Ontogenetic appearance and disappearance of tyrosine hydroxylase and catecholamines in the rat embryo

(neural crest/development/immunohistochemistry)

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ABSTRACT The ontogenetic pattern of noradrenergic differentiation in rat embryonic autonomic neuroblasts was defined in vivo. Noradrenergic specialization was examined by documenting the immunohistochemical appearance of tyrosine hydroxylase (Tyr-OH; tyrosine 3-monooxygenase; L-tyrosine-3-monooxygenase; EC 1.14.16.2) and the development of histochemistry due to catecholamine (CA). Tyr-OH and CA were undetectable in the dorsal neural crest or the ventrally migrating crest cells and first appeared at 12.5 days of gestation (36-37 somite stage) in sympathetic cells that had formed sympathetic ganglion primordia. Fluorescence intensity and the number of fluorescent cells increased progressively thereafter. In addition, Tyr-OH and CA transiently appeared in scattered presumptive neuroblasts in the gut. The enzyme and transmitter were first detectable at 11.5 days of gestation and thereafter decreased progressively so that, by 14.5 days, only rare cells were encountered. There was remarkable synchrony in the appearance and disappearance of Tyr-OH and CA. These observations suggest that a number of noradrenergic transmitter mechanisms develop simultaneously in the differentiating neuroblast. The relevance of these results to the elucidation of developmental regulatory mechanisms is discussed.

The neural crest and its derivatives have been used extensively to study developmental mechanisms that regulate neuronal differentiation. The neural crest arises in the edges of the neural fold during the neurula stage of embryonic development (1). As the neural plate closes to form the neural tube, the crest cells assume a dorsal midline position from which extensive migration occurs throughout the embryo (2). The crest cells migrate dorsolaterally, under the ectoderm and ventrolaterally within the mesoderm, to give rise to autonomic and sensory neurons, chromatophores, non-neural cells of the peripheral nervous system, chromaffin cells, calcitonin-producing cells, and mesenchymal derivatives of the cephalic region (3, 4). Hereafter, virtually all the work performed on neural crest has been restricted to avian and amphibian systems. The regulation of neural crest differentiation in mammalian embryos remains to be defined and the mechanisms that govern the neurotransmitter fate of embryonic autonomic neuroblasts in mammals are unclear (5, 6). Our approach to this problem involves documentation of the initial ontogenetic appearance of neurotransmitter mechanisms in presumptive autonomic neuroblasts in the embryo.

We have studied the appearance of noradrenergic characteristics in the rat embryo. Specifically, we document the appearance of tyrosine hydroxylase (Tyr-OH; EC 1.14.16.2) and of catecholamine (CA) fluorescence itself. Tyr-OH is the rate-limiting enzyme in catecholamine biosynthesis (7), is highly localized to noradrenergic neurons in postnatal sympathetic ganglia (8), and is a sensitive index of growth and differentiation of sympathetic neurons (9). The development of Tyr-OH in neurons of the postnatal rat is subject to anterograde trans-synaptic regulation (9-12), retrograde trans-synaptic regulation by target organs (13, 14), and regulation by nerve growth factor (14, 15). The factors that govern Tyr-OH development in the rat embryo are undefined. The availability of a specific antibody to Tyr-OH (16) has allowed us to define the ontogenetic appearance of this transmitter enzyme in the rat embryo. Using immunohistochemical techniques, we demonstrate that Tyr-OH is initially detectable in sympathetic primordia at 12.5 days of gestation and in scattered cells in the gut at 11.5 days of gestation. Fluorescence histochemical techniques were also used to define the developmental appearance of CA. Temporally and morphologically, the appearance of CA is virtually congruent with that of Tyr-OH. The developmental patterns of these indices of noradrenergic differentiation are described.

