Chapter 2

Appearance and maturation of neurotransmitter channels during retinal regeneration

1. Introduction

Functional neural differentiation of the central nervous system (CNS) includes the expression of voltage-gated ion channels specific for the activity of each type of neurons, and the expression of neurotransmitters and their receptors responsible for the synaptic transmission between neurons. The appearance and maturation of voltage- and/or transmitter-gated ion channels have been studied in the developing CNS including retina (Dupont et al., 1987; LoTurco et al., 1991; Spitzer, 1991; Blanton and Kriegstein, 1992; Skaliora et al., 1993; Rörig and Grantyn, 1994; Liets and Chalupa, 2001). The majority of these studies suggest that voltage- and transmitter-gated ion channels are expressed at early stages of development, even well before functional synaptic activity. Furthermore, several lines of evidence suggest that neurotransmitter systems are not only involved in information processing, but may also act as regulatory signals in many developmental events such as proliferation, cell death, cell differentiation and synapse formation (Mattson, 1988; Lipton and Karter, 1989; McDonald and Johnston, 1990; Lauder, 1993; Ben-Ari et al., 1997; Gleason and Spitzer, 1998; Owens and Kriegstein, 2002).

Certain species of adult amphibians, such as newts and salamanders, possess the ability to regenerate a new functional retina from the retinal pigment epithelium following the complete removal of the original retina (Hasegawa 1958; Keefe 1973a). Such retinal regeneration, as well as retinal development, may be a useful system for understanding the mechanisms of cytodifferentiation and the genesis of neural circuitry in the CNS. However, studies on functional differentiation of neurons during retinal regeneration have been limited (Kaneko and Saito, 1992; Chiba and Saito, 1995b).

In solitary ganglion cells dissociated from newt retinas at different stages of regeneration, the appearance and maturation of the voltage- and transmitter-

gated ion channels have been reported (Kaneko and Saito, 1992; Chiba and Saito, 1995b). However, the use of dissociated cells provides certain technical disadvantages, such as possible contamination by other neurons and loss of dendritic processes by the dissociation procedures. Furthermore, the presence of various regenerating stages even in single retinas makes it difficult to draw a firm conclusion on the basis of an electrophysiological analysis of dissociated cells.

In this study, I prepared living slice preparations from the adult newt retina at different stages of regeneration and examined the appearance and maturation of excitatory and inhibitory neurotransmitter-sensitivity in ganglion cells with whole-cell patch-clamp methods. This living slice preparation method makes us possible to reduce the variability in results due to differences in stage of regeneration. All of the cells examined were visualized by Lucifer Yellow injection through the recording electrode. This approach enables us to correlate the morphological development of ganglion cells with their functional differentiation during regeneration. It also allows us to compare the sequence of morphological and functional differentiation of ganglion cells in regenerating retina of adult newt with that of the ganglion cell differentiation in developing retina of other vertebrates.

2. Materials and Methods

2.1. Slice preparation

Adult newts (*Cynops phyrrhogaster*) were anesthetized with 0.1% FA100 (4-allyl-2-methoexyphenol; Tanabe, Japan). The neural retina together with the lens from one eye was surgically removed and the other eye was left intact as a control (Kaneko and Saito, 1992). The operated animals were maintained in a moist chamber at about 22°C and allowed to recover. They were sacrificed on selected post-operative days under anesthesia. Procedures to make retinal slices were the same as those in Chapter 1. Slice retinas were transferred to a perfusion chamber (about 1 ml volume) and fixed on the bottom of the chamber by a small amount of vaseline at both ends of the filter paper (Fig. 18). The chamber was put on the stage of an upright microscope (Axioscope; Carl Zeiss, Germany). Slices were continuously superfused at a flow rate of about 2 ml/min with control solution (solution A in Table 3), delivered into the experimental chamber by gravity and viewed through a 40x water-immersion objective with differential interference contrast (Nomarski) optics.

