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Ontogenic attendance of neuropeptides in the embryo chicken retina

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Summary. We have examined the ontogeny of somatostatin-, Glucagon-, Vasoactive Intestinal Polypeptide-, Substance P-, Neuropeptide Y, and Calcitonin gene-related peptide-like structures in the chicken retina by immunocytochemistry. Neuroblastic cells containing Substance P-like immunoreactivity (IR) first appeared at embryonic day 5 in the peripheral portion of the retina. Somatostatin-like immunoreactivity was detected as early as embryonic day 11 in the innermost level of the inner neuroblastic layer. The distribution pattern of amacrine cells containing Vasoactive Intestinal Peptide-like immunoreactivity was similar to that for Neuropeptide Y- and Calcitonin gene-related peptide-like immunoreactive cells. These three types of IR cell appeared at embryonic day 13. Glucagon-like immunoreactive cells first appeared in the retina at embryonic day 15, in the innermost part of the inner nuclear layer. From the 13th to 15th day of incubation, the number and intensity of Calcitonin gene-related peptide-, Somatostatin-, Neuropeptide Y- and Substance P-like immunoreactive cells increased and then decreased progressively before hatching. Glucagon immunoreactive cells increased in number on the last day before hatching. After embryonic day 15, the amacrine cells containing Vasoactive intestinal peptide-like immunoreactivity decreased notably in number. Our study showed that development of these immunoreactive structures was different for each neuropeptide. These differences in development may reflect the diverse neurophysiological roles of these neuroactive peptides, which could act as neurotransmitters/neuromodulators at the chick retinal level. Their presence may indicate roles as neuronal differentiation or growth factors.

Key words: Retina, Neuropetides, Embryo, Differentiation, Immunocytochemistry

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Introduction

The retina, a brain outgrowth, contains several different neuronal systems, which produce and/or store various substances. In the vertebrate central nervous system, the neuroretina has been widely used as a useful model for both the functioning of neural circuits and their embryological development.

In addition to classical neurotransmitters, a number of neuroactive peptides have been located in the retina and especially in the amacrine cells (Brecha and Karten, 1983; Ehinger, 1983; Das et al., 1985; Ekman and Tornqvist, 1985; Kiyama et al., 1985; Brunn et al., 1986; Ishimoto et al., 1986; Marc, 1986; Keiser et al., 1988; Wu and Lam, 1988; Li et al., 1990; Prada et al., 2000). However, the functions of these peptides are still to be entirely determined (Fdez-Trujillo et al., 1996), although it is reasonable to suspect that they act as neurotransmitters or neuromodulators.

Immunocytochemical techniques have provided a precise means of visualizing and identifying these neuronal populations in the chick retina. In this work, we have examined, using immunocytochemistry, the ontogeny of several chick retina neuropeptides, specifically Somatostatin (SMT), Vasoactive intestinal polypeptide (VIP), Substance P (SP), Glucagon (GLC), Neuropeptide Y (NPY) and Calcitonin gene-related peptide (CGRP), that can act as either neurotransmitters or neuromodulators at this level.

The seven major classes of cells of the vertebrate retina are generated from a pool of multipotent retinal progenitor cells (RPCs). In contrast to other well-studied regions of the CNS, retinal cell diversification is apparently not achieved by spatial pre-patterning into distinct progenitor domains, but rather by the sequential production of cell types in a definitive retinogenetic order. Cell fate decision is made during or after the terminal cell division, beginning a migration process toward the definitive localization (Ramón y Cajal, 1898; Layer and Makovitzky, 1990; Mey and Thanos, 1992; Genis et al., 1993; Mack and Fernald, 1993; Snow and Robson, 1994; Marquardt and Gruss, 2002; Prada et al., 2002). In this migration phase, extrinsic and intrinsic

factors can influence fate choice, according to a more recent model whereby both the progenitor cells and the environment change over time (Cepko et al., 1996). Those factors would include extracellular matrix proteins (Lemmon et al., 1992), neurotrophic factors as shown for rod photoreceptors (Ezzeddine et al., 1997), as well as neural cell adhesion and recognition molecules (Rutishauser and Jessel, 1988). The molecular mechanisms mediating the retinogenic potential of RPCs seem to be mediated by the expression of a set of transcription factors. Several studies implicate the Pax6, Pax2 and So/Six gene family in having played a crucial ancestral role in visual system development (Pichaud and Desplan, 2002).

Moreover, neurotransmitters and neuromodulators constitute other important factors, which have been associated not only with neurotrophic functions but also with processes of differentiation. Since the classical reports of Buznikov and Schmukler (1981) and Wallace (1982) on the influence of serotonin in neural tube development of the chicken, many neuroregulators have been proposed with these functions. The insulin-like growth factors (IGFI and IFG-II), which are usually found in a complex with specific binding proteins (IGFBPs), play a role in embryonic growth (Kyriakis et al., 1987; Bassas et al., 1989). The appearance of IGFBP-2 in ocular tissue suggests a role for this in development of the cornea and retina (Schoen and Chader, 1997).

Lankford et al. (1988) reported their belief that neurotransmitters might be a fundamental element necessary in neuroblast differentiation. Neuroblasts synthesize neurotransmitters during the embryonic period, as shown by the appearance of enzymes and precursors. Glutamate, gamma-aminobutyric acid, opioid peptides, VIP and pituitary adenylate cyclase-activating peptide (PACAP) are likely to play a role in down-regulating proliferation in the developing central nervous system. The presence of neurotransmitters would make neural circuits functional, whereas the differentiating neuroblasts prepare the circuits for their definitive role. In this context, the presence of neurotransmitters during intermediate stages has been explained both as a formative period for the neuroblasts to adopt their definitive positions in the neural circuit, and as an initiation of the enzyme synthesis response. This mechanism has been proposed as the basis of a selection model where neuroblasts select or reject the possible neural targets (Lankford et al., 1988; Cameron et al., 1998). The proliferation and cell fate determination in the developing brain are regulated extrinsically by complex interactions between a relatively large number of growth factors and neurotransmitters. The development of definitive functional circuits will be the result, in large part, of the secretion of neuropeptides, which act by autocrine or paracrine mechanisms.

It has recently been found that in the development of birds, neural cells decrease the synthesis of neurotransmitters until the moments just prior to hatching. This explains why only small amounts of immunoreactive binding to neurotransmitters were identified in those synthesizing neurones (for example, in amacrine cells). Moreover, ganglion and bipolar cells do not synthesize but rather accumulate many of the neuromodulators that they use, such as serotonin, which is synthesized in amacrine cells (Wilhelm et al., 1993). These ganglion and bipolar cells did not appear immunostained. Thus the retinal circuits are electrically functional before hatching, but in a manner different from when light stimulates the retina for the first time (Thomas and Iuvone, 1991; Espinar et al, 1994).

Certainly, the coexistence of multiple neuropeptides in retinal neurones would increase the number of ways they might act to influence neurotransmission in the retina. However, there are few studies that show the presence of neuropeptides in embryonic chicken retina. This work complements existing data on the morphology and location of immunoreactive cellular and fibrillar elements in respect to the chicken retina.

Materials and methods

Histological specimens

Thirty chick embryos were incubated in a humid chamber at 36 °C. Embryos were removed every two days, from the fifth day of incubation to one day post-hatching (day 22). When specimens were large enough, they were sacrificed by quickly cutting the neck to avoid possible pain. We analyzed two specimens for every studied stage. Eye globes were fixed in Bouin liquid for four hours, dehydrated using graded alcohols and benzol, and embedded in medium-paraffin by routine procedures. Serial sections were cut at 5 μm and placed on albumin-coated glass slides. They were counterstained with modified hematoxylin of Harris-VOF according to Gutierrez et al. (1963).

