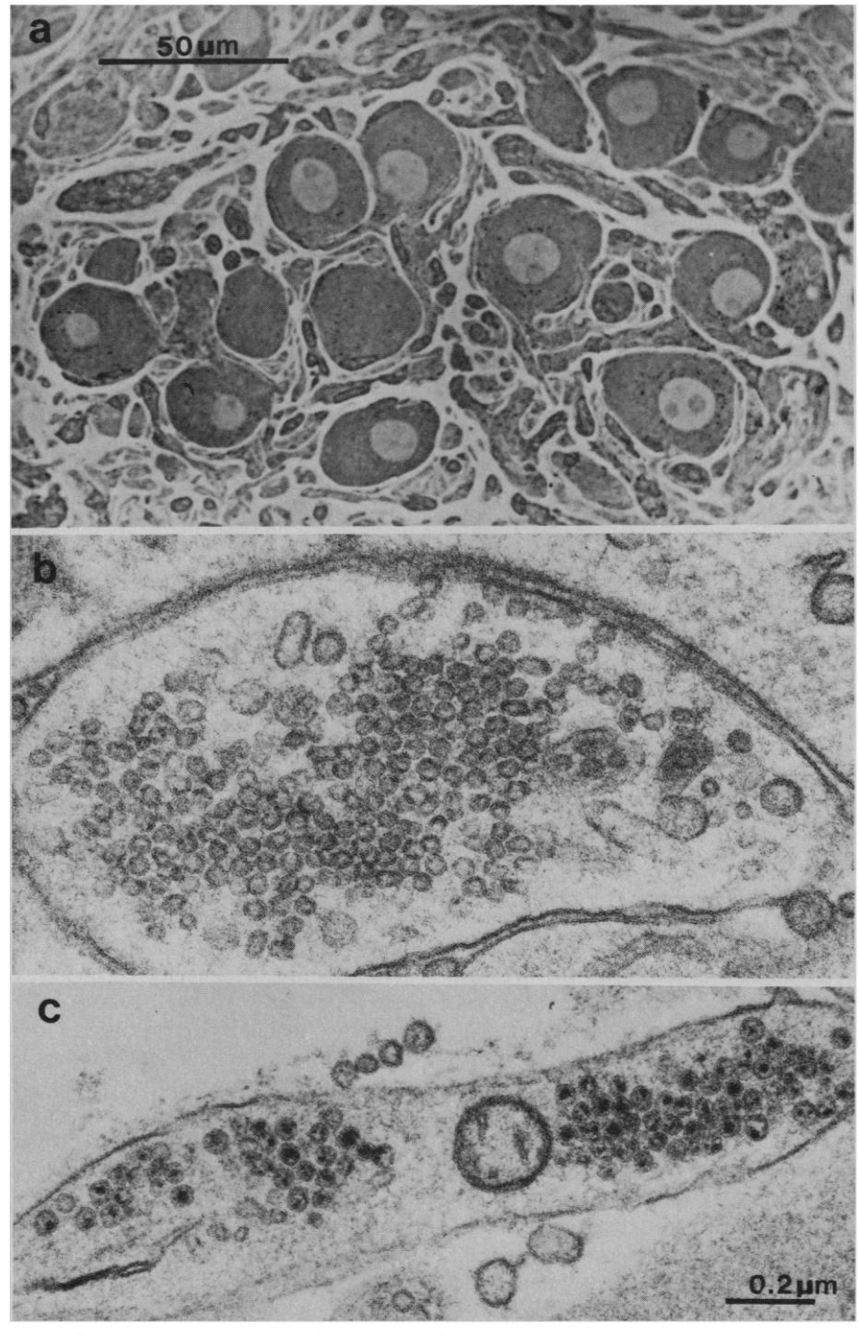

Fig. 2. Demonstration of the age dependence of the adrenergic to cholinergic "shift" in autonomic neurons. Cells from the superior cervical ganglion grown as an explant culture with supporting cells. This type of culture can be prepared from rats of all ages, but only those cultures prepared from neonatal or young rat pups accrue choline acetyltransferase activity (*10, 22*). (a) This explant, taken from an adult rat and grown for 1 month in culture was prepared for electron microscopy, but sectioned at one-half μm, stained with toluidine blue, and photographed by light microscopy. (b) Synaptic vesicle cytochemistry in a ganglion taken from a neonatal rat and grown for 1 month in culture. As with the dissociated neurons shown in Fig. 1, synapses are characterized by clear synaptic vesicles after this period of time in culture. Fixation as described in Fig. 1b. Magnification as in (c). (c) Synaptic vesicle cytochemistry in culture prepared from an adult rat and grown for 1 month. The majority of the vesicles in axonal varicosities are dense-cored, correlating with the lack of choline acetyltransferase accrual in these cultures, evidence that an adrenergic to cholinergic shift is not occurring in cultures prepared from mature rats.

the absence of any other cell type exhibit identifiable characteristics of adrenergic morphology by 1 week or less in vitro. Such cultures composed entirely of sympathetic neurons without supporting cells still develop significant choline acetyltransferase activity and a high percentage of cholinergic synaptic interactions when grown in the appropriate medium (*24*).

These experiments leave little doubt that a shift from adrenergic to cholinergic function was occurring within individual neurons cultured from the rat SCG. A central question then became whether this type of neuronal plasticity was peculiar to autonomic neurons in tissue culture. The adrenergic neurons of this ganglion isolated from their preganglionic synaptic input or their visceral targets in vivo have not been shown to develop cholinergic characteristics. Also, the postganglionic fibers from sympathetic ganglia of adult mammals have not been observed to form junctions with skeletal muscles in vivo (*25*). It thus seemed reasonable that the "transmitter-shift" capability observed in cultures (which are routinely prepared from perinatal animals) might be expressed only during a circumscribed developmental period. We have shown that the developmental age of the rat adrenergic neuron when put into culture influences whether this neuron can adjust its transmitter production and add cholinergic mechanisms (*11, 26*). These neurons exhibited the greatest capacity for the development of cholinergic mechanisms when cultured during the first days after birth; at this time the axons of many of these neurons are beginning to interact with