III General discussion

The vertebrate retina is an attractive model for studies of physiology, metabolism, development and regeneration (Stefanelli et al., 1967; Sheffield and Moscona, 1970; Dowing, 1987; Rodieck, 1998). This is made possible because of a limited number of cell classes, easy, intact removal from the back of the eye, and its conserved anatomical organization throughout all vertebrate classes. Moreover, it is one of the few regions of CNS capable of regeneration after mechanical disruption.

In early retinal development, neuroepithelial cells on the inner wall of the optic cup proliferate actively and differentiate into various retinal neurons. Differentiated cell types extend their processes to terminate in particular layers and to establish specific synaptic connections with their target cells (synaptogenesis), so as to form subsets of functional neural circuitry. One of the most important steps during synaptogenesis is the choice of neurotransmitter. Neurons can communicate with target cells through the release of transmitters. Appropriate function in the mature system requires the neurotransmitter released from presynaptic terminals to match the receptors present on the surface of the postsynaptic cells.

Acetylcholine (ACh) is widely accepted as a putative neurotransmitter in vertebrate retina (for reviews, see Neal, 1983; Hutchins, 1987). The investigation of ACh as a retinal transmitter has been approached by a variety of biochemical, pharmacological, physiological and anatomical techniques. ACh is synthesized in cholinergic amacrine cells by cholineacetyltransferase (ChAT) and stored in the presynaptic vesicles. ACh stored within vesicles is released by exocytosis in response to depolarization of the presynaptic nerve

terminals, and interacts with acetylcholine receptors (AChRs) on postsynaptic neurons, subpopulation of amacrine and ganglion cells. Acetylcholinesterase (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 (Fig. 4).

In Chapter I, I examined whether there is a cholinergic system in the adult newt retina. The results indicated that ChAT and AChE were localized mainly in subpopulations of amacrine cells, and ACh receptors (AChRs), especially mAChRs were present mainly on the ganglion cells. nAChR markers stained the IPL uniformly, but not any cell bodies. However, before coming to a definitive conclusion of diffuse distribution of nAChRs in the IPL, I need to identify specific nAChRs in the retina by using new markers specifically designed for the newt retina. Since there was evidence for the existence of a cholinergic system in the adult newt retina, like other vertebrate retinas, particular attention was directed to its appearance and maturation during retinal development and regeneration. Here, I provided evidence for similalites between development and regeneration processes.

1 Development versus regeneration

Figure 29, shows a schematic summary diagram illustrating the morphological properties of the newt retinas at each regeneration stage (A) in comparison with those at development stages (B) and the time course of appearance of ChAT, AChE, and AChR markers during development and regeneration (C).

In the developing newt retina, ChAT immunoreactivity first became detectable in somata around the presumptive IPL. At the same time, a

single ChAT-ir IPL band which later resolved into two bands appeared (Fig. 17). The same staining pattern was observed in the regenerating retina (Fig. 24), indicating that in both developing and regenerating retinas, cholinergic neurons seem to differentiate just before or at the time of synaptogenesis.

AChE reaction product in both developing and regenerating retinas appeared in somata located in the most proximal levels of the central retina before ChAT-ir neurons became detectable (Fig. 18 and 25). The onset of AChE activity earlier than ChAT immunoreactivity has been also reported in the developing chick retina (Spira et al., 1987). During the course of development and regeneration of the newt retina, AChE-positive somata in the vitreal surface of the retina disappeared and the remaining AChE-positive somata were located close to the IPL. This may suggest the migration of the early developed AChE-positive cells to their terminal positions (Fig. 18B and 25B). Alternatively, it is also possible to suppose that early developed cells lost their AChE activity during development and regeneration, and that new AChE-positive cells related to cholinergic neurotransmission appeared at the beginning of synaptogenesis.

mAChR immunoreactivity first became detectable in presumptive ganglion cells at embryonic stage 31 or intermediate-II regenerating stage, before ChAT-ir neurons appeared and well before synaptogenesis (Fig. 29C). The appearance of the neurotransmitter receptors before the formation of synaptic structures or the appearance of synthetic enzymes has been reported in the developing retinas of other species (Sheffield and Fischman, 1970; Hughes and LaVelle, 1974; Yamashita and Fukuda, 1993; Chiba et al., 1997). In the newt regenerating retina, GABA receptors could be also detected before the synthesis of transmitter GABA (Chiba et al., 1997).