Immunocytochemical techniques

The sections were de-paraffinized in xylol and rehydrated in a degraded alcohol series and distilled water. Endogenous peroxidase and pseudo-peroxidase activity was inhibited with treatment for 30 min in 3% H_2O_2 solution. Sections were washed twice in phosphate buffered saline (PBS, Sigma P-4417) 0.1 M and pH 7.4 for 5 min and incubated with the primary antibody for 36 h at 4 °C. Next, sections were washed twice in PBS. The avidin-biotinylated peroxidase complex (Biogenex Supersensitive Multilink HRP) was used in the next procedure: 30 min in secondary biotinylated antibody at a dilution of 1/50 at room temperature; twice in PBS for 5 min; 30 min in 1/50 diluted streptavidin-peroxidase complex at room temperature; and twice in PBS for 5 min.

For color development, a solution of 10mg/15ml Diaminobenzidine (3,3' Diaminobenzidine, Sigma D-

5905) in Phosphate Buffer Saline (Sigma T-5030) was employed in the presence of 20 ml/10ml H_2O_2, for 10 min. Afterwards, sections were dehydrated and coverslips were mounted with Entellan (Merck 7961).

We used rabbit polyclonal antibodies against: synthetic VIP (Biogenex HP44-5P) at a dilution of 1/200; human NPY bound to thyroglobulin (Boerhinger-Mannheim Biochemical 1428608) at a dilution of 1/100; synthetic Substance P (Biogenex AR069-5R) at a dilution of 1/200; synthetic Somatostatin (Biogenex AR-042-5R) at a dilution of 1/500; and human CGRP (Boerhinger-Mannheim Biochemical 1295241) at a dilution of 1/100. We also used a mouse monoclonal antibody against pig Glucagon (Sigma G-2654) at a dilution of 1/500. Negative controls included incubation of some sections in a PBS solution without primary antibodies or excluding each time one step in the avidin-biotin complex method. Positive controls were made in antrum-duodenum tissue, where a normal histological staining was shown.

We performed morphometric analysis by videomicroscopy in combination with the MIP 4 advanced software (CID –Consulting Imaging Digital S.L., Spain). We estimated the following parameters: the area of stained cells, the number of stained and unstained cells, and the number of stained cells per unit area. No statistical analysis was considered.

Results

In our study, we have examined the ontogeny of 5P-NPY-, 5MT -, VIP-, GLC- and CGRP-like immunoreactive structures in the developmental chicken retina. The histological controls showed a normal development of the different retinal layers with an adequate appearance of the differentiated neural and glial cells (Marquardt and Gruss, 2002; Prada et. al, 2002). No specific staining was observed in the immunocytochemical controls. No specific immunoreactivity was detected before embryonic day 5 in any of the examined embryos.

Glucagon

Embryonic chicken neuroretina showed a few scattered cells exhibiting Glucagon-like immunoreactivity but the distribution pattern of these cells became clear at the end of embryonic development. Glucagon-immunoreactive (IR) structures were first observed on the 15th day of incubation (S-41, stage of Hamburguer-Hamilton, 1951) as immunostained cellular bodies (Fig. 1). These IR-neuroblasts were located in the innermost half of the inner nuclear layer (INL). The localization and typology of these IR neuroblasts resembled amacrine-differentiating cells which had just occupied this position (Mey and Thanos, 1992; Prada et. al., 2002). These GLC-IR cells had a mean diameter of 37 µm, and they were present in the primitive central retina in a very small proportion (1 per 1000 cells). The amacrine cells ended the migration process at S-39, and immediately began their differentiation. These results remained unchanged until the last day before hatching when the glucagon immunoreactivity appeared in amacrine cells situated near the border with the inner plexiform layer (IPL).

The GLC-IR cells increased in number (7% in the central retina, 15% in the peripheral retina) and we observed IR-processes arising from some cells. The fibers of these neurones entered the IPL and ramified in the outer strata of this IPL (sublamina 1). These IR-fibers were detected from S-44 (19th day of incubation) since the IPL is assembled between the 17th and 19th day of incubation (Mey and Thanos, 1992; Marquardt and Gruss, 2002). One day before hatching, these varicose fibres could easily be traced for relatively long distances. From their morphology, size (48 µm) and typology, the amacrine GLC-IR cells appeared to correspond to those named by Ramon y Cajal (1898) as horizontal amacrines or short axonal association amacrine cells (Fig. 2).

Substance P

We noted that there were relatively few cells displaying SP immunoreactivity throughout the entire period of development. SP-IR cells were first observed on the 5th day of incubation (S-27) at the peripheral portion of the primitive neuroblastic layer, and they resembled postmitotic migrating neuroblasts. SP-IR cells were small (mean neuroblast diameter: 26mm) and were present in a very small proportion (less than 1 per 2000 cells). At this stage, the ganglion cell layer was not demarcated and the inward cell migration was in progress towards the inner portion of the retina. The migrational cells at this stage could be those that later differentiated into ganglion cells (Mey and Thanos, 1992; Genis et al., 1993; Snow and Robson 1994; Marquardt and Gruss, 2002). On the 7th day of incubation (S-30), there were few SP-IR neuroblasts located at the recently organised ganglion cell layer.

From S-30 we noted some small SP-IR cells (20 µm) in the inner neuroblastic layer (INbL) (Fig. 3), but not significantly more numerous (less than 1 per 2000 cells per field), and with no differences observed between center and periphery. The immunoreactivity was stronger in unsharpened neuroblasts located in the inner half of this layer. These observations remained unchanged until the 15th day of incubation. Then, the immunoreactivity was observed in medium size single cells (37 µm) that had moved to the INbL inner border, but the number of cells displaying immunoreactivity was unchanged. These neuroblasts were moving to the inner border of inner nuclear layer, where they would differentiate into amacrine cells. The SP-IR cells possessed rounded bodies, with unstained nucleus and long cones of stained cytoplasm directed to the IPL (42 µm). These cones were the progression zones of these migrating neuroblasts, which may later differentiate as

amacrine cells (Fig. 4). No stained dendritic prolongations were seen from either amacrine or ganglion cells at the IPL.

The maximum numbers of SP-IR cells (1 per 1500 cells) appearing at the ganglion cell layer was seen in the chick retina from the 15th day of incubation and all subsequent stages studied.

NPY

The occurrence, numbers and distribution of NPY-IR neurones in the chick retina were found to increase with age of development. The first appearance of NPY immunoreactivity was in some cellular bodies at the INbL, on the 13th day of incubation (S-39). The morphology and position of these IR neuroblasts resembled those of undifferentiated postmitotic cells, which migrated to the inner portion of the INbL. They were small isolated cells (25 µm), relatively few in number and more frequent in the central retina (1 per 1000 cells per field in central retina, 1 per 2000 cells per field in peripheral retina).

