



## Neurochemical phenotype and birthdating of specific cell populations in the chick retina

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### ABSTRACT

The chick embryo is one of the most traditional models in developing neuroscience and its visual system has been one of the most exhaustively studied. The retina has been used as a model for studying the development of the nervous system. Here, we describe the morphological features that characterize each stage of the retina development and studies of the neurogenesis period of some specific neurochemical subpopulations of retinal cells by using a combination of immunohistochemistry and autoradiography of tritiated-thymidine. It could be concluded that the proliferation period of dopaminergic, GABAergic, cholinergic and GABAergic cells does not follow a common rule of the neurogenesis. In addition, some specific neurochemical cell groups can have a restrict proliferation period when compared to the total cell population.

**Key words:** developing, neurogenesis, neurotransmitter systems, ontogenesis, proliferation.

### INTRODUCTION

Cells in the chick retina are generated during specific periods in a regulated profile, and some cell populations overlap the periods of their neurogenesis, as occurs in other species (Marquardt and Gruss 2002, Martins and Pearson 2008). The chick retina has been widely used as a model for studying the development of the nervous system, particularly because: (a) it is a tissue where the access is simplified by the fact that it is located out of the neuro axis, although it is part of the central nervous system; (b) it has a pattern of synaptic organization and a development similar to other central structures; (c) it shows a highly ordered histological organization; (d) it has almost all, if not all, neurotransmitters found in the brain; (e) pre- and post-synaptic components of

the neurotransmitter circuitries are present in the tissue. Since the diversity of neurotransmitters is extremely high, and as the beginning of the expression of the machinery responsible for the synthesis, release and recognition of many neurotransmitters is not synchronous, different neurochemical phenotypes may be generated during the same proliferating periods or only during a restricted developmental interval. Thus, it is very important to associate the histogenesis of this tissue with the neurogenesis of specific cellular types.

### NEUROCHEMICAL DIVERSITY OF CELL TYPES IN THE CHICK RETINA

With transmitter uptake studies, immunohistochemistry or cytotoxic lesions, almost all known neurotransmitters and neuromodulators have been demonstrated to be present in the vertebrate retina (Ehrlich and Morgan 1980, Kiyama et al. 1985, Spira et al. 1987, Tung et

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al. 1990). In particular, the heterogeneous population of amacrine cells employs a wide spectrum of synaptic transduction modulators, including substance P, dopamine, enkephalin, vasoactive intestinal polypeptide, adenosine, glucagon, somatostatin, serotonin, avian pancreatic polypeptide, acetylcholine, neurotensin, neuropeptide Y, corticotrophin releasing factor, glutamate and GABA (Kiyama et al. 1985, Fukuda et al. 1981, Spira et al. 1987, Hokoç et al. 1990, Paes de Carvalho and de Mello 1982, de Carvalho et al. 1992, Gardino et al. 1993, Kalloniatis and Fletcher 1993, Thoreson and Witkovsky 1999, Sun and Crossland 2000).

The retina has two basic circuitries for light analysis: one formed by photoreceptors, bipolar and ganglion cells that represent the vertical pathway, and the other formed by horizontal and amacrine cells, called lateral pathway, which modulates the vertical pathway of visual information. At a glance, the retina seems to have simplified circuitries because of the small number of neuronal types composing the tissue (only five in the chick retinal tissue: photoreceptors, horizontal, bipolar, amacrine and ganglion cells) and one main glial type: Müller cells. However, it is not that simple. In general, morphological, electrophysiological and neurochemical parameters are used to classify the many cell subtypes of the retina. Each cell type subdivides into subtypes that make specific connections to other specific cells contributing to different functions of the retina. Even in the chick, both types of photoreceptors (cones and rods) are necessarily functional in different light conditions. There are at least four distinct subtypes of horizontal cells, and it has been suggested that there may be more than thirty different types of amacrine cells (Fischer et al. 1998b, 1999, 2005, 2006, 2007, Masland 2001, 2004).

#### HISTOGENESIS OF THE CHICK RETINA

The eyes arise from the posterior part of the forebrain, the diencephalon, while the optic tecta derive from the dorsal portion of the midbrain. An invagination of the primary optic vesicle leads to a bilaminated structure, the eye cup or the secondary optic vesicle. The neural retina derives from the thickened inner layer of this primordial eye, while the outer layer, in contact with aggregating mesenchyme that will later form the sclera and the choroid coat, also gives rise to the retinal pigmented

epithelium (RPE). At the beginning of retinal development all cells are multipotent (Hyer et al. 1998), and even embryonic day 4.5 (E4.5) epithelial cells can be induced to transdifferentiate into neural phenotypes (Coulombre and Coulombre 1965).

Proliferation of RPE ceases early while stem cells continue to divide in the presumptive neural retina. The basic patterns of mitosis, vertical cellular migration and differentiation resemble the processes found throughout the early neural tube (Mey and Thanos 2000). The pattern of mitotic activity and, consequently, the maturation of the retina, follow a central to peripheral and temporal to nasal gradient (Rager et al. 1993).

After the report of the embryological origin of the neural retina, morphological features that characterize each embryonic stage established by Hamburger and Hamilton (1951; referred as H-H) will be revised as follow.