The significance of earlier appearance of mAChRs remains unclear. The proposed role for mAChRs is related to the morphogenesis of the eye (Oettling et al., 1988; Yamashita and Fukuda, 1993). Yamashita et al (1994) described a mAChR-mediated increase in $[Ca^{2+}]_i$ at the stage of optic cup formation in the chick embryo and subsequent decline of the muscarinic Ca^{2+} mobilization. Our recent prelimiary studies using 'live' slice preparations of regenerating newt retina indicated that exogenous muscarine similarly induces an increase in $[Ca^{2+}]_i$ in intermediate-II regenerating retina. At this stage, however, retinal cells could not synthesize ACh. Therefore, ACh may be brought over from other tissues to the retina through the blood circulation. Ca^{2+} is an important second messenger that regulate a broad spectrum of intracellular events. The generation of cytoplasmic Ca^{2+} signals involves various molecular cascades that differ among cell types. In the CNS, research has concentrated on the role and mechanism of Ca^{2+} signalling in neurons. It is clear now that changes in the cytoplasmic Ca^{2+} concentration are essential for neurotransmitter release and neuronal development (Kater and Mills, 1990; Spitzer et al., 1995). Further studies of mAChR-mediated increase in $[Ca^{2+}]_i$ are likely to lead to a better understanding of the function of the earlier appearance of mAChRs during development and regeneration.

2 Possible non-cholinergic functions of AChE

The present results in agreement with those obtained from a variety of other retinas suggest that AChE activity does not occur exclusively at cholinergic synapses. Over the last twenty years evidence has accumulated that AChE may have a novel, non-cholinergic role in both the CNS, including retina, and in peripheral tissue (Greenfield, 1984;

Layer, 1990; Greenfield, 1991; Greenfield, 1992; Appleyard, 1992; Layer et al., 1993; Jones, 1995; Layer and Willbold, 1995; Holmes et al., 1997). This notion has developed from several basic observations. For example, AChE can be localized in areas where there is little or no ACh (Silver, 1974; Hutchins, 1987; Criswell and Brandon, 1993; Hutchins et al., 1995). AChE activity can be detected in the developing retina at early embryonic stages long before synaptogenesis (Spira et al., 1987). The AChE protein can exist not only as a membrane-bound form but also as several soluble forms, at least one of which can be secreted by growing neurites (Chubb and Smith, 1975; Kristt, 1989; Robertson et al., 1991; Appleyard, 1992; Massoulié et al., 1993). AChE is also expressed by growing neurites in vitro (Gahwiler and Hefti, 1984). AChE may also be function as a peptidase to degrade components of the extracellular matrix, and then act to degrade neuroactive peptides after synaptogenesis (Chubb and Millar, 1984; Pourcho and Osman, 1986b; Goebel and Pourcho, 1992). Furthermore, it has been suggested that the non-enzymatic site of the AChE molecule may have an adhesive function and regulate neurite growth of chicken tectal cells or retinal explant in culture (Layer et al., 1993).

3 Future perspective

When all of data presented here are considered as a whole, the following three notions arise:

(1) Cholinergic functions may start at the earliest in intermediate-III regenerating retina, Electroretinogram (ERG) with a- and b-waves similar to those recorded from eyes of adult animals has been recorded from regenerating retinas around this stage, although the amplitude of the ERG was small (Lam, 1977;

Sarthy and Lam, 1983).

(2) The time course of appearance of ChAT, AChE and AChR markers in regenerating retina proceed with the same sequence as that in the developing retina. This raises a major question of what mechanisms are responsible for pre-programming this common sequence during both development and regeneration.

(3) In particular, the early appearance of AChE activity in both developing and regenerating newt retinas, as in some other parts of the CNS, would necessitate in the future consideration of the possible significance of this molecule to cellular differentiation, organization and synaptogenesis.