MATERIALS AND METHODS

Preparation of Antiserum. Tyr-OH was purified from human pheochromocytoma by a recently described procedure (16) and was used for the production of antibodies in rabbits. Approximately 0.2-0.3 mg of purified human Tyr-OH was subjected to polyacrylamide disc gel electrophoresis; 50-100 μg of protein was loaded on each gel. The protein bands were cut from the gel, extruded through a 20-gauge needle, and allowed to stand in an equal volume of 0.9% NaCl for several hours at 4°C. An equal volume of complete Freund's adjuvant was added and mixed thoroughly, and this mixture was injected into the foot pads of rabbits. The procedure was repeated every 2 or 4 weeks for 3-6 months by intramuscular injection. Control preimmune serum was obtained from each rabbit. Immunoelectrophoretic analysis of the antiserum against purified human Tyr-OH or against partially purified enzyme showed a single precipitation arc. Immunotitration revealed that antiserum to human pheochromocytoma Tyr-OH crossreacted with bovine adrenal Tyr-OH and with rat adrenal and striatal Tyr-OH.

Preparation of Embryos. Embryos of pregnant Sprague-Dawley rats were used. Embryonic age was computed from the day after mating, which was considered day 0.5 of gestation. Pregnant rats were sacrificed daily, between 10.5 and 14.5 days of gestation, by exposure to ether vapor, and the embryos were removed. The developmental stage was determined by counting somites, so that for each embryo, gestational age and somite number were defined. For routine histological staining with hematoxylin and eosin, embryos were fixed in Bouin's fluid and embedded in paraffin.

Abbreviations: Tyr-OH, tyrosine hydroxylase (EC 1.14.16.2; tyrosine 3-monooxygenase); CA, catecholamine; P1/NaCl/Triton, phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100.
Immunohistochemical Techniques. Uteri were rapidly dissected, under a dissecting microscope, in ice-cold 2% formaldehyde (freshly prepared from paraformaldehyde) in 0.05 M sodium phosphate (pH 7.4). Embryos were fixed for 24 hr at 4°C, washed for 12-24 hr in several changes of ice-cold 0.1 M sodium phosphate (pH 7.4), dehydrated in alcohol, and embedded in polyethylene glycol 1000 as described (17). Serial 10-μm sections were cut, floated either on a 5% glycerol solution or on a 0.1% gelatin solution to remove the polyethylene glycol (17), mounted on slides, and dried at 37°C.

Tyr-OH was visualized by using the indirect immunofluorescence method of Coons (18). The sections were hydrated with phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100 (P/NaCl/Triton) to decrease the nonspecific binding of antibodies (19) and were incubated for 30 min at room temperature in the specific anti-Tyr-OH antiserum diluted 1:100 with P/NaCl/Triton. After three 10-min washes in P/NaCl/Triton, the sections were incubated for 30 min in fluorescein isothiocyanate-conjugated IgG (Miles-Yeda, Ltd.) diluted 1:16, washed, and mounted in glycerin/phosphate-buffered saline, 4:1 (vol/vol).

Specificity of the immunohistochemical fluorescence was verified in all embryos by substituting preimmune serum for specific Tyr-OH antiserum.

Catecholamine Histofluorescence. Embryos were removed from the uterus in ice-cold 0.1 M sodium phosphate (pH 7.4), freeze-dried overnight after somites were counted, and proceeded for formaldehyde-induced fluorescence by minor modifications of published techniques (20). Tissues were examined with a Leitz Orthoplan fluorescence microscope.

RESULTS

Immunohistochemical Appearance of Tyr-OH in Ganglion Primordia. Early in embryonic development, neural crest cells migrate from the dorsal aspect of the neural tube to form primordia of the sympathetic ganglia. Migration takes place at the time of closure of the neural tube, which begins at 10 days of gestation in the rat embryo (21); at birth, on the 21st day, the definitive sympathetic chains are already present. To define precisely the time of appearance of the sympathetic ganglion

