

Chapter 1

Disappearance of gap junction channels and
the appearance and maturation of voltage-gated
Na⁺ channels during retinal regeneration

1. Introduction

Gap junctional communication is widespread in early vertebrate embryogenesis and has often been proposed to play an important role in developmental events, such as induction, pattern formation and morphogenetic gradients (for reviews, see Guthrie and Gilula, 1989; Kandler and Katz, 1995). In fact, interference of gap junctional communication in early amphibian embryos results in specific developmental defects (Warner et al., 1984). The presence of gap junctional communication in the vertebrate CNS including retina has been well documented by electrical measurements as well as dye coupling (Connors et al., 1983; LoTurco and Kriegstein, 1991; for review, see Vaney, 1994). Morphological and physiological studies on the CNS suggest that the degree of gap junctional communication is not a static phenomenon, but changes during development. For example, in the embryonic neocortex, gap junctional coupling is pronounced in early developmental stages, but decline as development progresses (Connors et al., 1983; LoTurco and Kriegstein, 1991; Peinado et al., 1993a; Kandler and Katz, 1995; Bittman et al., 1997). In the mammalian retina, electron microscopic studies show that the abundant gap junctions in early embryonic retina decreases during development (Dixon and Cronly-Dillon, 1972; Fujisawa et al., 1976). However, the functional significances of gap junctions between progenitor cells and their disappearance during development are largely unknown.

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 (Stone, 1950b; Hasegawa, 1958; Keefe, 1973a). Such a retinal regeneration may be a useful system for understanding the mechanisms of cytodifferentiation and the genesis of neural circuitry in the CNS. In the early regenerating newt retina, it has been reported that progenitor cells were

electrically inexcitable and strongly coupled each other through gap junctions (Chiba and Saito, 2000). Recently, Umino and Saito (2002) also found that a gap junctional channel protein Connexin 43 (Cx 43) was abundant in progenitor cells and that the amount of Cx43 decreased during retinal regeneration. Does the loss of Cx43 during regeneration correlate with the loss of electrical and/or tracer coupling and does the loss of gap junctional coupling correlate with cellular differentiation in the regenerating retina?

In the present study, I prepared living slice preparations from the newt retina at different stages of regeneration and examined changes in the gap junctional coupling with cellular differentiation using whole-cell patch-clamp methods. I focused on the ganglion cell differentiation because ganglion cells are the first retinal neurons to differentiate during retinal regeneration (Cheon et al., 1998), as well as during retinal development in most vertebrate embryos (for review, see Altshuler et al., 1991). I also focused on ganglion cells because they express voltage-gated ion channels, such as Na⁺ channels, which are easily identified by whole-cell current recordings. To confirm the presence or absence of gap junctional coupling, I injected cells having voltage-gated currents with Lucifer Yellow and/or biocytin. Here, I provide morphological and physiological evidence showing that cellular differentiation correlates with loss of gap junctions between progenitor cells in the regenerating retina and that new gap junctions are recreated between mature ganglion cells.

2. Materials and Methods

2.1. Preparations of retinal slice

Adult newts (*Cynops pyrrhogaster*) were obtained from a commercial supplier and reared in a moist chamber at room temperature under a natural day-night cycle. Animals were fed pieces of liver.

Under anesthesia with 0.1% FA100 (4-allyl-2-methoxyphenol; 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. Animals were kept in the dark for more than 60 min during anesthesia. Surgical removal of the original retina was operated as follows (Fig. 4). The dorsal half of the eye was cut open along the corneo-scleral junction and the lens was removed. The neural retina was detached from RPE by a gentle stream of newt saline solution through a 1 ml syringe and removed by cutting the optic nerve. The eye flap consisting of iris and cornea was gently returned to its original position. The operated animals were maintained in a moist chamber at about 22°C under a lighting cycle of approximately 12 hours light: 12 hours dark and allowed to recover. They were sacrificed on selected post-operative days under anesthesia.