On the 17th day of incubation (S-43), IR cell bodies were seen within the limits of the inner portion of the INL. These IR-neuroblasts presented a similar position and typology to differentiated amacrine cells. At this stage, the differentiation process of the amacrine cells was in progress and the dendritic prolongations were penetrating the IPL to establish synaptic relationships with ganglion cells (Layer and Makovitzky, 1990; Mey and Thanos, 1992; Marquardt and Gruss, 2002; Prada et. al., 2002). The IR-amacrine cells were not widely distributed (1 per 1000 cells per field in central retina); they appeared isolated and spatially arranged (every 2

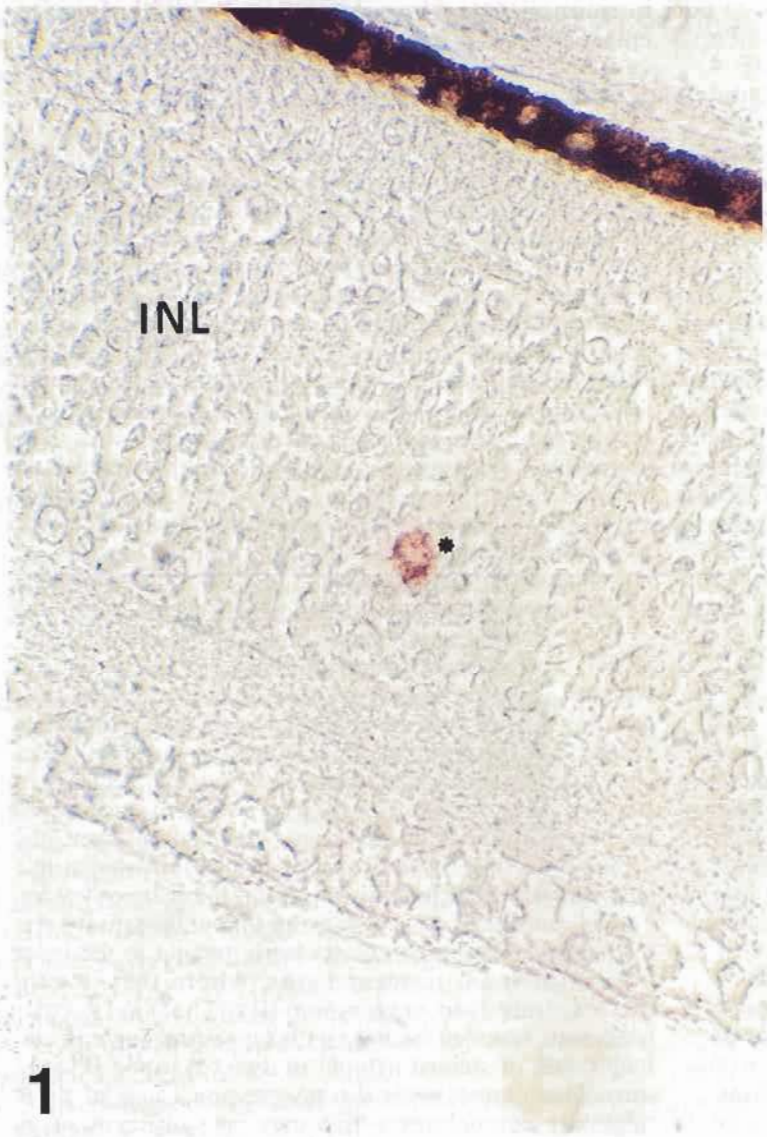

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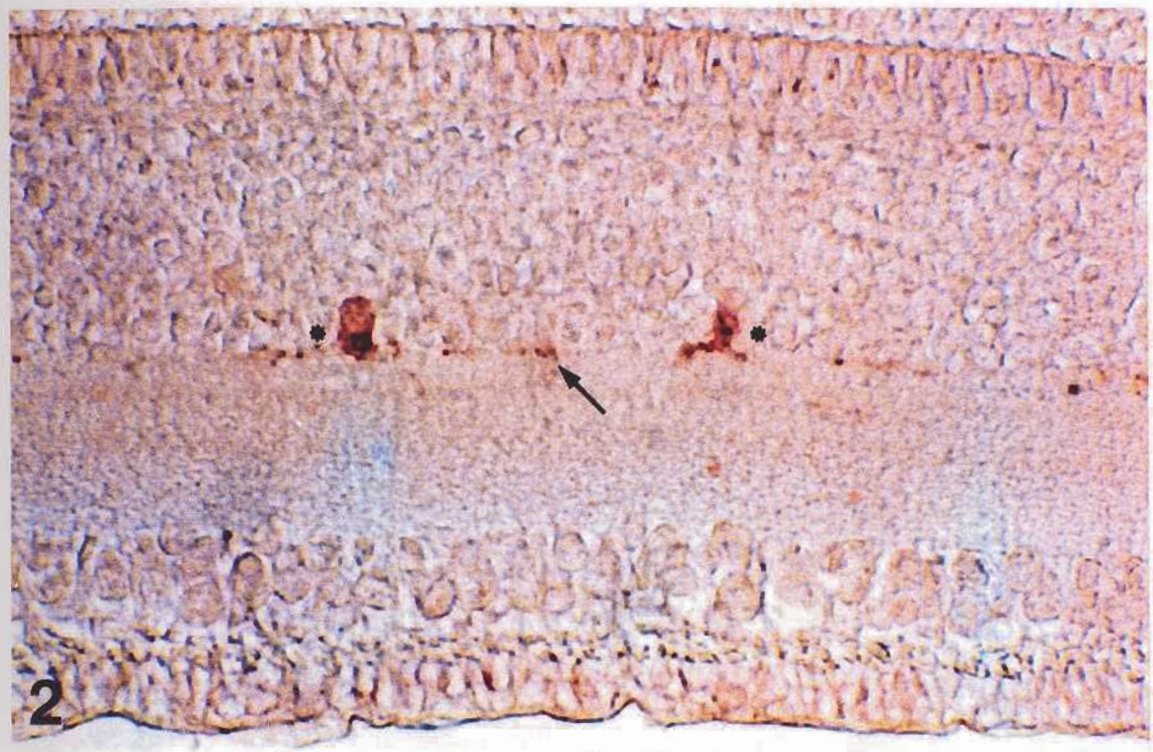
Fig. 1. Isolated Glucagon-like IR neuroblast (*) in the inner nuclear layer (INL) of embryo chicken retina at the15th day of incubation. x 630

mm). These neuroblasts possessed IR neuritic processes, which divided in close proximity to one to another at sublaminae 1, 2 and 5 of the IPL.

At S-44 (19th day of incubation), NPY immunoreactivity was present in a few cell bodies in amacrine cells and at the ganglion cell layers (especially



