The expression of neuropeptide Y immunoreactivity in the avian sympathoadrenal system conforms with two models of coexpression development for neurons and chromaffin cells

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Summary

We have studied the expression and development of neuropeptide Y-like immunoreactivity (NPY-LI) in the sympathoadrenal system of the chicken using single and double immunocytochemical techniques and radioimmunoassay. NPY-LI is expressed by neurons of the paravertebral sympathetic ganglia and by chromaffin cells of the adrenal gland in embryonic and adult chickens. The peptide is coexpressed with catecholaminergic properties in neurons. In chromaffin cells, it is also expressed with immunoreactivity to somatostatin and serotonin.

We have used the expression of NPY-LI to analyze how cells that coexpress two or more neuroactive substances arrive at their final phenotype. Our results suggest that the ontogeny of coexpression in neurons of the avian paravertebral sympathetic ganglia occurs in a sequential pattern, where the expression of the peptide follows the initial expression of the "classical neurotransmitter". In contrast, in chromaffin cells, expression of the peptide occurs concomitantly with expression of catecholaminergic properties or soon after. Initially, coexpression of several neuroactive substances occurs, but this is followed by further specialization where the expression of one peptide prevails over the other. We believe that the two models of coexpression shown by our results can be used to describe the ontogeny of coexpression in other cells of the nervous system.

Key words: NPY, sympathoadrenal system, neurons, chromaffin.

Introduction

Since the peptide somatostatin (SS) was detected in sympathetic noradrenergic neurons by Hökfelt and his colleagues (1977), it has been found that numerous cells of vertebrate and invertebrate nervous systems coexpress neuroactive substances. The partners in this coexistence usually comprise a neuropeptide and a "classical neurotransmitter" such as catecholamines or acetylcholine. For example, in sympathetic neurons, vasoactive intestinal polypeptide (VIP) has been found to coexist with acetylcholine (Lundberg et al., 1981; Johansson and Lundberg, 1981; Landis and Keefe, 1983) and SS has been found to coexist with catecholamines (Hökfelt et al., 1977; Lundberg et al., 1982). Coexistence of two classical neurotransmitters, or of two or more neuropeptides and a classical neurotransmitter, has also been documented (Hökfelt et al., 1980, 1987). To a large extent, the studies of coexpression of neuroactive substances consist of descriptions of the cellular phenotypes or of the fiber pathways in adult organisms. Less attention has been focused on the development of the coexistence. In most systems, it is unclear whether the substances appear in a concomitant or sequential pattern and whether, once present, they are modulated in a coordinated fashion during embryonic and postnatal development.

We and others have used the avian sympathoadrenal system to study the initial expression and development of peptides in neurons and chromaffin cells of the peripheral nervous system (Garcia-Arrarás et al., 1984, 1987, 1988; Fontaine-Perus, 1984; Maxwell et al., 1984; New and Mudge, 1986; Ross et al., 1990). The development of the avian sympathoadrenal system has been well described in terms of the expression of catecholaminergic properties. The neural crest cells that migrate to the area dorsolateral to the aorta are known to form the primary sympathetic ganglion chain between the third and fourth day of incubation and the adrenal gland primordia about 24 hours after (see Smith et al., 1981; Le Douarin and Smith, 1983). With the formation of both tissues by the aggregation of the neural crest cells, the first signs of biochemical differentiation appear. Cells express various catecholaminergic properties, including catecholamine histofluorescence and the presence of tyrosine hydroxylase (TH) mRNA, immunoreactivity and enzyme activity (Enemar et al., 1965; Cohen, 1972; Kirby and Gilmore, 1976; Allan and Newgreen, 1977; Coillard et al., 1978, 1979; Rothman et al., 1978; Jonakait et al., 1988; Fauquet and Ziller, 1989). Dopamine, noradrenaline and adrenaline are found in the sympathetic ganglia of 7-day chick embryos (Ross et al., 1990).

