Chapter I

Localization of the cholinergic system in the normal adult newt retina

1 Introduction

The acetylcholine (ACh) system (cholinergic system) is one of the important neurotransmitter systems in the CNS including the retina (for reviews, see Neal, 1983; Hutchins, 1987). This system includes ACh as the neurotransmitter, its receptors, choline acetyltransferase (ChAT) that catalyzes the synthesis of ACh, and acetylcholinesterase (AChE) that hydrolyzes ACh. Figure 4 shows a schematic diagram of a typical cholinergic synapse. ACh is synthesized in the presynaptic cells by ChAT and stored in subcellular structures, called synaptic vesicles. The vesicles can be released by exocytosis in response to depolarization of the presynaptic nerve terminals, and the released ACh interacts with acetylcholine receptors (AChRs) on the postsynaptic cells. There are two main type of AChRs: nicotinic and muscarinic (not shown). AChE hydrolyzes the ester bond of ACh to yield choline and acetate. Choline is then taken back up into the presynaptic terminals to be used in the re-synthesis of new ACh. ACh, ChAT and AChE have all been demonstrated, either biochemically or histochemically, to be present in retinas of all vertebrate species examined (Lam, 1972; Masland et al., 1984; Pourcho and Osman, 1986a, b; Voigt, 1986; Hutchins, 1987, White et al., 1990, 1991; Layer and Willbold, 1994). Furthermore, ACh receptors (AChRs) have been shown to be present in both plexiform layers in the retina of various vertebrates, in histochemical (Vogel and Nirenberg, 1976; Sugiyama et al., 1977; Yazulla and Schmidt, 1977; Hamassaki-Britto et al., 1994b) or electrophysiological studies (Masland and Ames, 1976; Lipton et al., 1987; Schmidt et al., 1987; Yazejian and Fain, 1993; Yamashita et al., 1994). In the adult newt retina, Dickson et al. (1971) have demonstrated ultrastructural localization of AChE activity in the synaptic membranes of the

horizontal and bipolar cells. Using radiochemical technique, Sarthy and Lam (1983) have reported that ChAT activity is present in the adult newt retina. Furthermore, they have described that a rise in the ChAT activity in regenerating newt retina corresponds with an increase in the synthesis and accumulation of ACh. The cholinergic system in the adult newt retina and its genesis during retinal development and regeneration have not been systematically investigated further. To better understand the functional organization of this system in normal retina, and during development and regeneration, it is necessary to demonstrate the distribution of ChAT, AChE and AChRs. Therefore, in this study, I have investigated their distribution in the mature newt retina by using immunocytochemistry and histochemistry. I found that the cholinergic neurons are present in the adult newt retina, as described in other vertebrate retinas studied. However, the localizations of ChAT, AChE and AChRs are not exactly the same in the IPL and OPL.

2 Materials and Methods

2.1 Preparation of the normal adult retina

Adult newts (*Cynops pyrrhogaster*) were maintained at room temperature under a natural day-night cycle. The animals were sacrificed following anesthesia in 0.1% FA100 (4-allyl-2-methoxyphenol; Tanabe, Ohsaka, Japan) solution for at least 30 min. Each eyeball was removed from the orbit and fixed for about 12 h at 4 ℃ in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Eyeballs were rinsed in 0.1 M PB for 30 min at 4℃. They were equilibrated in 30% sucrose in 0.1 M PB until they sank to the bottom at 4℃, frozen in an embedding medium (O.C.T. compound, Miles Inc. Elkhart, USA), and then cryosectioned at about 17 μm thickness. The transverse sections were thaw-mounted onto gelatine-coated cover-glasses, air-dried, and stored in the freezer until immunocytochemical or histochemical use.