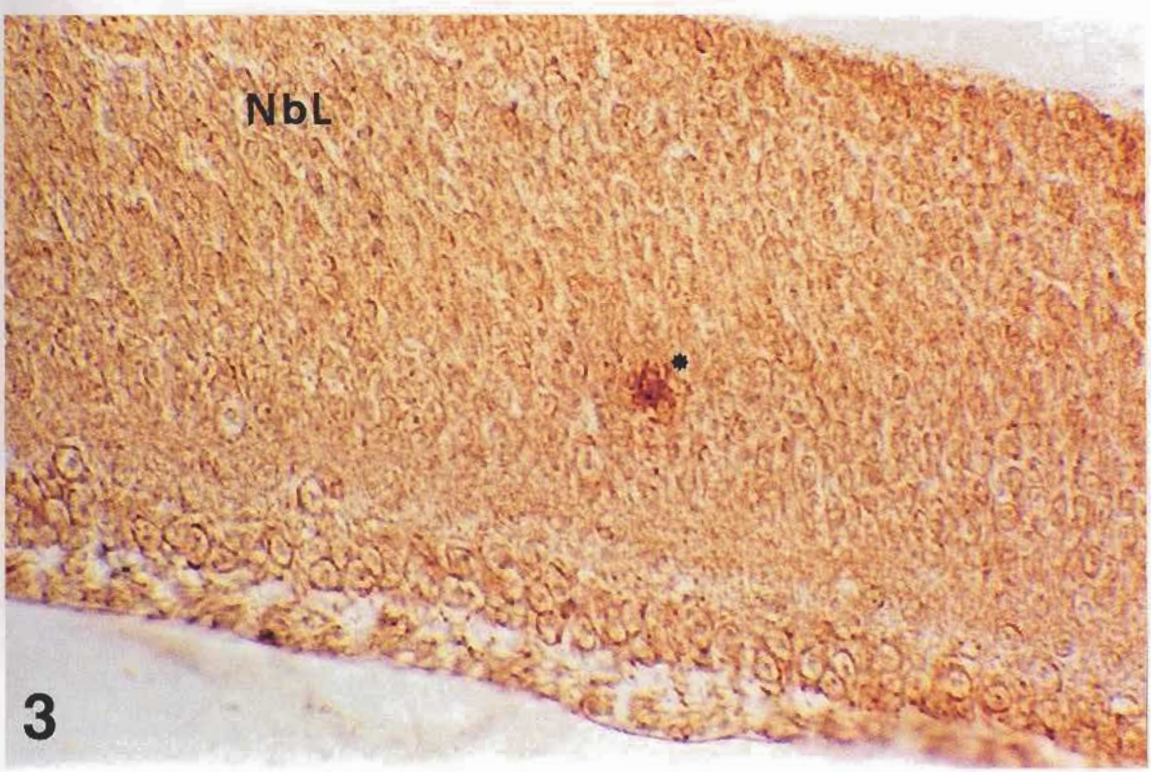


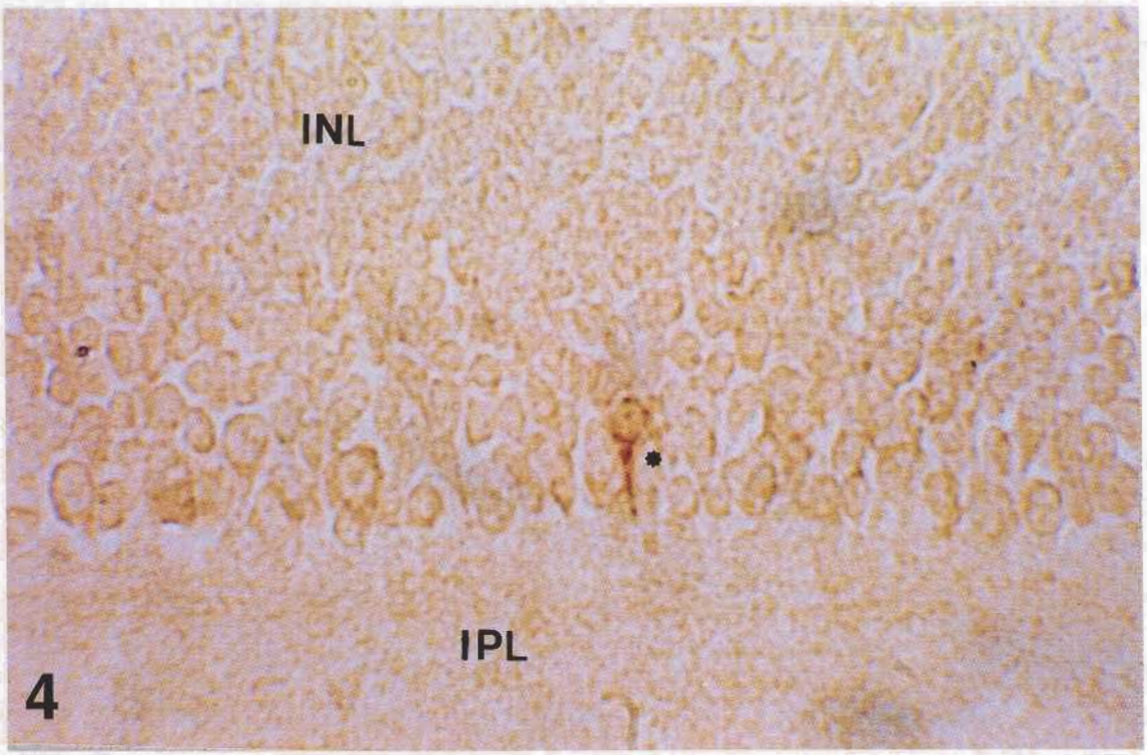
Fig. 2. Pre-hatching chicken retina. Glucagon-like immunoreactive amacrine cells (*) with varicose fibres in sublamina 1 (arrow) x 630

Fig. 3: Substance P-like immunoreactive neuroblast (*) in the primitive neuroblastic layer (NbL) of embryo chicken retina at the 7th day of incubation. x 630

in the larger sized cell of ganglion cell layer of about 48mm). The same immunoreactive pattern was shown at the IPL (Figs. 5, 6).

Somatostatin

The somatostatin-like immunoreactivity was



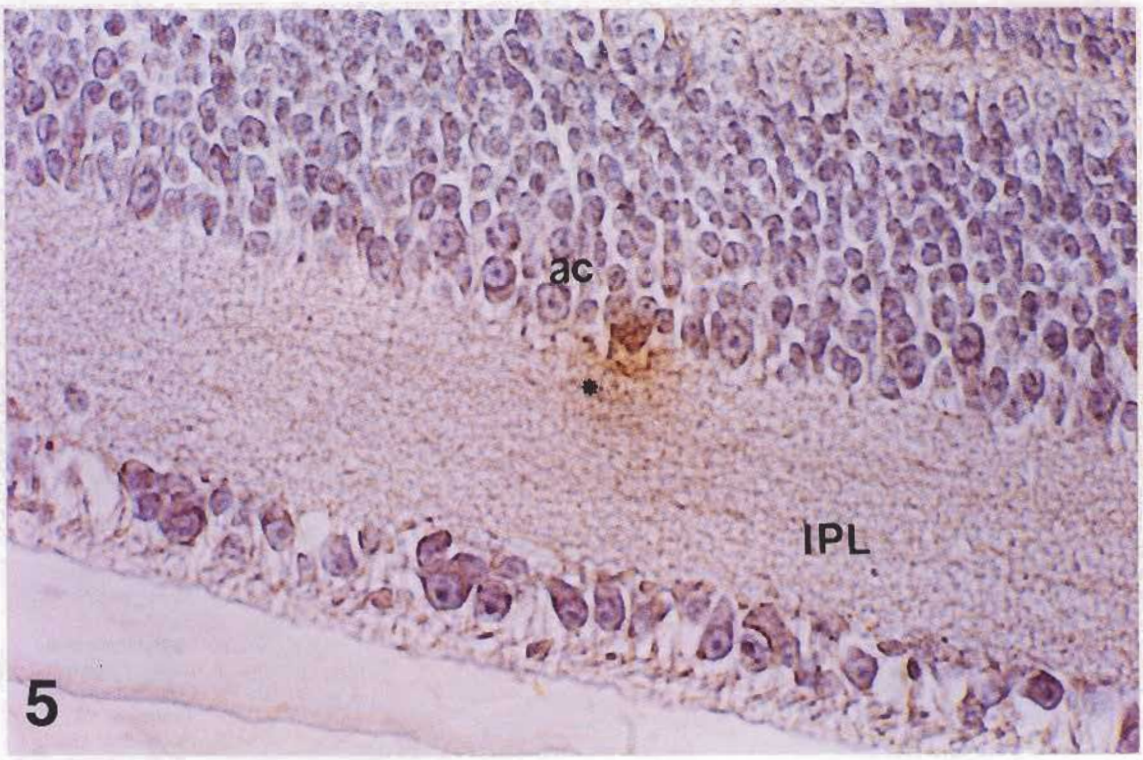
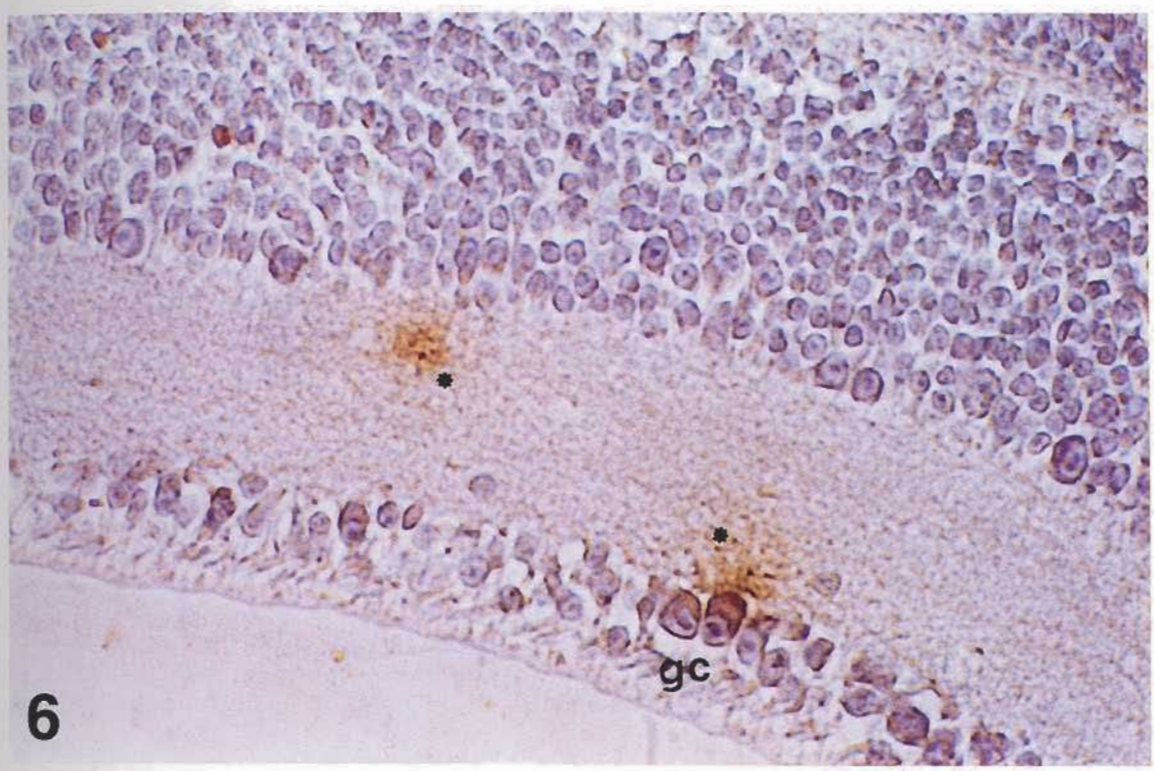