#### EMBRYONIC DAY 3-5 (E5 = H-H STAGE 25)

Around E3 or E4, the prospective neural retina becomes invaginated and located apposed onto the future RPE appearing as a two-layered optic cup. The pigmentation of the eye begins after E3 (Hamburger and Hamilton 1951). At E4 or E5, Müller fibers are present in the fundic region and Müller endfeet begin to form the internal limiting membrane (Coulombre 1955). The cellular strata of the retina derive from several radial migrations and the cessation of these neuroblast movements at various levels. The structural differentiation is evident beginning on E4 when the prospective retinal ganglion cells (RGCs) round up, increase in size, and begin to send out the axons that will form the optic fiber layer (OFL). At E5, the tissue shows a ventricular zone and a neuroblastic layer (NBL) (Fig. 1A-B). The former, near the future RPE, contains mainly proliferating cells, and the latter contains cells with elongated shape that, after their final mitosis (in the ventricular zone), migrate towards the vitreal side and differentiate into neurons or glial cells. In the innermost neuroblastic layer (iNBL), many round shaped cells can be visualized near the vitreal side, mainly formed by RGCs. Newborn horizontal cells migrate bi-directionally from their site of birth, close to the ventricular side, to the adjacent (vitreal) side of the neuroepithelium. At this point, they

align just next to the prospective ganglion cell layer before migrating back again to their final laminar position in the external part of the future inner nuclear layer. The bi-directional migration occurs between Hamburger and Hamilton stages 24 and 33, which are equivalent to embryonic days 4.5 and 8 (Edqvist and Hallböök 2004).

Finally, in this period, a very high density of apoptotic nuclei appears in the neuroepithelium of the central retina representing the cell death related to early proliferative retinal stages (Chavarría et al. 2007).

#### EMBRYONIC DAY 6-7 (H-H STAGES 29-31)

Because of the intense early proliferation of ganglion cells cited above, at E6 the ganglion cell layer (GCL) is constituted of two or three rows of cell bodies (Fig. 1C-D) extending over most central retina (Coulombre 1955). A clear segregation of retinal strata begins at about E6 with the appearance of the GCL, OFL and NBL (Spence and Robson 1989, Meller and Tetzlaff 1976). The first axonal process from RGC begins to arrive at the optic tectum at E6 (DeLong and Coulombre 1965, LaVail and Cowan 1971). In this period, it has been described a low incidence of cell death in both NBL and RGC (Chavarría et al. 2007).

#### EMBRYONIC DAY 8 (H-H STAGE 34)

At E8, very distinguishable from early retinas, the inner plexiform layer (IPL) appears in the central region of the retina (Fig. 2A) even though the GCL still appears with three rows of cell bodies. Furthermore, Müller fibers and the outer limiting membrane are found throughout the neural retina as can be observed with 2M6, a glial marker specific for chick retina (Schlosshauer et al. 1991) (Fig. 2B-C). The two plexiform layers of the mature retina result from neurite growth and dendritic branching that takes place during all the embryonic life.

#### EMBRYONIC DAY 9-10 (H-H STAGES 35-36)

The retina of animals on the 9<sup>th</sup> day of incubation is very typical because the outer plexiform layer (OPL) makes its appearance (Fig. 2D-E). Besides that, between E9 (Fig. 2D-E) and E10 (Fig. 2F-G), displaced amacrine cells occupy a transitory intraplexiform position in the IPL, forming a discontinuous one-cell-thick layer (Fig. 2D-G; Génis-Gálvez et al. 1977). These authors sug-

gested that the unusual position of the displaced amacrine cells in the IPL could be related to a growth in the thickness of the IPL between the displaced amacrine and the ganglion cells during the 9<sup>th</sup> and 10<sup>th</sup> days of incubation. This would lead to their appearance in an intraplexiform position. Then, the ulterior rapid elongation of a cytoplasmic stalk between the bushy arborization and the soma explains the apparent disappearance of those cells in that intraplexiform position, and the displaced amacrine cells become mixed with the ganglion cells. Thus, as we can see in Figure 2 (D-E), on the 9<sup>th</sup> day of incubation, the cell bodies are in the center of the IPL and, on the 10<sup>th</sup> day, the nucleus adopts a position nearer the GCL (Fig. 2F-G). Finally, it is not before E9 that photoreceptor neuroblasts show a round shaped apical process beyond the outer limiting membrane into the subretinal space (Fig. 2D-E), and the extension of inner segments of photoreceptors is the first indication of their morphogenesis (Olson 1979, Meller 1984 *apud* Mey and Thanos 2000).

At E10-E11, the number of ganglion cell axons is estimated at 4 million, but 40% of them will be eliminated up to E18 and, then, remain constant (Rager and Rager 1978). Probably because of the peak of cell death period, at E9, GCL does not appear with three rows of cell bodies anymore (Fig. 2E; Cook et al. 1998, Chavarría et al. 2007).

