

MORPHOLOGICAL AND BIOCHEMICAL STUDIES ON THE DEVELOPMENT OF CHOLINERGIC PROPERTIES IN CULTURED SYMPATHETIC NEURONS

II. Dependence on Postnatal Age

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ABSTRACT

Superior cervical ganglion (SCG) neurons taken from perinatal rats and dissociated in culture develop cholinergic properties. This report examines this "plasticity" of neurotransmitter function with regard to its dependence on the stage of neuronal development. Explants of SCG from rats ranging in age from 2 d to adult were cultured, and the number of neurons surviving after 6 wk in culture was evaluated. The activities of choline acetyltransferase (ChAc) and DOPA decarboxylase (DDC) were assayed for each age group over time in culture, and the cytochemistry of the synaptic vesicle population was studied after norepinephrine loading and KMnO_4 fixation.

The specific activity of ChAc in all explants fell during the first 3–4 d in culture (secondary to degeneration of presynaptic terminals), with an increase during the next 30 d in explants from all age groups except in those from the 22-d-old and adult rats. The highest activity found after 1 mo in culture was in explants from 2-d-old rats (62.5 mmol per kg dry wt per h); the lowest was in explants from adults (1.3 mmol per kg dry wt per h). After 1 mo in vitro, there were no significant differences in DDC activity among explants from animals of any age (~220 mmol per kg dry wt per h). Co-culture of the SCG explants with heart muscle increased even further the ChAc activity in explants from 2-d-old rats but not in explants from 16-d-old and 6.5-wk-old animals.

The cytochemistry of the synaptic vesicle population in 1-mo-old cultures correlated well with the ChAc activity; when the ChAc activity was high, the proportion of synaptic vesicles with clear centers was 71–88%. In explants from adult animals, only 12% of the vesicles contained clear centers.

From these data we conclude that the maturity of the SCG neuron influences the degree to which it is able to adjust its neurotransmitter mechanisms. That the axons of this neuron are interacting with target tissues during the time that

neurotransmitter plasticity is retained suggests that interaction with the target may play a role in the determination of transmitter type.

KEY WORDS postnatal age · sympathetic neurons · choline acetyltransferase · synaptic vesicle · neurotransmitter · tissue culture

The ability of cultured superior cervical ganglion (SCG) neurons dissociated from perinatal rats to develop a number of cholinergic functions is now well established. With maturation in culture there is increasing synthesis of acetylcholine (30), with a demonstrable increase in choline acetyltransferase activity (ChAc) (13, 28, 31). By physiological analysis these dissociated neurons have been found to form cholinergic synaptic contacts with each other (16, 26) that become more complex with increasing time in culture (13, 25, 36). The cytochemistry of the synaptic vesicle population is consistent with the biochemical and physiological data. Clear synaptic vesicles predominate in cultures with high ChAc activity (13) or in neurons demonstrated to be cholinergic (17). Cytochemical analyses of the synaptic vesicle content in maturing cultures indicate that initially the cultured neuronal population uniformly exhibits adrenergic characteristics, with a gradual shift to cholinergic cytochemical characteristics (14). The anatomical data suggest that during the transition the neurons express adrenergic and cholinergic mechanisms simultaneously. The existence of such "dual-function" neurons has been demonstrated physiologically (8).

That a basic characteristic of a neuron, namely, its neurotransmitter production, could show this "plasticity" is somewhat surprising in that SCG neurons have virtually completed mitotic division at the time they are dissociated for growth in culture (9). It is of interest, therefore, to know whether this capability is limited to early development, or whether it is expressed throughout the lifetime of the neuron. This question follows logically in view of well-known examples of a critical period during which the environment of the neuron can substantially influence its development. The mammalian and amphibian visual systems have been particularly well studied in this regard (for a review, see reference 12).

To address this question, explants of the SCG were taken from rats ranging in age from 2 d to adult and grown in culture. Activity of ChAc was determined at intervals, and the vesicle population

was studied cytochemically. Preliminary reports of these studies have appeared previously (33, 34).

MATERIALS AND METHODS

Culture Procedures

SCG were removed from 2-, 5-, 10-, 15-, and 22-d-old and adult rats of either sex. The loose connective tissue capsule was removed, and each ganglion was divided into explants of similar size. Each ganglion from 2-10-d-old rats was divided into four explants, and the larger ganglia from 15- and 22-d-old rats were divided into six pieces. Each adult-rat SCG yielded 10-12 explants. Three to four explants were cultured in 24-mm collagen-coated Aclar dishes and stored in Falcon (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) Petri dishes (3). The cultures were fed three times weekly with medium containing 62% Eagle's Minimum Essential Medium (Grand Island Biological Co., Grand Island, N. Y.), 24% human placental serum (HPS), 10% chick embryo extract (EE), 3% 1.1 M glucose, 1% glutamine (200 mM) with 25 U/ml of nerve growth factor (NGF) as prepared and assayed in the companion paper (14). These levels of HPS and EE have been demonstrated to foster the development of cholinergic properties in SCG neurons in vitro (31). Incubation was carried out in a 5% CO₂ humidified atmosphere at 35°C. The nonneuronal cells that proliferate under these culture conditions have also been shown to promote cholinergic mechanisms (28, 31).

For co-culturing with heart tissue, ganglia from 20-d embryos, 2- and 16-d-old pups and 47-d-old rats were removed and divided into explants on the basis of size and grown on collagen-coated dishes as described above. Two SCG explants from each age group were grown in the presence of two explants of perinatal rat atrium. Irregular spontaneous contractions were observed in about one-third of the atrial explants. Control explants were grown without atrium. Cultures were maintained in the medium described above, and supporting cells were allowed to proliferate. After 4 wk, cultures were stopped for biochemical determinations as described below.

