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

I. Correlative Changes in Choline Acetyltransferase and Synaptic Vesicle Cytochemistry

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ABSTRACT

Under certain culture conditions, neonatal rat superior cervical ganglion neurons display not only a number of expected adrenergic characteristics but, paradoxically, also certain cholinergic functions such as the development of hexamethonium-sensitive synaptic contacts and accumulation of choline acetyltransferase (ChAc). The purpose of this study was to determine whether the entire population of cultured neurons was aquiring cholinergic capabilities, or whether this phenomenon was restricted to a subpopulation. After 1–6 and 8 wk in culture, neurons were fixed in $KMnO_4$ after incubation in norepinephrine and prepared for electron microscope analysis of synaptic vesicle content to determine whether vesicles were dense cored or clear. ChAc, acetylcholinesterase (AChE), and DOPA-decarboxylase (DDC) activities were assayed in sister cultures.

In the period from 1 to 8 wk in culture, the average ChAc activity per neuron increased 1,100-fold, and the DDC and AChE activities increased 20- and 30-fold, respectively. After 1 wk in culture, 48 of 50 synaptic boutons contained predominantly dense-cored vesicles, but by 8 wk the synaptic vesicle population was predominantly of the clear type. At intermediate times, the vesicle population in many boutons was mixed. The morphology of the synaptic contacts on neuronal surfaces was that characteristic of autonomic systems, with no definite clustering of the vesicles adjacent to the area of contact. Increased vesicle size correlated with increasing age in culture and the presence of a dense core.

Considering these data along with available physiological studies, we conclude that these cultures contain one population of neurons that is initially adrenergic. Over time, under conditions of this culture system, this population develops

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cholinergic mechanisms. That a neuron may, at a given time, express both cholinergic and adrenergic mechanisms is suggested by the approximately equal numbers of clear and dense-cored vesicles in the boutons found at the intermediate times.

KEY WORDS sympathetic neuron · choline acetyltransferase · synaptic vesicle · neurotransmitter · tissue culture

Since the description of a method for culturing dissociated principal neurons from the superior cervical ganglion (SCG) of neonatal rats (2), anatomical, biochemical, and physiological studies of such preparations have provided both expected and unexpected results. Expected were studies demonstrating certain adrenergic characteristics. Early biochemical investigations showed that synthesis and accumulation of catecholamines occurred in a pattern similar to that observed in vivo (27, 28, 29). In addition, these dissociated SCG neurons were not only able to take up exogenous norepinephrine but could also be induced by depolarization to release the transmitter in a Ca^{+}-dependent fashion (5, 39). Early anatomical evidence indicated, as was expected for adrenergic neurons, the presence of small, dense-cored vesicles in both varicosities and endings in contact with neuronal soma. These could be demonstrated by aldehyde fixation after 5-hydroxydopamine (5-OHDA) incubation or by $KMnO_4$ fixation after norepinephrine (NE) incubation (10, 18, 23, 41). Unexpected, however, was the finding that the contacts between the SCG neurons were not only excitatory but also cholinergic (22, 33, 34, 35, 52). Depending on culture conditions, neurons in comparable cultures were able to synthesize and accumulate not only catecholamines but also acetylcholine as well as show increasing ChAc activity (18, 36, 37, 38, 44). In addition, cholinergic synapses were demonstrated between SCG neurons and both skeletal and cardiac muscle (14, 32, 53).

Although an explanation for such paradoxical findings might be the selection of the small (5%) subpopulation of cholinergic neurons thought to be present in the SCG (56), the stable neuronal number beginning early in vitro (8, 18) argued against the loss of an adrenergic population. In addition, the synaptic vesicle population was noted to be dense cored in 1-wk-old cultures and was found to be predominantly clear at 8 wk (18). It could thus be argued that one population of neurons was present that displayed uniformly adrenergic cytochemical characteristics during the first week in culture, with a subsequent shift to cholinergic properties. Biochemical evidence of an increase in ChAc activity and physiological evidence for the development of cholinergic interactions over the same time period in culture argued strongly for a single "shifting" population (18). These data did not rule out, however, the presence of an unexpressed multipotential precursor population that matured over time. Therefore, a detailed analysis of the vesicle-containing boutons present at intermediate times in culture was undertaken. Boutons containing a mixed population of clear and dense-cored vesicles at intermediate times would argue strongly for an initial adrenergic population that was in the process of becoming uniformly cholinergic. An alternative approach, the study of the morphological, biochemical, and physiological properties of individual neurons in minicultures, has been reported (14, 23, 42).

MATERIALS AND METHODS

Culture Procedures

Dissociated SCG neurons were obtained from perinatal rats and established in culture by the method described by Bray (2). Mechanically dissociated neurons were plated onto heat-molded and collagen-coated Aclar (Corning Glass Works, Science Products Div., Corning, N. Y.) dishes placed in Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) (4). The initial medium contained 76% Leibovitz medium (L-15) (Grand Island Biological Co., Grand Island, N.Y.), 10% human placental serum (HPS), 3% 1.1 M glucose, 10% 0.15 M KCl, 0.6% Methocel (Dow 65, HG Premium 4000 CPS, Dow Chemical Co., Midland, Mich.) and 25 U/ml of nerve growth factor (NGF). The initial incubation was at 35.5°C in a humidified atmosphere without CO_2. After 2 d, the cultures were changed to a standard medium containing 62% Eagle's Minimal Essential Medium (Grand Island Biological Co., Grand Island, N. Y.) with 1% 200 mM glutamine, 24% HPS, 10% chick embryo extract (EE), 3% 1.1 M glucose and 25 U/ml of NGF prepared as described in reference 1 and assayed as described in reference 50. The cultures were maintained at 35.5°C in a 5% CO_2, humidified atmosphere and refed three times a week. The diameter of neurons was measured in intact cultures. Only those neurons with spherical profiles were measured (the majority of the neurons present); neurons that had flattened out on the substrate and that had irregular contours were not included.