#### EMBRYONIC DAY 11-13 (H-H STAGES 37-39)

Comparing two different ages, at E10 and E13 (Figs. 2F-G and 3A), the IPL increases in thickness and it will continue to grow, presumably up to adult life (Fig. 4). In addition, displaced amacrine cells are not in an intraplexiform position anymore, and are present in the GCL after E12 or E13 (Hamburger and Hamilton 1951). Concerning the programmed cell death, the density of TUNEL-positive nuclei shows a new increase affecting in the inner nuclear layer (INL) with a peak at E11 (Chavarría et al. 2007). At E12, all axonal processes from RGC reached the optic tectum. Soon after, at E13, the GCL contains mainly one row of cells (Fig. 3A-B) due to cell death, as mentioned above. At the same day, on E13, the first functional synapses are observed in the IPL (Hughes and LaVelle 1974, Hering and Kröger 1996). Photoreceptors mature late in the retinal devel-

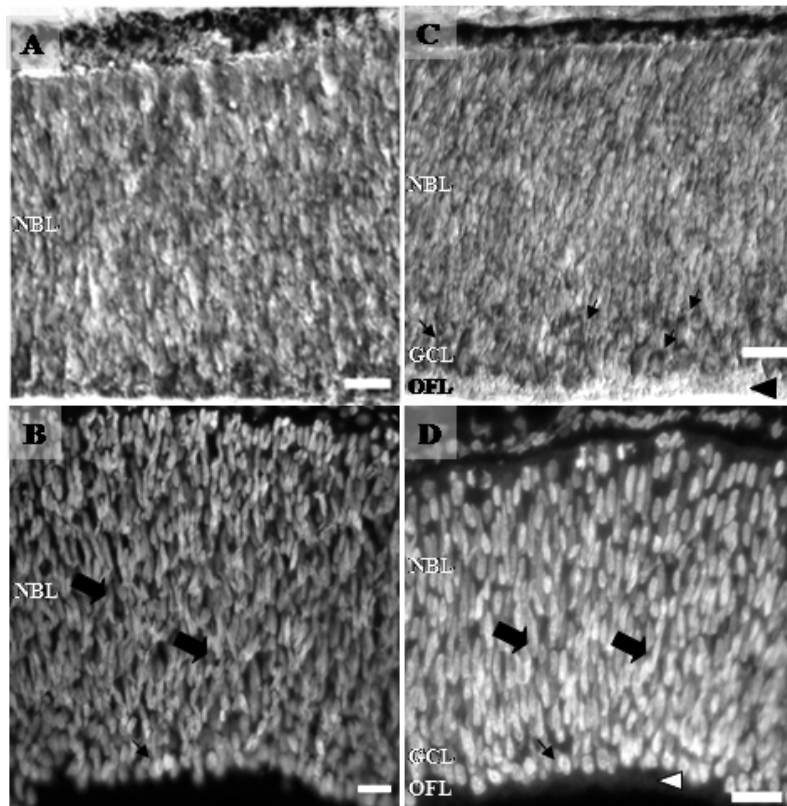


Fig. 1 – Photomicrographs of retinal sections from E5 (A and B) and E6 (C and D). Retinal sections stained for cresyl violet (A and C) and DAPI (B and D). In C, arrows point to round shaped cells of the future ganglion cell layer (GCL), and black arrowhead shows the optic fiber layer (OFL) region. In B and D, elongated (thick arrows) and round shaped nucleus (thin arrows) in separate layers; neuroblastic (NBL) and presumptive ganglion cell layer (GCL) can be visualized. In D, white arrowhead points to the OFL. Calibration bars = 20  $\mu\text{m}$ .

opment. The first receptor potentials are detected only after E13, and with the progress of the embryonic age, their increasing amplitude could be correlated with the appearance of photoreceptor outer segments that begin on the 15<sup>th</sup> day (Meller and Tetzlaff 1976, Hanawa et al. 1976).

#### EMBRYONIC DAY 14-16 (STAGE H-H 40-42)

Around E14, horizontal cells undergo some morphological changes and, from one of their main dendrites, a single axon arises of these cells. With Golgi silver impregnation, synaptic spines can be distinguished 2 days later on dendrites and axon terminals (Génis-Galvéz et al. 1981). In parallel, oligodendrocyte precursors are observed in the retina around E14 and one day later oligodendrocyte processes multiply and begin to envelop thicker axons. However, as at E18, only 3% of the axons of the optic nerve are ensheathed, and most of the

myelination takes place during the early postnatal period (Rager 1976).

It is on the 15<sup>th</sup> day that rod and cone outer segments appear (Fig. 3C, white arrow), and both photoreceptor types begin a very rapid increase in length. Although the extension of RGCs spine-like process to the IPL begins after E7, the morphological development of these cells is completed around E16. From this age, the retina tissue would only increase in complexity and thickness.