Cytochemistry and Electron Microscopy

After 4-4.5 wk in vitro, cultures from all rat ages were rinsed in Liebovitz (L-15) medium (Grand Island Biological Co.) and incubated for 30 min at 35°C with 10⁻⁵ M norepinephrine (NE) freshly prepared in L-15 that contained sodium ascorbate (0.2 mg/ml) at pH 7.3. Careful rinsing in L-15 preceded fixation with 3% KMnO₄ in Mg⁺⁺ Krebs-Ringers PO₄ (pH 7.2) at 35°C. The cultures were then transferred to 4°C for 1 h. After maleate buffer rinses (0.1 M, pH 5.2, 4°C), cultures were stained *en bloc* with 1% uranyl acetate in maleate buffer (0.1 M, pH 5.2, 4°C) for 45 min. Another maleate buffer rinse was followed by dehydration in alcohol and propylene oxide (PO). After 3-4 h in 1:1 PO:Epon-Araldite (Electron Microscopy Sciences, Fort Washington, Pa.), the cultures were embedded in Epon-Araldite (EA).

Fields containing fascicles of neurites radiating from the explant were chosen and marked with a diamond scorer. Blocks containing the scored area (~1 mm in diameter) were mounted in a way that allowed sectioning parallel to the collagen, and 0.5-1- μ m sections were taken until the level containing the neurites was entered. Thin sections (60-70 nm) were obtained by sampling

at 1- μ m intervals for 10–12 μ m. Sections were taken up on Formvar-coated 150-mesh grids and viewed in a Philips 300 electron microscope without further staining.

Sections selected for photography were separated by a minimum of 2 μ m, and fascicles of neurites were surveyed for vesicle-containing varicosities. Only varicosities were studied because somal endings were seen infrequently and only on the few single neurons separated from the explant proper (27). Moreover, we show in the first paper of this series (14) that the vesicle population of varicosities and somal endings is remarkably similar. Several blocks for each age group and a minimum of three levels per block were sampled. At a given level, consecutive varicosities containing a minimum of 10 clustered vesicles were photographed. 25 varicosities were obtained for each age. All negatives were printed to achieve a standard magnification of $\times 102,000$.

Classification and Analysis of Vesicle Type

Prints of all varicosities were coded, and the vesicles in each varicosity were classified by two independent observers as being dense cored, clear, or indeterminate. Only those vesicles between 40 and 70 nm in diameter with a clearly discernible unit membrane were included. 525 synaptic vesicles within 25 varicosities were analyzed for each rat age. The data were compiled from each observer to give the percent of vesicles categorized as clear for each age group. The percent of vesicles considered clear in each varicosity was also calculated. Thus, for each age group and for each observer, the number of varicosities containing 0–20%, >20–40%, >40–60%, >60–80%, or >80–100% clear vesicles was tabulated.

Estimation of Neuronal Number

At 42–48 d in vitro, 3–4 sister cultures from each age group were rinsed three times in L-15 and fixed overnight in dilute Karnovsky's solution (pH 7.3, 4°C) (15). After three rinses in 0.1 M cacodylate buffer (pH 7.3, 4°C to ambient), lipid extraction in chloroform:methanol (2:1) was carried out with two initial 10-min rinses followed by 1.5 h in fresh chloroform-methanol. The cultures were hydrated and stained in 0.25% thionine. Dehydration was followed by PO, PO:EA (1:1), and finally embedding in EA. The neuronal number in each explant was estimated by two independent observers using a Zeiss Universal Microscope with 16–25 \times objectives. As an aid in these counts, 100-mesh grids were firmly fixed over each explant to subdivide them into smaller areas. Grid bars proved not to obscure visualization because they were in a different plane of focus. Our original intent was to obtain the neuronal counts by serial section of the explants using the procedure outlined above. This proved to be impractical, and, since direct counting was possible, the method just described was used.

Enzyme Assays

At time points from 3 d to 6.5 wk in vitro cultures that were sister to those used for cytochemistry were rinsed with L-15 and, after excess medium was removed by blotting, were frozen and stored at -80°C . Cultures were freeze-dried under vacuum at -40°C (21) for 3 d. Explants were dissected free from the supporting cell and neuritic outgrowth and from the collagen, weighed on a quartz-fiber balance, and homogenized in 36 μ l of 50 mM sodium phosphate buffer (pH 7.6) that contained 0.1% bovine serum albumin and 0.1% Triton X-100. Intact ganglia for in vivo (or zero time) enzyme activity determination were treated in the same way, except that ganglia from older animals were

homogenized in larger volumes to keep the weight-volume ratio constant. Homogenates were stored at -80°C until assayed. DDC activity was determined by a modification of the method of McCaman et al (24). ChAc activity was determined radiometrically from the same homogenates described above by a modification (32) of published methods (7, 23). Enzyme activity is expressed relative to the dry weight of the explant \pm SEM.

RESULTS

General Description of Cultures—Light and Electron Microscopy (EM)

The general pattern of growth was the same for all age groups. A halo of neurites relatively free of other cell types initially emanated from the explant proper, followed by an advancing front of non-neuronal cells consisting of both Schwann cells and fibroblasts. As the cultures matured, the non-neuronal cells overtook the growing neurites and after 3–4 wk in culture a confluent layer of non-neuronal cells covered the dish. Around the periphery of the explant, individual neurons could easily be visualized in the living state.

The course of development, however, varied greatly as the age of the rat at explantation increased. Explants from older rats demonstrated a definite and sometimes rather distressing delay in the onset of neuritic growth: the older the rat the more pronounced the delay. Thus, in SCG from adult rats, 4–6 d elapsed before substantial neuritic outgrowth indicated that the neurons were viable, although remarkably little overt necrosis was noted. Later, however, the neuritic outgrowth was quite vigorous and seemed to “catch up,” at least to some degree, to the explants from younger rats. Although it has not been systematically studied, the ultimate length of neurites from adult rat explants was less than that of neurites from perinatal rats. Fig. 1 illustrates four explants taken from an 8-wk-old rat and kept 40 d in vitro. Unlike the series of explants described above, this culture was treated with antimetabolites (39) to inhibit cell division and thereby reduce the number of non-neuronal cells. The reduced number of non-neuronal cells allowed a clear visualization of the neuritic outgrowth, which fit well within the confines of the culture dish. The outgrowth from four similar SCG explants taken from a perinatal animal easily covered an entire dish (see Fig. 1 in reference 6).