Cytochemistry and Electron Microscopy

At weekly intervals of from 1–6 wk and at 8 wk, sister cultures were fixed by three methods. Cultures were rinsed with L-15 and incubated for 30 min at 35°C in freshly made 10^{-5} M NE or 5-

OHDA in L-15 with added ascorbate (0.2 mg/ml). pH was adjusted to 7.3. Three rinses with L-15 (35°C) were followed by either aldehyde or $KMnO_4$ fixation. The three incubation-fixation combinations were (*a*) NE–$KMnO_4$, (*b*) 5-OHDA–$KMnO_4$, and (*c*) 5-OHDA–aldehyde.

3% $KMnO_4$ in Mg^{++} Krebs-Ringer PO_4 solution (pH 7.2) was added initially at 35°C. The cultures were then transferred to 4°C for 1 h. Maleate buffer rinses (0.1 M, pH 5.2) were followed by 1% uranyl acetate in maleate buffer (pH 5.2, 4°C) for 45 min. After three rinses in maleate buffer (4°C to ambient), the cultures were dehydrated in alcohol and propylene oxide. After 3–4 h in 1:1 propylene oxide:Epon-Araldite (Electron Microscopy Sciences, Fort Washington, Pa.), the cultures were embedded in a thin layer of Epon-Araldite covering the culture dish bottom. Aldehyde fixation was begun in dilute Karnovsky's solution (20) (35°C, pH 7.3) for 30 min with transfer to full-strength Karnovsky's solution at room temperature for 3 h. After three rinses in 0.1 M cacodylate buffer (pH 7.3), the cultures were fixed further in 2% OsO_4 in 0.1 M cacodylate buffer (7.3) for 45 min. Both the aldehyde and osmium fixatives contained 0.05% $CaCl_2$. After OsO_4 fixation and three rinses in maleate buffer (pH 5.2), the cultures were stained *en bloc* in uranyl acetate, dehydrated, and embedded as described above.

Three or more separate cultures were fixed at each time period. Fields including several clusters of 2–6 neurons and bridging bundles of neurites were chosen with the aid of a light microscope and marked with a diamond scorer. Blocks were mounted in a way that allowed sectioning parallel to the collagen, and semithin sections (.5–1.0 μm) were taken until the level containing the neurites and cells was just entered. Thin sections (60–70 nm) were then taken at 1-μm intervals for 10–12 μm. Aldehyde-fixed material was stained with 1% lead citrate before electron microscopy, whereas $KMnO_4$ material received no further treatment.

The analysis of synaptic vesicle cytochemistry was carried out on the $KMnO_4$ material. Sections selected for photography were separated by a minimum of 2 μm. One group of boutons comprised vesicle-containing somal contacts (including those on proximal dendrites), and the second comprised varicosities found in the bundles of neurites between neuronal clusters. Only those boutons containing 10 or more vesicles were selected for analysis. For those boutons located on soma or dendrites, close membrane apposition but not membrane specialization was required, particularly since $KMnO_4$ shows this poorly. 25 boutons of both somal and varicosity types were photographed for each time period. A minimum of two blocks and two to three levels per block were sampled for each time period. A variance-component analysis was performed with data from two culture dishes fixed at the same time. The variance component associated with the dish was found to be negligible, justifying treating each site within the dish as an independent sample. At a given level, consecutive boutons were photographed without selection, except as noted above. All negatives were printed at a standard magnification of 2.5 to give a final magnification of 102,150.

Classification and Statistical Analysis of Vesicle Type

To evaluate the types of vesicles present in the boutons, the prints were coded, and the vesicles in each of 350 boutons (175 somal contacts and 175 varicosities) were classified independently by three observers as dense cored, clear, or indeterminate. Only those vesicles between 40 and 70 nm in diameter with a unit membrane were included.

The data were analyzed using standard statistical routines. An analysis of variance was made that treated the observers as a repeated-measure factor. The interactions between observer and other factors were found to be negligible, which justified using means calculated from all of the observers in subsequent analyses.

Analysis of variance was used to compare the percent of clear vesicles over time in vitro and over bouton type, i.e., somal vs. varicosity. Duncan's multiple-range test was used to test for significant differences in the percent of vesicles judged to be clear in pairs of weeks. Student's *t* test was used to compare percent judged clear with bouton type at each of the seven times.

Vesicle Size

Twelve somal boutons, at 1 and 8 wk in culture, were randomly chosen for analysis of vesicle size. All were fixed in $KMnO_4$ after incubation in NE. Prints were made at an approximate magnification of 300,000, with exact magnifications being determined for use in the PDP-12 computer (Digital Equipment Corp., Maynard, Mass.) described below. The magnification of the electron microscope was checked periodically and found to change <0.3% at the magnification used. All vesicles studied at 1 wk were dense cored. Those studied at 8 wk included a smaller group of vesicles containing dense cores sharing the same boutons as the clear vesicles. Only those measuring 40–70 nm in diameter with a unit membrane were studied.