FIG. 1. (A) Midthoracic transverse section of the 30-somite embryo (12 days of gestation). At this stage, a few neural crest derivatives, with basophilic cytoplasm, have begun to aggregate laterally to each aortic branch (A), forming sympathetic ganglion primordia (arrows). NT, neural tube; S, somite; NC, notochord; G, lumen of gut primordium. (Hematoxylin and eosin; bar = 100 μm.) (B and C) Immunohistochemical demonstration of Tyr-OH in transverse sections. (B) At lumbar level of 27-somite embryo (11.5 days of gestation). Specific Tyr-OH fluorescence is not detectable in the dorsal embryonic structures or in neural crest cells which are migrating ventrally at this stage. A, aorta. (Bar = 100 μm.) (C) At cervical level of the 36-somite embryo (12.5 days of gestation). Sympathetic ganglion primordia, dorsolateral to each aortic branch, exhibit specific Tyr-OH immunofluorescence. Blood cells around the neural tube and sensory ganglia emit fluorescence, judged as nonspecific because it also occurred in control sections incubated with preimmune serum. (Bar = 200 μm.) (D, E, and F) High-power views of early sympathetic ganglia demonstrating Tyr-OH. (D) Sympathetic primordium from section shown in (C). Specific Tyr-OH immunofluorescence is restricted to the cytoplasm of the sympathoblasts. Large, dark nuclei are surrounded by fluorescent cytoplasm. Blood cells in the aorta exhibit nonspecific fluorescence. (Bar = 50 μm.) (E) Sympathetic ganglion from 13.5-day embryo. Sympathoblasts exhibit specific Tyr-OH fluorescence and are more numerous and densely packed than at the previous stage. Several short Tyr-OH-positive processes are visible at the lower left border of the ganglion. (Bar = 50 μm.) (F) Control section, at same anatomic level as in E, incubated with preimmune serum from the same embryo. No fluorescence is detectable. (Bar = 50 μm.)
primordia, we examined serial sections of embryos at various developmental stages after staining with hematoxylin and eosin. The primitive sympathetic ganglia were initially identifiable at the cervical and thoracic levels of 27- to 30-somite embryos (between 11.5 and 12 days of gestation). The rudimentary ganglia consisted of small aggregates of cells, containing basophilic cytoplasm, located lateral to the dorsal aorta (Fig. 1A).

To determine whether neural crest cells contained detectable Tyr-OH before and during migration, embryos were subjected to fluorescence immunohistochemical analysis at the 17- and 28-somite stages, corresponding to 10.5 and 11.5 days of gestation, respectively. In embryos of these gestational ages, no fluorescence was detectable in any of the axial or periaxial structures: the neural tube, somites, notochord, and surrounding mesenchyme were devoid of specific green fluorescence (Fig. 1B).

Tyr-OH immunofluorescence was first present in sympathetic primordia of 36- to 37-somite embryos (12.5 days of gestation), half a day after initial aggregation of the sympathoblasts themselves. Small clusters of fluorescent cells were located dorsolateral to the aorta, throughout most of the length of the embryos (Fig. 1C). The sympathoblasts, containing large round nuclei, exhibited specific fluorescence restricted to the cytoplasm (Fig. 1D). In several instances, short, Tyr-OH-positive, cytoplasmic processes were identified. The sympathoblasts were more numerous and more densely packed at the cervical and rostral thoracic levels than more caudally. Similarly, intracellular fluorescence intensity decreased in a rostrocaudal direction.

Subsequently, at 13.5 days of gestation, the number of Tyr-OH-positive cells in sympathetic ganglia increased dramatically, particularly at the cervical and thoracic levels (Fig. 1E). In contrast, lumbar ganglia contained fewer Tyr-OH-positive cells with lower intensity of fluorescence than at more rostral levels. Sympathetic cells possessed only short processes; no identifiable axon outgrowth was observed at this stage. Use of preimmune serum as a control, instead of the specific antiserum, resulted in the total absence of fluorescence in the sympathetic Anlage at all stages examined (Fig. 1F).

**DISCUSSION**

The present studies were undertaken to define the temporal pattern of neurotransmitter differentiation in mammalian embryonic neuroblasts in vivo. Noradrenergic specialization

**Ontogeny of Catecholamine Fluorescence in Ganglion Primordia.** Development of catecholamine fluorescence in sympathoblasts was virtually synchronous with that of Tyr-OH and was undetectable at the level of presumptive sympathetic ganglia in 11.5-day embryos. Moreover, the neural tube, notochord, and other dorsal mesenchymal structures were also devoid of catecholamines. Fluorescence was first detectable in the sympathetic primordia of embryos at 12.5 days of gestation (Fig. 2A), corresponding precisely to the appearance of Tyr-OH (compare Figs. 1D-F and 2). The sympathoblasts exhibited intense yellow-green fluorescence localized to a small ring of cytoplasm surrounding the large round nucleus; fluorescence was also apparent in the cytoplasmic processes (Fig. 2B). As observed with Tyr-OH, fluorescent cell number and fluorescence intensity decreased in the rostrocaudal direction.

