Chapter III

Appearance and maturation of the cholinergic system during retinal regeneration

1 Introduction

Since Charles Bonnet (1781) originally found that a small normal-appearing eye would reconstitute one year after partial removal of the original eye in the adult salamander, the capacity of retinal regeneration has been studied in a wide variety of vertebrates. These studies showed that many vertebrates can regenerate a neural retina after deprivation of the original retina some time during normal development. However, this ability is lost early in embryonic life in most animals. On the other hand, certain species of adult urodele amphibians, such as newts and salamanders, possess the ability to regenerate a fully functional retina following the complete removal or destruction of the original retina.

The process of retinal regeneration in newts and salamanders has been studied morphologically (Wachs, 1920; Stone, 1950b; Hasegawa, 1958; Keefe, 1973a), immunohistochemically (Klein et al., 1990; Bugra et al., 1992; Negishi et al., 1992; Saito et al., 1994; Chiba et al., 1997; Cheon et al., 1998), biochemically (Sarthy and Lam, 1983; Chiba et al., 1997), and electrophysiologically (Dabagian and Oganessian, 1970; Lam, 1977; Kaneko and Saito, 1992; Kaneko et al., 1993; Chiba and Saito, 1995; Sakai and Saito, 1997). The consensus of most investigators is that regeneration occurs mainly from transdifferentiation of retinal pigment epithelial (RPE) cells into neurons and partly from intrinsic neuroblasts located at the retinal margin. Transdifferentiation (also known as metaplasia) is an alteration of the state of differentiation of cells that have already differentiated during embryonic development.

It is fascinating that after removing the entire retina, RPE cells can discard the pigment granules and dedifferentiate into neuroepithelial

cells which proliferate, differentiate into various retinal neurons and reform a functional neural network. While the neural network of the retina has been restored, axons growing out from newly differentiated ganglion cells extend as new optic nerve fibers, invade the stump of the old optic nerve, and follow it to reinnervate the optic tectum. Eventually, normal retinotectal projections are reestablished, and vision is restored after a period of several months (Grafstein and Burgen, 1964; Cronly-Dillon, 1968; Lam, 1977). Such retinal regeneration, as well retinal development, promises to be a useful system for not only understanding metaplasia, but also examining the mechanisms of cytodifferentiation and the genesis of neural circuitry in the CNS.

As described in the General Introduction, functional differentiation of neural circuitry in the developing CNS includes the genesis of the neurotransmitter systems. The acetylcholine (ACh) neurotransmitter system has been considered as one of the "classical" neurotransmitter systems found in the proximal retina of the adult vertebrates (Dowling, 1987). Development of the cholinergic system has been biochemically and morphologically investigated in the retina of various vertebrate species including newt (Chapter II).

In contrast to studies of the cholinergic system during retinal development, studies of this system during regeneration have been limited, probably because most vertebrate species, except newts and salamanders, do not possess the ability to regenerate a functional retina in their adult life. Using biochemical techniques together with electrophysiology, Sarthy and Lam (1983) have reported in the regenerating retina of adult newt, that the time course of appearance of ChAT activity correlated well with increase in the synthesis of ACh and with changes in amplitude of electroretinogram (ERG). In contrast,

appearance and maturation of AChE and AChRs during regeneration have not previously been investigated. Furthermore, there is no comparision of the behaviour of the cholinergic system during the processes of development and regeneration.

The purpose of the present study was to examine the time course of the appearance and subsequent maturation of the cholinergic system during regeneration of the newt retina, using immunocytochemistry and histochemistry. The results indicated that AChE and mAChRs appear before the synthesis of ACh and well before the period of synaptogenesis, and that nAChRs appear at the time of synaptogenesis. Furthermore, the overall sequence of appearance of the molecular components of the cholinergic system during regeneration seems very similar to that observed during development.