The developmental patterns of expression of SS and the
monoamine serotonin (5HT), which are present in catecholaminergic cells, have been described in the avian system (García-Arrarás et al., 1984; Maxwell et al., 1984; García-Arrarás and Martínez, 1990). In this system, immunoactivity to SS and 5HT coexist in chromaffin cells of the adrenal gland and in cells of the sympathetic ganglia. The expression of these molecules increases with embryonic development in the adrenal gland but decrease in the sympathetic ganglia. However, SS is only one of several neuropeptides expressed by cells of the vertebrate sympathoadrenal system. Other neuropeptides, such as neuropeptide Y (NPY), have been shown to coexist together with catecholamines or with catecholamine-related properties in neurons of the sympathetic ganglia and in chromaffin cells of the adrenal gland (Lundberg et al., 1982, 1983; Hökfelt et al., 1980, 1987). We have shown that NPY-like immunoreactivity (-LI) is present in the avian sympathoadrenal system (García-Arrarás et al., 1989). Thus, the avian sympathoadrenal system could be used to study coexpression of multiple neuroactive substances at different stages of development.

Moreover, in the avian system, the biochemical specialization of the cells is closely associated with the morphological phenotype. For example, SS is found in small intensely fluorescent (SIF)-like cells and chromaffin cells, which are catecholaminergic, but not in neurons (García-Arrarás et al., 1984; Maxwell et al., 1984; García-Arrarás and Martínez, 1990; New and Mudge, 1986). However, vasoactive intestinal polypeptide (VIP) has been detected in non-catecholaminergic neurons, but not in the other cell types of the system (Hayashi et al., 1985). NPY has not been described in avian principal neurons, but in most vertebrates where it has been studied, NPY is found in catecholaminergic (noradrenergic) neurons of the sympathetic ganglia (Lundberg et al., 1982; Hökfelt et al., 1980).

Several questions arise on the expression of NPY in the avian sympathoadrenal system that address directly the issue of coexpression development in vertebrate systems. What is the timing of the initial expression and how does it relate to the expression of the other neuroactive substances in the system? What is the developmental pattern of expression? Is NPY also associated with a specific phenotype?

Materials and Methods

Animals

Fertilized eggs were obtained from commercial sources (Ochoa Superchick, Rio Piedras, PR) and raised in a turning incubator at 38°C. Some of the chickens were hatched and kept in the departmental animal facilities for up to three months as previously described (García-Arrarás and Martínez, 1990). The term newly hatched was used for chicks used within the first three days after hatching.

Tissue preparation

Twelve-day or older embryos and post-hatched chickens were killed by decapitation. The adrenal gland and the lumbosacral sympathetic chain were rapidly dissected out and placed in 4% paraformaldehyde (Hartman, 1973) or in picric acid-formaldehyde (Zamboni) mixture (Keast et al., 1984). Younger embryos (4-8 days) were placed directly in the fixatives. In some cases, embryos of 5-18 days of age were sectioned at trunk level, most of the viscera were removed and the trunk region, including the sympathetic ganglia and adrenal gland was placed in the fixatives.

Embryos or tissues fixed in 4% paraformaldehyde were left for 1 hour at 4°C, then rinsed three times in phosphate-buffered saline (PBS) and placed in 30% sucrose-PBS at 4°C for 18-36 hours. Tissues fixed in Zamboni were left overnight in the fixative, then dehydrated in a series of alcohols, cleared in xylene and rehydrated before being placed in 30% sucrose-PBS.

Tissues or embryos were mounted in embedding medium (OCT, Miles, Inc., Elkhart, IN) and 10-12 μm sections were cut. Sections were picked up in chrome-alum gelatin or polylysine subbed slides and left to dry under cold air for 1-2 hours. In some cases, the sections were left in a desiccator with calcium chloride for 1-2 days before use.