Fig. 4: Substance P-like immunoreactive amacrine cell (*) in the inner part of inner nuclear layer (INL). It possesses an immunoreactive straight process toward the inner plexiform layer (IPL). Primitive neuroretina at the 15th day of incubation. x 630

Fig. 5. NPY immunoreactive amacrine cell (ac) with a finely immunostained arborisation (*) in the outer portion of the inner plexiform layer (IPL). Chicken embryo retina at the 19th day of incubation. Counterstained with haematoxylin. x 630

detected as early as stage 37 of embryonic development. Specifically, stained structures were observed exclusively in the innermost level of the inner neuroblastic layer at this stage. The number of immunoreactive cells was fewer than that found in later stages, but they were more frequent in the central retina



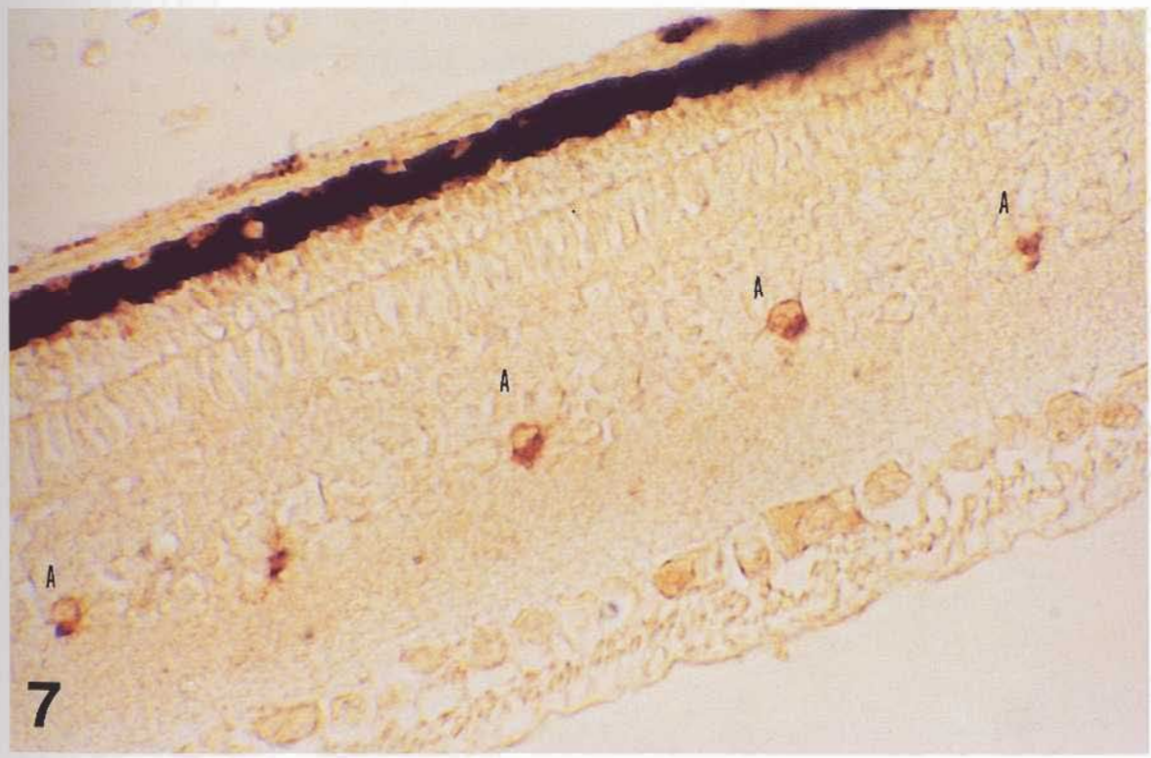


Fig. 6. Chicken embryo retina at the 19th day of incubation. NPY-like immunoreactive ganglion cell (gc). Immunoreactive arborisations at the sublaminae 2 and 5 of the inner plexiform layer are observed. Counterstained with haematoxylin. x 630

Fig. 7. Pre-hatching chicken retina. Somatostatin-like immunoreactive amacrine cells (A) orderly located at the inner border of the inner nuclear layer, which has a similar cellular morphology. x 630

(1 per 1000 cells per field).

On the 13th day of incubation, immunoreactivity appeared in isolated cellular bodies at the ganglion cell and inner neuroblastic layers. Arborization of the processes from the cell soma was rare, and no immunoreactive fibers were detected at this stage. Those IR cells were medium-sized neuroblasts of about 40 µm in diameter.

The distribution pattern of SMT-IR cells in later stages showed that these cells in the INL were moving from the outer to the inner part of this layer and, on the last day of incubation, the SMT immunoreactivity was exclusively present in amacrine cells (Fig. 7). It was possible to distinguish direct processes from amacrine cellular bodies towards the IPL. The SMT immunoreactivity appeared as fine positive granules in the first three sublaminae of the IPL, and was strongest in sublamina 3 (Fig. 8). Amacrine-IR cells were of large volume (45-60 µm), with an unstained nucleus and they were spatially and uniformly located (separations of 90-320 µm, with no center-to-periphery gradient observed).

The SMT-IR structures increased in number at the ganglion cell layer. The rate of increase was particularly high between the 13th and 15th days of incubation (from 1 per 2000 cells per field, to 1 per 1000 cells per field) and decreased progressively until time of hatching (from 1 per 1000 cells per field, to zero). These periods were also associated with the histological arrangement of the IPL, and the increasing and decreasing phases showed a center-to-periphery gradient.