#### LATE DEVELOPMENT TO POST-HATCH

The chicken retina contains one type of rods and four types of cones, each characterized by an opsin with different spectral absorbance: rhodopsin and red-, green-, blue-, and violet-cone opsin (Araki et al. 1990, Okano et al. 1992). In Figure 4(A), in post-hatched retinas, it is possible to see the outer and inner segments of photore-

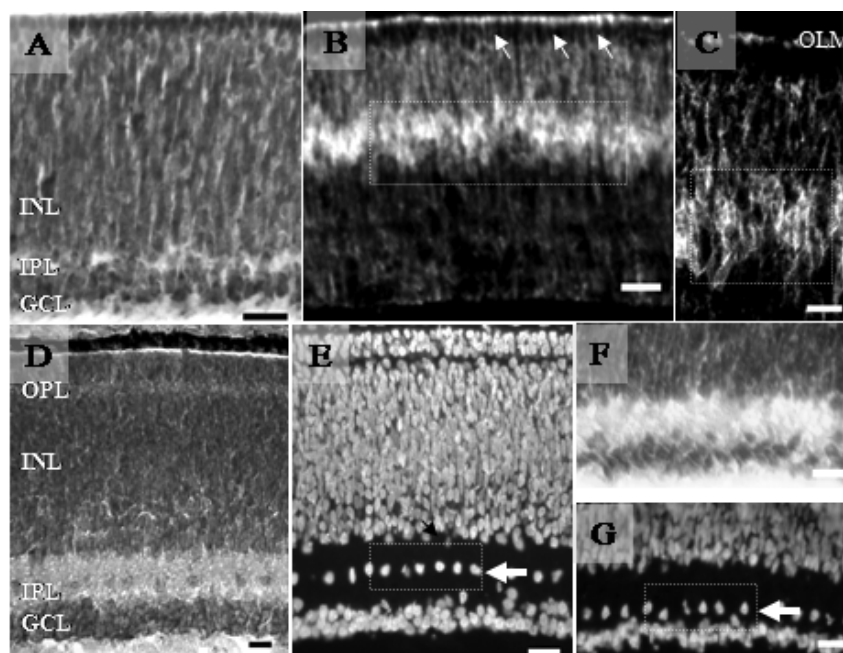


Fig. 2 – Photomicrographs of retinal sections from E8-E10 animals. In A, retinal section from E8 stained for cresyl violet (A) and immunoreactivity to 2M6, a specific Müller cell marker of the chick retina (B and C). In A, a clear segregation between the GCL and the inner nuclear layer (INL) is observed by the presence of the presumptive inner plexiform layer (IPL). In B and C, white arrows point to Müller cells endfeet, and soma cells take the middle region of INL (white rectangle). At E9 (D and E) and E10 (F and G), retinal sections stained for cresyl violet (D and F) and DAPI (E and G). At E9, the future outer plexiform layer (OPL) is present, turning the retina into a three nuclear layer tissue. The displaced amacrine cells in the characteristic intraplexiform position in the IPL, forming a discontinuous one-cell-thick layer in both ages (E9 and E10, white rectangles and arrows). Note the difference of displaced amacrine cells position in E9 and E10. OLM, outer limiting membrane. Calibration bars = 20  $\mu$ m.

ceptors. The chick retina has a specialized area (Ehrlich 1981), called area centralis, functionally similar to the primate fovea with the highest visual acuity enriched in red and green cones and no rods (Cepko 1996). During the development, red and green opsins are expressed first (E14), then rhodopsin follows (E15), and finally the blue and violet opsins (E16) (Bruhn and Cepko 1996). The development of rod and cone photoreceptors is similar, although rod outer segments are formed earlier. First, synapses between bipolar cells and photoreceptors are not observed in the OPL before E17 (Hughes and LaVelle 1974, Meller 1984 *apud* Mey and Thanos 2000). In accordance to that, the normal electrical response to light stimulation develops between E17 and P3 (Hanawa et al. 1976). In fact, the electroretinogram shows a mature profile only at E19. At that time, 2 days before hatching, light evokes beak clapping and movements of the eyelids (Oppenheim 1968, Rager 1979 *apud* Mey and Thanos 2000). Concerning to the cell death,

none TUNEL-positive nuclei are seen in the GCL and INL at E16 and E18, respectively (Chavarría et al. 2007).

Finally, in post-hatched retinas, the outer limiting membrane (OLM) (Fig. 4A, thick arrow) and some cells in the middle of IPL, called intraplexiform cells (Fig. 4B-C), can also be visualized.

#### NEUROGENESIS OF RETINAL CELLS

As discussed above, retinal neuroblasts leave the cell cycle in a specific order with respect to their prospective cell type: the first to be generated are retinal ganglion cells, followed by photoreceptors, then amacrine cells, horizontal cells and bipolar cells, which are the last neurons to leave the cell cycle in the chick retina. The production of photoreceptors continues over a longer period (Prada et al. 1991, Fekete et al. 1994, Meller 1984 *apud* Mey and Thanos 2000). Those specific sequences of cell proliferation are quite conserved in several vertebrate species (Marquardt and Gruss 2002, Martins and