2.2. Whole-cell patch-clamp recording in retinal slices

Whole-cell current recordings were performed in ruptured-patch mode (Hamill et al., 1981) from cells in the retinal slice, using an Axopatch-1D amplifier (Axon Instruments). Patch pipettes (about 1-2 μm in tip diameter) filled with a solution contained (in mM): 110 CsF, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 2 ATP-Na_2, 0.5 GTP-Na_3, 0.01% Lucifer Yellow CH. The pH was adjusted to 7.5 with NMDG. The osmolarity was adjusted to 255 mOsmo. Pipette tip resistances ranged from 5 to 7 MΩ in the external solutions. The membrane potentials in all recordings were corrected for the liquid junction potential at the patch pipette tip (–8 mV). The reference electrode was

connected to the bath via a 3 M KCl agar bridge. Other experimental and recording procedures were basically the same as those given in chapter 1.

Experiments were performed at room temperature, around 23°C. Command voltage protocols were generated by using pCLAMP 5.5.1 software (Axon Instruments).

All cells were depolarized from a holding potential of –100 mV to test potentials between –60 and +25 mV in 5 mV increments. Each step pulse of 8 ms duration was applied every 1 s. Sampling frequency was typically 100 kHz. Capacitive and leakage currents were reduced by the P/N subtraction protocol (number of P/N sub-pulses, –5; subpulse holding amplitude, –60 mV). The output cut-off frequency was 10 kHz.

Whole-cell currents activated by glutamate analogs were recorded at a holding potential of –80 mV; whole-cell currents activated by inhibitory neurotransmitters were recorded at a holding potential of 0 mV. Amino acids-induced current records were sampled at 25-100 Hz and low-pass filtered at 2 kHz. Current data were processed by 4 kHz Gaussian digital filter, and stored in a hard disk of a computer (433/M, Dell) and DAT recorder (RD-125T, TEAC). Statistical values in the text are represented as the mean ± standard error (SE).

To obtain the current-voltage (I/V) relationships for whole-cell currents activated by glutamate analogs and inhibitory neurotransmitters, currents were recorded by "ramping" the command voltage from –100 mV to +50 mV at a rate of 150 mV/sec and *vice versa*. Voltage ramp to the depolarizing direction often evoked transient inward currents in cells. Therefore, the I/V curves were obtained during voltage ramp to the hyperpolarizing direction. The I/V curves for drug-induced currents were constructed by subtracting the averages of two to three ramps without drugs from those with drugs.

2.3. Experimental solutions and drug applications

I examined the effects of drugs to cells by using a combination of two drug application systems, bath application system and 'Y-tube' system (Fig. 18). In bath application system, retinal slices were continuously superfused with either the control or experimental bath solutions (Table 3) which were fed into the experiment chamber by gravity and withdrawn by a vacuum. Excitatory amino acid analogs, AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and NMDA (N-methyl-D-aspartic acid), and inhibitory amino acids, GABA (γ-aminobutyric acid) and glycine were applied to the patch-clamped cells using the 'Y-tube' system (Murase et al, 1989). AMPA was dissolved into bath solution B or C, and NMDA was dissolved in bath solution D or E. GABA and glycine were dissolved in bath solution F. The control and drug-containing solutions described above were contained in 50 ml test tubes and connected with the inlet of the Y-tube (teflon tube, 0.63 mm in inner diameter) through pinch valves. The test tubes were kept at a positive pressure with nitrogen, controlled by pico-pump (PV820, World Precision Instruments, Inc., Sarasota, FL). The outlet of the Y-tube was led to a drainage bottle through an electromagnetic valve (EXAK-3, Takasago Electronic, Nagoya, Japan). The drainage bottle was kept at a negative pressure by air pump (NUP-2, Iuchi, Osaka). The tip of the Y-tube (Microfil, 100 μm i.d. and about 20 mm in length) was placed 200-300 μm away from the cell being recorded from. The 'Y-tube' system works as follows. At first, the pinch valve of the control solution and the electromagnetic valve are opened, so that the solution is sent into a drainage bottle. After obtaining a whole-cell voltage-clamp recording, the pinch valve for the control solution is closed and the pinch valve for an appropriate drug solution is open. Closure of the electromagnetic valve causes the drug solution to be released from the tip of the Y-tube onto the cell. Reopening of the electromagnetic valve causes the Y-tube to draw the drug solution into the

drainage bottle. Opening and closing of the electromagnetic valve were controlled by pCLAMP 5.5.1 software. Test solutions reached the cell recorded from within 0.5 sec after closure of the electromagnetic valve.