CGRP

Embryonic chicken retina showed a few scattered structures exhibiting CGRP-like immunoreactivity (1 per 2000 cells per field). These were detected as early as the 13th day of incubation in the whole inner neuroblastic layer. From the 13th to 15th day of incubation, the number and intensity of immunostaining of the CGRP-IR cells increased (from 1 per 2000 to 1 per 1500 cells per field, with a center-to-periphery gradient). We specifically observed stained neuroblasts at the inner nuclear and ganglion cell layers. Those IR-neuroblasts present at the INL resembled migrating undifferentiated neuroblasts, which had the capacity to become amacrine cells. They had a rounded stained body, with unstained cytoplasmic cone directed near the inner portion of the INL (45-50 µm in size). Meanwhile, the CGRP-IR cells present in the ganglion cell layer had finished their process of differentiation. They had a voluminous nucleus (70 mm) and were related to the amacrine-differentiating cells.

On the 15th day of incubation, CGRP-IR cells continued to increase considerably in number (1 per 500 cells per field, more frequent in central retina) and were particularly numerous in the ganglion cell layer. We noted CGRP-IR cells in the INL, which presumably were amacrine cells. From S-43 to immediately after hatching, the CGRP-IR cells seemed to have decreased in number and they were sparsely distributed (separations of 400-500 µm), lightly stained amacrine

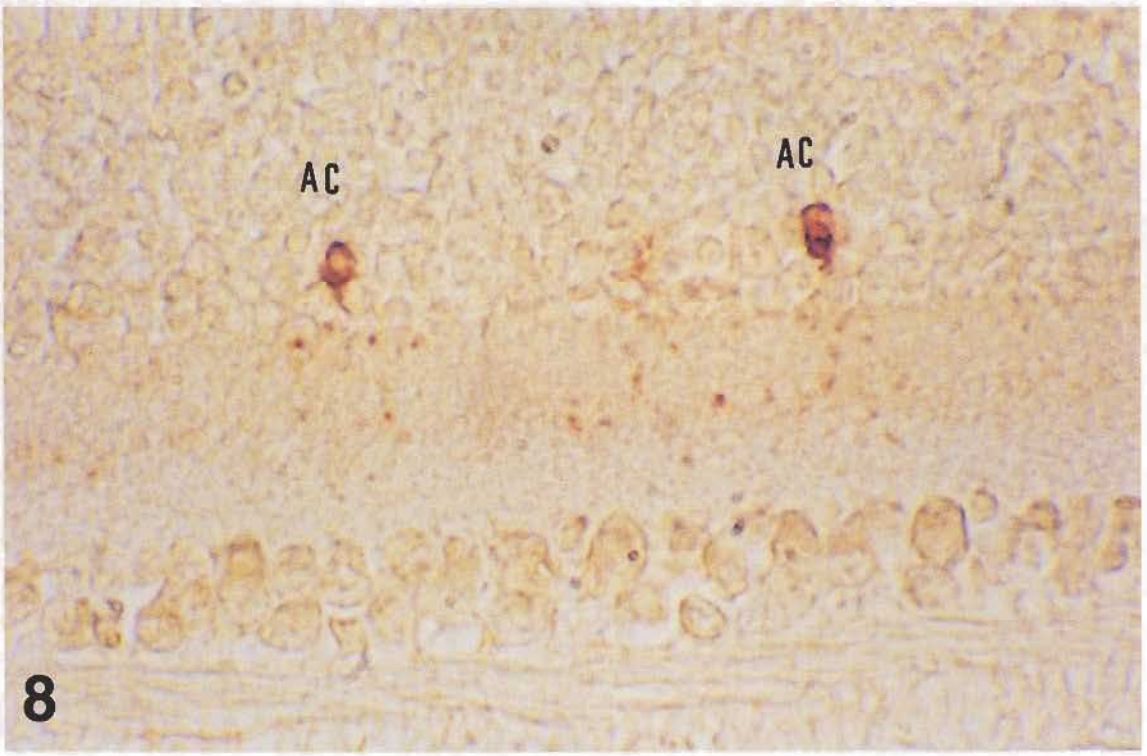
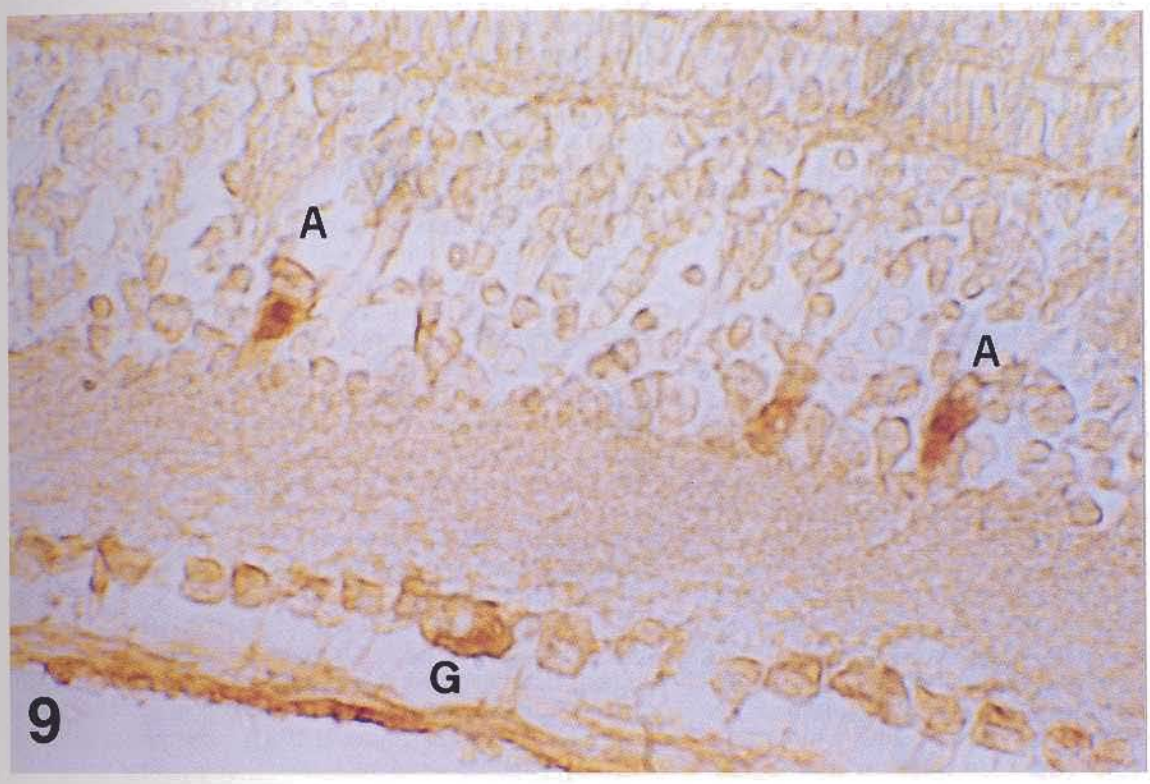


Fig. 8. With respect to Somatostatin-like immunoreactive amacrine cells (AC), we can observe a fine positive granulation in the sublaminae 1, 2 and 3 of the IPL that is most intense in sublamina 3. x 630

and probably ganglion cells (Fig. 9). We did not observe specific staining in dendritic prolongations at the IPL.