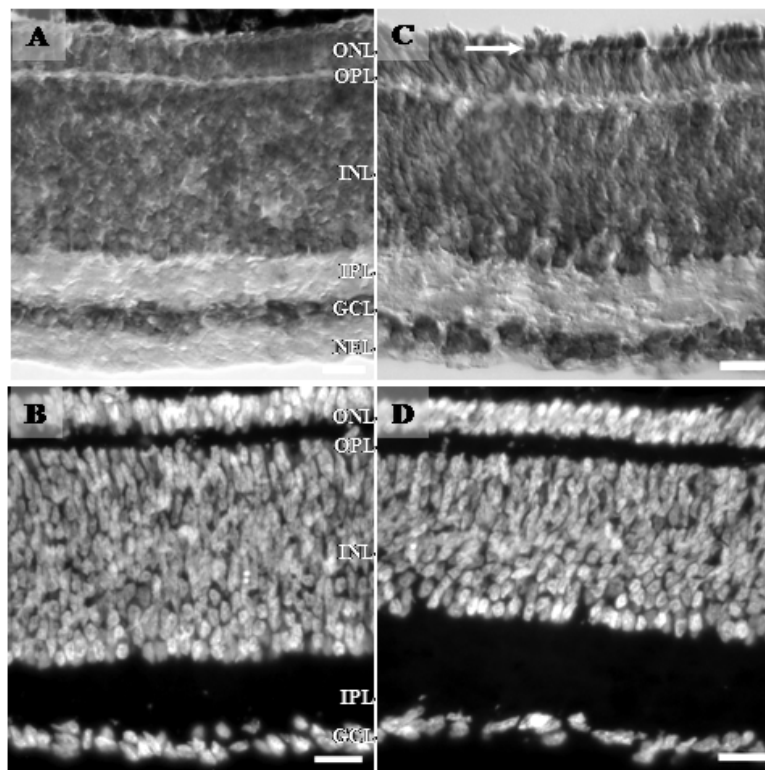


Fig. 3 – Photomicrographs of retinal sections from E13 (A and B) and E15 (C and D) stained for cresyl violet (A and C) and DAPI (B and D). In C, an arrow points to the outer segments of photoreceptors. Note the round shaped cells in the innermost regions of the INL and the growing of the plexiform layers. GCL is almost constituted by a one cell row (C and D). Calibration bars = 20  $\mu\text{m}$ .

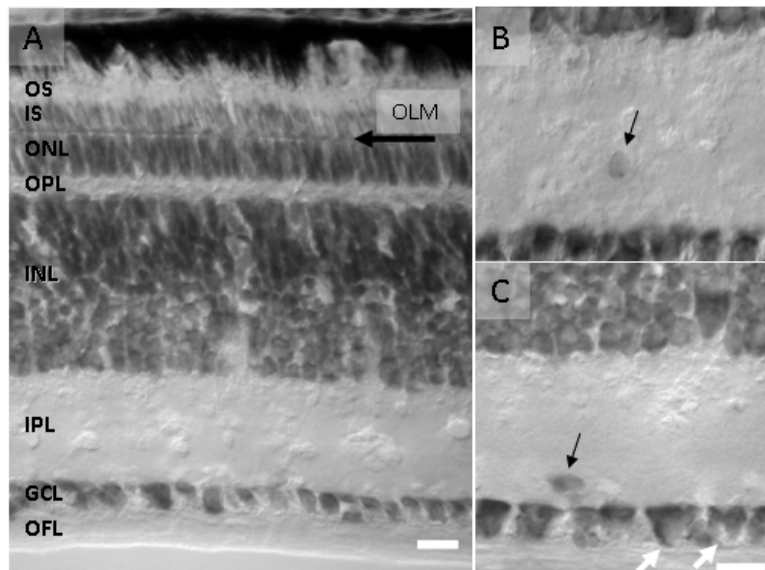


Fig. 4 – Photomicrographs of retinal sections of post-hatched animals stained for cresyl violet. In A, all retinal layers are clearly visualized, including the inner and outer segments segregation. An arrow points to the OLM as a line separating the ONL and the photoreceptors segments. In B and C, rounded cells (arrows) rarely observed in the middle of the IPL, called intraplexiform cells. GCL is constituted by a one cell row with cells of different sizes (white arrows). Calibration bars = 20  $\mu\text{m}$ .

Pearson 2008). In addition, the neurogenesis sequence occurs in a stereotypical order, but shows a strong overlap (Kahn 1973, Spence and Robson 1989, Prada et al. 1991). However, a question remains to be clarified: would there be a specific temporal order for neurogenesis of different neurochemical subpopulations of a specific cell type in the chick retina? Efforts to answer this question have been made through studies involving the neurogenesis of dopaminergic, GABAergic, cholinergic and GABAergic cells using the association of immunohistochemical and autoradiography of tritiated thymidine techniques in the chick retina. The main methodology to evaluate neurogenesis is the cumulative approach, developed by Fujita and Horii (1963). Originally, it was described with the use of [3H]-thymidine as a tracer. Nowadays, this problem can also be approached with the use of BrdU (Farah 2004). Either 3H-thymidine or BrdU have to be applied for a prolonged period to label all the cells that go through S-phase after tracer administration. So, for neurogenesis in avian retina, the tracer is injected once *in ovo* on different embryonic days (E1-E11), and the tissue is dissected at the end of the incubation period (around E19) when the retina is considered to be morphologically mature and the programmed cell death is finished. After that, retinas are analyzed for the presence of the tracer, and two populations are found: labeled cells (those that passed through S-phase while the tracer was available) and unlabeled cells (those that completed their terminal S-phase before the initiation of the cumulative labeling).

