

## Chapter II

### Appearance and maturation of the cholinergic system during retinal development

# 1 Introduction

The vertebrate retina is an attractive tissue for studies of neurogenesis, cytodifferentiation and genesis of neural circuitry in the central nervous system (CNS). The reasons for this include the following: (1) both retina and brain are derived from a common embryonic origin, the neural tube; (2) the retina has a regular laminar organization like the cerebral cortex, but the developing and mature architecture of the retina are relatively simple and more accessible than any other part of the CNS; and (3) most of the neurotransmitters in the CNS are identified in the retina. Moreover, morphology and physiology of the major classes of retinal neurons, and their sub-types, have been well characterized over the years (for reviews, see Ramón y Cajal, 1892; Dowling, 1987; Massey and Redburn, 1987).

As described in the General Introduction, one of the important components of the functional differentiation of the neural circuitry of the retina that occur during development is genesis of the various neurotransmitter systems. In particular, development of the cholinergic neurotransmitter system has been investigated biochemically, histochemically and morphologically in retinas of many species, including mammals (Puro et al., 1982; Dann, 1989; Hutchins et al., 1995), chick (Crisanti-Combes et al., 1978; Spira et al., 1987; von Bartheld et al., 1991; Layer et al., 1997), and amphibians (Ma and Grant, 1978, 1984). In some of these studies, it has been suggested that the onset and distribution of ChAT are not necessarily the same as those of AChE (Levey et al., 1983b; Greenfield, 1984; Hutchins, 1987) and AChRs (Hutchins, 1994; Wassélius et al., 1998). Therefore, it has been speculated that AChE and AChRs may have additional functions that are not dependent upon cholinergic transmission (Kristt, 1989;

Layer, 1991; Robertson et al., 1991).

In Chapter I, the presence of a cholinergic system including ChAT, AChE and AChRs in the adult newt retina was demonstrated using immunocytochemical and histochemical techniques. In the present study, the time courses of appearance and maturation of the cholinergic system during development of newt retina have been investigated. The results indicate that (1) ChAT and nAChR appear at the beginning of the period of synaptogenesis, whereas (2) AChE and mAChR appear before the synthesis of ACh and the expression of nAChR, and well before the period of synaptogenesis. These findings raise an important possibility that AChE and mAChR may play a non-cholinergic role in the developing retina.

## 2 Materials and Methods

### 2.1 Preparation of developing retina

Gravid females were collected in late autumn and kept in polyethylene containers at 10°C under a natural day-night cycle. Fertilized eggs were obtained after artificial induction of spawning by injection of 50 µl human chorionic gonatropin (Teikokuzouki R-3000, Tokyo, Japan) on alternate days for 7 to 10 days. The eggs were collected and kept at about 25°C until embryos reached appropriate stages. The eggs or larvae were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M PB at pH 7.4, and then rinsed in 0.1 M PB for 30 min at 4°C. They were equilibrated in 30% sucrose in 0.1 M PB, frozen in an embedding medium (O.C.T. compound, Miles Inc. Elkhart, USA), and cryosectioned at about 17 µm thickness.

The embryonic stages were characterized according to the criteria proposed by Ichikawa and Kajishima (1965). Developing retinas from embryonic stages 25 to 49 were examined immunocytochemically and histochemically.

### 2.2 Immunocytochemistry and histochemistry

A goat polyclonal antibody against ChAT was used for the localization of cholinergic neurons. A rat monoclonal antibody against the m2 subunit was used for the localization of mAChRs. Rat monoclonal antibody against the  $\alpha 3$  subunit and a biotin-conjugated  $\alpha$ -bungarotoxin were used for the localization of nAChRs. For localization of AChE activity in the retina, a histochemical method described by Karnovsky and Roots (1964) technique was used.

Immunocytochemical, and  $\alpha$ -BTX and AChE histochemical protocols used in the present study are outlined in Figs. 5, 6, and 7, respectively.

## 3 Results

### 3.1 Definition of stages of developing retinas

Figure 15 shows cross-sections of the newt embryo at the level of the eyes at various stages of development. At the embryonic stage 26, eyecup formation occurred (Fig. 15A). The retina without the lens was composed mainly of progenitor cells of elongated or oval shape at this stage. Before the embryonic stage 32, the lens appeared, and the neural retina was segregated from the pigmented epithelium (Fig. 15B). During the subsequent stages of development, the neural retina became thicker with the formation of the synaptic layers (Fig. 15C and D).