AMPA, NMDA, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), DL-AP7 (DL-2-amino-7-phosphonoheptanoic acid), bicuculline ((–)-bicuculline methobromide), strychnine and cyclothiazide were obtained from Sigma/RBI Chemical. GABA, glycine and picrotoxin were purchased from Wako (Osaka). Stock solutions of picrotoxin and cyclothiazide were dissolved in dimethyl sulfoxide (DMSO). The stock solution of bicuculline was dissolved in 0.05 N HCl. The final concentration of DMSO and HCl were always kept below 0.07%.

2.4. Intracellular Staining

To visualize the morphology of cells from which currents were recorded, all pipette solutions contained 0.01% Lucifer Yellow CH (dipotassium salt, Sigma/RBI Chemical). Lucifer Yellow dye diffused gradually into the cell during whole-cell recordings. The dye-injected cells were displayed on a video monitor through a CCD camera (C9585; Hamamatsu Photonix, Shizuoka, Japan), captured with a frame grabber, and stored on computer hard disk (Macintosh 4400/200). The morphological properties of these cells were analyzed using Photoshop 3.0 (Adobe) software.

2.5. Definition of regenerating stages

Regenerating retinas have been divided into five stages ('early', 'intermediate-I, -II and -III', and 'late') as described in Chapter 1 (Fig. 8B).

3. Results

I first examined the responsiveness of mature ganglion cells to excitatory and inhibitory amino acids: AMPA, NMDA, GABA and glycine. Since more than half of the cells in the ganglion cell layer (GCL) in the adult newt retina are displaced amacrine cells (Ball and Dickson, 1983), whole-cell currents were mainly recorded from cells located near the vitreal side of the retina where ganglion cells are present at relatively higher population densities than amacrine cells (Cheon et al., 1998). I then examined the appearance and maturation of these amino acid-induced responses during retinal regeneration. All experiments were performed on retinal slices using whole-cell patch-clamp, using the experimental solutions listed in Table 3. AMPA-, GABA- and glycine-induced currents were recorded while suppressing synaptic transmission with $CoCl_2$ (solutions B, C, and F). NMDA-induced currents were recorded in the absence of $CoCl_2$ (solutions D and E), because they were attenuated by Co^{2+} ions. AMPA- and NMDA-induced currents were obtained in the presence of both GABA and glycine receptors antagonists (solutions B-E). AMPA-induced currents were obtained in the presence of cyclothiazide (solutions B and C) to inhibit desensitization, and NMDA-induced currents were obtained in the presence of glycine and absence of Mg^{2+} ions (solutions D and E) to enhance the currents.

3.1. Whole-cell current recordings from ganglion cells in control retina

Whole-cell currents activated by excitatory amino acid analogs (AMPA and NMDA) and inhibitory amino acids (GABA and glycine) were recorded from ganglion cells in the living slice preparation of control retina (Fig. 19). Figure 19A shows representative responses of a single ganglion cell to application of 100 μM AMPA (upper trace) in solution B and 250 μM NMDA (lower trace) in

solution D and the morphology of the cell that these currents were recorded from (top panel). This cell is likely to be an ON-OFF ganglion cell, because of the localization of soma in the GCL and dendritic ramification in both proximal and distal parts of the inner plexiform layer. Both drugs produced an inward current at a holding potential of –80 mV. Regardless of the ganglion cell type, all cells examined responded to both 100 μM AMPA and 250 μM NMDA. The mean peak amplitudes of AMPA- and NMDA-induced currents were 281±28 pA (n=22) and 91±23 pA (n=15) respectively at a holding potential of –80 mV. Figure 19B shows representative responses of a ganglion cell to application of 30 μM GABA (upper trace) and 30 μM glycine (lower trace) in solution F. This cell is probably also an ON-OFF ganglion cell (top panel), because of the localization of soma and dendritic ramification pattern in the IPL. Both drugs produced an outward current at a holding potential of 0 mV. Regardless the ganglion cell types, all cells examined responded to both 30 μM GABA and 30 μM glycine and the mean peak response amplitudes were 80±19 pA (n=27) and 88±22 pA (n=29) at the holding potential of 0 mV, respectively. The amount of currents obtained above are plotted in Figure 23 for comparison with those obtained in regenerating retinas.