VIP

The maximum number of VIP-IR structures was



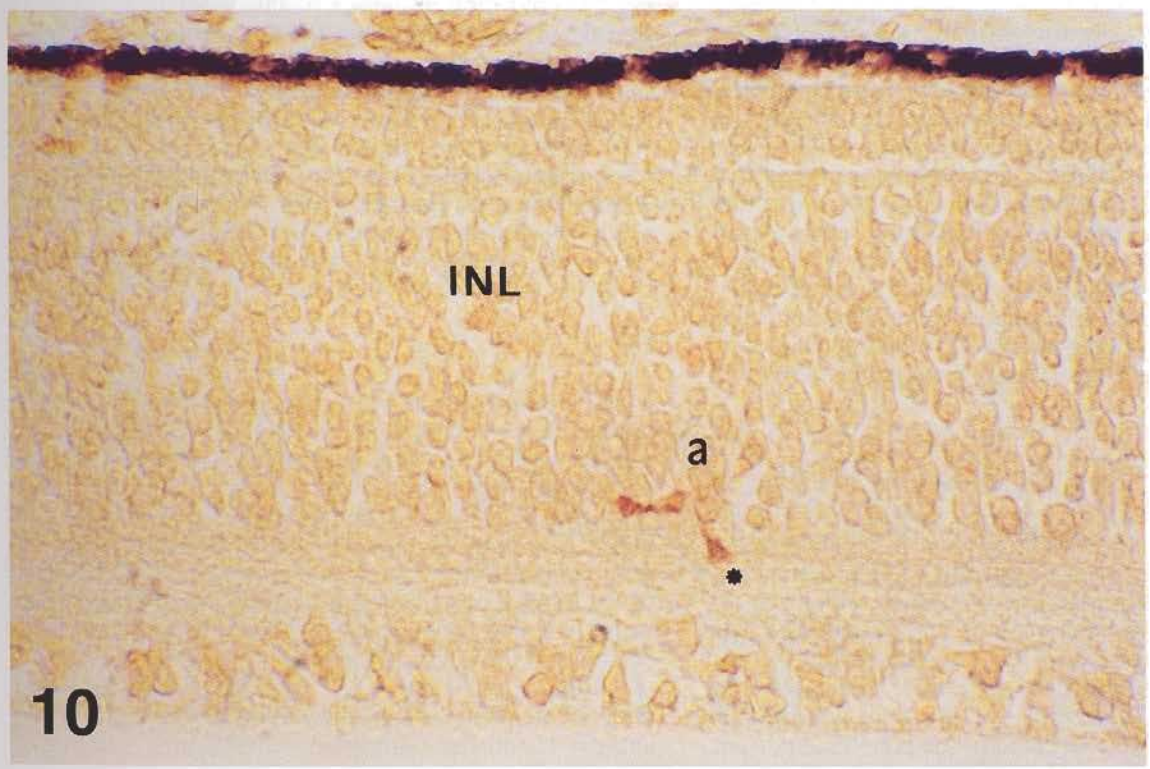


Fig. 9. Embryo chicken retina at the 21st day of incubation. CGRP-like immunoreactive amacrine (A) and ganglion cells are observed (G), sparsely distributed and lightly stained. x 630

Fig. 10. Embryo chicken retina at the 15th day of incubation. We find immunoreactive VIP-like neuroblasts in the inner border of the inner nuclear layer (INL), resembling differentiating amacrine cells (a). These neuroblasts possess a straight immunoreactive process toward the inner plexiform layer (*). x 630

seen in the retina of the chicks at the end of the second week of incubation. On the 13th day of development, VIP-IR cells were located deeper in the INbL. These IR neuroblasts were undifferentiating postmitotic cells that comprised the INbL before their migration process. On the 15th day of incubation, VIP-IR cells were moving from the medial to the inner part of the INL.

It was possible to observe some isolated neuroblasts with a single short process reaching down into the IPL, where they seemed to locate at the outermost part of this layer (Fig. 10). The typology and disposition of these IR-cells resembled that of recently differentiated amacrine cells, which in this stage of organization were beginning to make definitive contact with the ganglion cells at the IPL. From this stage to the end of the incubation period, the VIP immunoreactivity decreased notably in intensity.

Discussion

Glucagon

The immunocytochemistry and distribution of glucagon in the adult chicken retina have previously been described by Katayama et al. (1985a). These authors showed that GLC-IR cell bodies were present only in amacrine cells of the INL. The cells give off processes to the C1, C3 and C5 sublayers of the IPL.

Our findings are that GLC immunoreactivity does not appear to be present in the chicken retina prior to embryonic day 15 (S-41). Immunoreactive amacrine cells were observed from this stage to the end of development. Some cells possessed a single-labeled fiber oriented inwardly toward the C1 sublayer of the IPL, but we did not find immunoreactivity in any of the other sublayers. There are several reports on the appearance of glucagon immunoreactive structures in embryo chicken neuroretina (Fischer et al., 1998, 1999; Feldkaemper et al., 2000). These authors proposed that the glucagon-containing amacrine cells are associated with the growth of the eye, by mediating lens-induced changes. From results previously reported, it had been proposed that these changes take place in late embryonic stages. Thus, we think this induction process must be produced early in the development, based on this early appearance of immunoreactive amacrine cells for glucagon.

Substance P

Amacrine cells showing substance P-like immunoreactivity have been reported in chicken retina by many authors (Ishimoto et al., 1982; Millar and Chubb, 1984; Katayama et al., 1985a,b). Tahara et al. (1986) examined the ontogeny of the system with SP-like immunoreactivity in the chicken retina by the indirect immunofluorescence method. These authors reported that amacrine cells containing SP immunoreactivity first appeared in the peripheral portion of the developing retina at embryonic day 7, and that no immunoreactive fibers were detected at this stage.

At S-8 the SP-IR cells increased markedly in number, and SP-IR fibers first appeared in the periphery. Between S-10 and S-14, the SP-IR cells were located at the inner part of the INbL and the fibers formed a fiber plexus at the C1, C3 and C5 sublaminae. At S-19, inward cell migration had almost finished. The maximum number of SP-IR fibers in the IPL was seen in the retina of the chicks immediately after hatching. In addition, our results confirm the presence of SP-IR amacrine cells, which were similar in morphology and topographical distribution to those observed by Tahara et al. (1986).

However, we did not see dendritic processes extending from SP-IR cell somata into the IPL. We believe that the immunofluorescence method used by Tahara et al. allowed them to observe fine varicose immunoreactive fibers. However, these IR-fibers are difficult to observe with optical microscopy and they are usually beyond the resolution limits. May be embeding and deembeding paraffin techniques it is known as a cause to reduce the antigen accessibility.

In the adult chicken, numerous SP-IR cells are detected at the innermost portions of the INL (Tornqvist et al., 1981; Ehrlich et al., 1987). In the ganglion cell layer, a population of moderately labeled SP-IR neurones is also present (Tornqvist et al., 1981). These authors demonstrated that immunoreactive fibers were distributed in the sublaminae 1, 3 and 5 of the INL, and were most dense in lamina 1. It has been proposed that these subpopulations of substance P-containing ganglion cells extend their synapses to the superficial layer of tectal neurons (Yamagata and Sanes, 1995). The similarity between the latter stages of embryonic development, and the adult chicken retina is significant.

Substance P has been reported to be an important cell proliferation factor for the retinal pigment epithelium (RPE) in the human retina (Kishi et al., 1996). This is in agreement with our results, where we observed SP-immunoreactivity as early as the 5th day of incubation. The SP would stimulate the RPE growth by autocrine and paracrine methods.

NPY

The occurrence and distribution of NPY-IR neurones in the chick retina were similar to those of other neurotransmitters/neuromodulators commented on previously in our results. We showed NPY immunoreactivity in apparent ganglion cells and amacrine cells, from the intermediate stages of development to the end of the embryonic period. Many of the labeled cells observed between the 13th and 15th embryonic days were in the process of migration to their positions in the innermost layers.