Developing retinas were divided into four groups on the basis of their morphological properties (Fig. 16):

- (1) Retinas at embryonic stages between 25 and 26, which are a few cell thick, predominantly consisting of progenitor cells of elongated shape;
- (2) retinas at embryonic stages between 30 and 32, consisting mainly of progenitor cells, except at the most proximal retinal region where rounded cells, probably ganglion cells, appear;
- (3) retinas at embryonic stages between 36 and 39, which are multiple layers of cells, just before or at the beginning of the formation of the synaptic layers;
- (4) retinas at embryonic stages above 40 demonstrating a characteristic penta-laminar array (three nuclear layers and two synaptic layers).

## 3.2 Choline acetyltransferase

The time course of appearance and subsequent changes in the ChAT immunoreactivity during development were examined on sections of eye/retina from embryonic stages 25 to 49. Figure 17A shows a sample retinal section at embryonic stage 36. No ChAT immunoreactivity was detectable at this developmental stage. Weak ChAT-ir cells became detectable at the position of the presumptive IPL in the developing retina at stage 39, just before the formation of synaptic layers (data not shown). Figure 17B shows the ChAT-ir pattern in the retina at stage 42. The retina started to differentiate into inner and outer plexiform layers around this stage. A ChAT-ir IPL band was apparent in the central part of the retina; the intensity of the ChAT immunoreactivity decreased towards the periphery. At the same time, two types of ChAT-ir somata closely apposed to either side of the IPL became distinguishable (indicated by an arrow and arrowhead). Figure 17C shows the pattern of ChAT-ir cells in the retina at embryonic stage 46. The ChAT-ir IPL band became more intense and was also apparent at the peripheral part of retina. During subsequent development, at embryonic stage 49, the single immunoreactive band in the IPL resolved into two bands (Fig. 17D, arrows). The density of the two types of ChAT-ir amacrine cells close to either side of the IPL appeared higher in the central retina than in the periphery, as in the mature retina. The apparent ChAT-staining in the OPL was probably nonspecific since it was still present even after omitting the primary antibody.

### 3.3 Acetylcholinesterase

Figure 18A shows a cross-section of the eye/retina at embryonic stage 31. The retina was composed mainly of ovoidal progenitor cells. At such a stage, AChE-positive cells could first be detected in the most proximal region of the retina (arrowheads). Figure 18B shows a section of the retina at embryonic stage 36. Number of AChE-positive somata increased by this stage, and some of them were observed at more distal levels of the retina. During subsequent development, the retina became thicker and synaptic layers appeared. Figure 18C shows a section of the retina at embryonic stage 42. The AChE-positive IPL band was observed in the central part of the retina as a single band, and its intensity decreased towards the periphery. At this stage of development, the AChE reaction product in the vitreal region disappeared, but stained somata became detectable on either side of the IPL (indicated by an arrow and arrowhead). The OPL was also weakly stained at this stage. Figure 18D shows a section of the retina at embryonic stage 49. Three types of AChE-positive somata were distinguishable, two of them in the INL (arrowheads) and one in the GCL (arrow). This AChE staining pattern was almost identical to the situation observed previously in the mature retina (Chapter I), except that almost all layers of the IPL were AChE-positive. With further advancement of development, the width of the IPL became thicker and the AChE-positive band became restricted approximately to the distal half of the IPL (data not shown).



## 3.4 Acetylcholine receptors

3.4.1 Muscarinic acetylcholine receptors (mAChRs): The time course of appearance and subsequent changes in the mAChR immunoreactivity was examined during development (Fig. 19). mAChR-ir cells were detected first at the most proximal region of the retina at embryonic stage 31 (Fig. 19A, arrowheads). The neural retina at this stage was composed mainly of ovoidal progenitor cells, and the mAChR-ir somata were oval in shape. Figure 19B shows a retina at embryonic stage 39 that is about to form the IPL in the central area. By this stage, the mAChR-ir somata became round and increased in number in rows, a few-cells-thick, at the proximal region of the retina. During the following period of development, the IPL appeared, separating the GCL and INL. Retinas at embryonic stage 42 contained a large number of intensely immunoreactive somata in the GCL and a small number of less immunoreactive somata at the INL/IPL border (Fig. 19C, arrowhead). At the same time, mAChR immunoreactivity became detectable in somata at the INL/OPL border (arrows) where horizontal cells are usually present. Figure 19D shows the mAChR-ir pattern of the retina at embryonic stage 49. The mAChR-ir pattern at this stage was similar to that in the mature retina, except that the two mAChR-ir bands were not clearly detected within the IPL. Also, the immunoreactivity of horizontal cell-like somata became very weak.