2.2 Immunocytochemistry

Antibodies against choline acetyltransferase (ChAT), muscarinic acetylcholine receptor (mAChR), and nicotinic acetylcholine receptor (nAChR) were obtained commercially. The immunocytochemical protocol (avidin-biotin-peroxidase protocol) used is illustrated in Fig. 5. Briefly, wholemount retinas or retinal sections were rinsed in 0.02 M phosphate buffer saline (PBS), 1 % Triton X-100 in 0.02 M PBS, and 0.02 M PBS for 15 min each at 4℃ before incubation in the primary antibodies. Polyclonal goat anti-ChAT (Chemicon International INC, Temecula, CA) was diluted 1:100 in a mixture of 3% normal rabbit

serum (Vector Labs, Burlingame, CA), 5% bovine serum albumin (Gibco Labs, Gran Island, NY), and 0.3% Triton X-100 in 0.02 M PBS. Rat monoclonal anti-m2 mAChR (Chemicon International INC, Temecula, CA) and rat monoclonal anti-α3 and anti-α8 subunits for nAChRs (Research Biochemicals International, MA, USA) were diluted 1: 200 in a mixture of 3% normal goat serum (Vector Labs.), 5% bovine serum albumin (Gibco Labs, Gran Island, NY), and 0.3% Triton X-100 in 0.02 M PBS. The incubation period for the primary antibodies was 24-48 h for cryosections and 4 days for wholemount retinas at 4℃.

All incubations were done in a humidity-tight box with agitation. To control for possible non-specific binding of the antiserum, some tissue sections were incubated omitting the primary antibodies from the incubation solution. The retinal sections or wholemount retinas were washed with PBS and Triton X-100 as described above. Secondary antibody for ChAT was affinity-purified biotinylated rabbit anti-goat IgG (H+L, Vector Labs.), diluted 1:300 with 3% normal rabbit serum, 5% bovine serum albumin, and 0.3% Triton X-100 in 0.02 M PBS. Secondary antibodies for mAChRs and nAChRs were affinity-purified biotinylated goat anti-rat IgG (H+L, Vector Labs.), diluted 1:300 with 3% normal goat serum, 5% bovine serum albumin, and 0.3% Triton X-100 in 0.02 M PBS. The retinal preparations were incubated with these antibodies for 1.5 h (sections) or 2 days (wholemounts) at room temperature. They were washed in PBS and Triton X-100, and then incubated in a mixture of Avidin and Biotin Complex (Vectastain, Vector Labs.) diluted 1:100 in 0.02 M PBS for 1.5 h (sections) or 2 days (wholemounts) at room temperature. They were washed again in PBS and Triton-X 100, and then visualized with a DAB substrate Kit (Vector Labs.) for 3-10 min. This reaction was stopped by immersing the tissue in ice-cold PBS, followed by a PBS wash. The retinal sections

or wholemount retinas were mounted in glycerine-PBS solution. The ChAT-immunoreactive (ChAT-ir), mAChR-immunoreactive (mAChR-ir) and nAChR-immunoreactive (nAChR-ir) neurons were examined by light microscopy.

2.3 α-bungarotoxin binding histochemistry

In some experiments, nAChR was localized by using biotin-conjugated α-bungarotoxin (Molecular Probes Inc. Eugene, Oregon). This histochemical protocol is shown in Fig. 6. Briefly, retinal sections were washed in 0.1 M PB for 30 min at room temperature, and then incubated with 10 μM biotin-conjugated α-BTX, 2mg/ml bovine serum albumin in 0.1 M PB for 12 h at room temperature. They were washed with 0.02 M PBS, 1 % Triton X-100 in 0.02 M PBS, and 0.02 M PBS for 15 min each at room temperature , and then incubated in a mixture of Avidin and Biotin Complex (Vectastain, Vector Labs.) diluted 1:100 in 0.02 M PBS for 1.5 h at room temperature. The retinal sections were washed again in PBS and Triton X-100, and then visualized with a DAB substrate Kit (Vector Labs.) for 3-10 min at room temperature. This reaction was stopped by immersing the tissue in ice-cold PBS, followed by a PBS wash. The α-BTX binding sites were examined by light microscopy.