The method of embedding used (see above) allows the selection of specific regions of the culture for EM observations. The *inset* in Fig. 1 is a

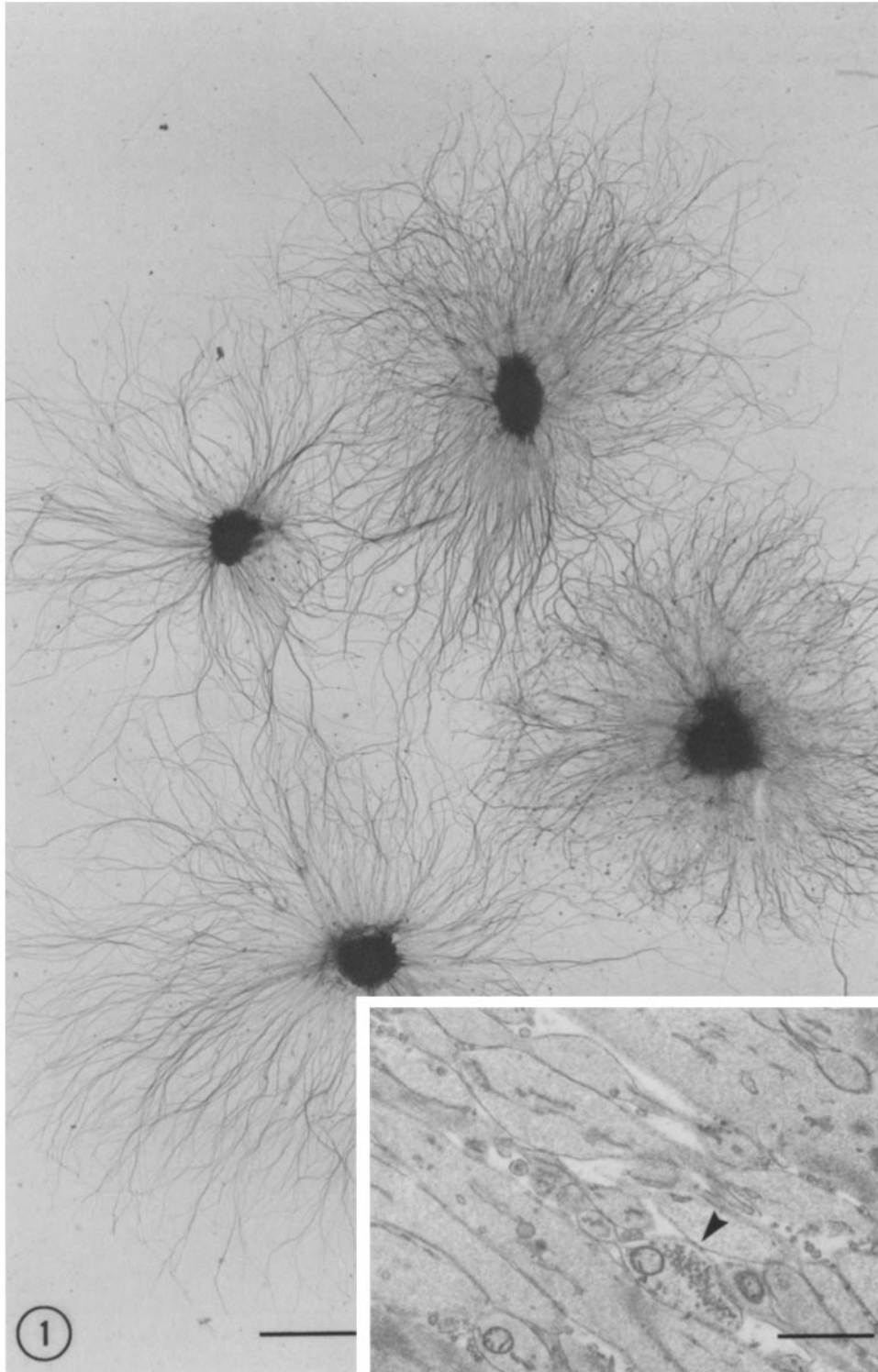


FIGURE 1 Light micrograph of four explants of superior cervical ganglion taken from 8-wk-old rats and grown in culture for 40 d. The number of nonneuronal cells were reduced by treatment with antimetabolites. Sudan Black stain after OsO_4 fixation. Bar, 1 mm. $\times 14$. *Inset* is a low-power electron micrograph of a field of neurites growing out from an explant. The processes show periodic dilations (varicosities), some of which contain clusters of small vesicles (arrowhead). 10-d-old rat. 4 wk in vitro. KMnO_4 fixation. Bar, 1 μm . $\times 14,000$.