their peripheral targets—such as iris, smooth muscle, and pineal gland (*27, 28*). Neurons cultured from rats after 3 weeks of age showed a greatly decreased ability to synthesize choline acetyltransferase, which is routinely measured as an index of the degree of "cholinergicity" in our culture system. These observations led us to speculate that a "specifying" signal from the adrenergic targets of these neurons begins its influence during the early postnatal period and that the result of this signal is the commitment to adrenergic function, which characterizes most neurons of this ganglion in vivo (*11*). These new data established that the "routine" cultures of perinatal rat autonomic neurons may have been fortuitously prepared at a time when this neuron in the animal retains an option in the final selection of the transmitter.

The reports of cholinergic interaction between these sympathetic neurons raised other questions as well. Are the cultured neurons assuming an intermediate state of differentiation in culture and, contrary to the generally accepted principle, utilizing two very different chemicals in their transmission processes? The fact that adrenergic neurons were adding cholinergic characteristics over a period of several weeks in culture had been observed in several laboratories (*7, 10*). Whether adrenergic characteristics are concomitantly reduced is still unknown; several reports (*29–31*) suggest that at least certain adrenergic functions are reduced as cholinergic mechanisms accrue. Our own cytochemical study (*22*) of synaptic profiles formed in cultures undergoing a cholinergic "shift" indicated that, when first established in culture, synaptic profiles (or the varicosities forming periodic dilations on autonomic nerve fibers) are uniformly of the adrenergic type, that is, containing dense-cored vesicles after potassium permanganate fixation (Fig. 1). If these adrenergic characteristics were expressed, during the first few days in culture, by a subpopulation of neurons, and the developing cholinergic characteristics were progressively expressed by an originally quiescent neuronal subpopulation, then as the culture matured and cholinergic characteristics appeared one would expect the proportion of definitively adrenergic synaptic profiles to decrease as increasing numbers of endings with the clear vesicles characteristic of cholinergic terminals made their appearance. Such a result was not found; over several weeks in culture there were increasing numbers of endings containing a mixture of dense-cored and clear vesicles. After 8 weeks in culture, however, the most commonly observed synaptic ending did not contain a mixture of vesicle types but instead contained primarily clear vesicles. Thus, as judged from this extensive cytochemical characterization, the shift was from adrenergic function through an intermediate state to cholinergic function.