2.4 Acetylcholinesterase histochemistry

For localization of acetylcholinesterase (AChE) activity in the retina, I used a histochemical method described by Karnovsky and Roots (1964). This histochemical protocol is shown in Fig. 7. Briefly, retinal sections were washed in 0.1 M sodium acetate buffer at pH 6.0 (three washes, 15 min each), and then preincubated in non-

chromogenic substrate solution with 10^{-4} M tetraisopropylpyrophosphoramide (iso-OMPA, Sigma, St. Louis, MO) to inhibit nonspecific cholinergic (butyrylcholinesterase, BChE, E.C.3.1.1.8) activities. After washing, selective staining of AChE was performed by incubation of sections for 2-3 h at 37℃ in the presence of 2.56 mM acetylthiocholine iodide (Sigma, St. Louis, MO) as a substrate. The reaction was terminated by rinsing in two changes of 71 mM Na_2SO_4, 0.1 M sodium acetate buffer at pH 6.0 for 20 min at 25℃ and then followed by a PBS wash. The retinal sections were mounted in glycerine-PBS solution. The AChE activity in the retina was examined by light microscopy. The specificity of the enzyme reactions was tested by pre-treating the tissues in 10^{-4} M physostigmine (eserine, Sigma, St. Louis, MO), in 10^{-4} M BW 284C51 (Sigma, St. Louis, MO).

3 Results

3.1 Cellular organization of the normal newt retina

Figure 8A shows a transverse section of the adult newt retina. The newt retina has a laminar organization that is fundamentally the same as that of other vertebrate retinas. Fig. 8B shows a schematic diagram of the retina in which five basic types of neurons have been identified by intracellular staining (Niino, 1993). Three layers of cell bodies include the outer nuclear layer (ONL) in which somata of the photoreceptor cells are located, the inner nuclear layer (INL) in which horizontal, bipolar and amacrine cell somata are located, and the ganglion cell layer (GCL) in which somata of the ganglion cells and displaced amacrine cells are located. Two synaptic layers are the outer plexiform layer (OPL) where photoreceptor terminals, horizontal cell processes and bipolar cell dendrites make synaptic contacts, and the inner plexiform layer (IPL) where bipolar cell terminals, amacrine cell processes and ganglion cell dendrites make synaptic contacts.

3.2 Choline acetyltransferase immunoreactivity

The distribution, density and stratification of ChAT-immunoreactive (ChAT-ir) cells were examined in frozen sections and wholemount retinas. Two types of ChAT-ir cells were identified according to the position of their somata and their dendritic stratification pattern in the IPL (Fig.9). Type I cells had somata at the border between the INL and IPL (INL/IPL border). They appeared to be a subtype of conventional amacrine cells, because of their location in the INL. Type II cells had somata at the border between the IPL and GCL (IPL/GCL border).

They are probably displaced amacrine cells, because many GABA-ir displaced amacrine cells are also located at the IPL/GCL border (Chiba et al.,1997). It has also been shown that more than half of the somata in the GCL of the newt retina are displaced amacrine cells (Ball and Dickson, 1983).

At least two ChAT-ir bands were clearly seen within the IPL, at relative depths of 0-15% and 45-60% (defining the INL/IPL border as 0% and the IPL/GCL border as 100%) (Fig. 9). The two bands at the most distal level and lower middle level of the IPL probably correspond to dendrites of conventional and displaced amacrine cells, respectively. The staining seen in the inner segment of photoreceptor cells and the OPL was also present in control sections of the retina without primary antibody (data not shown) and, therefore, it was concluded to be nonspecific.