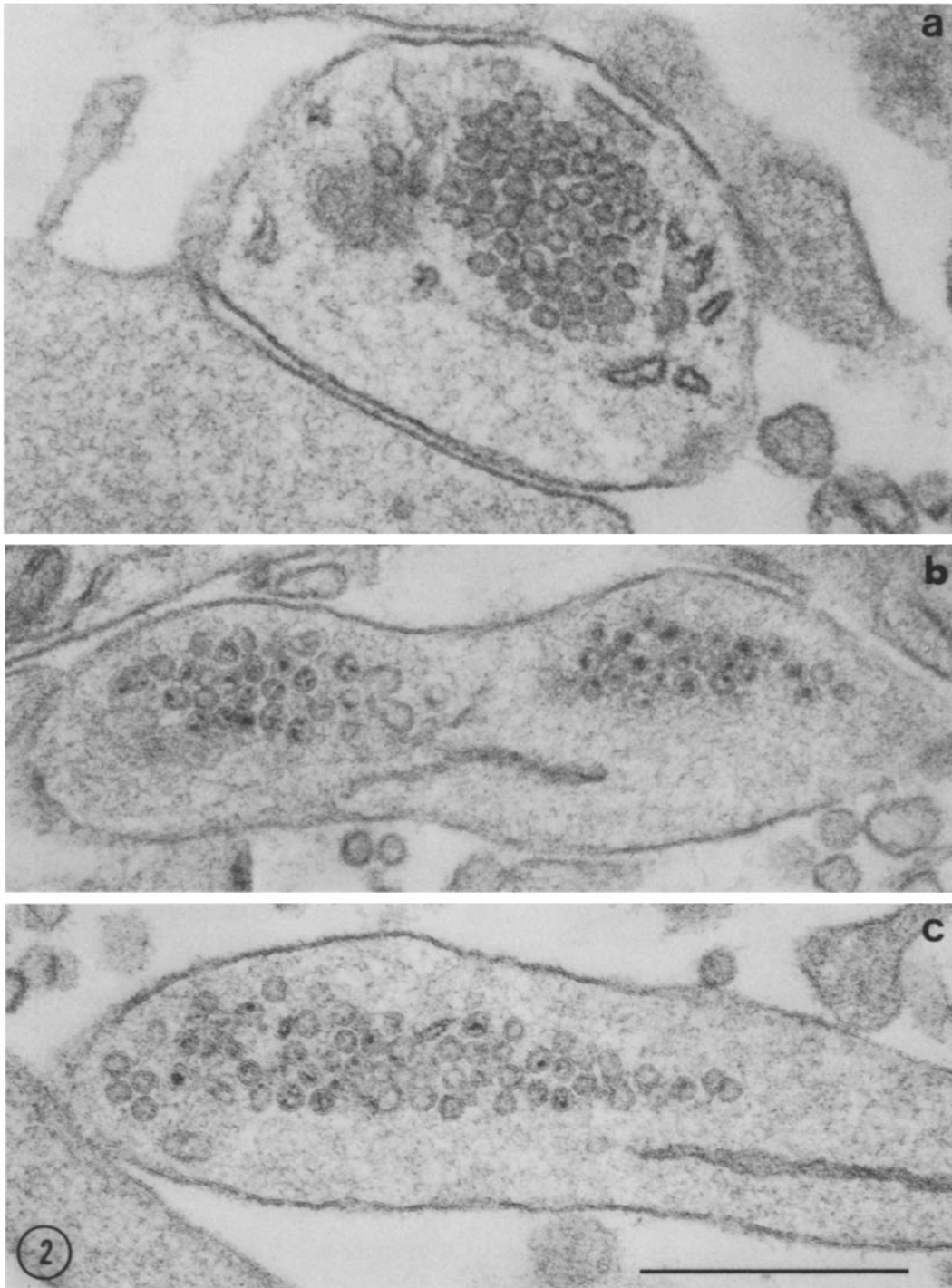


FIGURE 2 Electron micrographs of vesicle-containing varicosities from explants of superior cervical ganglia taken from rats of different ages and fixed after 4–4.5 wk in culture. (a) 2-d-old rat. The vesicle population is predominantly of the clear core type. (b) Adult rat. This profile contains two clusters of dense-cored vesicles. (c) 22-d-old rat. In neurons of intermediate age the vesicle profile is mixed, with both clear and dense-cored vesicles present. (a–c) KMnO_4 fixation after preincubation with norepinephrine. Bar, $0.5 \mu\text{m}$. $\times 80,000$.

low power electron micrograph of bundles of neurites in which vesicle-containing varicosities were located. After 4–4.5 wk in culture, the vesicle population in varicosities from explants from 2-d-old rat pups was predominantly clear (Fig. 2*a*), whereas the vesicle population from adult explants was strikingly dense cored (Fig. 2*b*). Profiles with a mixed population of vesicles (40–60% clear) were seen, especially in cultures of explants from 15- and 22-d-old rats (Fig. 2*c*).

Neuronal Counts

The average number of neurons per explant is given in Table I for each of the age groups. Knowing the approximate number of explants obtained from the SCG at each age and knowing the number of neurons the SCG contains *in vivo* (10), we could calculate an estimate of the percent survival for each age (Table I). For 2-d-old rat SCG, 45 d *in vitro*, the estimated survival was 23%. For adult rat explants at 42 d *in vitro*, the survival was 26%. Possible reasons for the substantially lower survival (8%) observed for 5-d pups are given in the Discussion.

Vesicle Population

The percent of the 525 vesicles categorized as clear after 4–4.5 wk in culture is shown in Fig. 3 for each age group. The data from observers *A* and *B*, when plotted separately, show close agreement for all age groups. The vesicle population of 2-d-old rat pups was 73 and 69% clear (*A* and *B*, respectively). The comparable numbers for 5- and 10-d-old rats were 89 and 88% and 83 and 81%, respectively. The proportion of clear vesicles, however, decreased rather sharply for 15-d-old rats to 38 and 36% and dropped slightly lower to 34 and 31% for 22-d-old rats. Values for adult rat explants

were even lower, with only 14 and 10% clear vesicles, as determined by the two observers.