This study also provided data indicating the essential morphological similarity of the adrenergic and cholinergic autonomic neuron. Without specific cytochemical staining of the synaptic vesicle cores, the two types of neurons are indistinguishable. The architecture of their terminals and varicosities, the size of their synaptic vesicles, and the positioning of these vesicles within the synaptic profile are similar. Electron microscopists making studies in vivo of autonomic ganglia that contain an admixture of adrenergic and cholinergic neurons (such as the pelvic ganglia of the rat) have noted the ultrastructural similarity between these two neuronal types (*32*).

Tissue culture experiments of both similar and quite different design have shown that, by specific adjustments of culture conditions, it is possible to obtain cultures of perinatal rat SCG neurons that synthesize varying portions of catecholamines and acetylcholine (*29, 31, 33*). Cultures synthesizing predominantly catecholamines had a low incidence of cholinergic synaptic interactions, and dense-cored synaptic vesicles predominated. As the amount of acetylcholine synthesized and the percentage of cholinergic synaptic interaction increased, fewer vesicles contained dense cores. In discussing these observations, Landis (*9*) raised the question whether two populations of neurons were present, one exclusively adrenergic and one cholinergic. This question was pursued by studying single isolated sympathetic neurons (*9, 34*). Microcultures were prepared in which rat SCG neurons were grown on small islands of previously cultured rat heart cells. On myocyte islands containing a single neuron it was possible, by physiological and pharmacological methods, to identify this neuron as either adrenergic, cholinergic, or dual function. The adrenergic neurons had a propranolol-sensitive excitatory effect on the cultured myocytes. Electron microscopy after potassium permanganate fixation identified the synaptic vesicles of these neurons as predominantly dense-cored. In contrast, the solitary cholinergic neurons produced myocyte hyperpolarization with cessation of spontaneous activity, an effect that was blocked reversibly by atropine. The synaptic vesicles of these cholinergic neurons contained few dense cores, and these were seen only after prior incubation with 5-hydroxydopamine. Stimulation of the dual function neurons first inhibited and then excited myocyte activity. Inhibition could be blocked by atropine and excitation by propranolol. One-third of the terminals in these dual function neurons contained a small number (1 to 4 percent) of dense-cored vesicles. Seven such dual function neurons were identified, a striking demonstration that a single neuron could in fact function with two neurotransmitters. Landis (*9*) and Furshpan *et al.* (*34*) discuss several unanswered questions, including whether such neurons are, in fact, synthesizing (as opposed to taking up and storing) both transmitters and how stable this capability might be. Because most cultures were studied after less than 1 month in vitro and were taken from perinatal rats, the question arises whether,

after several months in culture (under conditions which foster development of cholinergic characteristics), neurons observed to exhibit dual capabilities earlier would complete a shift to cholinergic function.