Measurements of the density of ChAT-ir somata were made in wholemount retinas. The density of ChAT-ir amacrine cells tended to be higher in the central retina than in the periphery. The total number of ChAT-ir cells near the central area ranged from 300 to 450 cells/mm^2 with a mean value of 363 ± 59 (SD; n=6). Typical distributions of ChAT-ir conventional amacrine cells and displaced amacrine cells are shown at different focal planes in Fig. 10. In Fig. 10A, the displaced amacrine cells (three representative cases being indicated by arrowheads) are in focus, whereas in Fig. 10B the conventional amacrine cells (three of them indicated by arrows) are in focus. Soma size of the two cell types was in the range 11-13 μm in diameter which is a common size range for cells in both the INL and the GCL. The peak density of both cell types was in the range 170-248 cells/mm^2 with a mean value of 205 ± 28 cells/mm^2 (SD) for conventional amacrine cells and in the range 125-184 cells/mm^2 with a mean value of 153 ± 20

cells/mm^2 (SD) for the displaced amacrine cells (Table 1).

3.3 Acetylcholinesterase activity

Figure 11 shows the histochemical localization of the AChE reaction product. The AChE staining pattern changed with the incubation time in substrate staining solution. The AChE reaction was first detected within the IPL, as a single band at a depth of 20-40% (Fig. 11A). With further increase in incubation time, the AChE-positive band became thicker in width and occupied approximately 0-60% of the IPL as a single broad band (Fig. 11B). At the same time, AChE-positive somata were detected on either side of the IPL. They were usually stained intensely in their basal parts. The two cell types were distinguished by the location of their somata in the INL. One cell type has somata closely apposed to the INL/IPL border (arrow) where the ChAT-ir conventional amacrine cells are present. The somata of the other cell type lie one or half cell body away from the INL/IPL border (arrowheads). A third cell type has somata in the GCL at the IPL/GCL border where the ChAT-ir displaced amacrine cells are present. The OPL also became AChE-positive with longer incubation time. Preincubation of tissue sections in iso-OMPA to inhibit nonspecific cholinesterase failed to eliminate the staining reaction, indicating that the enzymatic activity observed was specific to AChE.

3.4 Acetylcholine receptor immunoreactivity

It is necessary to characterize the postsynaptic targets for cholinergic neurons and identify AChRs that mediate specific postsynaptic responses. The AChRs are classified into nicotinic AChRs

(nAChR) and muscarinic AChRs (mAChR). The location of some examples of these AChRs in the mature retina was investigated by immunocytochemistry and histochemistry.

3.4.1 Muscarinic acetylcholine receptors (mAChRs): Figure 12 shows the immunoreactivity specific to m2 AChRs. Intense mAChR immunoreactivity was detectable in a large number of somata at the most proximal level in the GCL. These are probably ganglion cells, because of their localization. This may be supported by the fact that the mAChR-ir pattern was similar to the immunoreactive staining obtained with Na^+ channel antibody as a marker of ganglion cells (Cheon et al., 1998). Relatively weak immunoreactivity was also present in two cell types in the INL; one cell type lying at the INL/IPL border (arrow), and another one lying in one or two cell bodies away from the INL/IPL border (arrowhead). A particular cell type indicated by an arrowhead, showed a primary dendrite sending processes into the most distal region of the IPL. mAChR immunoreactivity was also detected within the IPL, at depths of 0-15% and 85-100% as spotty staining rather than continuous bands. Some varicosties and a few somata (probably belonging to horizontal cell) closely apposed to the OPL (double arrow) were also weakly stained.

3.4.2 Nicotinic acetylcholine receptors (nAChRs): Three nAChR markers were used: α-bungarotoxin (α-BTX) and two antibodies against nAChR subunits α3 and α8. The α3 subunit of nAChRs is one of the α-BTX-insensitive nicotinic receptor subunit, which bind nicotinic ligands with high affinity and do not interact with α-BTX (Luetje et al., 1990; Deneris et al., 1991; Whithing, 1991). On the other hand, the α8 subunit of nAChRs is one of the α-BTX-sensitive nicotinic