Antisera

We prepared an antisera against NPY by following the procedure described by Harlow and Lane (1988). In brief, 0.5 mg of NPY (Peninsula Labs, Belmont, CA) was coupled to 1.5 mg of bovine serum albumin (Sigma) with 0.1% glutaraldehyde for one hour. The reaction mixture was then dialysed against phosphate-buffered saline (PBS) for 24 hours, with three changes of the dialysis solution. The dialysate was divided into three aliquots of 1 ml each and frozen until use. One aliquot of the BSA-NPY conjugate was emulsified in complete Freund’s adjuvant (1:1) and used to immunize two rabbits (New Zealand White). Each rabbit received 1 ml of the emulsion, half of it injected subcutaneously in the neck region, and the other half injected intraperitoneally. Six weeks after the initial injection, a booster, another aliquot, was given. Sera were drawn 7 and 14 days after the second injection. A second booster was given 5.5 months later and antisera were collected 7, 14 and 20 days afterwards. The sera were assayed by immunocytochemical reactivity on sections of chicken intestine. Both rabbits produced antibodies after the second and third injections that labelled endocrine cells in the mucosa and nerve fibers in the circular muscle expressing NPY-like immunoreactivities, a positive control.

Four other anti-NPY sera were tested. Two were obtained from commercial sources (Amersham, Arlington Heights, IL and Cambridge Research Biochemicals, Cambridge, England) and the two others were generous gifts from Dr. M. F. Beal (Boston, MA) and from Dr Julia Polak (London, England). Their pattern of immunocytochemical labelling in the sympathoadrenal tissues was similar to the ones produced by our antibodies, but all, except the sera from Dr Polak, produced a weaker signal.

Immunohistochemistry

(A) Single labelling

Sections were treated with non-immune goat or bovine serum at a dilution of 1/50 for 15 min. rinsed three times in PBS and covered with the antisera. In some cases the tissues were treated with PBS-0.1% Triton X-100 for 15 minutes before the addition of the antibody. The primary antibody was left for 18-30 hours at room temperature in a humid chamber, after which the sections were rinsed twice with PBS and treated with the secondary antibody, goat anti-rabbit coupled to fluorescein isothiocyanate (GAR-FITC, Tago, Burlingame, CA) at a dilution of 1/50 for one hour. The sections were rinsed three times with PBS, mounted with buffered glycerol and observed with a Leitz Laborlux fluorescence microscope equipped with an 12/3 filter.

For controls, our antibodies were preincubated with 1 μg/ml NPY during 24 hours prior to treating the sections. Sections of adrenal gland and sympathetic ganglia from newly hatched chicks stained after preincubation of the antibodies with peptide showed no immune reaction. Additional controls were done by omitting the primary antibody from the procedure.
B) Double labelling

Tissue preparation for double labelling have been described previously (García-Arrarás and Martínez, 1990). For NPY/SS double labelling, the primary incubation was done with a mixture of mouse monoclonal anti-SS (gift of Dr L. Chun) at a final dilution of 1/500 and our NPY antiseraum at a final dilution of 1/1000. In order to label the catecholaminergic cells, we used a monoclonal antibody against tyrosine hydroxylase (TH) (Fauquet and Ziller, 1989), the rate-limiting enzyme in catecholamine biosynthesis. For NPY/TH double labelling, the primary incubation was done with a mixture the mouse monoclonal anti-TH culture supernatant (gift of Dr M. Fauquet) and our NPY antisierum at a final dilution of 1/10 and 1/1000, respectively. Double labelling for SS/TH was performed using the mouse TH monoclonal and a rabbit sera against SS that has been described previously (García-Arrarás et al., 1984).

The secondary antibody mixture for both double labelings consisted of goat anti-mouse coupled to fluorescein isothiocyanate (GAM-FITC, KPL, Gaithersburg, MD) and goat anti-rabbit coupled to rhodamine (GAR-RHOD, Tago) each at a final dilution of 1/50. Similar results were obtained with a secondary antibody mixture of GAM-RHOD (KPL) and GAR-FITC (Tago) each at a final dilution of 1/50. The rhodamine label was observed using a N2 filter in the Leitz microscope.