Fluorescence intensity increased with embryonic age and was markedly increased by 13.5 days of gestation, paralleling the development of Tyr-OH. The aortic plexus was also easily identified at this stage. No fluorescence was observed in the neural tube or notochord.

**Appearance and Disappearance of Tyr-OH and CA in Cells of the Gut.** As described above, our attempts to define the earliest appearance of Tyr-OH immunoreactivity involved examination of presympathetic embryos. No fluorescence was ever observed in the dorsal embryonic structures. Surprisingly, however, Tyr-OH-positive cells transiently appeared in the gut. In embryos at the 28-somite stage (11.5 days of gestation), a few cells exhibiting specific Tyr-OH fluorescence were encountered in the mesenchyme of the gut. These contained large nuclei, and fluorescence was restricted to the cytoplasm and cytoplasmic processes (Fig. 3A). This unexpected result was confirmed and extended by observations during subsequent stages of development. In 12.5-day embryos, the number of Tyr-OH-positive cells in the gut mesenchyme had increased dramatically. These cells exhibited the same morphological characteristics observed at the previous stage, but their cytoplasmic processes were longer (Fig. 3B). The cells were predominantly localized to the distal portion of the esophagus and the stomach. Some cells were also encountered in the proximal part of the intestine. These cells tended to occur singly or in groups of two. By 13.5 days of gestation, most of these fluorescent cells were no longer observed, but a few were still encountered in the mesenchyme of the esophagus and intestine.

As described for the sympathetic ganglia, there was remarkable temporal coincidence of the appearance of Tyr-OH and CA in the gut cells. CA-containing cells were not present in the gut mesenchyme of 11.5-day embryos. However, at 12.5 days of gestation, numerous isolated cells exhibiting specific CA fluorescence were present throughout the gut. These cells were morphologically indistinguishable from the Tyr-OH-positive cells within the gut. They also possessed long fluorescent processes and large nuclei (Fig. 3C). Fluorescence intensity of the gut cells was less than that in the sympathoblasts at comparable developmental stages. CA-containing cells disappeared at the same stage as did the Tyr-OH-positive cells. In 13.5-day embryos, the number of CA-containing cells was markedly reduced in the gut (Fig. 3D). However, rare cells were still visible in the gut of embryos at 14.5 days of gestation. In these last two stages, the intensity of fluorescence in these cells was much decreased and was far less than that in the sympathetic neuroblasts.
was examined, because extremely sensitive and specific methods exist for demonstration of the enzyme gene-product Tyr-OH and the CA neurotransmitters. Simultaneous examination of Tyr-OH and CA provided insights unobtainable by study of either character alone.

Tyr-OH immunoreactivity and CA fluorescence developed in sympathetic primordia, as expected, and, in addition, transiently appeared in a population of cells within the gut wall. Most probably, these gut cells originated from the crest and were forerunners of the enteric ganglia, as indicated by time of appearance, morphologic features, and localization (see below for details).

There was a striking degree of synchrony in the immunohistochemical appearance of Tyr-OH and the histochemical appearance of CA. In sympathoblasts, the enzyme and the transmitter initially appeared in embryos at age 12.5 days, corresponding to the 36- to 37-somite stage. Similarly, in the presumptive neuroblasts of the gut, Tyr-OH and CA developed simultaneously. Tyr-OH and CA were initially detectable in the gut cells at 11.5 days of gestation (28-somite stage). Perhaps more dramatically, Tyr-OH and CA fluorescence also appeared at the same time in the gut neuroblasts. These observations suggest that a number of noradrenergic transmitter mechanisms develop simultaneously in the differentiating neuroblast. The synchronous appearance of rate-limiting enzyme and neurotransmitter suggests that at least several transmitter characters are operative simultaneously. CA fluorescence requires the presence of at least Tyr-OH and L-aromatic amino acid decarboxylase (dopa decarboxylase) for the biosynthesis of dopamine. The presence of intense CA fluorescence in perikarya may also imply the activation of high-affinity reuptake processes and storage mechanisms, although this remains to be demonstrated. These inferences suggest that the neuron responds to appropriate internal or external stimuli by production of several transmitter-related molecules. Consequently, the synthesis of a number of transmitter-related gene products may occur simultaneously in response to developmental signal(s). However, these contentions contrast with earlier work in the chicken embryo which indirectly suggested that CA biosynthetic enzymes appeared in a sequential fashion (22). The whole chicken embryo converted $[^3H]$tyrosine to L-$[^3H]$dopa on day 1 of incubation, to dopamine on day 2, and to norepinephrine on day 4. However, since these experiments were performed in whole embryos, it is unclear whether different cell populations were responsible for different biosynthetic reactions. Nevertheless, additional studies will be required to determine whether a wide array of transmitter traits appear simultaneously in response to appropriate regulatory cues.