The percent of varicosities containing an increasing percent (in increments of 20%) of clear vesicles was determined for each observer for each age group. The average percent could then be calculated for each increment group for each rat age group. Thus, for 2-d-old rats, after 4 wk in culture, an average of 66% (17 and 16 varicosities; observers *A* and *B*) were considered to contain from 80 to 100% clear vesicles. When plotted as a bar graph (Fig. 4), the vesicle characteristics in explants from 2-, 5-, and 10-d-old rats can be seen to have a very similar pattern, that is, the majority of varicosities (66, 84, and 74%, respectively) have

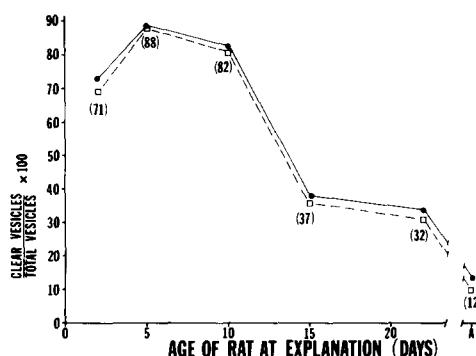


FIGURE 3 Percent of clear vesicles vs. age of rat at explantation of superior cervical ganglion. Cultures were analyzed after 4–4.5 wk *in vitro*. Vesicles in 25 varicosities from each age group were classified as clear or dense cored by two independent observers *A* (●) and *B* (□). Values for each observer are plotted separately, and the average percent is given in parentheses. Explants from adult rats (*A*) contain synapses with a low percent of clear vesicles, i.e., a predominance of dense-cored vesicles.

TABLE I
Neuronal Survival as a Function of Age of Rat at Explantation

Age of rat	Explants analyzed	Neurons per SCG <i>in vivo</i> *	Explants per SCG	Neurons/explant at explantation	In vitro age of explant	Estimated neuronal survival		
						Average No./explant ± SEM	% ± SEM	
<i>d</i>					<i>d</i>			
2	7	21,000	4	5,250	45	1,181 ± 145	23 ± 3	
5	12	21,000	4	5,250	48	435 ± 85	8 ± 2	
10	3	17,000	4	4,250	43	404 ± 144	10 ± 4	
22	9	17,000	6	2,833	48	448 ± 66	16 ± 2	
Adult	7	12,500	10	1,250	42	324 ± 55	26 ± 4	

* See reference 10.

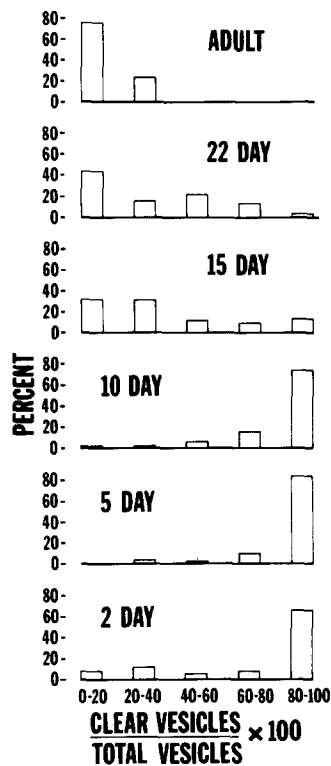


FIGURE 4 The percent of varicosities containing clear vesicles (expressed as percent of all vesicles present) is plotted for all age groups from 2-d-old pups to fully mature rats. The data from the analyses by two independent observers of vesicle-containing varicosities in cultures after 4–4.5 wk in culture were averaged.

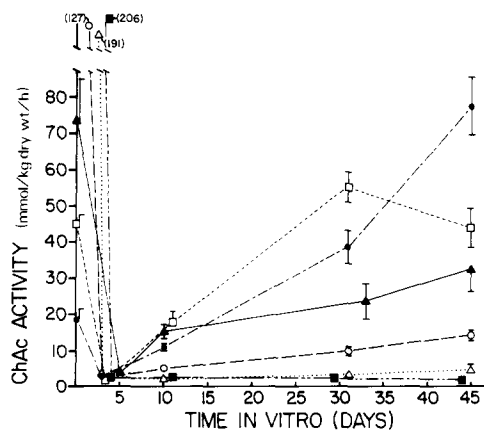
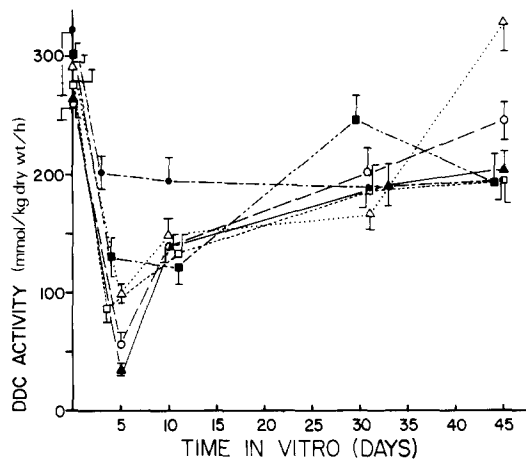
between 80 and 100% clear vesicles. Some varicosities in all three groups, however, have a low proportion of clear vesicles (i.e., a high proportion of dense-cored vesicles).

In the explants from 15- and 22-d-old rats, a distinct change was seen in the pattern of distribution of vesicle types, with an increasing number of varicosities containing 40% or less clear vesicles and a greater number of varicosities (12 and 22%, respectively) containing a mixed population of vesicles, i.e., 40–60% clear. Finally, explants from adults had no varicosity with >40% clear vesicles. Thus, the synaptic vesicle population in SCG neurons in explants taken from adult rats does not make the dramatic shift in cytochemistry that characterizes the perinatal neuron in long-term culture.

The variation in DDC activity with time in culture is given in Fig. 5 for explants from rats of different ages. DDC activities in SCG explants

from 2-, 5-, 10-, 12-, and 22-d-old pups and from adults first decreased, then leveled off or increased during the 4 wk after the decrease, beyond which time there were no significant differences among the explants. The DDC activity in explants after 30 d in culture stabilized at ~200–250 mmol per kg dry wt per h (Fig. 5), which is about 70% of the activity in adult ganglia in vivo.

As would be expected with the degeneration of preganglionic cholinergic terminals, the specific activity of ChAc in SCG explants fell during the first 3–4 d in culture to between 1 and 3 mmol per kg dry wt per h (Fig. 6). The ChAc activity subsequently increased in explants taken from rats of



FIGURES 5 and 6 Changes in DDC activity (Fig. 5) and ChAc activity (Fig. 6) in SCG explants with time in culture. Explants were obtained from 2- (●), 5- (□), 10- (▲), 15- (○), 22- (△) d and adult (■) rats. Enzyme activity is expressed relative to the dry weight of the explant ± SEM. Activity at zero time is the in vivo activity in the ganglion.

all ages, except in those cultured from adult animals. The activity in explants from adult animals after 45 d in culture was significantly less than the activity remaining 4 d after explanation. There was only a slight increase in ChAc activity in explants from 22-d-old rats.