In order to verify that the secondary antibodies were not cross-reacting with the inappropriate primary antibodies, sections with NPY antisierum were treated with the GAM-FITC secondary antibody and with the GAM-FITC/GAR-RHOD mixture. Similarly, sections with anti-SS or anti-TH were treated with the secondary antibody mixture and GAR-RHOD to check for cross-reactivity. Additional controls for possible cross-reactivity were done by treating sections with the primary antibody mixture followed by either GAM-FITC or GAR-RHOD in order to see if there was any possible interaction of the primary antibodies. The controls for double labelling showed that none of the secondary antibodies cross-react with the inappropriate primary antibodies. For example, when the NPY (rabbit) antibody was incubated with GAM-FITC alone or together with GAR-RHOD, the fluorescent cells were only observed by using the rhodamine filter and not with the FITC filter.

In order to rule out the possibility that steric hindrance from one of the antibodies prevents the binding of the second one to the same cell population, we performed double labelling experiments using our two antibodies against the SS molecule, one made in rabbit (García-Arrarás et al., 1984) and the other the mouse monoclonal. When both GAM-FITC and GAR-RHOD secondary antibodies were applied after the combined primary antibodies, the same cells were labelled with both markers.

Radioimmunoassays (RIA)

Tissues for RIA were dissected and placed in Tyrode solution at 4°C. Most of the extraction and RIA procedures have been described previously (García-Arrarás et al., 1984). In brief, tissues were extracted in 200-500 µl of 2 M acetic acid, ruptured with ultrasound (Microson Cell Disruptor, Structure Probes, Inc. West Chester, PA), boiled for 10 minutes, and freeze dried. Prior to lyophilization, an aliquot was removed for protein analysis and frozen at −20°C until used. Protein was measured using Bio-Rad assay.

The RIA was done using 100 µl of the sample, 100 µl of the radioactive tracer (NPY 125, Dupont de Nemours, Wilmington, DE) and 100 µl of the antibody all dissolved in the RIA buffer (García-Arrarás et al., 1984). For the radioimmunoassays, the serum used was obtained from the second bleed after the third injection of rabbit no. 4 (NPY 3-12s). Of all the sera, this serum consistently gave the best results for RIA and was used at a dilution of 1/40,000 for the RIA. Free and bound radioactive tracer were separated by centrifugation at 2000 g for 20 minutes after precipitation with 1 ml of propanol and 10 µl of normal horse serum. The radioactivity in the precipitate was measured in a Beckman Gamma Counter. Standard curves were prepared using synthetic peptide (Sigma). Our NPY RIA shows a 50% binding at about 500 pg NPY and detects levels of the peptide down to 80 pg. When we compare the slope of the binding curve from serial dilutions of our sympathetic or adrenal extracts with synthetic NPY standard curve, we observe a strong similarity indicating that the molecule present in the avian sympathoadrenal system is NPY or a very similar molecule.

Results

Immunohistochemical detection of cells expressing NPY-LI

No NPY-LI was observed in 3.5- and 4-day embryos, which already show TH expression in the lumbar sympathetic primordia (data not shown). Cells expressing NPY-LI first appear in the sympathetic ganglia between day 5 and 6 of incubation. At this stage, however, it is difficult to distinguish individual cells. Instead, the ganglia are generally immunofluorescent and only a few cells can be seen (Fig. 1A). As the embryo develops, the number of distinct cells expressing NPY-LI increases and their neuronal morphology is evident; they have a large soma (10-15 µm diameter) with immunoreactivity localized around the nucleus (Fig. 1B). Axon-like processes are sometimes observed extending from these cells. During embryonic development, the cells expressing NPY-LI are distributed throughout the ganglion and most of the immunoreactivity is observed in the perinuclear or Golgi area. The pattern of NPY-LI expression in the post-hatched animal does not differ from the late embryonic stages. Fibers expressing NPY-LI become more conspicuous as the embryo ages and some of the fibers can be observed surrounding the sympathetic neurons.