#### BIRTH DATING SPECIFIC RETINAL CELLS

Several reports have addressed the neurogenesis period of specific neurochemical subpopulations of retinal cells (Gardino et al. 1993, 1996, da Costa Calaza et al. 2000, Barros et al. 2003) by using a combination of immunohistochemistry and the cumulative method (Fujita and Horii 1963). As the [3H]-thymidine injected *in ovo* remains available to be incorporated into the DNA for several days, immunolabeled cells without [3H]-thymidine, i.e. without autoradiographical silver grains, are judged to have been generated prior the time of [3H]-thymidine injection. On the other hand, double-labeled cells (immunolabeled cells showing autoradiographical silver grains) are considered to be in mitotic division and,

therefore, are judged to have not been generated at the time of the [3H]-thymidine injection.

#### DOPAMINERGIC AMACRINE CELLS

The most abundant catecholamine in the chick retina, as in some other species, is dopamine (Ehinger 1978). The retinal dopaminergic amacrine cells, visualized by the expression of the enzyme tyrosine hydroxylase, show a shorter neurogenesis period, between E3 and E7 (Gardino et al. 1993). According to the literature, the generation of amacrine cells begins at E3 and is concluded at E9 (Fujita and Horii 1963, Kahn 1974, Prada et al. 1991). In rat retina, Evans and Battelle (1987) have reported that the dopaminergic amacrine cells are generated during embryonic ages 16-20, whereas the first detection of TH was reported at post-natal day 3 (PN3), it means several days after the end of neurogenesis period. However, Wu and Cepko (1993), by using a modified staining method, have detected TH immunoreactive cells in retinal whole-mount preparations at embryonic day 19. Therefore, in rats, it seems that TH detection is almost simultaneously to the dopaminergic cell neurogenesis period. On the other hand, in the avian retina, it has been demonstrated that TH expression is indeed delayed in 4-5 days in relation to the end of the proliferation period (Gardino et al. 1993). In addition, these authors demonstrated that the specific dopaminergic amacrine cell subpopulation displays a more restrict neurogenesis period than the general amacrine cell population (Fig. 5). Similarly, TH-immunoreactive cells appear late during the development of the cat retina (Mitrofanis et al. 1988) and at the end of neurogenesis of the total amacrine cell population in this species (Robinson 1988). This delay could reflect the existence of a narrow window during the development when undifferentiated dopaminergic cells are capable of being influenced by specific signals. It is well known that the activity of catecholaminergic enzymes is regulated by hormonal and neural inputs, but the mechanism that underlies the induction of TH expression is only now beginning to be solved. Signals conveyed by cAMP are important to determine the TH phenotype (Guimarães et al. 2001). More recently, Borba et al. (2005) have shown that PACAP is an important endogenous peptide influencing the definition of the tyrosine hydroxy-

lase phenotype of retinal dopaminergic cells within this narrow window of development. Finally, the neurogenesis period of dopaminergic cells in the chick retina ends when the width of the IPL rapidly increases (Coulombre 1955), and the TH immunoreactive cells are first detected after amacrine migration to their final position (Prada et al. 1987). In addition, the first detection of TH positive cells is temporally coincident with some of the above cited events of the chick retina development, for example the beginning of synaptogenesis in the IPL and the maximum activation of dopamine-dependent adenylyl cyclase activity (Ventura et al. 1984). Nevertheless, the factors responsible for dopaminergic specific cell proliferation remain unsolved.

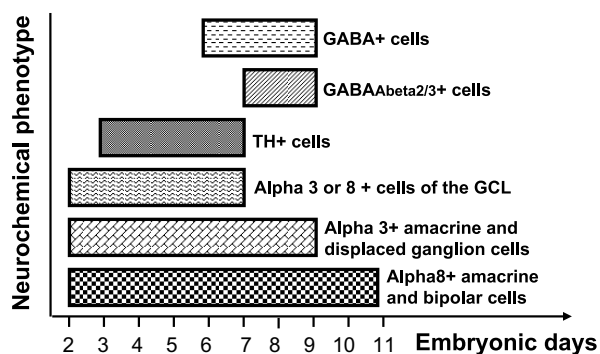


Fig. 5 – Schematic histogram of neurogenesis periods of different neurochemical cell subpopulations. Note that subpopulations of amacrine cells, for example, expressing different phenotypes, can have different neurogenesis periods (compare alpha8+ amacrine cells and TH+ cells).

#### CHOLINOCEPTIVE CELLS

The chick retina contains a prominent cholinergic system, which includes three populations of cholinergic amacrine cells that could be identified by the expression of choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine (ACh) (Millar et al. 1985, Spira et al. 1987). Both ChAT and acetylcholinesterase (AChE), the enzyme that metabolizes ACh, are found very early in the developing tissue and are also present in the mature retina (Shen et al. 1956, Spira et al. 1987). These cholinergic markers are found in amacrine cells in the INL (cholinergic cell type I and III) and in displaced amacrine in the GCL (cholinergic cell type II) since E6.5 (ChAT) and E4.5 (AChE). Other aspects of the cholinergic system, such as mod-

ulation of choline acetyltransferase activity and regulation of vesicular acetylcholine transporter (Loureiro-Santos et al. 2001, 2002, Prado et al. 2002), have also been studied.