Retinal slices of control or regenerating retinas were prepared as illustrated in Figure 5. First of all, the eyeball was enucleated and put on a filter paper (0.42 μm pore size; Millipore, Bedford, MA) cornea side up (Fig. 5A), and cut radially into four sectors from above. The lens was removed to expose the retina (Fig. 5B), and the retina was wetted with a newt saline solution containing 0.1% bovine serum albumin (Life Technologies, NY). New filter paper was mounted on it (Fig. 5C), and the sclera and choroid were removed. The retina mounted on the filter paper (Fig. 5D) was sliced, at a thickness of 400 μm with a Mellwain tissue chopper (The Mickle Lab. Engineering Co. Ltd, England) (Fig. 5E). Slices of retina were transferred to a perfusion chamber (about 2 ml

volume) and fixed on the bottom of the chamber by a small amount of vaseline at both ends of the filter paper (Fig. 5F). 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 solutions delivered into the chamber by gravity and viewed through a 40X water-immersion objective with differential interference contrast (Nomarski) optics.

Figure 6A shows a photomicrograph of a living slice preparation of a control retina under Nomarski optics. The newt retina has a laminar organization that is fundamentally the same as that of other vertebrate retinas (Fig. 2). Figure 6B shows a schematic diagram of the retina in which five basic types of neurons have been identified by intracellular staining (Umino et al., 2003).

2.2. Experimental solutions and drugs

For gap-junction current recordings, retinal slices were superfused with an external solution having the following composition (in mM): 100 NaCl, 3.7 KCl, 3 CaCl₂, 1 MgCl₂, 18 TEA-Cl, 2 4-aminopyridine (4-AP), 3 CoCl₂, 5 HEPES. pH was adjusted to 7.5 with 0.3 N N-methyl-D-glucamine (NMDG). This solution effectively minimized the nonjunctional currents flowing through ion channels, such as K⁺ and Ca²⁺ channels, in the cell membrane, and made it possible for us to analyze gap-junction currents. In some experiments, octanol (500 μM), halothane (2 mM) or 18 β-glycyrrhetic acid (18 β-GA, 10 μM) was mixed with the above experimental solution just before use. For voltage-gated Na⁺ current recordings, retinal slices were superfused by the external solution (in mM): 100 NaCl, 24 NMDG-HCl, 3.7 KCl, 3 CaCl₂, 1 MgCl₂, 5 HEPES. pH was adjusted to 7.5 with 0.3N NMDG. CoCl₂ was omitted in the solution, because it attenuated voltage-gated Na⁺ currents. For elimination of

the Na⁺ current, NaCl in the above external solution was replaced by an equimolar NMDG-HCl.

2.3. Electrophysiological recordings

Whole-cell currents were recorded from cells in the retinal slice using the ruptured-patch technique (Hamill et al., 1981) using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). The output cut-off frequency was 10 kHz (−3 dB, four-pole; Bessel). Patch pipettes (about 1-2 μm in tip diameter) were filled with a solution that contained (in mM): 110 CsF, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 10 TEA-Cl, 10 HEPES, 2.5 ATP-Mg₃, 0.01% K₂-Lucifer Yellow (LY) and/or 0.5 mg/ml biocytin (Sigma/RBI Chemical, St. Louis, Mo. USA). pH was adjusted to 7.5 with NMDG. The osmolarity was adjusted to 255 mOsmo. Pipette tip resistances ranged from 5 to 10 MΩ in the external solution. 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. Experiments were performed at room temperature, around 23°C.

To record gap-junction current, cells were initially held close to the resting potential to minimize the voltage difference between a clamped cell and its surrounding cells. The cell was then voltage-clamped to test voltages from hyperpolarizing to depolarizing voltages in 10 mV increments. Each step pulse of 525 ms duration was applied every 1 s. Sampling frequency was typically 1.4 kHz. Capacitive and leakage artifacts in recordings were not subtracted. The output cut-off frequency was 10 kHz.

To recording voltage-gated Na⁺ current, 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.

Current data were acquired using pCLAMP 5.5.1 software (Axon Instruments), stored in a hard disk of a computer (433/M, Dell) and DAT recorder (RD-125T, TEAC, Tokyo). Statistical values in the text are given as the mean \pm standard error (S.E.).