Brunn et al. (1986, 1993) have previously described NPY-IR neurones in the adult chicken retina. These authors found discontinuous NPY-IR nerve fibers in

sublaminae 1, 3 and 5 of the IPL, composed of very fine varicose processes. Cell bodies displaying NPY immunoreactivity were seen in the inner half of the INL. In the present study we have found that the occurrence, numbers and distribution of NPY-IR neurones in the developmental chicken retina increased in line with age of development. NPY-IR neurones in both the latter embryonic days and the adult chicken showed similar patterns of distribution.

NPY immunoreactivity has previously been described in the retinal amacrine cells and IPL of several species (Brunn et al., 1984, 1985; Brunn and Ehinger, 1993; Osborne et al., 1985). However, there is considerable variability in the immunological staining in different species, with different cytological patterns (Brunn et al., 1986; Brunn and Ehinger, 1993).

We have found no previous references to NPY-IR structures in embryonic retina. This neuromodulator has not been implicated as a differentiating or growth factor. On the other hand, it is well known that NPY is widely distributed in the central nervous system, as well as playing an important role in blood flow through its vasoconstrictor property, but the chicken retina is a non-vascular tissue. We think the major NPY-IR presence found during the 17th and 19th days of incubation must be associated with the establishment of contact between amacrine and ganglion cells at the IPL. NPY is not a proliferation factor for RPE in the embryo chicken retina (Kishi et al., 1996)

Somatostatin

Tornqvist et al. (1982), Watt et al. (1985, 1991, 1994), Ishimoto et al. (1986), Hamano et al. (1989) and Li et al. (1990) reported the presence of amacrine cells containing SOM in the adult chicken retina. These cells send processes to the C1, C3, C4 and C5 sublaminae of the IPL.

Our results showed that the distribution pattern of SOM in late embryonic retina is similar to the distribution pattern in adult chicken. Labeled processes of amacrine cells did not ramify extensively, unlike in the adult.

There are few reports about SOM as either a trophic or a differentiating factor. This neuromodulator seems to be present during the differentiation process of amacrine cells. Therefore, its incidence may be related to this preliminary period when enzyme synthesis appears in the neuroblasts (Lankford et al., 1988).

CGRP

In the adult chicken retina, CGRP-IR cells are seen in the inner part of the INL. A single process from the soma ascends to the IPL, where it ramifies extensively (Kiyama et al., 1985b, Tohyama et al., 1987). Our immunocytochemical analysis of the maturation of CGRP-IR structures indicated that these cells appear as early as the 13th day of incubation in the whole inner neuroblastic layer. From the 13th to 15th day of incubation, the CGRP-IR cells increased in number and intensity, and this was particularly so in the ganglion cell layer. During the subsequent days, the CGRP-IR cells decreased in number. Immediately after hatching, the CGRP-IR amacrine cells increased again in number, but no labeled dendrites could be seen arising from the neurones in the IPL.

As with SOM, there was no evidence that this neuromodulator participates in growth stages.

VIP

Fukuda et al. (1981) investigated the distribution of vasoactive intestinal polypeptide-like immunoreactivity in the adult chicken retina. VIP positive cells were found throughout the chicken retina. Most of them were located in the proximal portion of the INL and the processes from these cells were directed to the IPL

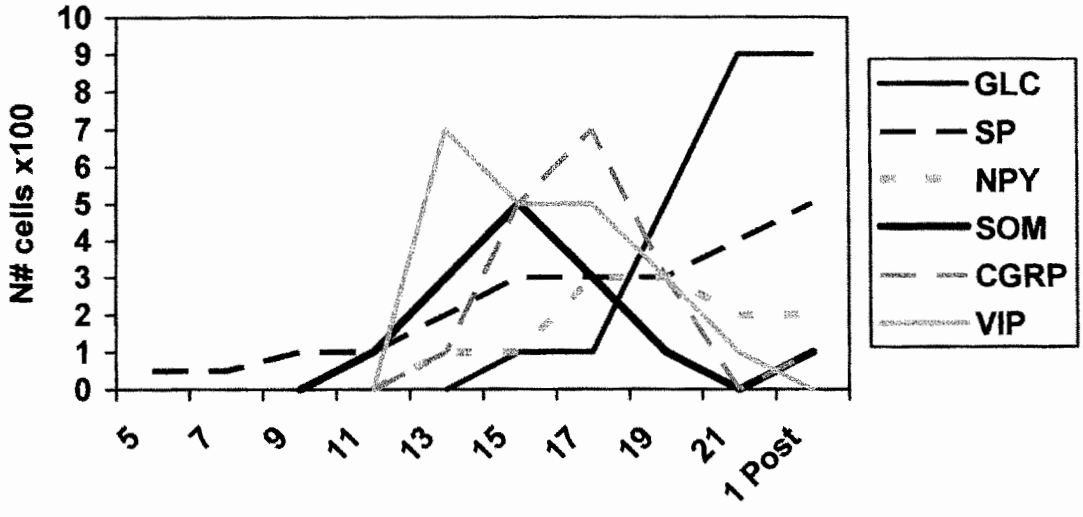


Table 1. Number of IR-cells per 100

where they ramified, suggesting that VIP-positive cells located in this region are probably amacrine cells.

We noted that, in the retina of the embryo chicken, the maximum number of VIP-IR structures was seen at the end of the second week of incubation. This is in agreement with the study made by Fernandez-Trujillo et al. in 1989.

Although the VIP positive neuroblasts were not especially abundant, the progressive formation of a pattern could clearly be seen, resembling that observed in the adult chicken retina by Lankford et al. in 1988 and other authors. On the 15th day of incubation, VIP-IR cells were moving from the outer to the inner part of the INL, and some short dendritic processes extending down into the IPL could be seen.

Recently a novel neuropeptide (pituitary adenylate cyclase-activating polypeptide, PACAP), related structurally to the VIP, has been described as an important differentiating and growth factor in the mammalian central nervous system (Nielsen et al, 1998; Waschek et al., 1998; DiCicco et al., 2000; Parisi et al., 2002) and human fetal retina (Olianas et al., 1997). VIP itself has been reported as a neurodifferentiating agent, with a potent influence in cell division in the early development of the central nervous system (Gressens et al., 1997). However, VIP did not appear in either the early optic cup or the mitogenic stages in the primitive neuroretina. For this reason, we consider VIP to be a differentiation factor for the later stages of differentiating amacrine cells, rather than having a premature mitogenic role.

As a general reflection on the results, we have shown a mechanism in which several neuromodulators, known to be present in adult chicken retina, appear progressively during the embryo neurodifferentiation process.

Broadly, these neurotransmitters/neuromodulators usually emerged during the second week of incubation (Table 1). They were related to neuroblasts that progressively migrated from the INbL to later occupy the inner portion of the INL. The IR-neurons had a typology, disposition, and neuritic prolongation that allowed us to identify them as amacrine cells. The IR-amacrine cells showed a tendency to increase during the IPL establishment stages, and in some cases stained apparent ganglion cells also appeared. Later, just prior to hatching, IR-neurones decreased until the post-hatching stage.

The incorporation of immunoreactive fibers in the IPL can be understood as a selection mechanism for the synaptic contacts that will be seen in the adult period. This hypothesis, ventured by Lankford et al. in 1988, considers it possible that neurotransmitters appear first in embryonic periods, before the synapses are functional, and that they could play an important role in neuronal differentiation.

Our results suggest that the neuropeptides present in the developing retina may play a formative role that precedes their functions as neurotransmitters/modulators, and that this is indicated by their early presence in retinal development.

With the exceptions found in this study, no research on chick retinal development has previously been reported, and to our knowledge, no neuropeptides apart from SP have so far been located in the embryo chick retina.

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