A number of the above observations suggested the possibility that critical signaling from the peripheral fields may be involved in the differentiation of transmitter mechanisms in autonomic neurons. This possibility seemed additionally attractive when Patterson and Chun (*31*) clearly demonstrated that a factor capable of inducing the cholinergic shift was produced and released by an autonomic target, that is, tissue cultured from the fetal rat heart. These investigations were extensions of studies on the influence of nonneuronal cells on the development of sympathetic neurons, and in particular on the production of neurotransmitters (*30*). Cultures of dissociated SCG neurons from perinatal rats, grown in the absence of nonneuronal cells (as achieved by varying the medium and the incubation conditions), were found to synthesize virtually no acetylcholine. Addition of a nonneuronal cell population to similar cultures resulted in increased synthesis of acetylcholine (100- to 1000-fold) as well as choline acetyltransferase (1000-fold). Under these same conditions, an increase in physiologically detectable cholinergic interactions has been noted. The nonneuronal cell lines were not all equally effective in increasing acetylcholine or choline acetyltransferase production. A second approach was to add to cultured neurons variable proportions of culture medium conditioned by the presence of nonneuronal cells. The results were similar to those obtained in cultures actually containing the nonneuronal cells. Conditioned medium in increasing amounts resulted in increasing synthesis of acetylcholine. Some types of rat cells conditioned the medium more effectively than others; several nonrat cell types were less effective. No differences in cell survival or health were noted between cultures that remained adrenergic in the absence of nonneuronal cells or conditioned medium, and those induced to be cholinergic by the presence of these factors. Conclusions derived from these experiments include: (i) the presence of certain nonneuronal cells or a soluble factor can influence transmitter production in the immature sympathetic neurons, and (ii) there is some specificity in this effect in that not all nonneuronal cell sources are equally effective in providing the appropriate environment or soluble factor.

A number of questions are pertinent. What might be the relationship between the inducing factor contained in conditioned medium and that factor (or factors) possibly produced by the target tissues of a neuron in vivo? By what mechanism (nuclear or cytoplasmic) might this factor influence neurotransmitter enzyme production in the postmitotic neuron? How might this factor be transferred from the target tissue to the developing neuron?

Relevant data are only available regarding the last of these questions. In considering the mechanism of transport of a specifying signal, it should be noted that the avidity for endocytotic uptake shown by both growing and more mature nerve fibers has been extensively documented (*35*). It is thus tempting to suggest that uptake of some signal from the peripheral innervation field of the maturing sympathetic neuron is needed to set the final switch for cholinergic or adrenergic differentiation. Such a mechanism would not be without precedent in developmental neurobiology. It is known that nerve growth factor, vital for adrenergic neuron development, is carried from the peripheral axonal fields to the cell soma by retrograde axoplasmic transport (*36*).

Of more direct relevance to the present case, however, are results of experiments on the properties of sensory neurons during early embryonic development. Some observations (*37*) indicate that the function of sensory neurons as primarily dorsal or ventral is imprinted during a critical period early in development. Sensory neurons contacting dorsal skin during this critical age will make connections within the spinal cord appropriate for neurons contacting dorsal regions. If the neuron, by experimental manipulation, is exposed instead to ventral skin during this critical period it will thereafter function as a ventral neuron. That is, stimulation of the skin containing its axonal branches will lead to a reflex directed to the ventral portion of the body even though the skin contacted has been transplanted to the back. Transplantation of skin after this critical period does not induce this change. In this instance, as with the autonomic neuron, the identity of the neuron as a sensory neuron is retained, but a primary functional characteristic is changed during a critical developmental period, apparently in response to interaction with target tissue.

Because (in general) most neurons cannot be characterized by unique biological molecules, there is no way of measuring what biochemical changes may occur at various stages in their differentiation. However, the type of positional information that the developing neuron possesses can often be measured. Such measurements in retinal ganglion cells indicate that positional information is imprinted on the neuron at the time of its last mitotic division (*37*). Because the transmitter in this neuron is not known, there is apparently no way to test whether a secondary shift in neuronal biochemical parameters may take place after this stage. For the autonomic neuron, however, we can measure the enzymes that synthesize the neurotransmitter, the cytochemistry of the synaptic endings, and the physiological aspects of synaptic function. These basic characteristics have been shown to change in cultures prepared from postmitotic neuronal populations during a limited period of early development. Thus, this postmitotic neuron can make major changes in the state of its differentiation.