The DDC and ChAc activities after 1 mo in vitro are shown in Fig. 7 as a function of age of animal at the time of explantation. A substantial difference in ChAc activity was observed, depending upon the in vivo age. The highest activity was present in explants from 2-d pups (62.5 mmol/kg dry wt/h), and the lowest activity was found in

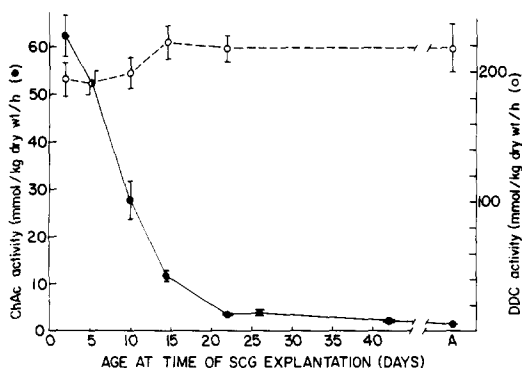


FIGURE 7 Variation with rat age in the activities of DDC and ChAc in SCG explants after 1 mo in culture. Values for 30- and 45-d points in Figs. 6 and 7 for each age were averaged to obtain as large a statistical population as possible. Values for explants from 2-, 5-, 10-, 15-, 22-, 26-, 42-d and adult rats represent averages \pm SEM of 31, 43, 22, 55, 23, 12, 21, and 15 explants, respectively.

explants from adult rats (1.3 mmol/kg dry wt/h).

The variation with rat age of DDC activity in SCG explants after 1 mo in culture differed considerably from the variation in ChAc activity in the same explants. Explants from animals aged 2 d or older showed no significant differences in DDC activity after 30 d or more in culture.

The activity of ChAc in SCG explants grown in the presence of atrium was higher than that in controls when the explants were taken from 20-d embryos and 2-d-old rats but not when taken from 16-d or 6.5-wk-old rats (Table II). Thus, although ChAc levels were higher in explants taken from perinatal animals, the levels of ChAc in explants taken from more mature animals remained low, even when grown for several weeks in the presence of heart tissue. DDC activity, however, was increased by the presence of atrium in SCG explants taken from rats of all ages. Therefore, for the two enzyme activities studied in co-cultures of atrium and SCG explants taken from 16-d and 6.5-wk-old rats, there was no change in ChAc activity and a doubling of the DDC activity.

DISCUSSION

We conclude from these results that with increasing age in vivo the SCG neuron becomes decreasingly able to assume cholinergic characteristics when it is placed in tissue culture. Data recently reported by Hill and Hendry (11) also support this conclusion. They observed that, after 14 d in culture (in the presence of NGF), whole SCG taken from 2-d-old rats demonstrated small amounts of ChAc activity, but ganglia taken from 21-d rat

TABLE II
Effect of Rat Age on the Change in ChAc and DDC Activities in SCG Explants Elicited by Co-culturing with Atrium

	Age of rat			
	20-d embryo	2 d	16 d	6.5 wk
ChAc				
SCG alone	35.0 \pm 5.0 (8)	62.4 \pm 15.0 (6)	6.85 \pm 0.9 (9)	1.75 \pm 0.42 (6)
SCG with atrium	59.1 \pm 10.0 (8)	126.0 \pm 17.0 (12)	6.43 \pm 1.1 (9)	2.24 \pm 0.76 (5)
	1.67 \times	2.02 \times^*	NS	(1.28 \times) NS
DDC				
SCG alone	85.9 \pm 25.7 (8)	202.0 \pm 41.0 (4)	166.0 \pm 38.0 (8)	179.0 \pm 33.0 (4)
SCG with atrium	106.1 \pm 9.97 (4)	394.0 \pm 64.0 (4)	334.0 \pm 40.0 (8)	355.0 \pm 82.0 (4)
	1.24 \times	1.95 \times^*	2.01 \times^*	1.98 \times

Values are averages (mmol/kg dry wt/h) \pm SEM. Number of explants is given in parentheses.

* $P \leq 0.05$.

\times , increase in enzyme activity with atrium.

NS, no significant change.

pups did not. Because whole ganglia were cultured in both cases, and because the ganglion from a 3-wk-old rat is several times as large as that from a 2-d-old rat, much more central necrosis can be expected in cultures from older rats. Because these assays were performed on a per ganglion basis (rather than per unit weight or per neuron) lessened neuronal survival might explain the decreased ChAc activity in the more mature ganglia. Concern over this variable led to our extensive efforts in this study to tabulate neuronal survival. Hill and Hendry (11) felt that their observations could be explained by the presence of undifferentiated neurons in the neonatal ganglion, which were influenced by culture conditions to express cholinergic properties; we have, in the first paper in this series, reviewed data indicating that the neonatal SCG is unlikely to contain neurons failing to express either adrenergic or cholinergic function (14).

We will here present arguments that this decrease in inducibility with age occurs within each individual neuron and does not represent selection, in certain of our cultures, of neurons with cholinergic characteristics. We will also address the question of whether the decreasing ability of the adult neuron to respond to culture conditions by expressing cholinergic characteristics is absolute or partial.

Consideration of Alternative Interpretations

Although this study leads us to conclude that the degree of neurotransmitter plasticity in SCG neurons is age dependent, several alternative interpretations of the results should be considered. These include: (a) a decreased neuronal survival in explants from adult because of the poor adaptability of mature neurons to culture conditions (Thus, the lower absolute neuronal numbers in the explants would explain the low ChAc activity observed.); (b) A selective proliferation of neurons capable of becoming cholinergic in the explants from young rats but not in explants from adult rats; and (c) a selective survival of a neuronal population with cholinergic capabilities in the explants from young but not from adult rats.