receptor subunit, which bind α-BTX with high affinity and nicotinic ligands with a lower affinity (Oswald and Freeman, 1981; Schoepter et al., 1990; Keyser et al., 1993). These three markers showed a somewhat different staining pattern. Figure 13A shows the binding sites of biotin-conjugated α-BTX, a specific nicotinic ligand at the vertebrate neuromuscular junction (Chang and Lee, 1963; Fertuck and Salpeter, 1974). α-BTX binding sites appeared in the OPL and the whole thickness of the IPL, but did not appear in any somata (Fig. 13A). Antibodies against α3 and α8 subunits of the nAChRs also stained both the OPL and IPL (Fig. 13B and C). In contrast to α-BTX, the α8 subunit antibody clearly stained radial processes (arrow) and end-feet (arrowhead) of Müller cells (Fig. 13C), while α3 subunit antibody lightly stained Müller cell-like endfeet (arrow in Fig. 13B).

4 Discussion

The results presented in this chapter demonstrated the presence of ChAT, AChE and AChRs in the adult newt retina. Their distribution patterns are summarized in Fig. 14. ChAT and AChE were localized mainly in subpopulations of amacrine cells, whilst mAChRs were present mainly on ganglion cells. nAChR markers were localized only in the IPL and OPL diffusely. However, there was no one-to-one correspondence between the two ChAT-ir bands in the IPL and the location of the AChE or mAChR bands (Fig. 14).

4.1 Localization of cholinergic neurons

ChAT is a reliable marker for retinal cholinergic neurons containing ACh (Graham, 1974; Eckenstein and Thoenen, 1982; Tumosa et al., 1984; Masland and Tauchi, 1986). Most vertebrate retinas include two or three types of cholinergic amacrine cells; generally two subpopulations of conventional amacrine cells in the INL and displaced amacrine cells in the GCL (Famiglietti, 1983; Tauchi and Masland, 1984, 1985; Ross et al., 1985; Pourcho and Osman, 1986a; Voigt, 1986; Famiglietti and Tumosa, 1987; Sandmann et al., 1997). The adult newt retina also has two types of ChAT-ir cells: The conventional amacrine cells whose somata are localized at the INL/IPL border, and displaced amacrine cells whose somata localized at the IPL/GCL border. These cells sent their dendrites in the outermost region of the IPL and the lower middle of the IPL as single bands, respectively (Fig. 9 and 14). Two ChAT-ir bands have commonly been reported in many vertebrate retinas including goldfish (Tumosa et al.,1984), chick (Millar et al., 1985; Spira et al., 1987; Reiss et al., 1996), cat and rat (Criswell and

Brandon, 1993), tree shrew (Conley et al., 1986; Sandmann et al., 1997), and rabbit (Famiglietti and Tumosa, 1987), although the localization of the band is not necessarily the same in all species. In the turtle retina, for example, additional ChAT-ir bands in the IPL have been reported (Guiloff and Kolb, 1992).

In the ChAT immunoreactivity experiment on the wholemount retinas, the spatial density of the ChAT-ir amacrine cells tended to be higher in the central retina than in the periphery. The density of ChAT-ir cells in central areas ranged from 300 to 450 cells/mm^2. This value is a about one-half of the density value of cholinergic amacrine cells in the rabbit, cat and rat retina (Vaney, 1984; Pourcho and Osman, 1986a; Voigt, 1986). The density ratio of the conventional (INL) to displaced (GCL) amacrine cells in the present study was approximately 1.3 : 1. This ratio in the newt retina was higher than that ($\leq$1) found in retinas of rabbit (Masland et al., 1984; Famiglietti et al., 1986), rat (Ross et al., 1985; Voigt, 1986), chick (Miller et al., 1987) and cat (Schmidt et al., 1985; Pourcho and Osman, 1986a).