Related Aspects of Autonomic Development in Higher Vertebrates

We have outlined above the extensive migrations that scatter neurons of neural crest origin widely throughout the body. The most extensive single group of autonomic neurons is established in relation to the smooth muscle of the gut, providing there the intrinsic neurons of the plexuses of Auerbach and Meissner (*12*). Other neurons of the parasympathetic portion of the ANS characteristically take up residence in the walls of other visceral structures such as the heart or in ganglia near these visceral structures (for example, the pelvic ganglia or cranial parasympathetic ganglia). As we have discussed above, many, but by no means all, neurons of this parasympathetic system are cholinergic. In contrast, many of the adrenergic neurons come to reside in the autonomic chain ganglia or the preaortic ganglia related to the mesenteric and renal arterial branches of the aorta. These neurons are thus located some distance from the visceral structures that they innervate. Therefore, if cell bodies of the neurons of sympathetic ganglia and the neurons of the intrinsic enteric plexus arrive at their points of permanent residence at about the same time during embryonic development, their axons have quite different distances to traverse in arriving at their visceral targets.

In the chick embryo, certain prospective sympathetic and parasympathetic neurons have been shown to arrive at their permanent places of residence at about the same time. For example, Juba (*38*), using Cajal's silver technique, was

able to stain autonomic ganglion cells within the gut wall as early as 69 hours from the beginning of incubation; the number of these cells was substantially increased during day 3 of development. Levi-Montalcini (*39*) noted that, in the 3-day chick embryo, nerve fiber bundles (rami communicans) can be seen connecting the spinal ventral roots with identifiable sympathetic primordia occupying the position of the sympathetic chain ganglia. It is likely that neural crest cells destined to provide these autonomic ganglia may have arrived in this paravertebral position even earlier. The experimental procedures of Andrew (*40*) allow a precise determination of the time of arrival of neuronal precursor cells in the presumptive gut of the chick embryo; progressively earlier excision of presumptive gut tissue with transplantation to the chorioallantoic membrane established that progenitors of intramural ganglion cells arrive in the presumptive gizzard and intestine shortly before the 12.5-somite stage (that is, at about 40 hours of incubation). Thus, neurons of the enteric plexus and neurons of sympathetic ganglia (or their precursors) are in place very early in embryonic development of the trunk of the chick. Taken together with the data considered below concerning the sequence of development of functional innervation there seems little question that the early arrival of the intrinsic neurons of the gut, and their close proximity to the visceral targets there, give these neurons first access to receptor sites located in the various tissues of the gut wall.

Detailed analyses have been undertaken on the order of arrival of autonomic innervation in the intestinal walls of the rabbit and the mouse. Using both anatomical and physiological techniques Gershon and Thompson (*41*) have determined that the earliest components of the developing innervation of the rabbit intestine were cholinergic. This innervation appeared on day 17 of gestation in the rabbit and on day 16 in the mouse. The hallmark of the arrival of adrenergic innervation, the specific uptake of norepinephrine, was first detected at day 21 of gestation in the rabbit; stores of norepinephrine could not be detected histochemically until days 26 to 28. They also note the primacy of the arrival and function of cholinergic nerves and conclude that, in fetal and newborn gut, the cholinergic excitatory innervation must function without adrenergic modulation. They also point out that this pattern in ontogeny is quite similar to the pattern of autonomic innervation seen in vertebrate phylogeny (discussed below).