In our studies it is possible that CA biosynthetic capacity developed sequentially over minutes to hours, instead of days, because our initial samples were obtained at daily intervals. Alternatively, the apparently synchronous appearance of Tyr-OH and CA could have reflected the relative sensitivities of our methods. This seems unlikely since the immunohistochemical and histofluorescent techniques differ theoretically and practically. We tentatively conclude, therefore, that, regardless of regulatory stimulus or location of neuroblast, several neurotransmitter characters develop synchronously in the differentiating neuron.

What developmental signals regulate differentiation into a
noradrenergic neuroblast? Tyr-OH and CA were undetectable in the dorsal neural crest itself or in the migrating crest derivatives. Similarly, a recent report of work in chicken embryos indicates that CAs are not detectable in migrating crest cells (23). In our studies, noradrenergic differentiation did not occur until 12.5 days of gestation, when sympathoblasts associated to form sympathetic ganglion primordia, consistent with previous observations (24). Thereafter, Tyr-OH and CA fluorescence intensity and fluorescent cell numbers increased progressively. These observations are consistent with recent work in chicken embryo, which suggests that appearance of CAs in presumptive sympathoblasts is due to interactions with somitic mesenchyme and therefore requires ventral crest migration (25). The evidence indicates that interactions of ventral neural tube, notochord, somite, and neuroblast are critical for CA differentiation (26), although a recent study (27) suggests that adrenergic fate may be predetermined in a subpopulation of crest cells. In our studies, the appearance and persistence of nonadrenergic specialization may also have been due to these interactions, because Tyr-OH and CA did not appear before or during migration. Also, the progressive increase in sympathoblast fluorescence may have been due to continued proximity to neural tube, notochord, and somite, as discussed below.

The unexpected appearance of Tyr-OH and CA in presumptive neuroblasts in the gut may help elucidate regulatory mechanisms. CA differentiation in the gut neuroblasts occurred at 11.5 days of gestation, a full day earlier than that in the ganglion primordia. Consequently, the gut may have been colonized by the first crest cells to migrate ventrally past somite and neural tube. Such a contention is consistent with observations in the chicken embryo which indicate that presumptive enteric ganglioblasts are the first to migrate ventrally from the truncal neural tube (28). CA differentiation may have been induced by interactions with somite and neural tube. Subsequently, however, in the environment of the gut, at a great distance from somite and neural tube, noradrenergic characters may have failed to persist. This may explain the disappearance of noradrenergic cells in the gut by approximately 14.5 days of gestation. Nevertheless, our results suggest that initial expression of adrenergic differentiation may not require localization to the region involved in “induction.” These observations imply that neuroblasts that migrate ventrally become noradrenergic, if only transiently, but that persistence of this type of transmitter differentiation depends on the environment of the definitive site. Such a contention is consistent with recent work that has demonstrated that expression of a given neuronal phenotype in transplanted crest is regulated by the target site (29–31). In our studies, the gut neuroblasts that developed transient noradrenergic characteristics may ultimately be destined to become enteric ganglion cells, which utilize other transmitters. We have not yet excluded the possibilities, however, that the gut neuroblasts either died or migrated out of the gut mesenchyme.

Regardless of the mechanisms regulating the fate of the gut cells, a number of our observations suggest that they are neural crest derivatives. These cells appear precisely at the time of crest migration and are restricted to the gut mesenchyme. They exhibit characteristic morphologic features, having large round nuclei and long cytoplasmic processes. We tentatively conclude, therefore, that these cells originate from neural crest and migrate in the gut to form the enteric ganglia.

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