The ChAT activity in the retina was first described by Lam (1972), working on isolated photoreceptors from turtle retina. He concluded that ACh may be a transmitter utilized by a subpopulation of turtle cones. On the other hand, Ross and McDougal (1976) could find very low ChAT activity in the photoreceptors and the OPL in mouse, rat, cat, pigeon, goldfish, turtle, rabbit, monkey and frog retinas. In the present study, there was no observable ChAT immunoreactivity in the OPL.

The AChE reaction product was localized in somata in both the INL and the GCL of the newt retina. At least three types of AChE-positive cells were observed according to the location of their somata. The AChE-positive somata directly apposed to either side of the IPL appeared to correspond to the two types of ChAT-ir amacrine cells.

However, double-staining of the chick retina for ChAT immunoreactivity and for AChE activity showed that AChE-positive cells are not always ChAT-ir (Millar et al., 1985). The additional type of AChE-positive somata lying one-half to one cell body away from the INL/ IPL border probably represents another subtype of amacrine cells because their primary dendrites could sometimes be traced into the IPL.

It has been reported that bipolar cells in sheep and rabbit retinas (Francis, 1953) or horizontal cells in the goldfish (Nichols et al., 1972) and rabbit retinas (Reale et al., 1971) are AChE-positive. Moreover, photoreceptors in the turtle retina synthesize ACh (Lam, 1972; Ross and McDougal, 1976; Sarthy and Lam, 1979). In the newt retina, Dickson et al. (1971) first demonstrated AChE activity in the horizontal and bipolar cell somata and membranes in the OPL. In the present study, however, although the OPL was weakly AChE-positive, no AChE activity was detected in somata of photoreceptors, horizontal cells or bipolar cells whose processes make synaptic connections in the OPL. Furthermore, the OPL was not ChAT-ir. It would appear, therefore, that the OPL in the newt retina may not involve cholinergic neurotransmission.

There is interspecies variation in terms of the number of AChE-positive bands, ranging from 2 to 5 bands, and their localizations in the IPL (Shen et al., 1956; Ma and Grant, 1984; Millar et al., 1985; Ross et al., 1985; Brandon, 1987; Hutchins, 1987; Hutchins and Hollyfield, 1987; Spira et al., 1987; Criswell and Brandon, 1992; Criswell and Brandon, 1993; Reiss et al., 1996). In cat retina, Pourcho and Osman (1986b) reported two AChE-positive bands which did not overlap with the two ChAT-ir bands, and suggested that AChE may participate in functions not directly related to cholinergic neurotransmission. On the other hand, in many other studies, two of the AChE-positive bands

were present at the same depth to those of ChAT-ir bands in trutle (Criswell and Brandon, 1992) and chick retinas (Millar et al., 1985; Reiss et al., 1996), and at depths in close proximity to those of ChAT-ir bands in rabbit (Brandon, 1987), cat and rat retinas (Ross et al., 1985; Criswell and Brandon, 1993), indicating that they are related to cholinergic transmission. The functional significant of additional AChE-positive bands which show no overlap with ChAT-ir bands is unknown. Over the last twenty years, however, evidence has accumulated that AChE may have a novel, non-cholinergic role in both the CNS including retina (Greenfield, 1984; Layer, 1990; Greenfield, 1991, 1992; Appleyard, 1992; Layer et al., 1993; Jones et al., 1995; Layer and Willbold, 1995; Holmes et al., 1997).

In the newt retina, there was no direct correlation between maximum AChE and ChAT localizations in the IPL. If the incubation period was relatively short, the AChE activity was detected at a relative depth of about 20-40% IPL where ChAT was not present. When the incubation period was prolonged, the AChE-positive band became thicker in width and overlapped with the two ChAT-ir bands (Fig. 14). Whether a broad AChE-positive band is due to diffusion of the products of enzymatic activity, or whether there is a real gradient of AChE activity in the distal half of the IPL is difficult to say at this stage.