Concerning cholinceptive aspects, nicotinic and muscarinic acetylcholine receptors are expressed in the chick retina (Vogel and Nirenberg 1976, Large et al. 1985, Hamassaki-Britto et al. 1994, Fischer et al. 1998a, da Costa Calaza et al. 2000). Muscarinic acetylcholine receptors modulate cell cycle and calcium signaling in the chick retinal ventricular zone (Pearson et al. 2002, Syed et al. 2004). Acting through nicotinic acetylcholine receptors, ACh seems to play important roles in neurite outgrowth of ganglion cells, dendritic filopodia motility and remodeling during synaptogenesis, and development of spontaneous rhythmic activity in retinal ganglion cells during the period in which their connectivity pattern is shaped (Wong et al. 1998, Wong and Wong 2001). Therefore, the neurogenesis pattern of the cholinergic system is an important parameter considered to establish the temporal relationship between histogenesis and definition of functional phenotypes.

Alpha 3 subunit nicotinic receptors are observed in amacrine, displaced ganglion cells, and cells in the ganglion cell layer. Alpha 8 subunits are seen in amacrine and bipolar cells, as well as in cells in the ganglion cell layer (Hamassaki-Britto et al. 1994). The first cells that exhibit either alpha 3 or alpha 8 immunolabeling are almost certainly presumptive ganglion cells. Thereafter, the expression of both subunits are seen in amacrine neurons, followed by alpha 8 subunit in bipolar cells and alpha 3 in displaced ganglion cells in the inner nuclear layer. These results reveal not only different developmental patterns of cells containing alpha 3 and alpha 8 nAChR subunits, but also indicate that both subunits are expressed in the chick retina before ChAT and well before retinal synaptogenesis (Araki et al. 1982). In addition, despite the fact that nAChRs alpha 3 and alpha 8 subunits have different time courses of development in the embryonic chick retina, it could also be suggested that the birth of cholinceptive cells followed the neurogenesis time course of specific cell types.

From neurogenesis studies, it was established that the alpha 3-positive neurons in the ganglion cell layer leave the cell cycle from E2 through E7 (Gardino et



al. 1996). Alpha 3-immunoreactive cells in the INL (amacrine and displaced ganglion cells) leave the cell cycle from E2 through E9. The alpha 8-positive cells in the ganglion cell layer are born between E1 and E7, and those in the inner nuclear layer (amacrine and bipolar cells) from E2 through E11. At least one difference was noticed between the neurogenesis of alpha 3- and alpha 8-positive neurons in the chick retina (Fig. 5). The cells in the ganglion cell layer that stained for alpha 3 begin to leave the cell cycle almost one day later than the alpha 8-positive neurons do. Furthermore, until E4, the alpha 3-positive cells were observed to leave the cell cycle at a slower rate than the one of alpha 8-positive cells. However, the expression of the alpha 3 subunit begins almost one day before (Hamassaki-Britto et al. 1994). These results suggest that the factors controlling the expression of alpha 3 and alpha 8 nAChR subunits in the chick retina appear to be relatively independent of those that control cell genesis. Indeed, the ontogenesis of alpha 3 and alpha 8 nAChR subunits in the chick retina is not related in any simple way to the neurogenesis of neurons bearing those receptor subunits. The neurogenesis rates of alpha 3- and alpha 8-positive amacrine cells also appear to differ slightly, although in this case no difference in their ontogenesis has been noticed in a previous study (Hamassaki-Britto et al. 1994). Finally, previously reported data suggest that the time of birth of cholinergic neurons in the chick retina follows the general pattern of cell generation in this tissue (Gardino et al. 1996).

#### GABAERGIC AND GABACEPTIVE CELLS

L-Glutamate (L-Glu) and  $\gamma$ -aminobutyric acid (GABA) are widely recognized as mediators of excitatory and inhibitory neurotransmissions, respectively, in the central nervous system, including the retina of many vertebrate species (Mosinger et al. 1986, Yazulla 1986, Massey and Redburn 1987, Thoreson and Witkovsky 1999). The GABAergic system is composed of a large population of cells, mainly constituted of amacrine neurons, with many important functions in the development and physiology of the retina (Catsicas and Mobbs 2001, Barnstable 1993, Ge et al. 2007, Luján et al. 2005, Sernagor et al. 2003, Tachibana and Kaneko 1988, Yazulla 1986). In fact, GABA is visualized in horizontal and

amacrine cells and in cells in the GCL, most of them containing glutamate, probably used as a precursor for GABA synthesis (Sun and Crossland 2000). Neurons expressing GABA in the chick retina appear at E6 and, at this period, GABA is mainly synthesized from putrescine (de Mello et al. 1976, Hokoç et al. 1990). The classical enzyme that synthesizes GABA from glutamate (glutamic decarboxylase, GAD) first appears only at E11 (Hokoç et al. 1990). After this stage, GABA is mainly synthesized by the classical pathway involving GAD.

As observed in several regions of the CNS (Ben-Ari 2002 for review), retinal GABA, acting on ionotropic receptors, also seems to be a depolarizing mediator at the beginning of the development (Yamashita and Fukuda 1993, Catsicas and Mobbs 2001). At E3, GABA is able to induce calcium influx by its depolarizing action (Yamashita and Fukuda 1993). Depolarization by GABA reaches a peak at E8 and undergoes a slight decrease around E12 (Catsicas and Mobbs 2001). Finally, at E14, GABA no more induces calcium influx and probably assumes its classical inhibitory function (Catsicas and Mobbs 2001).