2 Materials and Methods

2.1 Preparation of regenerating retina

Adult newts (*Cynops pyrrhogaster*) were deeply anesthetized with 0.1% 4-allyl-2-methoxyphenol solution (FA100; Tanabe, Ohsaka/ Japan) for at least 30 min in the dark before surgery under a dissecting microscope. Figure 22 shows a schematic diagram illustrating the surgical removal of the normal retina from an adult animal. The dorsal half of the eye was cut open along the corneo-scleral junction (Fig. 22B) and the lens was removed. The neural retina was detached from the RPE by a gentle stream of physiological saline through a 1ml, syringe and removed by cutting the optic nerve (Fig. 22C). The eye flap consisting of iris and cornea was gently replaced at its original position (Fig. 22D). The other eye was left intact as a control. The operated animals were maintained in a moist chamber at a temperature of about 22℃ and allowed to recover until experiment. They were killed on selected post-operative days (18-70 days) under anesthesia. Both control and operated eyeballs were removed from the orbit and fixed for about 12 h at 4℃ in 4% paraformaldehyde in 0.1 M phosphatebuffer (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, frozen in an embedding medium (O.C.T. compound, Miles Inc. Elkhart, IN), 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 and histochemistry

Antibodies against ChAT, m2 subunit of mAChR, and α3

subunits of nAChR were used for the localization of the cholinergic system components. nAChRs were also identified by a biotin-conjugated α-BTX. For localization of AChE activity in the retina, a histochemical method described by Karnovsky and Roots (1964) was used. The procedures of ChAT and AChR immunocytochemistry, and α-BTX and AChE histochemistry were the same as those described in Chapter I (Figs. 5-7).

3 Results

3.1 Definition of stages of regenerating retinas

Regenerating retinas have been divided into three broad groups ('early', 'intermediate' and 'late') on the basis of their morphological appearance (Kaneko and Saito, 1992; Chiba et al., 1997; Cheon et al., 1998). Figure 23 shows a schematic diagram illustrating morphological characteristic of the retina at each regenerating stage. An 'early'-regenerating retina was 1-2 cells thick, and comprises pigmented cells and progenitor cells with nonpigmented cytoplasm. An 'intermediate'-regenerating retina had multiple layers of cells with no apparent segregation of synaptic layers. In this study, the intermediate-regenerating retina was further divided into three sub-groups, I, II and III, to facilitate the correlative analyses and discussion. An 'intermediate-I' retina was 3-4 cells thick, consisting predominantly of progenitor cells. An 'intermediate-II' retina was 4-5 cells thick, consisting of a progenitor cell layer with only one row of rounded cells, probably ganglion cells, at the most proximal region of the retina (Cheon et al., 1998). An 'intermediate-III' retina was 5-7 cells thick, corresponding to the stage just before or at the beginning of formation of the synaptic layers. Photoreceptor cells start to differentiate at this stage of regeneration (Saito et al., 1994; Cheon et al., 1998). A 'late'-regenerating retina characteristically had a penta-laminar structure, having three nuclear layers and two synaptic layers. Unlike development, sections of a certain regenerating retina could sometimes possess morphological characteristics spanning two stages of regeneration, because of inherent variabilities of regeneration.

3.2 Choline acetyltransferase

The appearance and subsequent changes in ChAT immunoreactivity were examined in retinas from different regenerating stages (Fig. 24). Figure 24A shows a section of an 'intermediate-II'-regenerating retina. The retina was characterized by multi-layered cells with only an innermost row of rounded cells providing evidence of ganglion cell differentiation (Cheon et al., 1998). At this stage of regeneration, no apparent ChAT immunoreactivity was detectable. Figure 24B shows a transition area between the 'intermediate-III'- and the 'late'-regenerating retinas. ChAT-ir IPL was seen as a single band, and the two types of ChAT-ir somata of oval shape were detected on either side of the IPL (an arrow and arrowheads). Figure 24C shows an early period of the 'late'-regenerating retina. The single ChAT-ir IPL band became thicker in width and the number of the ChAT-ir somata increased. During subsequent regeneration, the single ChAT-ir IPL band resolved into two bands (Fig. 24D, arrowheads), thickening concomitantly with the IPL width. Thus, the overall ChAT-ir pattern, such as the density, distribution and shape of the ChAT-ir somata, and the location of the two ChAT-ir IPL bands, became comparable with those found in the mature retina. The staining seen in the OPL was probably nonspecific since it was still present in the mature retina without primary antibody.