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. In some experiments, to examine the dye and tracer coupling between cells, both LY and biocytin (Sigma/RBI Chemical) were iontophoretically injected into single current-recorded cells by repetitive negative current pulses (-1 nA, 100 Hz, for up to 10 min). LY-filled cells were viewed under a blue excitation light during injection. The retinal slices were transferred to a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) immediately after the injection, and left overnight at 4°C. Procedures of the immunohistochemistry for biocytin identification is shown diagrammatically in Figure 7. Briefly, the retinal slices were incubated in an avidin-biotin complex (ABC) solution (Vector Laboratories, Burlingame, CA) for 3-5 days at 4°C and then treated with 3,3'-diaminobenzidine-tetrahydrochloride (DAB) solution (Vector Laboratories). Translucent and fluorescent images of cells were displayed on a video monitor through a CCD camera (C9585; Hamamatsu Photonix, Shizuoka, Japan) stored on computer hard disk (Macintosh 4400/200). The morphological properties of these cells were analyzed using Photoshop 3.0 (Adobe, Mountain View, CA) software.

With this technique I could visualize the morphology of injected cells, as well as examine tracer coupling between an injected cell and its neighbors.

2.5. Definition of stages of regenerating retina

Regenerating retinas have been divided into five stages ('early', 'intermediate-I, -II, and -III', and 'late' stages) on their basis of the morphological appearance (Cheon and Saito, 1999). Figure 8A shows typical slice preparations of the retina at different stages of regeneration. Since images of cells are difficult to resolve from thick tissue sections, I show in Figure 8B schematic illustrations of the retina corresponding at each regenerating stage. An 'early' regenerating retina consists of 1-2 rows of mitotically active progenitor cells. An 'intermediate' regenerating retina has multiple layers of cells with no apparent segregation of synaptic layers, in which an 'intermediate-I' retina consists of 3-4 rows of progenitor cells, an 'intermediate-II' retina consists of several progenitor cell layers with one row of immature ganglion cells along the vitreal side, and an 'intermediate-III' retina corresponds to the stage just before, or at the beginning of, formation of the synaptic layers. A 'late' regenerating retina characteristically has a penta-laminar structure, consisting of three nuclear layers and two synaptic layers.

3. Results

3.1. *Electrical and tracer coupling between progenitor cells and its loss between premature ganglion cells*

In the ‘early’ regenerating newt retinas, mitotic progenitor cells are electrically inexcitable and strongly coupled each other through gap junctions (Chiba and Saito, 2000). Here, I examined changes in the electrical and tracer coupling of progenitor cells and differentiated ganglion cells. For this purpose, I targeted the voltage-gated Na⁺ current because it is a potential marker of ganglion cells that are the first retinal neurons to differentiate during regeneration (Cheon et al., 1998).

3.2. *‘Intermediate-I’ regenerating retina*

Figure 9 shows whole-cell voltage-clamp currents recorded from a progenitor cell in the ‘intermediate-I’ regenerating retina and its morphology. After establishing the whole-cell clamp mode, the cell was initially held close to the resting potential (−80 mV) to minimize the voltage difference between the clamped cell and the cells surrounding it. The cell was then voltage-clamped to test voltages between −160 and 0 mV in 10 mV increments (Fig. 9A, inset). Each step pulse of 525ms duration was applied every 1 s. In Figure 9A, the hyperpolarizing or depolarizing voltage steps produced currents that declined in amplitude from an initial “instantaneous” value (I_{inst}) immediately after the voltage step to a roughly constant, but smaller “steady-state” amplitude (I_{ss}). The I_{inst} value, measured at 25 ms (arrow), is plotted against the test voltage (V) in Figure 9B (filled circles). The I_{inst} -V curve is linear over the entire voltage range that I examined. The slope yields a conductance of about 62.0 nS, that is much greater than that (0.1 nS) recorded from an isolated solitary progenitor cell that was mechanically detached from its surrounding cells by a glass pipette

(Fig. 9B, open circles). The slope conductance of solitary progenitor cells under suppression of voltage-gated ion channels ranged from 0.06 to 0.49 nS (0.2 ± 0.1 nS, $n=8$) suggesting that whole-cell currents in intact progenitor cells may be driven by the junctional potential difference between the clamping cell and its neighbors. The cell examined electrophysiologically in Figure 9A was identified by injection of both Lucifer Yellow (LY) and biocytin. LY labeled a single cell that is oval in shape (Fig. 9C). After histochemical processing, I found that biocytin labeled not only the dye-injected cell but also surrounding cells, forming a cluster around the injected cell (Fig. 9D). The dye-injected cell was recognized by the deepest stain and its shape. Such a staining pattern was only observed when successful whole-cell attachments were made, and not when biocytin was injected into the extracellular space. Therefore, the presence of gap-junction currents between progenitor cells correlated with the presence of the tracer coupling.