To characterize the response of ganglion cells to AMPA, NMDA, GABA and glycine, whole-cell current-voltage (I-V) relationships were measured under voltage clamp (Fig. 20). In Figure 20A, the I-V curve for AMPA-induced current is linear and the reversal potential is about –10 mV (arrow) in solution B. The mean value of the reversal potential was –6±0.9 mV for 12 cells. This value was close to the value (–7 mV) obtained in dissociated spiking cells from adult newt retina (Chiba and Saito, 1995a). The ionic dependence of AMPA-induced currents was not examined in this study. However, the previous studies of my laboratory showed that the reversal potential value of AMPA-induced current shifts with the external Na^+ concentration. These results suggest that Na^+ is a

main ion component of the AMPA-induced currents. The I-V curve for NMDA-induced current was almost linear between –50 and +50 mV, but markedly rectified between –60 and –100 mV even in the absence of Mg^{2+} ions (Fig. 20A). The reversal potential was about –8 mV in this case (arrowhead). The mean value of the reversal potential was –4±0.7 mV for 7 cells in solution D. A similar I-V curve has been obtained in dissociated spiking cells from adult newt retina (Chiba and Saito, 1995a). The I-V curves for GABA- and glycine-induced currents showed outward rectification (Fig. 20B). The reversal potential were about –79 mV (arrow) and –75 mV (arrowhead) in these cases. The mean reversal potential for GABA- and glycine-induced currents was –66±2.3 mV for 8 cells and –66±1.9 mV for 11 cells in solution F, respectively. These values are close to the values (–65 mV) obtained in dissociated spiking cells of the adult newt retina (Chiba and Saito, 1995a). The ionic dependence of GABA- and glycine-induced currents was not examined in this study, but examined in the previous study in which the reversal potential value of GABA- and glycine-induced currents was modified by intrapipette Cl^- concentration in agreement of the theoretical values of Cl^- equilibrium potential calculated from Nernst equation with Cl^- activities. These results are consistent with activation of an anion-permeable conductance by GABA and glycine.

Effects of antagonists on AMPA, NMDA, GABA and glycine receptors were similar to those obtained in dissociated spiking cells (Chiba and Saito, 1995a) and regenerating ganglion cells of retinal-slice in this study (Fig. 25). Briefly, AMPA response was almost suppressed by CNQX (50 μM), an antagonist of AMPA receptors (n=4), and NMDA response was suppressed by DL-AP7 (100 μM), an antagonist of NMDA receptors (n=4). GABA responses were almost completely suppressed by bicuculline (20 μM), an antagonist of $GABA_A$ receptors (n=11), whereas glycine responses were completely suppressed by strychnine (2 μM), an antagonist of glycine receptors (n=11).

3.2. Whole-cell current recordings from ganglion cells in regenerating retinas

The appearance and maturation of responses to excitatory amino acid analogs and inhibitory amino acids were examined in living retinal-slices prepared at different stages of regeneration. None of the cells examined (n=28) at the 'early' and 'intermediate-I' regenerating retinas expressed any voltage-gated Na^+ currents (Table 1) or responded to any drugs (data not shown). Cells identified by LY fills were typically slender (Fig. 9, 10).

Voltage-gated Na^+ currents were first detected in cells with round somata located at the most proximal level of the 'intermediate-II' regenerating retina. They are probably premature ganglion cells, because of their location and because ganglion cells are the first retinal neurons to appear during both regeneration (Cheon et al., 1998) and development (Altshuler et al., 1991) of the vertebrate retina. Figure 21 shows whole-cell currents recorded from two presumed premature ganglion cells in 'intermediate-II' regenerating retinas and their morphology. Both cells had a round somata of about 18 μm in diameter with no processes (Fig. 21A, top panel) and a short process (Fig. 21B, top panel, arrowhead). Cell B is probably more differentiated than cell A, because the Na^+ current amplitude of cell B (first trace) is much larger than that of cell A (first trace), and because cell B has an axon-like process, whereas cell A did not. Furthermore, cell B responds to application of 100 μM AMPA in solution B, 30 μM GABA and 100 μM glycine in solution F (from third to fifth trace), but not to 250 μM NMDA in solution D (second trace), while cell A did not respond to any drugs.