The computer system has been described in detail (11), but the essential steps may be summarized as follows. A print and its exact magnification is identified to the computer. The print itself is placed on a Graf/Pen data tablet, and vesicles meeting the criteria described above are traced on the outer leaflet of the unit membrane with a stylus. As it travels around the circumference of the vesicles, the stylus emits a high frequency acoustic wave. The *X* and *Y* coordinates of the stylus are sampled at intervals by strip microphones on the left and upper edges of the tablet. For each of the vesicles of a given boutons, the computer calculates the perimeter, area, and equivalent diameter (i.e., the diameter of a circle of equal area). For a given class of vesicles, i.e., 8-wk clear vesicles, the computer gave the mean, variance, and standard deviation for perimeter, area, and equivalent diameter. With relative ease, data could thus be generated to study not only 1-wk vs. 8-wk vesicles, but also 1-wk dense-cored vesicles vs. 8-wk dense-cored vesicles or 8-wk dense-cored vesicles vs. 8-wk clear vesicles. The numbers of vesicles studied were 345, 357, and 85 for 1-wk dense-cored, 8-wk clear, and 8-wk dense-cored vesicles, respectively. A *t* value was calculated to test the statistical difference between various groups using the equation

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(VAR_1)(n_1 - 1) + (VAR_2)(n_2 - 1)}\ \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where $\bar{X}$ is the average, VAR is variance, and n is the number of vesicles in the sample.

Enzyme Methods

At approximately weekly intervals, cultures from the group studied morphologically were stopped for biochemical analysis. The enzymes for which sufficiently sensitive assays were available for use with small amounts of tissue included ChAc, AChE, and DDC. After the number of neurons was counted, each dish was rinsed several times in L-15 medium, excess medium was blotted, and the culture, including collagen substrate, was scraped into the center of the dish and quickly frozen on dry ice. After freeze-

drying at −40°C for 3 d (26), cultures were homogenized in 100 μl of 50 mM sodium phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin and 0.1% Triton X-100. Homogenates were stored at −80°C and aliquots were removed for enzymatic analysis.

ChAc activity was assayed by a modification (45, 46) of the methods of McCaman and Hunt (30) and Fonnum (13). DDC activity was assayed by a slight modification (the modification consisting primarily in the use of smaller volumes) of the method of McCaman et al. (31). AChE activity was determined by the method of Ross et al. (45). Enzyme activities determined from each culture homogenate are expressed relative to the number of neurons in that culture.

Although DDC (also known as L-aromatic amino acid decarboxylase) activity is found in many tissues other than adrenergic neurons, the enzyme was considered to be present only in neurons in this culture system. The cultures described here consisted of sympathetic neurons, Schwann cells, and fibroblasts. We have found that virtually no DDC activity could be detected in cultures of pure Schwann cells and fibroblasts.[1] Almost all of the DDC activity in the SCG in vivo also appears to be neuronal (16, 21). Therefore, the decarboxylase activity contributed by supporting cells in these cultures was insignificant.

RESULTS

General Description of Cultures

Within 24 h after dissociation, the neurons had already shown the outgrowth of neurites and the early formation of networks of processes between single or small clusters (3–6) of neurons. The number of neurons was stable after several days, the average being 2,160 ± 92 (SEM) in 31 cultures observed 7–53 d in vitro. No mitotic figures were observed in the neuronal population (18). Moreover, the small, intensely fluorescent interneuron of the SCG (see reference 12 for review) did not survive under these culture conditions (18, 41).

The neurons matured over several months, increasing in size from 22 ± 0.77 μm (SEM) at 1 wk in vitro to 49 ± 2.1 μm (SEM) at 8 wk, and the nuclei assumed a more central position. On the somal surface of the neurons, including proximal dendrites, vesicle-containing boutons (Figs. 2 *a–d* and 5 *a* and *b*) could be found. The endings were not found preferentially on either the collagen substrate side of the neuron or on the surface exposed to the medium. The neuritic bundles between neurons became more prominent with increasing age in culture and contained many processes along which were observed intermittent swellings or varicosities containing vesicles, mitochondria, and lysosomes. It was in such bundles that the varicosities were photographed.

Under the culture conditions used in this study, the nonneuronal cells, both fibroblasts and Schwann cells, survived and increased in number over the 8-wk period, never forming, however, a confluent layer over the culture dish.

Development of Enzyme Activity

Groups of cultures from the series that was studied morphologically and physiologically (see reference 18) were homogenized at approximately weekly intervals and assayed for ChAc, DDC, and AChE activities (Fig. 1). Between 7 and 53 d in vitro, average enzyme activity in picomoles per neuron per hour (± SEM) increased from 0.0083 ± 0.0009 to 9.17 for ChAc, from 0.77 ± 0.04 to 14.7 for DDC, and from 25 ± 2.9 to 763.0 for AChE. Measurement of ChAc in two other experiments showed a similar pattern of development in enzyme activity, despite the differential neuronal densities present.[1]

Vesicle Data

The number of vesicles counted in 25 somal boutons averaged 1,179, 1,045, 1,070, 1,344, 1,263, 1,691, and 1,458 for 1–6 and 8 wk, respectively. The average number of vesicles per bouton, therefore, was 47, 42, 43, 54, 50, 68, and 58 for the respective time periods. The comparable numbers for 25 varicosities for the respective times studied were 577, 711, 681, 969, 846, 877, and 911 vesicles,

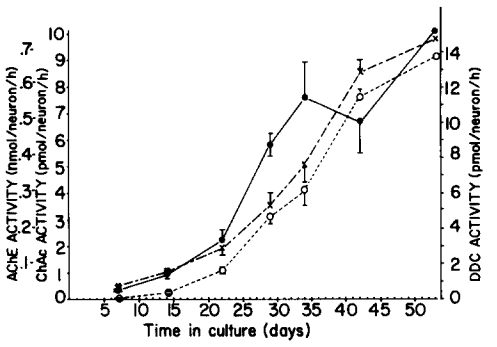

FIGURE 1 Increase in ChAc, AChE, and DDC activities in dissociated sympathetic neurons. Enzyme activities for all three were determined for each culture, and the average activity per neuron was calculated by dividing by the number of neurons in the respective cultures. The values for the time points between 7 and 53 d are means ± SEM of activities in 8, 8, 3, 3, 3, 4, and 2 cultures, respectively. The average number of neurons per culture was 2,160 ± 92 (n = 31). ChAc activities at 7 and 14 d were 0.0083 ± .009 and 0.239 ± .009 pmol/neuron/h, respectively. ChAc (○); AChE (●); DDC (×).