Figure 10 shows another example of electrical and tracer coupling between progenitor cells in the ‘intermediate-I’ regenerating retina. The cell was initially held close to the resting potential (-66 mV) and then voltage-clamped to a wide range of test voltages. Figure 10A shows that I_{inst} increased linearly with the test voltage, while the I_{ss} decreased in a voltage- and time-dependent manner. The I_{inst} and I_{ss} values, measured at 25 ms and 524 ms respectively, are plotted against the test voltage (V) in Figure 10B. The I_{inst} -V curve (filled circles) was linear over the entire range of test voltages. The slope yields a conductance of about 8.4 nS. The I_{ss} -V curve (open circles) is linear in the range between -86 and -56 mV with a slope similar to that of the I_{inst} -V curve. However, the data deviate from linearity for higher and lower clamp potentials. The conductance measured at 524 ms was 3.0 nS. The cell was identified morphologically by injection of both LY and biocytin. LY labeled a slender cell that is crooked (Fig. 10C), while biocytin labeled several cells surrounding the

injected cell (Fig. 10D).

To provide additional evidence that the whole-cell currents recorded from single progenitor cells in retinal slices are gap-junction currents, I tested the effect of gap junction blockers, 18β -GA, octanol and halothane on cells in the 'intermediate-I' regenerating retina. These drugs were applied for brief period (<1 min), because longer application impaired the recovery from drug effects. In Figure 11A, a cell was initially voltage-clamped close to the resting potential (-80 mV), and then stepped to test voltages between -180 and $+20$ mV in 50 mV increments. The current exhibited no voltage- or time-dependent changes with depolarizing steps but showed a slight voltage-dependency for hyperpolarizing steps. Before application of the drug (left), a maximum conductance of 40.6 nS was measured at 25 ms. Application of $10\ \mu\text{M}$ 18β -GA (middle) reduced the conductance to about 8.8 nS within 30 s. This effect partially recovered within 4 min after washout (right). Figure 11B shows the effect of octanol. A cell was initially voltage-clamped close to the resting potential (-72 mV) and then stepped to test voltages between -172 and $+28$ mV in 50 mV increments. The current exhibited a significant voltage- and time-dependent decline. Before application of the drug (left), the conductances measured at 25 ms and 524 ms were 11.4 nS and 4.7 nS, respectively. Application of octanol (middle) markedly reduced currents, with the slope conductance decreasing to 0.4 nS within 30 s. This effect partially recovered within 4 min after washout (right). A similar inhibitory effect of octanol was observed in other three cells yielding a mean conductance of 1.3 ± 0.4 nS ($n=4$). Halothane evoked inhibitory effects (data not shown) similar to those reported for progenitor cells in the 'early' regenerating retina (Chiba and Saito, 2000).

My criterion for the presence of gap-junction currents was that a cell must have a conductance greater than 0.5 nS which is the maximal conductance recorded from isolated progenitor cells. According to this criterion, twenty-five

(ca. 96%) of 26 cells possessed gap-junction currents that varied in magnitude from cell to cell. Estimates from the $I_{\text{inst}}-V$ curve yielded slope conductances ranging from 3.7 to 95.4 nS with a mean value of 34.3 ± 5.0 nS. As the difference between the test voltage and the resting potential increased, seven out of 25 cells exhibited a symmetrical voltage-dependent decline in current on either side of the resting potential with a mean slope conductance of 22.4 ± 5.8 nS. The remaining 18 cells that did not exhibit significant voltage dependency had a mean slope conductance value of 38.9 ± 6.3 nS. Twenty-two out of 25 cells that exhibited gap-junction currents were successfully stained with both LY and biocytin. LY labeled mostly single cells, while sixteen (ca. 73%) of 22 LY-labeled cells showed tracer coupling with their neighbors. None of 26 progenitor cells examined in the 'intermediate-I' regenerating retina exhibited voltage-gated inward currents, indicating that they are electrically inexcitable. The percentages of cells, which exhibited the inward current, gap-junction current and tracer coupling are listed in Table 1.