The first of these explanations, excessive neuronal loss, is unlikely because direct neuron counts (Table I) show similar survival in both the 2-d-old and adult groups. Although the average number of neurons in each explant from 2-d-old rats was greater than that found in explants from adult rats,

this does not account for the 50-fold difference in ChAc levels (see Table I).

Selective proliferation of cholinergic neurons also seems unlikely in that mitotic activity has virtually ceased at the time of explantation, even in the youngest explants (9). Moreover, in cultures of dissociated SCG, mitosis has not been observed in the neuronal population (5, 13), nor do the neurons show [³H]thymidine incorporation (22). Neuronal numbers in fact remain constant over time in culture (5, 13, 18).

The selective survival of neurons with cholinergic properties also seems untenable. The ChAc activity after 3–4 d in culture is uniformly low in explants from all age groups. Thus, no population of neurons synthesizing ChAc is present in neonatal SCG that is not present in the adult. Also, cytochemical studies on the dissociated SCG neuron have shown that a uniform population of adrenergic neurons is present early in cultures prepared either from neonatal (13, 14) or from adult rats.¹ A number of observers have noted a close correlation between cholinergic neurotransmitter enzyme activity and synaptic vesicle cytochemistry (13, 14, 17). Similarly, the predominantly dense-cored vesicles seen in cultures of adult neurons, even after extended periods in vitro, would correlate with the low ChAc enzyme activity. We would then argue that, in all age groups, the neurons are initially adrenergic, and that only neurons in explants taken from young rats undergo a significant shift to cholinergic function.

Neuronal Survival

Somewhat unexpected was the low (23%) survival for the neonatal neurons when cultured as explants. Although this represents an increase over the ~10–15% survival reported for mechanically dissociated SCG neurons (4, 29), it is not substantially better. We have observed that, of the culture methods used for perinatal SCG, trypsin dissociation gives the highest survival (often 50%, and as high as 70%) of the 21,000 neurons present in the neonatal SCG. Dissociated SCG neurons also have a target inasmuch as they make synaptic contacts among themselves, whereas few synapses are observed in an explant (27). Explantation might be expected to be less traumatic than dissociation for the individual neurons, but it has the possible disadvantage of decreased nutrient avail-

¹ Johnson, M. I. Unpublished observations.

ability, particularly if the explant is thick. The lower neuronal survival in explants from the 5-d-old and 22-d-old rats may indicate the increased susceptibility of these neurons at a time during which target tissue is contacted and normal neuronal loss occurs (10). Studies of *in vivo* systems indicate that lack of access to target tissue during this period of normal neuronal loss results in accentuated reduction of the neuronal population (see pp. 281–285 of reference 12 for a review).

Correlation of Morphology and Biochemistry

The pattern of change with rat age in the proportion of clear vesicles in varicosities (Fig. 3) corresponds to the pattern of change in ChAc activity developed by explants (Fig. 7). The highest proportions of clear vesicles and the highest ChAc activities were observed in explants from 2-, 5-, and 10-d-old rats. A marked decrease in both occurred in explants from 15- and 22-d-old rats. The lowest proportion of clear vesicles and the lowest ChAc activity were in explants from adult animals. The pattern of change in ChAc activity between explants from rats of different ages resembles the curve in Fig. 3 even more closely when the enzyme activity is expressed on a per neuron basis. From the average number of neurons per explant (Table I), we calculated ChAc activities to be 1.49, 1.79, 1.03, 0.12, and 0.06 pmol ACh synthesized per neuronal soma per h in explants from 2-d, 5-d, 10-d, 22-d, and adult rats, respectively. The highest ChAc activity, expressed on a per neuron basis, was in explants from 5-d-old rats, which corresponds to the highest percent clear vesicle composition (Fig. 3).

The calculated ChAc activity per neuronal soma (about 1.5 pmol per neuron per h at perinatal age) in explants correlates well with the activity per entire neuron in dissociated cultures (about 9 pmol per neuron per h (14)). The activity of ChAc/neuron in dissociated cultures is higher, representing the additional enzyme contained in processes and terminals not present in explant samples, which contain predominantly somata.

Influences on Transmitter "Shift"

The co-culture of heart muscle and explants of the SCG was designed to give SCG explants exposure to factors known to promote the development of cholinergic mechanisms. Conditioned medium taken from cultures of a variety of nonneuronal cells will induce dose-dependent acetylcho-

line synthesis, and heart muscle is one of the more potent sources (29). Our findings from the explants taken from perinatal rats confirm these observations. Stimulation of ChAc activity, however, was not achieved by the co-culture of heart muscle with SCG explants from 16-d or 6.5-wk-old rats. Thus, even under conditions that increased the ChAc activity twofold in neurons from perinatal animals, the neurons from adult animals showed little response.

Both *in vivo* and *in vitro* studies suggest that the depolarization provided by preganglionic input from the spinal cord may promote the acquisition and retention of adrenergic properties in developing SCG neurons (1, 38). In our study, however, explants taken from adult rats remained adrenergic even though preganglionic input was removed and culture conditions that promote cholinergic function were established. It could be argued that neurons in the explants from the older rats are not accessible to the signals in the culture medium, but the stimulation of DDC activity in all heart-SCG explants (Table II) counters this argument.