Concerning to the neurogenesis pattern, the profile of GABAergic amacrine and horizontal cells showed a very slow proliferation rate up to E6, when only 20% of GAD-positive cells were considered generated. Then, between E6 and E9, 80% of GAD expressing neurons are born (da Costa Calaza et al. 2000) (Fig. 5). Birth dates of GABAergic neurons were also determined in the rat retina by using BrdU or thymidine as markers (Lee et al. 1999, Silveira et al. 2007). In both cases, the rate of GABAergic cell generation peaked at E18 in both central and peripheral sectors of the retina, and displayed a homogenous generation rate from E14 to PN4, ending after that.

The early appearance of GABA during the retinal development raises the possibility that it could act as a factor modulating embryonic events, similar to other several neurotrophic factors (NGF, BDNF, NT3, etc) and molecules such as acetylcholine (Lipton et al. 1988), acetylcholinesterase (Blasina et al. 2000, Dupree and Bigbee 1996) and dopamine (Lankford et al. 1987, 1988, Reis et al. 2007). It has been shown that GABA expression occurs exactly at E6 (Hokoç et al. 1990) when most of GAD-IR cells starts to leave the cell cycle (da

Costa Calaza et al. 2000), and long before GAD expression (Hokoç et al. 1990). Considering GABA as a neurotrophic molecule and knowing its inhibitory effect on DNA synthesis (LoTurco et al. 1995), GABA could carry specific signals that are needed to arrest the mitotic cycle of GAD immunoreactive cells. Therefore, it could constitute a factor acting to control the proliferation of its own population. However, one cannot rule out the possibility of other substances influencing this process, and this possibility remains to be confirmed.

During the development, GABA functions are apparently mediated by ionotropic GABA receptors. GABA<sub>Aβ2-3</sub>, an important subunit of these receptors, is expressed in amacrine cells and in cells in the GCL (Barros et al. 2003). At E9, GABA<sub>Aβ2-3</sub> immunoreactivity was restricted to the inner plexiform layer, and the first cell bodies immunoreactive to GABA<sub>Aβ2-3</sub> were seen at E14. Thereafter, the number of cell bodies and the intensity of GABA<sub>Aβ2-3</sub> immunoreactivity increased until the adult pattern is established. Interestingly, GABA<sub>Aβ2-3</sub> amacrine positive cells are born in the same pattern as GAD positive cells, late in the neurogenesis period of the overall amacrine cell population (E7-E9) (Fig. 5).

#### CONCLUSION: NO REGULAR PLAN FOR BIRTH!

One interesting point that could arise from the data discussed above is that the neurogenesis period of the total cell population of the retina is not necessarily the same for different neurochemical cell subpopulations. Thus, although the neurogenesis of the total population of amacrine cells in chick retina takes place mainly from E2 to E9 (Fujita and Horii 1963, Kahn 1974, Spence and Robson 1989), subpopulations of amacrine cells expressing different phenotypes, like dopaminergic amacrine cells for example, can have distinct periods of neurogenesis, i.e. from E3 to E7 (Gardino et al. 1993, 1996, da Costa Calaza et al. 2000). By studying the ontogenesis and the neurogenesis of different retinal cell subpopulations, with distinct phenotypes, one could think that, for some of them, such as for the GABAergic system, there is a temporal window during the development, during which specific signal(s), responsible for their decision to leave the cell cycle, must be given. For the GABAergic system, it appears that there are im-

portant signals around E6, which determine the end of the proliferation period. We cannot exclude the collaboration of progenitor cells competent to respond to some signal, which is present before E6 at their retinal microenvironment. However, for a full comprehension of the signals that modulate the neurogenesis of each specific neurochemical system, further experiments must be done. If there is not a common plan for the birth of the overall population of retinal cell, we can conclude that, among the great diversity of neurochemical cell types in the retina, there may be several genetic or epigenetic signals, factors, molecules, or even a combination of these, which are responsible not only for phenotype definition, but also for the establishment of the end of the mitotic cycle for each neurochemical cell type. The identification of signals and the determination of the proliferation periods of each cell type of the retina could be important for future therapeutic interventions using stem cells.

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#### RESUMO

O embrião de galinha é um dos mais tradicionais modelos de estudos da neurociência do desenvolvimento e seu sistema visual tem sido um dos mais exaustivamente estudado. A retina tem sido utilizada como modelo para estudar o desenvolvimento do sistema nervoso. Aqui, nós descrevemos as características morfológicas que caracterizam cada estágio da retina em desenvolvimento e os estudos do período de neurogênese de algumas subpopulações de células neuroquimicamente específicas da retina usando uma combinação de imuno-

histoquímica e autoradiografia de timidina-tritiada. Conclui-se que o período de proliferação das células dopaminérgicas, GABAérgicas, colinoceptivas e GABAceptivas não segue uma regra comum. Além disso, alguns grupos celulares neuroquimicamente distintos podem ter um período de proliferação mais restrito quando comparado ao da população total destas células.

**Palavras-chave:** desenvolvimento, neurogênese, sistemas neurotransmissores, ontogênese, proliferação.

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