[1] Ross, C. D. Unpublished observations.

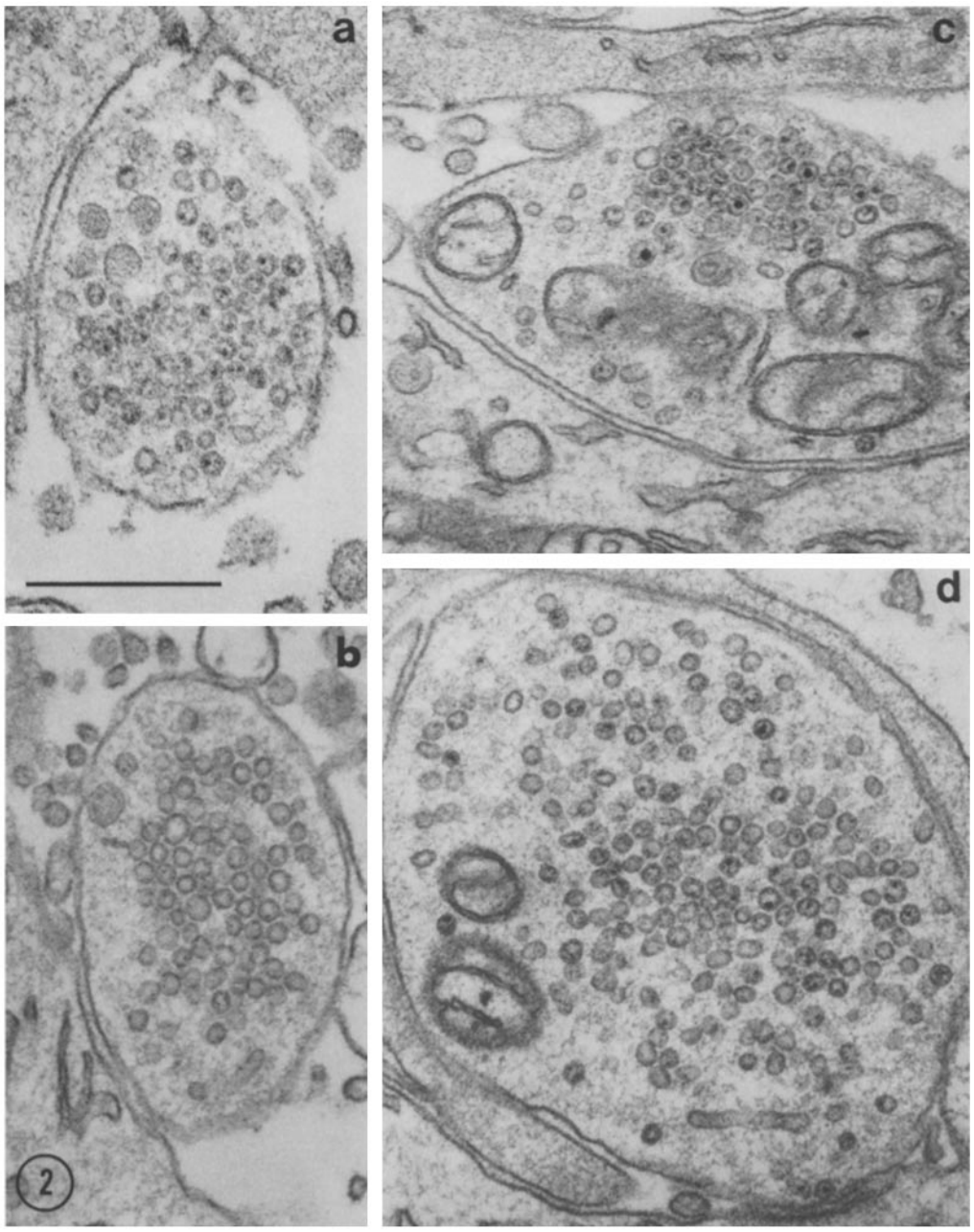

FIGURE 2 Electron micrographs of vesicle-containing boutons found on neuronal soma in cultures of dissociated superior cervical ganglia from perinatal rats. The specimens were fixed in $KMnO_4$ after incubation in norepinephrine. (*a*) Example of typical boutons found after 1 wk in vitro. The majority of vesicles are dense cored. (*b*) Example of the predominant bouton type after 8 wk in culture. The majority of vesicles have clear centers. (*c*) One of a few boutons containing predominantly dense-cored vesicles even after 8 wk in vitro. (*d*) A bouton containing a mixture of vesicle types (clear and dense-cored) found at an intermediate time (6 wk in vitro). (*a–d*) Bar, 0.5 μm. × 54,500.

with 23, 28, 27, 39, 34, 35, and 36 vesicles per bouton.

Examples of the vesicle-containing boutons found on neuronal soma or proximal dendrites are shown in Fig. 2. The majority of vesicles in all boutons found in cultures at 1 wk in vitro were dense cored (Fig. 2*a*). Boutons found after 8 wk in culture, in contrast, contained predominantly clear vesicles (Fig. 2*b*), although a few endings contained >20% dense-cored vesicles (Fig. 2*c*). At intermediate times in culture, boutons contained a mixture of both types of vesicles (Fig. 2*d*). The varicosities showed a similar shift in vesicle type.