The early development of the dual innervation of the chick heart has been reported in a series of studies by Pappano and associates (*42*). The heart is particularly well suited for this type of study because neural influences on its intrinsic beat are subject to detailed analysis. Physiological and pharmacological studies indicated that functional cholinergic inhibitory transmission appeared initially on day 12 of incubation; adrenergic acceleratory transmission appeared for the first time on day 21 of incubation, shortly before hatching. By anatomical analysis it has been possible to demonstrate that the nerve fibers of these two systems are present 5 to 8 days prior to the time that they are able to elicit demonstrable effects on heart muscle (*42*, *43*). This gap of several days, between the anatomical arrival of each type of nerve fiber and its demonstrable physiological effect, apparently relates to the relatively small amount of transmitter available for release from the very young nerve fiber. Considering both the evidence for the arrival of nerve endings and the evidence for the appearance of nerve transmission, cholinergic characteristics appear about nine embryonic days before adrenergic characteristics.

By these studies, the relatively late arrival of adrenergic nerve fibers in the innervation of gut and heart was established. With the introduction of the induced fluorescence method for catecholamines and the available tests for specific uptake systems, the time of arrival of adrenergic fibers in various organs and species has been studied. In different tissues of the rat, the ability to take up and accumulate norepinephrine as well as the outgrowth of sympathetic adrenergic nerves takes place during the period immediately before and after birth (*28*). With studies available from chick, mouse, and rabbit, the principle that adrenergic nerves invade their target tissues relatively late in development seems firmly established.

Related Aspects of the Phylogeny of the Autonomic Nervous System

From a review of the comparative vertebrate anatomy and pharmacology of the ANS, a generalized impression is gained that the cholinergic system appears earlier in phylogenetic development than the adrenergic system (*44*). Although the great diversification of vertebrate forms provides many exceptions to this generalization, in most autonomic systems of lower forms the cholinergic system is dominant, sometimes providing both parasympathetic and sympathetic innervation to smooth and cardiac muscles. With the increasing complexity of higher vertebrate forms, adrenergic innervation often either replaces the cholinergic innervation or is added to it.

Burnstock's review (*44*) of the form and function of autonomic neurons in vertebrates provides the following generalizations. In the lung, excitatory cholinergic innervation is of sympathetic origin in anuran amphibians and of vagal parasympathetic origin in reptiles and mammals. Noradrenergic sympathetic inhibitory fibers to the lung are found in reptiles, birds, and mammals; inhibitory fibers to the amphibian lung apparently release another transmitter. Although cholinergic innervation to the urinary bladder is present in all vertebrates, including the teleost fish, adrenergic fibers have been demonstrated only in vertebrates higher than fish. Inhibitory noradrenergic control of intrinsic neurons in the bladder is present in reptiles and mammals. Adrenergic innervation to the bladder from neurons in the bladder wall is well-developed only in mammals, being poorly developed and nonexistent in lower forms. The predominant sympathetic innervation to the stomach and intestine of fish is cholinergic and excitatory; inhibitory innervation to the stomach is apparently provided by neurons releasing a transmitter other than norepinephrine. Nervous control of the gut increases in complexity in higher forms with the aggregation of neurons into ganglia within the muscle wall. Noradrenergic sympathetic control of cholinergic neurons in the gut wall appears in the reptiles and is more highly developed in the mammals.

The primacy of cholinergic innervation is particularly well illustrated in the cardiovascular system. Autonomic innervation to the heart of cyclostome and elasmobranch fish is only cholinergic. Adrenergic innervation to the heart appears in the teleost fish but becomes more pronounced in reptiles and mammals. Adrenergic modulation of cardiac ganglion cells is found only in mammals. The innervation to the arteries of fish is primarily cholinergic, to the arteries of reptiles and birds primarily adrenergic, and to the arteries of mammals totally adrenergic. The innervation to the vascular bed of fish is only cholinergic; adrenergic innervation appears in the amphibians, and becomes dominant in mammals. The innervation to the iris in fish is cholinergic. Adrenergic innervation to the iris does not appear until the amphibians and becomes increasingly important in pupillary control

through reptiles and birds to the mammals.