In conclusion, the present results, in agreement with those in a variety of retinas, suggest that AChE activity does not occur exclusively at cholinergic synapses. Then, a question arises why are there AChE bands which are unrelated to cholinergic neurotransmission. Growing evidence suggests that non-cholinergic AChE may play a role as a neurotrophic agent in survival and development of the CNS including retina (Greenfield, 1984, 1992; Layer et al., 1993; Jones et al., 1995; Holmes et al., 1997).

4.2 Acetylcholine receptors (AChRs)

AChRs are divided into two categories: (1) Ionotropic receptors (nAChRs) that are activated selectively by nicotine-like ligands and (2) metabotropic receptors (mAChRs) that are selectively activated by muscarine-like ligands. Identification and localization of these AChRs have been investigated by many techniques including physiology (Bonaventure et al., 1989; Jardon et al., 1992; Papke et al., 1993), pharmacology (Langdon and Freeman, 1987; Deneris et al., 1991; Xie et al., 1992), immunohistochemistry(Sargent et al., 1989; Whiting et al., 1991; Britto et al., 1992; Keyser et al., 1993; Hamassaki-Britto et al., 1994a, b; McKinnon and Nathanson, 1995) and molecular biology (Bonner et al., 1987, 1988; Lindstrom et al., 1987; Peralta et al.,1987, 1988; Skorupa and Klein, 1993).

4.2.1 Localization of mAChRs: In mammals, five distinct mAChR isoforms (m1~m5) have been characterized in the CNS by molecular biology (Bonner et al., 1987, 1988; Peralta et al.,1987, 1988). Three mAChR subtypes (cm2, cm3, cm4), named accoding to sequence homology with their mammalian counterparts (Tietje et al., 1990; Tietje and Nathanson, 1991; Gadbut and Galper, 1994) were identified in chick retina (Fischer et al., 1998; McKinnon et al., 1998). Each mAChR was localized to distinct bands within the IPL, although the localization of the main bands was not necessarily the same for each subtype. The cm3 isoform was found in the OPL, whereas the cm2 and the cm4 isoforms were not. Furthermore, cm2 and cm3 were shown to be expressed in ganglion, bipolar, and photoreceptor cells, whereas cm4 was found in all cell types of the neural retina.

In the present study, a commercially available antibody against m2

was used and found to cross react with some epitopes in the adult newt retina. The anti-m2 antibody labelled somata in both the GCL and the INL, as well as the IPL. Intense immunoreactivity was observed in somata at the most proximal region in the GCL. These cells appeared to be ganglion cells because of their location in the GCL. This is also supported by the observation that the innermost cells in the GCL of the newt retina showed intense immunoreactivity to Na^+ channel antibody as a possible molecular marker for ganglion cells (Cheon et al., 1998). At present, whether a few mAChR-ir somata at INL/IPL border are conventional amacrine cells or displaced ganglion cells is uncertain. To confirm this, it is necessary to do either double staining of Na^+ channel-ir and mAChR-ir cells or retrograde labelling of ganglion cells with horseradish peroxidase before mAChR-immunoreactivity. In chick retina, cm2 isoform was expressed in numerous ganglion cells and amacrine cells (Fischer et al., 1998; McKinnon et al., 1998), and also weakly expressed in bipolar cells and photoreceptors (McKinnon et al., 1998). The significance of the mAChR immunoreactivity in those cells presently remains uncertain.

More than two mAChR-positive bands in the IPL have been revealed in the adult chick retina. However, these bands do not always correlate with the ChAT-ir bands (Sugiyama et al., 1977; Hutchins, 1987; Fischer et al., 1998; McKinnon et al., 1998). In the newt retina, this was also the case. The distal mAChR-ir band in the IPL at a depth of 0-15% showed overlap with one of the ChAT-ir bands, as well as the broad AChE-positive band, while the proximal mAChR-ir band at 85-100% depth showed no overlap with either the AChE-positive or ChAT-ir bands (Fig.14). A similar disagreement of localizations between ChAT and mAChRs was observed in the OPL of the newt retina as well as many other vertebrate retinas (Hutchins, 1987;

Fischer et al., 1998).