In the 'intermediate-II' regenerating retina, 17 (ca. 47%) of the 36 cells in which Na^+ current could be activated also responded to 100 μM AMPA. The peak amplitude of the AMPA-induced current ranged from 3 to 233 pA at a holding potential of –80 mV with a mean value of 58±17 pA. Fifteen (ca. 63%) of the 24 cells tested responded to 30 μM GABA. The other 9 cells in this group

did not respond even to 100 μM GABA. The peak amplitude of GABA-induced current ranged from 3 to 148 pA at a holding potential of 0 mV with a mean value of 52±17 pA. Thirteen (ca. 57%) of the 23 cells responded to 30 μM glycine. The peak amplitude of glycine-induced current ranged from 3 to 57 pA at a holding potential of 0 mV with a mean value of 22±5 pA. None of the cells (n=26) examined in 'intermediate-II' regenerating retinas responded to NMDA. The number of cells that responded to each ligand and the peak amplitude of ligand-gated currents are plotted in Figure 23.

NMDA-induced currents were first detected in the 'intermediate-III' regenerating retina. A typical example is shown in Figure 22A. The LY-labeled cell (top panel) exhibited a dendritic process to a presumptive IPL (arrow) and an axon-like protrusion (arrowhead). Application of 250 μM NMDA activated approximately 11 pA of inward current (first trace) at a holding potential of –80 mV. The NMDA-induced current declined sharply in amplitude during the application of the drug, perhaps due to receptor desensitization. The same cell responded to AMPA, GABA and glycine (second to fourth trace). All ganglion cells examined in the 'intermediate-III' regenerating retina responded to AMPA, GABA and glycine, while 3 (ca. 18%) of 17 ganglion cells responded to NMDA only. The mean peak amplitudes of AMPA- and NMDA-induced currents were 84±25 pA (n=18) and 8±2 pA (n=3) at a holding potential of –80 mV, respectively. The mean peak amplitudes of GABA- and glycine-induced currents were 71±15 pA (n=15) and 20±5 pA (n=16) at a holding potential of 0 mV, respectively. The number of cells that responded to each ligand and the peak amplitudes of ligand-gated currents are plotted in Figure 23.

Figure 22B shows whole-cell currents recorded from a ganglion cell in the 'late' regenerating retina and its morphology. The LY-labeled cell extended dendritic processes to the IPL (top panel, arrow). This cell is probably an OFF ganglion cell, based on the level of dendritic arbolization in the IPL (white

arrow in Fig. 22B). The response pattern of this cell to each drug application was fundamentally the same as that of matured cells. All ganglion cells examined in the 'late' regenerating retina responded to AMPA, GABA and glycine, while 28 (ca. 90%) of 31 cells responded to NMDA. The mean peak amplitudes of AMPA- and NMDA-induced currents were 212±28 pA (n=37) and 40±7 pA (n=31) at a holding potential of –80 mV, respectively, and those of GABA- and glycine-induced currents were 56±11 pA (n=18) and 65±13 pA (n=18) at a holding potential of 0 mV, respectively. These values are plotted in Figure 23.

3.3. The I-V relationships of ganglion cells in regenerating retinas

The I-V relationships for AMPA-, NMDA-, GABA- and glycine-induced currents in the regenerating retinas are shown in Figure 24. In Figure 24A, the I-V curve for AMPA response was obtained from a premature ganglion cell in the 'intermediate-II' regenerating retina. The curve displayed a slight outward rectification and a reversal potential of about –7 mV in this case. Figure 24B shows a comparison of the mean I-V curves for AMPA between premature ganglion cells (n=5) in the 'intermediate-II' regenerating retina and ganglion cells (n=12) in control retina. The I-V curve of mature ganglion cells (filled circle) was nearly linear, while that of premature ganglion cells (open circle) was somewhat voltage dependent. On the other hand, the mean reversal potential value (–6±0.4 mV) of premature ganglion cells was indistinguishable from that (–6±0.9 mV) of mature ganglion cells.