3.3 Acetylcholinesterase

The time course of appearance of the AChE reaction product was examined in retinas from different regenerating stages (Fig. 25). The 'early'- and 'intermediate-I'- regenerating retinas were not AChE-

positive (data not shown). AChE-positive cells were first detected in the most proximal region of the 'intermediate-II'-regenerating retina (Fig. 25A, arrowhead). Figure 25B shows a retinal section at the transition period between the intermediate-II and -III stages. AChE-positive somata appeared in many of the most proximal levels of the retina (arrowhead) and also in more distal retinal layers (arrow). Figure 25C shows a regenerating retina that is about to segregate into synaptic layers. AChE-positive somata were detected at either side of the presumptive IPL (arrowhead), but were no longer detectable near the vitreal surface. In the 'late'-regenerating retina, the AChE-positive IPL completely separated the INL and GCL as a single band (Fig. 25D, arrowhead; and Fig. 25E). As shown in Figure 25D, the AChE-positive somata were localized close to the IPL on either side. At the same time, the OPL was weakly stained (arrow). During subsequent regeneration, the retina thickened with an increase in the IPL width, and the AChE-positive band became intensive, occupying approximately 0-60% of the IPL width (Fig. 25E). The OPL also became intensely AChE-positive. Overall, this staining pattern was identical to that of the mature retina.

3.4 Acetylcholine receptors

3.4.1 Muscarinic acetylcholine receptors (mAChRs): The mAChR staining pattern in the regenerating retina was substantially the same as that in the developing retina (Fig. 26). The 'early'- and 'intermediate-I'-regenerating retinas were not mAChR-ir (data not shown). The mAChR-ir somata first became detectable at the most proximal level in the 'intermediate-II'-regenerating retina where a thin row of round presumptive ganglion cells was visible close to the

vitreal surface (Fig. 26A, arrowheads). The cell number increased to form a layer several cells thick in the 'intermediate-III'-regenerating retina where the IPL was about to form (Fig. 26B, arrowhead). Figure 26C shows an early stage of the 'late'-regenerating retina where the GCL and the INL have been separated by a thin IPL. The mAChR-ir somata in the GCL increased in number and a few somata close to the IPL in the INL became mAChR-ir (arrowheads). At the same time, somata in the horizontal cell layer clearly became mAChR-ir (arrows). With further advancement of regeneration, the whole layers of the retina thickened (Fig. 26D). Around this regenerating stage, two mAChR-ir bands became detectable within the IPL. On the other hand, the mAChR-ir somata in the horizontal cell layer became less immunoreactive, similar to that in the developing retina. These mAChR staining patterns in the 'late'-regenerating retina were identical to those of the mature retina.

3.4.2 Nicotinic acetylcholine receptors (nAChRs): The time course of appearance and subsequent changes in nAChRs were examined by using two markers; α-BTX and α3 subunit antibody. Figure 27 shows α-BTX binding sites in the retina from different regenerating stages. Figure 27A shows a transverse section of an intermediate-II regenerating retina, with no α-BTX binding sites. Weak α-BTX binding sites were first detected in the intermediate-III regenerating retina in the presumptive IPL (Fig. 27B, arrowhead). In the late regenerating retina, the IPL thickened and was uniformly stained by α-BTX (Fig. 27C and D). The OPL was also stained at this stage. None of cell bodies were α-BTX-positive. Figure 28 shows α3 immunoreactivity in the regenerating retina. α3-ir IPL became first detectable in the retina at the intermediate-III stage (Fig. 28B,

arrowhead); $\alpha3$-ir OPL was observed in the late stages (Fig. 28C and D). Overall $\alpha3$-ir pattern was similar to that observed by α-BTX binding pattern, except that the $\alpha3$ subunit antibody stained Müller cell-like endfeet (Fig. 28D, arrowheads).

4 Discussion

4.1 The onset of AChE activity in regenerating retina is earlier than that of ChAT immunoreactivity

ChAT activity during regeneration of adult newt retina was first examined by Sarthy and Lam (1983) using biochemical techniques. They reported that the appearance of ChAT paralleled ACh synthesis and preceded development of the ERG. In the present immunohistochemical study, two populations of ChAT-ir cells appeared along either side of the presumptive IPL just before the formation of synaptic layers. The cells are probably subpopulations of conventional and displaced amacrine cells, because of their localization being identical to the ChAT-ir amacrine cells in the control retina. As regeneration proceeded, ChAT-ir amacrine cells increased in number and arborized into two ChAT-ir bands in the IPL, like those in the control retina. Basically the same staining pattern was observed in the developing retina.