4.2.2 Localization of nAChRs: The snake toxin α-bungarotoxin (α-BTX) has initially been utilized as a marker for nAChR sites at vertebrate neuromuscular junctions (Fertuck and Salpeter,1974; Lentz et al., 1977) and in the CNS (Polz-Tejera et al., 1975; Lentz and Chester, 1977). In the vertebrate retina, α-BTX binding sites were described in both the IPL and the OPL of chick (Vogel and Nirenberg, 1976, Vogel et al., 1977), goldfish (Yazulla and Schmidt, 1976; Schwartz and Bok, 1979; Zucker and Yazulla, 1982), pigeon (Yazulla and Schmidt, 1977), turtle (Yazulla and Schmidt, 1976) and rabbit (Pourcho, 1979). Light microscopic localization of α-BTX binding sites in the newt retina was also restricted to the whole width of IPL and OPL; there was no binding to cell bodies.

In the OPL, Schwartz and Bok (1979) localized ^{125}I-α-BTX by electron microscopic autoradiography to the cell bodies and dendrites of bipolar cells in the goldfish retina. However, this may not reflect simply the localization of cholinergic synapses, because of the absence of ChAT in the OPL in the retinas of a wide variety of vertebrate (Ross and McDougal, 1976), as well as newt retina. In the IPL, three or four bands of ^{125}I-α-BTX binding sites have been observed in retinas of goldfish (Schwartz and Bok, 1979) and chick (Vogel and Nirenberg, 1976). In the newt retina, however, α-BTX binding sites appeared in the whole thickness of the IPL. It is unclear whether the observed α-BTX distribution in the IPL implicates the presence of 'diffuse' nicotinic synapses. Analysis by electrophysiology is needed to answer this issue.

In goldfish retina, a large number of nonsynaptic nAChR have been described by electron microscopic analysis of α-BTX-HRP binding. Nonsynaptic binding sites for α-BTX-HRP were seen on the

dendrites of ganglion, amacrine, and bipolar cells. Nonsynaptic nAChR are not restricted to the goldfish retina, but have been observed, for example, in the dorsal root ganglion (Schechter et al., 1978; Polz-Tejera et al., 1980) and in the OPL of pigeon retina (Yazulla and Schmidt, 1977). The function of nonsynaptic nAChRs in the retina is currently unclear.

The recent availability of antibodies and cDNA probes for nAChR subunits has made it possible to localize precisely the cholinoceptive neurons in the nervous system (review in Deneris et al., 1991; Hamassaki-Britto et al., 1994). In the chick retina, the antibody of the α3 subunit stained at least two bands within the IPL, and somata of ganglion, amacrine, and displaced ganglion cells (Whiting et al., 1991; Britto et al., 1992; Hamassaki-Britto et al., 1994; Araki et al., 1997). The antibody against the α8 subunit stained the whole thickness of the IPL, and somata of ganglion, amacrine, and bipolar cells (Britto et al., 1992; Keyser et al., 1993; Hamassaki-Britto et al., 1994). Immunohistochemistry with antibodies against the α3 and α8 subunits was undertaken in the newt retina. The α3 antibody stained the OPL and a whole thickness of the IPL with no apparent sublamination, and also stained Müller cell-like processes and endfeet. The α8 antibody stained clearly radial processes and endfeet of Müller cells as well as both the IPL and OPL. In contrast to the results obtained in the chick retina, however, the two molecular markers for nAChRs did not stain any somata in the three nuclear layers.

When all of the data presented here are considered as a whole, the most distal region in the IPL where ChAT, AChE and mAChR overlap, may play at least some role in cholinergic neurotransmission in the adult newt retina. A more precise characterization of the cholinergic system in the IPL must await use of many other molecular markers

specific for mAChR and nAChR subtypes (Hamassaki-Britto et al, 1994; McKinnon et al., 1998; Fischer et al., 1998).