Using data from all three observers to obtain an average, the percent of vesicles considered clear in somal boutons after 1 wk in culture was 6.5 (Fig. 3). By 2 wk in culture, this value had increased to 34.9%. At intermediate times, that is, from 3 and 5 wk, the percent of vesicles considered clear was between 40 and 60, whereas, after 6 wk, >60% were considered clear. A similar pattern was found for the vesicles in the varicosities: 6.9% were clear at 1 wk in culture, 39.3% at 2 wk, and >60% in this group were clear by 5 wk, (in contrast to 6 wk for somal boutons).

Fig. 4 shows the percent of the somal boutons found to contain clear vesicles at each time in culture. After 1 wk in culture, 97% of the boutons contained 20% clear vesicles or less. 3% of the boutons contained >20 but <40% clear vesicles; no somal bouton contained >40% clear vesicles at 1 wk. In contrast, at 8 wk, 60% of the boutons contained >80% clear vesicles, 16% contained <40% clear vesicles, and 24% contained 40–80% clear vesicles. The most striking change occurred between 1 and 2 wk. At 2 wk, the percent of boutons containing 20% clear vesicles or less had decreased to 45% (from the 97% at 1 wk); 13% of the boutons already had >80% clear vesicles (compared to none at 1 wk; and 42% had a clear vesicle population of between 20 and 80%. It was thus established that as early as after 2 wk in culture a shift occurred toward boutons containing a mixture of vesicle types.

The comparable data for the varicosities given in Fig. 4 show a similar pattern, with 95% of the boutons containing <20% at 1 wk and 55% of the varicosities having >80% clear vesicles by 8 wk. The distribution of boutons at intermediate times again shows an early shift toward an increasing percent of clear vesicles by 2 wk, with an increasing proportion of the boutons containing >80% clear vesicles occurring between 2 and 6 wk in culture. Unlike the somal boutons, however, 3% of the varicosities contained >40% clear vesicles after 1 wk in culture.

Statistical Analysis of Vesicle Counts

Results of the Duncan multiple-range test comparing the percent clear vesicles at various times in culture are given for both somal boutons and varicosities in Table I. For both groups, the percent clear vesicles at 1 wk was significantly different from that at any of the following weeks. Week 2

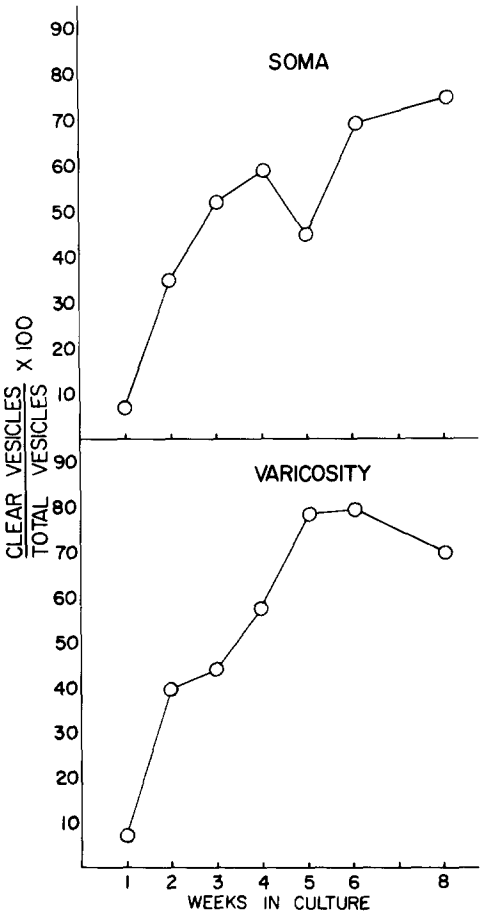

FIGURE 3 Percent of total vesicles considered to be clear after 1–6 and 8 wk in culture. Vesicle-containing boutons found either on somal surfaces (*SOMA*) or in bundles of neurites (*VARICOSITY*) were analyzed for vesicle type. Each point represents 25 boutons containing a total of 500–1,700 vesicles, depending on the length of time in culture. The percent is the average obtained from three independent observers.

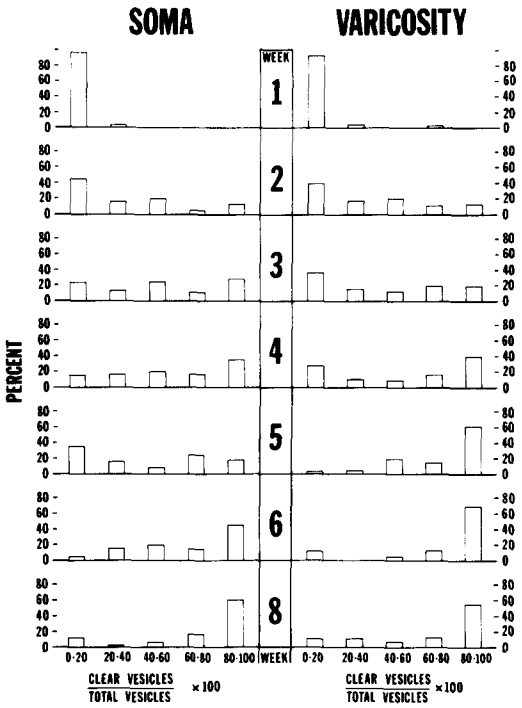

FIGURE 4 Bar graph showing the percent of boutons containing differing percents of clear vesicles at each time point for somal boutons (*SOMA*) and for varicosities found in neuritic bundles (*VARICOSITY*). After 1 wk, for both bouton types, >90% of the boutons contained <20% clear vesicles, i.e., a high percent of dense-cored vesicles. In the succeeding weeks, and after as early as 2 wk, a shift toward an increasing percent of clear vesicles is seen for both bouton types, with a significant number of boutons containing a mixture of vesicles, i.e., between 20 and 80% were clear. After 8 wk in culture, clear vesicles predominate in the majority of boutons.