In the adrenal gland, NPY-LI also appears in cells between day 5 and 6 of incubation (Fig. 2A). At this early stage, few cells are immunoreactive. The number of immunoreactive cells increases as the embryo develops and later stages they can be seen to form the cords of chromaffin cells inside the adrenal gland (Fig. 2B, C). The cells expressing NPY-LI are distributed uniformly throughout the gland, including the areas in the periphery and those adjacent to blood vessels that are present near the gland. In older embryos and in the newly hatched chick, not all chromaffin cells appear to be immunoreactive for NPY, since, when adjacent sections are compared for TH and NPY-LI, the number of cells expressing TH is far greater than those expressing NPY-LI. Cells expressing NPY are found during embryonic and post-hatching development of the adrenal gland in chickens up to three months old, the last stage studied.

Quantification of NPY-LI by radioimmunoassay

In both the sympathetic ganglia and the adrenal gland the net amount of NPY-LI increases from embryonic day 10 to 3 months post-hatching. In the sympathetic ganglion, the amount goes from 0.25 ± 0.06 s.e.m., n=6 ng/chain at E-10 to 25 ± 3.5 s.e.m., n=6 ng/chain at 3 months, while in the adrenal the increase is from 0.15 ± 0.04 s.e.m., n=6 ng/gland to 125 ± 13 s.e.m., n=6 ng/gland at the same stages. When the ratio of NPY-LI/protein content of the sym-
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Fig. 1. Immunocytochemical labeling for neuropeptide Y-like immunoreactivity (NPY-LI) in chicken paravertebral sympathetic ganglia. (A) Although in the early stages weak NPY-LI is found throughout the ganglia certain cells with a neuronal morphology are already present in the 7-day embryo. (B) As the embryo ages the number of cells expressing NPY-LI increases and the immunoreactivity is found in the perinuclear area of the somata in the newly hatched chick. Scale bars (A) 15 μm, (B) 25 μm.

Determination of coexpression of NPY with TH and SS by double labelling
In order to verify that NPY-LI was being expressed by catecholaminergic cells, we performed double labelling experiments using antisera made in two different species. In order to be able to observe changes in the patterns of coexpression, double labelling was performed at two different stages, early embryonic (10-day) and newly hatched. In both embryonic...
and newly hatched sympathetic ganglia, as was observed with single labelling, not all neurons expressed NPY-LI. However, all the neurons that expressed NPY-LI also expressed TH (Fig. 5). When the percentage of TH immunoreactive neurons expressing NPY-LI is compared between E-11 and newly hatched chicks no significant difference is found (Table 1). By counting the number of TH-positive cells and the number of neurons observed in phase contrast in sections of newly hatched chick ganglia, we have determined that 59% (±3 s.e.m., n=16) of the neurons in the sympathetic ganglia are cathecolaminergic. If 70% of these catecholaminergic neurons also express NPY-LI then about 40% of all the neurons in the ganglia express NPY-LI. The other cell type present in the ganglia are SIF-like cells which are strongly TH immunoreactive. Most of these cells, whether in embryos or post-hatched stages, did not express the NPY-LI, but in several cases one of the cells within a cluster did express NPY-LI (data not shown).

In the adrenal gland of the newly hatched chick, all cells expressing NPY-LI also expressed immunoreactivity for the catecholaminergic enzyme (Fig. 6). As was observed in the sympathetic ganglia, not all the chromaffin cells in the adrenal gland expressed the peptide. It was estimated by counting cells in several regions of the gland that, at this stage, about half of the chromaffin cells express NPY-LI (Table 2). Coexpression of NPY-LI and TH in the early

Table 1. Developmental expression of NPY-LI in sympathetic neurons

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<th>Stage</th>
<th>NPY/TH (%)</th>
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<tr>
<td>E-11</td>
<td>70% (±5.8)</td>
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<tr>
<td>Newly hatched</td>
<td>60% (±4.8)</td>
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The numbers represent the mean (±s.e.m.) of at least six sections from three different organs for each stage. Cells immunoreactive to TH were chosen at random, 123 for E-11 and 310 for newly hatched, and analyzed to determine what percentage of them expressed NPY-LI. Student's t-test shows no significant difference between the two stages.
embryo was also evident. At this stage, the percentage of cells within the medullary cords that coexpress NPY-LI and TH is much higher. The double-labelled cells are distributed homogeneously throughout the gland although clusters of cells expressing only TH were usually found near the gland periphery.