3.3. 'Intermediate-II' regenerating retina

In the 'intermediate-II' regenerating retina, ganglion cells start to differentiate at the most proximal level of the retina, while other levels consist mostly of mitotic progenitor cells. Figure 12 presents a family of currents recorded from a progenitor cell in the 'intermediate-II' regenerating retina together with its morphology. The cell was initially held close to the resting potential (-63 mV) and then voltage-clamped to various test voltages (Fig. 12A, inset). In this case, a prominent voltage-dependent decline in gap-junction currents was observed. The conductance measured at 25 ms and 524 ms were 2 nS and 0.3 nS, respectively. Figure 12B shows a fluorescence micrograph of the LY-labeled cell that was recorded in Figure 12A, and Fig. 12C shows a light microscope of tracer-coupled cells. The photomicrograph in Fig. 12D, obtained

by horizontal sectioning the retinal slice in Fig. 12C, shows a cluster of biocytin-labeled cells in which the LY-labeled cell is indicated by an arrow. The asymmetric tracer-coupling around the LY-injected cell may result from cell damage caused by slicing the retina or from closing of gap junctions under the condition that tracer was injected. Sixteen (80%) of 20 progenitor cells examined in the 'intermediate-II' regenerating retina showed gap-junction currents. The slope conductance measured at 25 ms ranged from 1.2 to 57.1 nS with a mean value of 18.8 ± 4.4 nS ($n=16$). Twelve (ca. 71%) of 17 cells that were successfully stained with LY showed tracer coupling. These values are listed in Table 1.

Figure 13 shows whole-cell currents recorded from a cell with a round soma located in the most proximal layer of the 'intermediate-II' regenerating retina together with its morphology. In Figure 13A, I initially held the cell close to the resting potential (-18 mV) and then voltage-clamped it to various test voltages (inset). I found the current flowing across the cell membrane to be much smaller than that recorded from progenitor cells. The conductance at 25 ms was only about 0.4 nS. When the same cell was held at -100 mV and stepped to various voltages (Fig. 13B, inset), a transient inward current was activated by steps more positive than -40 mV, suggesting that the cell is a premature ganglion cell. The inward current reached a maximum value of about 410 pA during steps to -10 mV and decreased in amplitude at more positive test voltages. Labeling by both LY and biocytin injection revealed a single cell with no processes (Fig. 13C) and no tracer coupling with neighboring cells (Fig. 13D). Thirteen (ca. 81%) of 16 cells with a round soma examined in the 'intermediate-II' regenerating retina showed the transient inward current. The slope conductances for these premature ganglion cells ranged between 0.03 and 0.44 nS with a mean value of 0.2 ± 0.1 nS ($n=16$), suggesting that they did not possess gap-junction currents. None of these cells exhibited significant tracer coupling

(Table 1).

3.4. *'Intermediate-III' and 'late' regenerating retinas*

The presence or absence of gap-junction currents and/or tracer coupling was examined by LY- and biocytin-injection after current recording from cells located at the most proximal level of the 'intermediate-III' (n=9) and 'late' regenerating retinas (n=11). All cells examined exhibited voltage-gated transient inward currents and none of these cells possessed significant gap-junction current and tracer coupling (Table 1).

3.5. *Peripheral retina*

In fish and amphibians, the peripheral retina is analogous to the developing or regenerating retina because it contains a small cluster of intrinsic progenitor cells at the ciliary marginal zone (CMZ). This zone provides new cells of all types throughout the animal's life (Johns, 1977; Wetts and Fraser, 1988). The CMZ in the regenerating newt retina is wide enough to investigate the electrical properties of progenitor cells (Negishi et al., 1992). Figure 14 shows a family of currents recorded from a cell at the CMZ in the 'Intermediate-III' regenerating retina and its morphology. The cell was initially held close to the resting potential (-63 mV) and then voltage-clamped to various test voltages (Fig. 14A, inset). The current was nearly proportional in amplitude to voltage steps over a wide range of negative and positive voltages and showed a slight time-dependent decline for hyperpolarizing steps to membrane potentials more negative than -123 mV. LY injection labeled a single cell (Fig. 14B), while biocytin diffused into neighboring cells (Fig. 14C). Because the thick slice blurs the image, I show schematically in Figure 14D the outline of the peripheral retina and localization of biocytin.