was significantly different from week 4 in both groups, but only in the somal bouton group was it different from week 3. Starting at week 4, although the absolute percent of clear vesicles continued to increase, there was considerable overlap. Thus, for varicosities, weeks 4 and 8 are in a set and 5, 6, and 8 are in another set. In the somal bouton group, week 5 proved to be aberrant, being with week 2 in one set and weeks 3 and 4 in another. Review of the week-5 samples showed no difference in number of nonneuronal cells and/or neuronal numbers that might have explained this deviation.

Vesicle Size

The average perimeter, area, and equivalent diameter with standard deviations for vesicles analyzed after 1 and 8 wk in culture are summarized in Table II. The average diameter for 1-wk dense-cored vesicles was 49.0 ± 4.5 nm and was significantly different ($P < 0.001$) from either that for 8-wk dense-cored vesicles (53.0 ± 4.2 nm) or that for 8-wk clear vesicles (50.8 ± 5.0 nm). In addition, 8-wk dense-cored vesicles were found to be significantly larger than 8-wk clear vesicles ($P < 0.001$). Thus, not only was there an increase in vesicle size with maturation in culture, but, at 8 wk in culture, there was a small but significant difference in the size of the vesicles, depending on whether there was a dense core present.

Morphology of Vesicle Containing Profiles

No morphological differences within the neuronal soma were seen with either aldehyde or $KMnO_4$ fixation, even in cultures in which a significant shift toward cholinergic function had occurred. This observation is consistent with in vivo studies on "mixed" ganglia that contain both sympathetic and parasympathetic neurons (19, 57). The morphology of the vesicle-containing profiles was similar whether the profiles contained dense-cored or clear vesicles and was that characteristic of the autonomic nervous system (43). Illustrated in Fig. 5 *a* are consecutive and connected varicosities taken from a culture after 6 wk. They are apposed to a neuronal soma, and the vesicles show clustering but no tendency to gather adjacent to the site of presumed contact (membrane specializations are not apparent in this $KMnO_4$-fixed material). This autonomic characteristic was present not only in samples studied after 1 wk in culture, but also in those studied after 6 and 8 wk. This was also true of aldehyde-fixed material, although membrane specialization and occasional

TABLE I

Homogeneous Subsets for Percent of Clear Vesicles with Regard to Weeks In Vitro for Somal Boutons and Varicosities

Somal boutons		Varicosities	
Subset	Weeks	Subset	Weeks
A_1	8, 6	A_2	8, 6, 5
B_1	6, 4	B_2	8, 4
C_1	5, 4, 3	C_2	4, 3
D_1	5, 2	D_2	3, 2
E_1	1	E_2	1
$F = 18.39$		$F = 23.70$	
$P < 0.0001$		$P < 0.0001$	

TABLE II

Vesicle Size after 1 and 8 wk in Culture

Group	*n*	Perimeter		Area		Equivalent diameter	
		Average	SD	Average	SD	Average	SD
		nm		*nm*^{2}		*nm*	
1 wk dense-cored	345	157.5	15.0	1899.3	344.5	49.0	4.5
8 wk dense-cored	85	170.2	13.4	2222.6	351.3	53.0	4.2
8 wk clear	357	163.7	16.2	2046.8	405.9	50.8	5.0
8 wk total	442	169.2	15.7	2189.4	404.2	52.6	4.8

High-magnification electron micrographs of twelve somal boutons after 1 and 8 wk in culture were analyzed for vesicle size using a PDP-12 computer system (see Materials and Methods for detail). The Graf/Pen tablet of this system allowed the determination of the perimeter and area of each vesicle. The equivalent diameter is then calculated as the diameter of a circle of equal area.

adjacent clusters of vesicles could sometimes be demonstrated (Fig. 5 *b*). The observed similarity in morphology is consistent with in vivo studies showing that juxtaposed cholinergic and adrenergic endings in primate eccrine sweat glands have a similar morphological appearance, with the exception of the presence (in adrenergic endings) or absence (in cholinergic endings) of granules in the vesicle population after 5-OHDA loading (49).

DISCUSSION

We conclude that SCG neutrons cultured from perinatal rats are initially a uniformly adrenergic population, to which accrue cholinergic neurotransmitter properties under the culture conditions employed. Because the method of mechanical dissociation used by us and by others (7, 37) gives only a 10–15% neuronal survival, the selection of a small number (5%) of initially cholinergic neurons (56) is a possible explanation for the observed transmitter shift. Evidence from several laboratories, however, supports our conclusion that the initial population of neurons is adrenergic. We observed that early in culture the ChAc activity is very low and that synaptic vesicle cytochemistry, after NE incubation, reveals a uniform population of dense-cored vesicles. Similarly, autoradiographic studies after tritiated-NE incubation showed silver grains over every cell (51). Moreover, under culture conditions that prevent the cholinergic shift, all neurons are demonstrated to be capable of NE synthesis (42).