An I-V curve for NMDA-induced current was obtained from a ganglion cell in the 'intermediate-III' regenerating retina (Fig. 24C). The curve was almost linear between –30 and +50 mV with the reversal potential of about –5 mV in this case. However, it became markedly nonlinear when the membrane was further hyperpolarized. Figure 24D shows a comparison of the mean I-V curves

for NMDA response between ganglion cells (n=10) in the 'intermediate-III' and 'late' regenerating retina and ganglion cells (n=7) in control retina. The curve in the regenerating retinas tended to rectify towards hyperpolarizing direction more than that in control retinas. The mean reversal potential value of NMDA response (–4±1.0 mV), on the other hand, was indistinguishable from that (–4±0.7 mV) in control retinas.

Figure 24E shows the I-V curves for GABA- and glycine-induced currents which were obtained from a premature ganglion cell in the 'intermediate-II' regenerating retina. Both curves rectified outwardly. The reversal potential in this case was about –69 mV for GABA response and –67 mV for glycine response. The mean reversal potential values of GABA and glycine responses were –66±3.2 mV (n=7) and –66±2.5 mV (n=8), respectively, in the 'intermediate-II' regenerating retinas. They were indistinguishable from –66±2.3 mV (n=8) for GABA and –66±1.9 mV (n=11) for glycine in control retinas. Furthermore, the mean I-V curves for GABA and glycine in premature ganglion cells were similar to those in mature ganglion cells (data not shown).

3.4. Effects of amino acid receptor antagonists on ganglion cells in regenerating retinas

Bath application of 50 μM CNQX (solution C) completely suppressed the 100 μM AMPA-induced current recorded from premature ganglion cells in the 'intermediate-II' regenerating retina (Fig. 25A, compare the left and middle traces). The response recovered partially 10 min after washing (Fig. 25A, right). The amount of suppression was 92.8±2.3% (n=4). Bath application of 100 μM DL-AP7 (solution E) reduced the amplitude of 250 μM NMDA-induced currents recorded from ganglion cells in the 'late' regenerating retinas (Fig. 25B, middle). The response amplitude recovered almost completely after 9 min of extensive washing (Fig. 25B, right). The amount of suppression was 69.2±2.3%

(n=4). Both GABA- and glycine-induced currents were recorded from a premature ganglion cell in the 'intermediate-II' regenerating retina (Fig. 25C and D, left). These appeared to be mediated by ionotropic GABA and glycine receptors, as 20 μM bicuculline suppressed almost completely the response to 30 μM GABA (Fig. 25C, middle) and 2 μM strychnine completely suppressed the response to 30 μM glycine (Fig. 25D, middle). The effects of these GABA and glycine antagonists were always reversible (Fig. 25C and D, right). The amount of suppression of GABA-induced current by bicuculline was 93.5±3.0% (n=8). The amount of suppression of glycine-induced current by strychnine was 94.8±3.0% (n=7).

4. Discussion

Using living slice preparations from newt retinas at different stages of regeneration, I examined the appearance and maturation of excitatory and inhibitory amino acid responses by whole-cell patch-clamp techniques. AMPA, $GABA_A$ and glycine receptors were expressed simultaneously in many premature ganglion cells with Na^+ channel expression well before formation of the IPL, while NMDA receptors were expressed in some ganglion cells just before the IPL formation. Pharmacological properties and reversal potential values of the excitatory and inhibitory amino acid responses did not change substantially between premature ganglion cells and mature ganglion cells. However, somewhat different degrees of rectification were noticed in the voltage dependence of AMPA- and NMDA-induced currents recorded from premature and mature ganglion cells.