It thus appears that with the increasing complexities of higher phylogenetic forms, adrenergic sympathetic innervation becomes more important. In some cases adrenergic innervation, only rudimentary or nonexistent in lower forms, is the primary innervation to a structure in mammals. During phylogeny cholinergic innervation, which provides the dominant (or sometimes the only) autonomic innervation to structures in lower forms, becomes increasingly antagonized or modulated by the adrenergic system. This supports the view that the autonomic innervation to structures of lower animals is primarily cholinergic and that this system is therefore phylogenetically more primitive than the adrenergic system.

Discussion

The division of the vertebrate ANS into sympathetic (thoracolumbar) and parasympathetic (craniosacral) components originated with Gaskell and with Langley (*45*). The sympathetic nerves were considered to supply all visceral parts of the body; nerves supplying only certain special visceral structures were thus logically termed parasympathetic. This division was also engendered by the recognition that certain pharmacological agents (for example, adrenaline) produced effects similar to those observed after stimulating sympathetic nerves while others (such as pilocarpine) produced effects like those produced by stimulating parasympathetic nerves. From the earlier terminology for the sites of origin of the preganglionic nerve fibers to these two components (tecto-bulbar-sacral and thoracico-lumbar) we derive the modern terms, craniosacral and thoracolumbar. We have now come to generally view these two divisions of the peripheral ANS not only as very different in position within the body but also very different in terms of the major neurotransmitters employed by the "principal" second order neurons.

One must also consider, however, the basic similarities between the two major portions of the ANS. These similarities, some of which we have discussed and documented, include the following. (i) All autonomic neurons are now thought to have a common embryological source in the neural crest. (ii) Neural crest tissues serving as sources for sympathetic neurons may, if transplanted elsewhere in the early embryo, form parasympathetic neurons. (iii) Sympathetic and parasympathetic neurons are often intimately mixed within the same autonomic ganglion. (iv) Without special cytochemical staining to reveal neurotransmitters or their related enzymes, these cells present essentially identical morphology by light and electron microscopy. Are we then to view the ANS as fundamentally one system or two?

It seems reasonable to suggest that cells of the ANS are initially of one general type and that at some stage in their development they become subdivided into subpopulations, including the commonly recognized adrenergic and cholinergic principal cells. The data presented support the following relevant conclusions. (i) The transmitter employed by the neural crest cell which will become an autonomic neuron is not fixed before or during its migration, but is determined by the final environment of the neuron. (ii) Postmitotic autonomic neurons, expressing morphological and biochemical characteristics of adrenergic neurons, become cholinergic in function when placed in tissue culture during a critical period of their differentiation. (iii) This shift in transmitter may be influenced by the presence of target tissue, by certain non-neuronal cell lines, as well as by soluble factors. (iv) In several species and organ systems cholinergic innervation develops approximately one full embryonic week before adrenergic innervation and, for a period of several days, provides the only innervation to that organ.

Conclusion

The data summarized above suggest that the following hypothesis best explains the stepwise development of the autonomic nervous system. The development of the principal neuron of the autonomic nervous system takes place in at least two major steps. In the first step its designation as autonomic occurs early in neural crest development, providing the autonomic precursor cells with the information necessary so that both its soma (by migration) and its terminals (by axonal growth) will assume correct positions in the embryo. Although expression of neuronal type in terms of transmitter synthesis may occur at this stage, it does not represent a final commitment to a specific differentiated state. In a second stage in differentiation the axons sample the periphery with which they are in contact, encountering there signals instrumental in determining which transmitter the neuron will employ. The first of the neuronal populations to contact peripheral visceral targets occupies the most preferred or available sites; in several well-studied cases the earliest known innervation is cholinergic. Autonomic neurons whose axons arrive in visceral target fields later occupy alternate sites; in several cases the late arriving axons are known to develop adrenergic function. This does not imply manifold options for the autonomic neuron. By its earlier nature, and the various positions it will assume in the embryo, it remains immutably autonomic. In a second later step in differentiation, by some form of nurture from its peripheral field, the autonomic neuron elects to express either adrenergic or cholinergic function, and at this stage it becomes committed permanently to the expression of this phenotypic characteristic.