The cytochemistry of vesicle-containing profiles in explants taken from 2-, 5-, or 10-d-old rats and cultured for 4 wk most closely resembles the pattern for perinatal dissociated neurons after 8 wk in culture (13, 14), possibly indicating a faster rate of shift in synaptic vesicle cytochemistry in the explants as compared with networks of neurons. The explants from 15- and 22-d-old animals show a scattered pattern, with some varicosities containing predominantly clear vesicles, others containing predominantly dense cored vesicles, and a significantly number showing a mixture. This pattern most resembles that seen in the dissociated neurons after 3–5 wk in culture. We interpret this to mean that some neurons in 2- and 3-wk-old rat SCG are no longer capable of shifting transmitters and, therefore, have a low proportion of clear vesicles, whereas others show a shift with a number of varicosities exhibiting >60% clear synaptic vesicles. Those with a mixture of vesicle types, therefore, might represent neurons capable of expressing both cholinergic and adrenergic mechanisms, i.e., those dual in function. The question remains, however, whether these explants, if allowed to mature in culture longer than 4–4.5 wk, might show an increase in the number of varicosities with predominantly clear vesicles. The dual-function perinatal neurons identified physiologically (8) were studied at relatively early times in culture, and it was not determined whether these neurons

would eventually complete the shift and express only cholinergic function.

The pattern in synaptic varicosities in explants from the adult animals after 4 wk in vitro, (i.e., a pattern of predominantly dense-cored synaptic vesicles) resembles the pattern in dissociated neurons from perinatal animals at 1 wk in culture (13, 14). Correlation with low ChAc activity indicates that in neither case is there substantial cholinergic function. This would argue that the pattern for 15- and 22-d-old pups may be more a function of animal age at the time of explantation than of time in culture. Explants from adult animals cultured for more than 4 wk in vitro are currently under study to determine whether a more prolonged exposure to these culture conditions will produce a shift even in the adult explants.

Implication for Development

This study demonstrates that SCG neurons taken from rats of various ages and grown as explants are, with increasing age of the rat from which they are taken, progressively less able to assume cholinergic characteristics than are SCG neurons removed from perinatal rats. Explants from adult rats have a synaptic vesicle population that is strikingly dense-cored, and, although they have measurable ChAc activity, it is only 1/60 of that in explants from 2-d-old pups after comparable time in culture. Furthermore, this progressive decline in the acquisition of cholinergic function, as Figs. 3, 4, and 7 all illustrate, occurs rather rapidly in the first several weeks after birth. This is the same period of time during which innervation of target organs by the rat SCG is proceeding (see p. 1412 of reference 2 for a review). Iris innervation begins 2-3 d postnatally, and mature innervation is not present until 3-4 wk after birth. Similarly, mature adrenergic innervation in the pineal body is observed 2-3 wk postnatally. It has also been observed that the longer dissociated SCG neurons are kept in culture under conditions that maintain adrenergic function, the less capable they are of assuming cholinergic function (30).

The evidence from experimental embryology also indicates that the target organ may play a major role in the determination of the appropriate neurotransmitter. LeDouarin and Teillet (19) found that when neural crest anlagen normally destined to provide sympathetic neurons are transplanted more rostrally, they may be induced by exposure to the altered environment to provide

parasympathetic neurons. Conversely, the neural crest anlagen normally forming parasympathetic neurons may be induced to form cells of the sympathetic chain and adrenal medulla if transplanted to the midtrunk region. A biological marker made it possible to follow the migrating cells and to identify the neurons at their destination. When the migratory pathway was bypassed by direct transplantation of potentially sympathetic neurons to the gut, a shift to cholinergic function was again observed. These workers concluded that these neurons became parasympathetic and, therefore, that the differentiation of the autonomic neuroblasts may be determined by the environment of the neurons at the end of their migration.

These observations from experimental embryology and the results from tissue culture studies on the SCG neuron led us to propose a two-step hypothesis to explain certain aspects of the development of the autonomic neuron (2). According to this hypothesis, the first stage of development includes the appropriate migration and final positioning of the neuron. Only after assuming its permanent position does the neuron make a final choice of neurotransmitter. During this second stage the environment plays an important role by interacting with the neuron, presumably via its growing axon. This interaction may influence the developing neuron over an extended period. Thus, ciliary ganglion neurons that have completed migration and have already formed the ganglion display adrenergic characteristics if transplanted into younger embryos in which they undertake a second migration (20). These studies were performed on early embryos; the data presented in this paper indicate that the period of neurotransmitter plasticity continues for several weeks postnatally in the rat. The results from explants from adult rats indicate, however, that this capability may not be retained indefinitely.

Can Dissociated Adult Neurons Develop Some Cholinergic Function?

The question remains, however, whether, under certain conditions, such as dissociated cell culture, adult adrenergic neurons can be induced to express some cholinergic characteristics. Could the low but detectable activity of ChAc in adult explants mean that some ability to develop or maintain cholinergic mechanisms remains indefinitely? Was the ChAc activity detectable in adult explants

present only in a few cholinergic neurons (a question discussed above), or did all the neurons retain some cholinergic characteristics? Techniques have recently been developed that use sequential and prolonged enzyme incubations to achieve dissociation of neurons from the SCG of 250–350-g rats. This allows the use of intracellular electrophysiological recordings, perhaps the most sensitive method of detecting cholinergic function. In addition, these dissociated neurons from older rats can be co-cultured with skeletal muscle, a potent inducer of cholinergic mechanisms (30). Our most recent studies of dissociated adult neurons have produced evidence that SCG neurons from even 10- and 12.5-wk-old rats (average weights, 316 and 326 g, respectively) are capable of forming hexamethonium-sensitive synaptic contacts among themselves and curare-sensitive junctions with skeletal muscle (35, 36, 37). Inasmuch as cytochemical study of these cultures reveals a mixed population of synaptic vesicles, the question of whether these neurons retain both cholinergic and adrenergic functions and, thus, demonstrate dual function arises. Alternatively, there still may be an age beyond which the neurotransmitter shift cannot occur, but the extended period of plasticity may be even longer than is suggested by the explant studies. Current studies are directed toward determining whether the cultured adult SCG neuron becomes cholinergic or “dual function” and whether SCG neurons from rats older than those studied so far are incapable of the neurotransmitter shift under any circumstances. Whatever the results of further studies in the dissociated system may be, there is now clear evidence that the environment is a major influence in neurotransmitter selection in the postmitotic sympathetic neuron.

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