Another possible explanation, that a small number of cholinergic precursors proliferate to dominate the neuronal population with time in culture, is ruled out by the following observations: (*a*) essentially no neuronal mitotic activity is present in the SCG at birth or shortly thereafter, for, although NGF administered in vivo to neonatal rats increases the number of neurons per ganglion, it does so by increased survival of neurons normally destined to die and not by increasing mitosis in a precursor population (17); (*b*) neuronal division in cultures from perinatal animals is not observed (8, 18); (*c*) the neurons do not incorporate [^{3}H]thymidine (27); and (*d*) significant neuronal death is not observed after the initial few days in vitro, and stable neuronal numbers over time in culture have been well documented (8, 18).

None of these arguments, however, completely rules out the question raised in the Introduction of an initially unexpressed precursor population. Could the accrual of cholinergic characteristics be explained by the presence of potentially cholinergic neurons that remain functionally "silent" initially and begin to form synaptic profiles and produce ChAc only after several weeks in culture? We believe that this explanation is unlikely because: (*a*) our study has shown that synaptic profiles of the mixed vesicle type predominate during intermediate times in culture, in contrast to the appearance of endings with predominantly clear vesicles that would be expected if a silent population of cholinergic neurons began to function; (*b*) where careful studies exist, cholinergic innervation has been observed to precede adrenergic innervation in embryonic development (see the extensive discussion in reference 3), and, thus, cholinergic neurons would be expected to be more, rather than less, advanced in synapse-forming ability during early stages of development; (*c*) autoradiograms of the early neuronal population after tritiated-NE uptake show all neurons to be labeled (51); and (*d*) when culture conditions favor the retention of

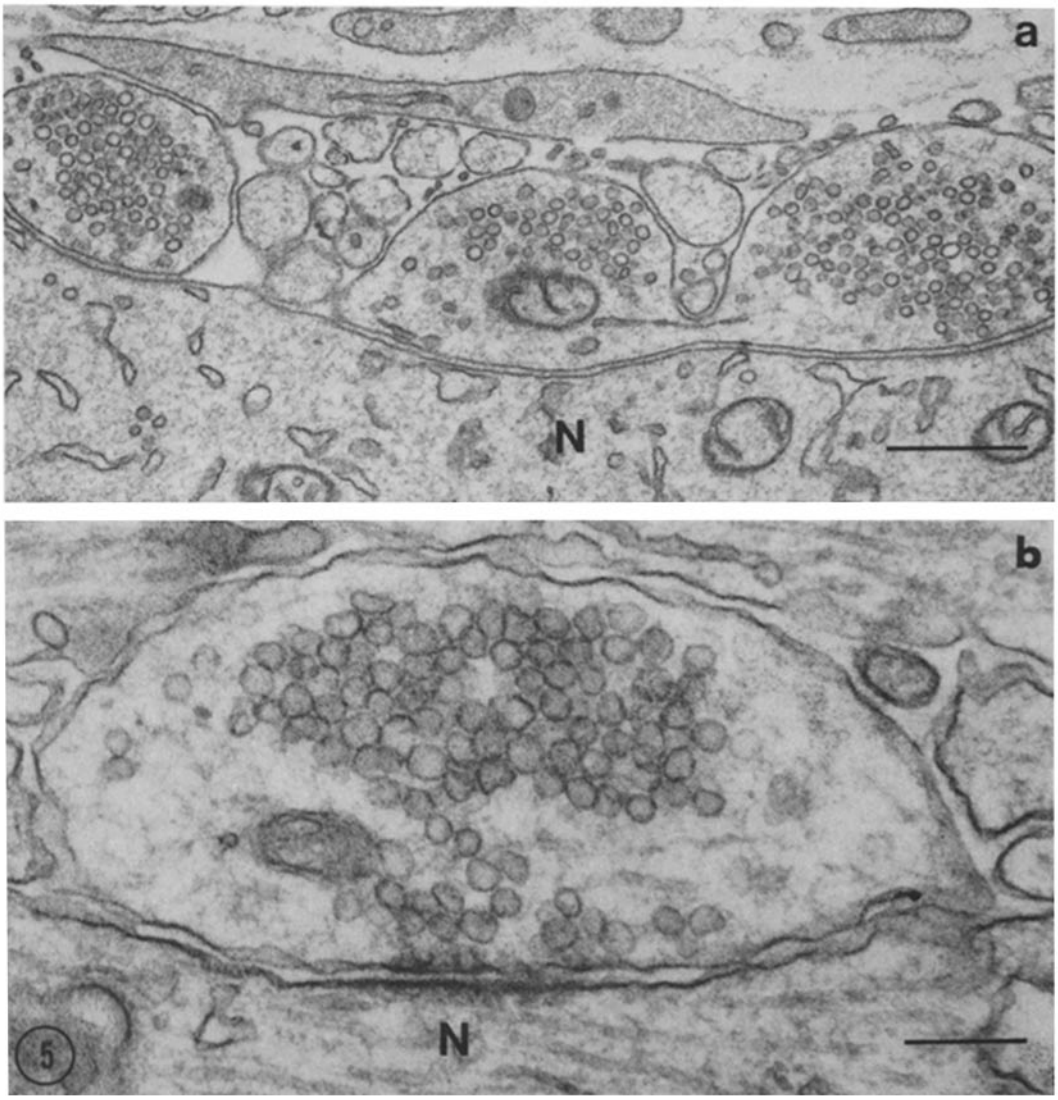

FIGURE 5 Electron micrographs illustrating the morphology of somal boutons found in cultures of dissociated superior cervical neurons from neonatal rats. (*a*) The two varicosities shown are connected, and a third was seen to be connected in an adjacent section. Although the vesicles are clustered, there is no tendency to gather near the presumed site of contact with the somal plasmalemma. In this $KMnO_4$-fixed material, no membrane specializations are seen. 6 wk in vitro. Bar, 1 μm. × 35,000. (*b*) Aldehyde fixation demonstrated that there was membrane specialization and an occasional adjacent vesicle cluster in some boutons. 2 wk in vitro. Bar, 0.2 μm. × 82,000.

adrenergic properties, cholinergic characteristics are suppressed (42, 54).