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NEWS AND COMMENT

East Coast Mystery Booms: A Scientific Suspense Tale

Since last December, the public, government officials, and some very distinguished scientists have been baffled by a series of booming noises heard off the East Coast of North America, mostly in southern Nova Scotia, New Jersey, and Charleston, South Carolina. Like any mystery, the boom incidents have attracted their share of nuts and spooks—the nuts include those who write the government about them, giving a return address of "Planet Jupiter"; the spooks seem to include representatives of the Central Intelligence Agency, who quietly have been inquiring around Washington about the possible cause of it all.

And then there are the scientists. Competing scientific views of the cause, or causes, of the booms came into the open recently when (i) two prominent scientists, who apparently believe that the booms could be precursors of an East Coast earthquake, considered giving a press conference but decided not to do so and, (ii) conflicting explanations were put forward at press conferences given first by the government's Naval Research Laboratory (NRL) and then by the Washington-based Federation of American Scientists (FAS).

On 3 March, the NRL announced that offshore military aircraft traveling at supersonic speeds for brief periods were probably responsible for the booms. Several days later, FAS director Jeremy J. Stone postulated a new phenomenon—namely that shock waves from the Concorde supersonic transport, which tripled its number of transatlantic flights just when the boom reports began, are transmitted at great speeds through the upper atmosphere and causing the booms. In the midst of all this, as these scientific sleuths have been crisscrossing each other on the scent of the mystery, presidential science adviser Frank Press seems to have been serving as an informal traffic cop.

As far as is known, the booms do not harm people or property. People simply report having heard a loud, sharp detonation, often when they are indoors. Most scientists who have looked over the boom reports agree that what is being heard is not direct sound but infrasound—the noise of the overpressure of a shock wave hitting a structure, such as a building. In most cases, instruments recording the events indicate that the shock wave is airborne and is not accompanied by seismic activity.

Apart from the recent spate of reliably reported East Coast booms, booming is a historical phenomenon. For hundreds of years sailors in the North Atlantic have heard booming noises and considered them harbingers of good weather; near Lake Seneca, New York, booms, known as the "Seneca guns," have been heard since historical times.

No single theory of their origin has been completely accepted by scientists, although such booms have been a subject of considerable interest. But those who operate sensitive acoustical instruments say the new series of East Coast booms is different. After a highly unusual boom in the Palisades region of New York on 2 December, other soundings from New Jersey, Charleston, New York, and New England were reported. In late December, science adviser Press asked the Department of Defense to look into their possible cause, and, in January, as the reports continued, the NRL began its 2-month investigation of the citizen reports.

The mystery booms also interested Thomas Gold, professor of astronomy at Cornell University, who is well known for his work on pulsars, and Gordon J. F. MacDonald, a prominent geophysicist who is, at present, a consultant to the Mitre Corporation.

Gold, who has been interested in methane as an indication of tectonic activity in the earth, and in the possibility that explosions of leaked, airborne methane are the cause of historical booms such as the "Seneca Guns," apparently was postulating that the East Coast booms were linked to methane explosions. Several associates of Gold's say that he thought that the East Coast Booms could presage a major quake in the area. Gold would not comment directly to *Science* on his earthquake hypothesis, but he did note that Charleston had suffered a major earthquake in 1886 after booming noises were reported in the region.

An associate of Gold's at Cornell, astronomer Carl Sagan, apparently put Gold and MacDonald in touch with FAS director Stone. Stone told *Science* that Gold and MacDonald were interested in publicizing their view of the booms and that, among other things, they talked of holding a press conference to warn of the boom-methane-earthquake possibility.

Stone says this aroused his interest in the booms and in particular correlations between Concorde flights and booms reported in Nova Scotia by an amateur group lead by a housewife named Hattie Perry. By late February, Stone was more and more convinced that Concorde was the cause, but the earthquake precursor theory was also still alive.

Meanwhile, the Naval Laboratory's

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