AChE-positive cells were detected first in somata located at the most proximal region in the intermediate-II regenerating retina. At this stage, ChAT-ir amacrine cells could not yet be observed. In the intermediate-III state, most AChE-positive cells were found along either side of presumptive IPL. From their localization, they are probably subpopulations of amacrine cells like those in many other vertebrate retina (Hutchins, 1987; Hutchins and Hollyfield, 1987; Spira et al., 1987; Reiss et al., 1996). In chick retina, it has been reported that AChE-positive and ChAT-ir amacrine cells are not always the same (Millar et al., 1985). Since ChAT is regarded as a reliable

marker for cholinergic neurons in the CNS, the cholinergic system may vertually mature after the intermediate-III stage (Fig. 29C).

In contrast to the presence of two ChAT-ir single bands in the IPL, AChE-positive IPL occupied the distal half of the IPL. Furthermore, AChE activity appeared before the appearance of ChAT-ir cells or the morphological development of the IPL. These results in agreement with those in developing retinas suggest that AChE activity does not exclusively occur at cholinergic synapses. As described in Chapter I and II, there is now growing evidence that AChE may play a role which is unrelated to cholinergic transmission. This point will be mentioned again in General Discussion.

4.2 The onset of mAChRs in regenerating retina is earlier than that of ChAT immunoreactivity

mAChR immunoreactivity, like AChE activity, first became detectable in somata located in the most proximal layer of the retina at intermediate-II stage, well before ChAT-ir neurons appeared. The mAChR-ir somata are probably ganglion cells, because Na^+ channel antibody, a possible marker of ganglion cells, was visible close to the vitreal surface at the same stage (Cheon et al., 1998). To confirm this, it is necessary to do double staining of Na^+ channel-ir and mAChR-ir cells. The appearance of the neurotransmitter receptors before the formation of synaptic structures or the appearance of synthetic enzymes has also been reported in the regenerating newt retina (Chiba et al., 1997) and in the developing retinas of other species (Sheffield and Fischman, 1970; Hughes and LaVelle, 1974; Yamashita and Fukuda, 1993).

Two mAChR-ir bands were observed in the IPL during regeneration. A distal band corresponded to one of two ChAT-ir bands. However, a

proximal band did not correspond to another ChAT-ir band. When the OPL began to form in regenerating newt retina, the mAChR immunoreactivity was transiently expressed in somata in the horizontal cell layer. This was also the case in developing retina (Fig. 19). Transient mAChR expression has been also described in the OPL of the developing ferret retina (Hutchins, 1994). However, the possible sources of ACh in the OPL is unknown in newt and ferret retinas, because ChAT has not been detected in this layer or in photoreceptors or horizontal cells (Lam, 1972; Kim et al., 1998). These results in agreement with those in developing retinas suggest that mAChRs may play a role which is unrelated to cholinergic synaptic transmission.

4.3 The onset of nAChRs in regenerating retina is later than that of mAChRs

The appearance and maturation of nAChRs have mostly been studied in the developing retina by using either α-BTX (Vogel and Nirenberg, 1976; Sugiyama et al., 1977; Voget et al., 1977) or molecular markers α3 and α8 subunits (Britto et al., 1992; Hamassaki-Britto et al., 1994). However, the present study is the first on the regenerating retina. Two nAChR markers, α-BTX and α3 subunit were used in this study and showed substantially the same staining pattern. They 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 (Fig. 29C).

nAChRs labeled with ^{125}I-α-BTX in the developing chick retina initially appeared as a thin layer of presumptive IPL. As the IPL thickened, four single α-BTX-positive bands became visible (Sugiyama

et al., 1977). Similar changes in the staining pattern in the IPL were observed for α3 and α8 immunohistochemistry (Britto, et al., 1992; Hamassaki-Britto, et al., 1994). In contrast to the result of chick retina, the IPL in the regenerating retina was uniformly stained with α-BTX binding sites and α3 immunohistochemistry. It is unclear whether the lack of the specific staining bands in the IPL is due to the sensitivity of detection or diffuse distribution of nAChRs through the IPL.