4.1. Onset of excitatory and inhibitory amino acid receptors

Progenitor cells in the 'early' and 'intermediate-I' regenerating retinas are typically slender in shape, and did not exhibit Na^+ currents (Fig. 8, 9, Table 1) and ligand-gated ion currents. Na^+ currents were first detected in presumed premature ganglion cells with round somata located in the most proximal layer of the 'intermediate-II' regenerating retina where ganglion cells differentiate. This confirmed the result obtained by immunohistochemistry using Na^+ channel antibody (Cheon et al., 1998). AMPA-, GABA-, and glycine-induced currents were detected in many of the premature ganglion cells that expressed Na^+ currents, but not all. This suggested that the onset of the above ligand-gated ion channels are expressed at the same time or slightly later than that of Na^+ channels. In the developing cat retina, however, it has been reported that

glutamate receptors in dissociated immature ganglion cells are established prior to the time when these neurons are capable of generating spiking activity (Skaliora et al., 1993). At present, I do not know whether the difference between the finding from developing cat retina and regenerating newt retina reflect differences between development and regeneration, amphibian and mammalian retinas, or technical differences.

NMDA-induced currents were first detected in ganglion cells of the 'intermediate-III' regenerating retina, corresponding to the stage just before, or at, the beginning of, synaptic layer formation. The onset of AMPA receptors prior to NMDA receptors in regenerating newt retina is in agreement with the ordered appearance of glutamate receptors in cultured hippocampal neurons of rat (Mattson and Kater, 1988) and retinal neurons of chick (Yamashita et al., 1994; Catsicas, et al., 2001), and in intact cells of the developing rabbit retina (Wong, 1995). The opposite sequence, namely, NMDA receptors followed by AMPA receptors, has been reported during development of rat hippocampal synapses (Ben-Ari et al., 1989; Durand et al., 1996), rat thalamocortical synapses (Isaac et al., 1997) and retinotectal synapses in *Xenopus* tadpoles (Wu et al., 1996). However, it has been also suggested that in the hippocampus earlier observation of NMDA receptors may not be due to the lack of functional AMPA receptors but instead may be attributed to the spillover of glutamate from a presynaptic bouton onto the postsynaptic site of an adjacent cell (Kullmann et al., 1996). Since NMDA receptors have a much higher affinity for glutamate than AMPA receptors (Patneau and Mayer, 1990), they will be preferentially activated by glutamate spillover. In any case, at least some excitatory and inhibitory neurotransmitter sensitivities seem to emerge prior to the onset of synaptogenesis both during development of the CNS (Blanton and Kriegstein, 1992) and regeneration of the newt retina (this study).

Development of excitatory synapses onto ganglion cells precedes the maturation of inhibitory afferent in postnatal rat retina (Rörig and Grantyn, 1993). This differs from the sequence found in developing rat hippocampus, where GABAergic synapses are established before glutamatergic synapses mature (Ben-Ari et al., 1989; Durand et al, 1996). Unlike either of these results, I have found that GABA and AMPA responses appear simultaneously in premature ganglion cells in the 'intermediate-II' regenerating retina, well before synapse formation. I have not yet tried to examine whether excitatory and inhibitory synapses fully form at the same time or not, and leave that point open to future investigation.

4.2. Maturational changes in excitatory and inhibitory amino acid receptors

A number of investigators have demonstrated that, in the developing retina (Somohano et al., 1988; Allcorn et al., 1996) and brain (McDonald et al., 1988; Insel et al., 1990, Miller et al., 1990), the density of both NMDA and nonNMDA receptors is maximal during early development and declines at later times. This has been partly explained as a result of naturally occuring cell death at the time of synaptogenesis (Hughes and LaVelle, 1975; Hughes and McLoon, 1979; Rager, 1980). Chiba and Saito have also seen such a maturational change in the excitatory amino acid receptors in their previous study on dissociated spiking cells from regenerating newt retina (Chiba and Saito, 1995b). The number of presumed ganglion cells that responded to AMPA and NMDA appeared to be maximal at the beginning of formation of the IPL and decreased during subsequent regeneration to numbers seen in the normal retina. Furthermore, the amount of AMPA-induced current was also maximal at the time of synaptogenesis, although NMDA-induced current persists. Many

apoptotic ganglion cells have also been observed in the 'late' regenerating retina (Kaneko et al., 1999b).