Several lines of biochemical evidence also indicate that the neurons in these cultures became cholinergic during their in vitro development. The ChAc activity per neuron, which is very low in the first few days in culture, increases substantially and after 53 d is comparable to that found in cholinergic cells such as the somata of spinal cord motor neurons (55). Using an estimated protein value of 10 ng per neuron (37), the specific activity of ChAc would be about 0.9 nmol/mg of protein/h, which is similar to that found in the uniformly cholinergic neurons of the ciliary ganglion (6). The

ratio of AChE activity to ChAc activity changed from over 3,000 at 7 d to 290 at 14 d, to ~150 at 22–34 d, and to 83 at 53 d. The lower ratio, characteristic of neurons that are cholinergic, is similar to that found in the ciliary ganglion after denervation (6) or that in central cholinergic structures such as the facial motor nucleus (15) and spinal cord motor neurons (55). Neurons were maintained in medium that contained HPS and EE, both of which stimulate, to some extent, DDC activity and, to a larger extent, ChAc activity (44). The medium also contained NGF, which has a permissive role in promoting the shift to cholinergic function (9). Supporting cells, both Schwann cells and fibroblasts, are proliferating. Studies have shown that these cells, as well as medium conditioned by them, stimulate ChAc activity (36, 37, 44).

The exact time at which the neurons begin to develop cholinergic mechanisms is not clear, but the data presented above and other studies (9, 38) point to an early event, occurring perhaps between weeks 1 and 2. The largest proportionate increase in ChAc activity with time occurs in this interval (although 90% of the absolute increase occurs after week 2). The increase in ChAc activity between days 7 and 14 was 29-fold; the increase during the remainder of the experiment, from days 14 to 53, was 39-fold. This early change corresponds to the change in composition of synaptic vesicles from a population of almost completely dense-cored vesicles at week 1 to a very mixed population of dense-cored and clear vesicles at week 2 (Figs. 3 and 4). The character of synaptic activity in this culture series has been studied electrophysiologically (18). The graph of the incidence of cholinergically driven cells with time in culture (Fig. 2 in reference 18) intersects the abscissa (0%) between 10 and 15 d in vitro. The activity of ChAc at 10–14 d in culture is ~0.1–0.2 pmol/neuron/h (Fig. 2). This low activity is similar to that found in developing ciliary ganglion at the stage in which cholinergic ganglionic transmission is first detected (6, 24). In both the in vivo ciliary ganglion and the in vitro sympathetic neurons, ChAc activity increased significantly after the onset of cholinergic transmission.

The paradox then is that these cultured neurons simultaneously demonstrate cholinergic function, with a predominantly clear vesicle population, and the ability to take up and release ^{3}H-NE (42, 51). This cannot be resolved until we know which adrenergic mechanisms continue to be expressed in these neurons. What we know now can be summarized as follows. The neurons remain autonomic in the morphology of their processes and in their synaptic structure (18, 23). In contrast to an increasing DDC activity, NE synthesis actually decreases under conditions that stimulate ACh synthesis (38). DDC is not an enzyme important in the regulation of NE synthesis, as tyrosine hydroxylase (TH) and dopamine β-hydroxylase are, and is apparently not under the same mechanism of control as the two latter enzymes (47, 48). Because TH is the rate-limiting enzyme in NE biosynthesis, it is under regulatory control, and decreasing TH activity over time could explain a decreasing NE synthesis in cultures acquiring cholinergic characteristics. Some adrenergic characteristics, however, such as DDC activity and NE uptake (5, 39, 51), may continue to be expressed because they are not under the same regulatory control as the rate of NE synthesis. Another adrenergic character, Ca^{++}-dependent NE release, is present in dissociated neurons (5, 39), but, because the dynamics of this release over time in culture have not yet been systematically studied, it is not known whether NE release continues to increase, as NE uptake does, or whether it decreases, as NE synthesis does, as the neurons acquire cholinergic characteristics.

In summary, we conclude that there may be a dissociation between the various adrenergic characteristics present in the SCG neuron to which have accrued certain cholinergic functions in culture. Such neurons present a problem in classification, that is, should they be called cholinergic or adrenergic? Some may have a dual function (14), but others that can be demonstrated to have certain cholinergic functions require further characterization of retained "adrenergic" characteristics.

In the present study in which $KMnO_4$ fixation was used for the vesicle analysis, there is no obvious difference in the morphology of vesicles with or without a dense core. There was, however, at 8 wk, a small but significant difference in vesicle size, with the dense-cored vesicle being larger. This difference cannot be ascribed to fixation artifact or small changes in the magnification of the electron microscope because the vesicles were in the same boutons and, therefore, were fixed and photographed simultaneously. A similar difference in size of vesicles with a dense core has been noted by others (40). The increase in size of vesicles

observed over time in culture was again small but statistically significant. In vivo measurements, in contrast, have shown a decrease with age in the size of synaptic vesicles in hamster cerebellum (25).

Little doubt remains that perinatal superior cervical ganglion neurons, when placed in culture, undergo a significant alteration in neurotransmitter metabolism and become cholinergic. The logical question now is whether this ability of perinatal neurons to shift their neurotransmitter is age dependent. We examine this question in the second paper in this series by biochemical and cytochemical analysis (similar to that performed in the present study) of explants of SCG taken from rats of increasing age, including fully mature rats.

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