3.4.2 Nicotinic acetylcholine receptors (nAChRs): The time course of appearance and subsequent changes in nAChRs were examined by using two markers;  $\alpha$ -BTX and an antibody against nAChR subunit,  $\alpha 3$ . Figure 20 shows the binding sites of biotin-conjugated  $\alpha$ -BTX. At embryonic stage 31, no  $\alpha$ -BTX binding sites was

detected (Fig. 20A).  $\alpha$ -BTX binding sites were detectable at the IPL and OPL in the central part of the retina at embryonic stage 42 (Fig. 20B, arrowheads).  $\alpha$ -BTX-positive IPL became thicker and progressed radially from central towards peripheral retina following stages of development (Fig. 20 C and D). None of cell bodies were  $\alpha$ -BTX-positive. This staining pattern is identical to that of the mature retina (cf. Fig. 13A).

Figure 21 shows  $\alpha 3$  nAChR immunoreactivity in the developing retina. At embryonic stage 31, no  $\alpha 3$  nAChR immunoreactivity was detected (Fig. 21A).  $\alpha 3$  nAChR-ir IPL and OPL became detectable at embryonic stage 42 (Fig. 21B, an arrowhead and arrow). Overall, the  $\alpha 3$  nAChR-ir pattern with further development stage was identical to the pattern of  $\alpha$ -BTX binding sites (Fig. 21C and D), except that the  $\alpha 3$  subunit antibody also stained Müller cell-like endfeet (Fig. 21D, arrow).

## 4 Discussion

### 4.1 The onset of AChE activity at an earlier embryonic stage than that of ChAT immunoreactivity

ChAT is regarded generally as a reliable marker for cholinergic neurons in the nervous system (Hutchins, 1987; Criswell and Brandon, 1993). An understanding of the developmental pattern of ChAT, therefore, may provide useful information concerning the potential importance of cholinergic system in the ontogenesis and functional maturation of the retina. In the developing *Xenopus* retina, ChAT activity appears before the morphological differentiation of the synapses, and its increased activity correlates with increasing synaptogenesis in both the IPL and OPL (Ma and Grant, 1978, 1984; Hutchins, 1987). The onset of ChAT-ir amacrine cells in the chick retina also occurs before the period of synaptogenesis (Spira et al., 1987). In the developing newt retina, however, the onset of the ChAT-ir amacrine cells almost coincided with the period of morphological development of the IPL.

In rat retina, horizontal cells transiently became ChAT-ir during early postnatal development stages (Kim et al., 1998). However, this was not the case in developing newt retina. In addition, ChAT immunoreactivity was not found in the OPL. Little ChAT activity in the photoreceptors and the OPL has been reported in a various retinas (Ross and McDougal, 1976).

AChE plays an established role in cholinergic transmission by hydrolyzing, and thus terminating the action of the transmitter ACh. However, the presence of AChE can not be used as a reliable criterion for localization of cholinergic synapses, because of the lack of

correspondance between ChAT and AChE (Lehmann and Fibiger, 1979; Pourcho and Osman, 1986b). In the developing newt retina, AChE-positive cells were detected first in the somata located at the most proximal level of the retina, before ChAT-ir cells could be detected and well before the morphological development of the IPL. The onset of AChE activity at an embryonic stage earlier than that for the ChAT immunoreactivity has also been reported in the developing chick retina (Spira et al., 1987). On the other hand, studies of the developing *Xenopus* retina indicated that the development of AChE activity closely parallels the development of ChAT immunoreactivity and correlates with the period of synaptogenesis (Ma and Grant, 1984).

Over the last decade it has become increasingly evident that AChE may have additional functions that are not dependent upon its synaptic transmission, in both the developing and mature nervous system (for review, see Appleyard, 1992). For example, in several brain regions, including the substantia nigra, there is a high concentration of AChE, while ChAT activity is disproportionately lower and there are few cholinergic synapses (Henderson and Greenfield, 1987). This is also the case in the newt retina, that is, AChE-positive OPL is ChAT-negative. Furthermore, in many vertebrate retinas including newt retina (Lehmann and Fibiger, 1979; Pourcho and Osman, 1986b; Criswell and Brandon, 1993; Hutchins et al., 1995; Reiss et al., 1996), the localizations of AChE and ChAT have been found not to be exactly the same in the IPL. The earlier appearance of AChE in the developing retina may also support the notice of a possible non-cholinergic function of this molecule, although its functional significance is unclear. One of the proposed novel roles of AChE could be related to neurite guidance, since inhibition of AChE *in vitro* changed neurite growth pattern (Layer et al., 1993; Jones et al., 1995). Further studies are

necessary to elucidate this point in the developing newt retina. In the General Discussion, possible non- cholinergic functions of AChE are reviewed in some detail.