Double labelling was also performed in order to corroborate previous experiments showing that most of the chromaffin cells in the early embryo coexpress SS-LI and TH and to ascertain whether NPY-LI and SS-LI were being expressed by the same cells in the adrenal gland. Chromaffin cells expressing SS-LI were found to be numerous in early embryos as well as in the newly hatched chickens (Fig. 7) with only a small reduction in the percentage of those expressing SS-LI occurring during embryonic development (Table 2). The coexpression of NPY- and SS-LI, however, undergoes a greater modification during development. In the newly hatched chick, it was observed that most of the cells expressing NPY-LI were different from those expressing SS-LI (Fig. 8). At this stage only 23% of the cells expressing SS-LI also expressed NPY-LI (Table 2) and a similar number of the cells expressing NPY-LI also expressed SS-LI. In most of the cells where coexpression occurred, only a weak immunofluorescence to one of the peptides was observed.

When double-labelling for NPY- and SS-LI was performed in the early stages of embryogenesis (10 days) it was found that clustered cells in the periphery of the gland that did not express NPY-LI did express SS-LI. However, most of the cells within the medullary cord expressed the two peptides.

<table>
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<th>Table 2. Developmental changes in chromaffin phenotypic populations</th>
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<td>SS/TH</td>
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<td>NPY/TH</td>
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The numbers represent the mean (±s.e.m.) of at least five sections from three different organisms for each stage. From each section 50 cells expressing TH were chosen at random and analyzed to determine what percentage of them expressed SS- or NPY-LI. NPY/SS counts were done by choosing 50 cells expressing SS-LI from each section and determining what percentage of them expressed NPY-LI.

*Significant differences between E-10 and newly hatched, P<0.001, Student's t-test.
neuropeptides (Table 2). Thus, the relative number of chromaffin cells coexpressing SS- and NPY-LI decreases during development of the embryo to the newly hatched chick.

Discussion

Expression of NPY-LI

The expression of NPY-LI in the post-hatched avian sympathoadrenal system corresponds to that described for other vertebrate species. Catecholaminergic neurons expressing NPY-LI are a consistent feature in the paravertebral sympathetic ganglia of all vertebrates studied to date, specifically mammals (Lundberg et al., 1982; Varndell et al., 1984; Majane et al., 1985) and amphibians (Morris et al., 1986; Horn et al., 1987; Kuramoto, 1987). NPY-LI is also found in SIF cells within the sympathetic ganglia of mammals (Jarvi et al., 1986). In the adrenal gland, NPY-LI has been found in most mammalian species (Lundberg et al., 1983; Varndell, 1984; Pruss et al., 1986; Schalling et al., 1988). Preferential coexistence with either noradrenaline or adrenaline has been reported depending on the species (see Pruss et al., 1986; Pelto-Huikko, 1989; Henion and Landis, 1990). No NPY-LI has been detected in chromaffin cells of amphibians, even in cases where it has been sought (Horn et al., 1987; Kuramoto, 1987). In avian embryos, we made a preliminary report on the presence of NPY-LI in chromaffin cells (García-Arrarás et al., 1988) and, in this report, we expand these observations.