In contrast, I have found in the present study that the number of AMPA- and NMDA-sensitive cells and the amount of current induced by these agonists gradually increases to the control value as regeneration proceeds. The discrepancy between the present and previous results may be attributable to the dissociation procedures. That is, AMPA receptors might be expressed to a large extent over somata well before the synaptogenesis and later become localized at post-synaptic sites on dendrites that are lost or truncated in the dissociation procedures. It remains to be determined whether the increase in AMPA- and NMDA-induced currents of ganglion cells during regeneration is due to an increased numbers of receptors or increases in single channel conductances, or both.

In the Rohon-Beard neurons of the *Xenopus* spinal cord (Bixby and Spitzer, 1982) and hippocampal neurons of rat brain (Ito and Cherubini, 1991), the sensitivity to glycine disappears after increasing at early stages of development, while GABA sensitivity persists. In regenerating newt retina, both GABA- and glycine-sensitive cells gradually increased in number to control levels, and the current amplitude induced by these amino acids also increased monotonically. A gradual increase in the receptor density has been reported for $GABA_A$ receptors in the developing chick brain (Enna et al., 1976) and glycine receptors in the chick embryo spinal cord (Zukin et al., 1975). A monotonic increase in $GABA_A$ sensitivity has also been reported in dissociated spiking cells during newt retinal regeneration (Chiba and Saito, 1995b).

4.3. Properties of excitatory and inhibitory amino acid-induced currents during regeneration

In the present study, pharmacological properties and reversal potential values of the excitatory and inhibitory amino acid responses did not change substantially between regenerating ganglion cells and mature ganglion cells. However, there were somewhat differences in voltage dependency of AMPA- and NMDA-induced currents between them, that is, the mean I-V curves for AMPA or NMDA in regenerating ganglion cells were more outward rectifying than those in mature ganglion cells (Fig. 24B and D). Differences of ionic dependency and membrane rectification between glutamate receptors of early embryonic neurons and those of mature neurons have been reported in the developing CNS. For example, Ca^{2+} permeable AMPA/KA receptors, which were usually not observed in mature neurons, have been reported in the developing rat retinal ganglion cells (Rörig and Grantyn, 1993) and embryonic rat hippocampal neurons in culture (Bochet et al., 1994). These receptors, in contrast to the I-V curve in premature ganglion cells of regenerating newt retina, displayed a strong inward rectification in the I-V curves. NMDA-induced inward currents in rat hippocampal neurons are less voltage-dependent in immature neurons than in adult neurons (Ben-Ari et al., 1988). The first NMDA receptors expressed in rat hippocampal neurons differ in their properties from "mature" NMDA receptors in that they are not sensitive to blockade by Mg^{2+} (Mattson and Kater, 1988; Ben-Ari et al., 1988). In the present study, however, NMDA-induced inward currents in regenerating ganglion cells were slightly more voltage-dependent than in mature ganglion cells.

It has been now considered that different characteristics of excitatory amino acid receptors in the CNS may result from the different composition of subunit proteins comprising glutamate receptors (for review, see Ozawa et al., 1998). At present, I did not analyze differences of I-V curves between regenerating and

mature ganglion cells. However, it is necessarily in the future to investigate whether these differences reflect differences in AMPA or NMDA receptor subunit composition.

4.4. Significance of early expression of neurotransmitter receptors

A growing number of studies have indicated that neurotransmitter system components are not only associated with synaptic transmission in the mature CNS, but may also influence many aspects of developmental processes (see reviews, Mattson, 1988; McDonald and Johnston, 1990; Lauder, 1993; Ben-Ari et al., 1997). Furthermore, the activation of neurotransmitter systems often accompanies changes in intracellular concentration of calcium ions ($[Ca^{2+}]_i$) which are important in regulating many cellular functions as one of the intracellular second messengers (Mattson, 1988; Lipton and Kater, 1989; Yamashita and Fukuda, 1993; Wong, 1995). In the present study, the appearance of the major excitatory and inhibitory amino acid receptors well before IPL formation supports the above notion. The recent preliminary studies of my laboratory using Ca^{2+} imaging techniques also showed that the activation of $GABA_A$ and non-NMDA receptors produces a rise in $[Ca^{2+}]_i$ of premature neurons in early regenerating newt retina (Ohmasa, 2000). This similarity in the differentiation of neurons in regenerating newt retina and developing retina would suggest that some common mechanisms may contribute to neuronal differentiation during both development and regeneration.