#### 4.2 The onset of mAChRs at an earlier embryonic stage than that of ChAT

In the present study, mAChR-ir cells were detected first in the somata located at the most proximal layer of the retina, before ChAT-ir cells could be detected. The appearance of the transmitter receptors earlier than the synthesis of the neurotransmitter has been reported in many embryonic tissues. For example, the presence of mAChRs in the limb bud prior to innervation has been described in the chick embryo (Schmidt, 1981). In the developing chick retina, mAChRs could be detected well before the synthesis of ACh (Vogel and Nienberg, 1976; Sugiyama et al., 1977; Hutchins, 1994). The functional significance of the earlier appearance of mAChRs in the developing retina is unclear. At present, information about possible non-synaptic function of mAChRs is fragmentary. It has been proposed on the basis of histochemical and biochemical studies in the CNS that there are two different phases of the development of the cholinergic system (Filogamo and Marchisio, 1971). The 'earlier' system appears transiently in all differentiating neuroblasts, whilst the 'later' one develops with the formation of cholinergic synapses in authentic cholinergic pathways. Molecular alteration of mAChRs during synaptogenesis has also been observed in the chick retina (Large et al., 1985; Marchisio et al., 1985). Furthermore, a regulatory function of the embryonic muscarinic system in morphogenesis has been suggested in the chick embryo (Oettling et al., 1988). In relation to the morphogenesis of the eye,

Yamashita and Fukuda (1993) have shown that exogenous ACh can produce a folding of the neural retina in the E3 chick embryo at the stage of optic cup formation that correlates with a mAChR-mediated increase in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). They further described the rapid decline of the muscarinic  $\text{Ca}^{2+}$  mobilization after the E5 stage (Yamashita et al., 1994). In future experiments, it would be interesting to clarify whether ACh-induced  $[\text{Ca}^{2+}]_i$  changes via mAChRs occur in 'live' slice preparations of developing newt retina in a stage-dependent manner using  $\text{Ca}^{2+}$  imaging techniques.

### 4.3 The later appearance of nAChRs than that of mAChRs

In the developing newt retina, the two nAChR markers used,  $\alpha$ -BTX and  $\alpha 3$ -specific antibody gave substantially the same staining pattern. Both appeared in the IPL and the OPL, but not in cell bodies. In contrast to the earlier appearance of the mAChR-ir cells, the onset of the nAChR-positive plexiform layers coincided temporally with the appearance of ChAT-ir amacrine cells (Fig. 29C).

The appearance and maturation of nAChRs have mostly been studied in the developing chick retina. It has been reported that  $^{125}\text{I}$ - $\alpha$ -BTX binds to ganglion cells which are 'born' on the 6th embryonic day, and then to the IPL and OPL which are formed after the 8th and 9th embryonic days, respectively (Vogel and Nirenberg, 1976; Vogel et al., 1977). Furthermore, most of the synapses mediated by nAChRs are formed at a later developmental stage in retina than those mediated by mAChRs (Sugiyama et al., 1977). The recent availability of antibodies and cDNA probes for subunits of nAChRs has made it possible to localize precisely the retinal cell types expressing these receptors (Britto

et al., 1992; Keyser et al., 1993; Hamassaki-Britto et al., 1994). Molecular analyses of  $\alpha 3$  and  $\alpha 8$  nAChR subunits have revealed in the developing chick retina that these subunits are first expressed in presumptive ganglion cells of the vitreal margin for  $\alpha 3$  and  $\alpha 8$  before ChAT-ir cells and well before synaptogenesis (Hamassaki-Britto et al., 1994).

nAChRs labeled with  $^{125}\text{I}$ - $\alpha$ -BTX in the developing chick retina were first distributed uniformly through the IPL by 11th embryonic day and resolved into four bands during subsequent development. Similar changes in the staining pattern in the IPL were observed for  $\alpha 3$  and  $\alpha 8$  immunohistochemistry. In contrast to the results from the chick retina, diffuse staining of the IPL throughout the development of newt retina was observed with  $\alpha$ -BTX binding sites and  $\alpha 3$  immunohistochemistry.