Developmental expression of neuropeptides in the avian sympathoadrenal system

The developmental expression of NPY-LI differs from that of the other two neuropeptides previously described in this system, SS and VIP. In the paravertebral sympathetic ganglia SS-LI appears early in the primordia but eventually decreases as the embryo ages and is never found in the principal neurons. Therefore, the levels of SS-LI/protein decrease as the embryo develops and as the cells expressing the peptide die or differentiate (Hayashi et al., 1983; Maxwell et al., 1984; García-Arrarás et al., 1984; New and Mudge, 1986; García-Arrarás and Martínez, 1990). The expression of NPY-LI is similar in the sympathetic ganglia to that of VIP-LI, which can be first detected by RIA at about 6 days of incubation and localized by immunocytochemistry to the neurons at about 10 days of incubation (Fontaine-Pérus, 1984; Hayashi et al., 1985; New and Mudge, 1986; García-Arrarás et al., 1987). However, VIP-LI is expressed by a noncatecholaminergic (supposedly cholinergic) cell subpopulation. The amount of VIP-LI expressed by the principal neurons, as seen for NPY-LI, increases during embryonic development (Hayashi et al., 1983; García-Arrarás et al., 1987). It could be argued that low, undetectable levels of NPY-LI are found in the sympathoblasts; however, circumstantial evidence does not support this idea. We have used different antibodies against NPY and none labels sympathoblasts. This differs from immunoreactivity to other peptides, such as SS (García-Arrarás et al., 1984; Maxwell et al., 1984) enkephalin and galanin (our own unpublished obser-
Fig. 8. Double labeling for NPY-LI (A, C) and SS (B, D) in the chicken adrenal gland. Cells expressing both peptides can be found within the adrenal gland of embryonic (11 days, A, B) and newly hatched chickens (C, D). However, as the embryo ages, the number of cells that coexpress both SS and NPY-LI become less common and a greater number of cells only express one or the other peptide. In the newly hatched chick cells expressing only NPY-LI (arrow) or only SS-LI (arrowhead) can be observed. Scale bar (A,B) 25 μm. (C,D) 15 μm.
reaching levels in the adult that are similar to the early embryonic stages (García-Arrarás et al., 1988).

Differential modulation of coexpressed peptides has been described before, mainly using rat superior cervical ganglia and adrenal chromaffin cells in culture (LaGamma et al., 1984; Kessler, 1985; Nawa and Sah, 1990; Nawa and Patterson, 1990). In the avian system, factors such as glucocorticoids, nerve growth factor, ciliary neurotrophic factor, electrical activity, cell density and others are known to affect the expression of the peptides (Hayashi et al., 1985; Ernsberger et al., 1989; García-Arrarás, 1991). It has been proposed that the high levels of glucocorticoids in the adrenal gland favor the expression of SS-LI (García-Arrarás et al., 1986); however, what factors are involved in the differential development of NPY-LI in the avian sympathoadrenal system remain to be explained.

**Developmental coexpression of neuroactive substances**

From our results together with those reported in the literature, we propose two models to describe the ontogeny of coexpression of neuroactive substances in the sympathoadrenal system. The first model involves the neurons of the paravertebral sympathetic ganglia and can be used to represent the coexpression of neuroactive substances in neurons. In this model, the acquisition of the coexpression occurs in a step-wise fashion. The neuroblasts express first catecholaminergic characteristics. A few days later, as the cells differentiate to the neuronal morphology, the expression of immunoreactivity to the neuropeptides, NPY and VIP, begins. A comparable developmental pattern occurs in the frog where paravertebral neurons express NPY-LI weeks after the expression of catecholaminergic properties (Stofer and Horn, 1990). Similarly, in the normal development of the innervation of the rat sweat glands, Landis and colleagues (1988) have found that VIP is expressed when the neurons are undergoing a change from adrenergic to cholinergic properties but not before. In addition, CGRP, another peptide expressed by these neurons appears even later than VIP. Thus, in the rat sympathetic neurons, the normal developmental expression of neuroactive substances also occurs in a sequential manner from adrenergic to cholinergic followed by VIP coexpression and then CGRP. Invertebrate neurons provide a third example of this phenomenon. In the American lobster and in the grasshopper some neurons are known to coexpress proctolin and serotonin immunoreactivities (Taghert and Goodman, 1984; Beltz and Kravitz, 1987). In both cases, 5HT immunoreactivity appears earlier in development than the peptide (see Beltz et al., 1990). Almost a decade ago, Black (1982) had already proposed that, in order for sympathetic neurons to arrive at their mature biochemical phenotype, they must pass through a state where both of them are expressed. Experiments are now in progress in our laboratory to determine the changes that are occurring in these subpopulations during the different developmental stages and the factors that are affecting the expression of each phenotype.

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