3.6. Control retina

Electrical coupling was not apparent in a total of 17 cells examined in mature unregenerated retinas. I successfully injected both LY and biocytin into 13 of 17 cells after recording their transient inward currents. Four (ca. 31%) of the 13 biocytin-labeled cells exhibited tracer coupling with neighboring cells (Table 1). An example is shown in Figure 15. LY labeled a single cell (Fig. 15A), while biocytin labeled at least four nonadjacent cells (Fig. 15B, arrowheads). One possibility is that poor electrical coupling among mature ganglion cells results from attenuation of the current flowing between dendrodendritic gap junctions and somatic recording site.

3.7. Appearance and maturation of ganglion cells during retinal regeneration

The development of the voltage-gated Na^+ currents and morphology of ganglion cells during regeneration was examined by whole-cell voltage clamp recording and intracellular injection of LY. In this experiment, the recording pipette was filled with Cs^+ -rich solution containing TEA, and the extracellular medium contained TEA and 4-AP. All cells examined were polarized from a holding potential of -100 mV to test voltages between -60 and $+25$ mV in 5 mV increments.

Figure 16 shows typical whole-cell currents and morphology of cells located in the most proximal level of the retina at different stages of regeneration. The transient inward currents were first detected in cells with a round soma in the 'intermediate-II' regenerating retina (Fig. 16A, upper trace). Forty-four (80%) of 55 presumed premature ganglion cells in the 'intermediate-II' regenerating retina exhibited the transient inward currents. They were completely diminished by removal of Na^+ from the external solution, indicating that they are carried by Na^+ ions (data not shown). The activation threshold of the voltage-gated Na^+ current ranged from -55 to -35 mV with a mean value of -45 ± 1 mV. The

maximum Na⁺ current ranged from 35 to 1540 pA with a mean value of 432±54 pA (n=44). These values are listed in Table 2 and plotted in Figure 17. The cell identified by LY injection in Figure 16A (lower panel) showed two short processes. Eleven cells that did not exhibit the Na⁺ current and 19 cells that exhibited the Na⁺ current showed no processes. The remaining 25 cells had one or two short processes and 3 of them exhibited an axon-like protrusion. The cell bodies (14-18 μm in diameter) of these premature ganglion cells at this regenerating stage were slightly larger than those (13-17 μm in diameter) at subsequent regenerating stages.

Figure 16B shows a cell in the 'intermediate-III' regenerating retina. All cells examined at this stage of regeneration exhibited voltage-gated Na⁺ currents and their activation threshold ranged from -60 to -35 mV with a mean value of -50±1 mV (n=32). The maximum Na⁺ current ranged from 129 to 1550 pA with a mean value of 711±76 pA (Table 2, Fig. 17). The cell recorded in Figure 16B (upper trace) was identified by LY injection in Figure 16B (lower panel). The cell showed two short processes. Twenty-seven out of 32 LY-labeled cells had one to three processes and some of them extended long processes of about 35 μm towards a presumptive IPL (data not shown). Only 6 cells exhibited an axon-like protrusion. Relatively poor staining may be result from axons that are too fine to fill with LY, axons that are severed during the slice preparation or inadvertent filling of displaced amacrine cells.

Figure 16C shows a cell in the 'late' regenerating retina. All cells examined at this stage of regeneration exhibited voltage-gated Na⁺ currents and their activation threshold ranged from -60 to -45 mV with a mean value of -55±1 mV (n=42). The maximum Na⁺ current ranged from 131 to 1480 pA with a mean value of 795±57 pA (Table 2, Fig. 17). The cell identified by LY injection (Fig. 16C) possessed a long process extending to the most proximal level of the IPL. All LY-labeled cells had one to three processes with or without

branching in the IPL. Axons were observed in 6 cells.

Figure 16D shows a cell in the control retinal slice. All cells examined exhibited voltage-gated Na⁺ currents and their activation threshold ranged from -60 to -45 mV with a mean value of -56 ± 1 mV (n=31). The maximum Na⁺ current ranged from 225 to 2850 pA with a mean value of 953 ± 88 pA (Table 2, Fig. 17). The cell identified by LY injection in Figure 16D showed long processes at both outer and inner most levels of the IPL. All LY-injected cells had stratified dendrites in the IPL (n=31). However, axons were observed in only 5 cells.

4. Discussion

4.1. Gap junctions between progenitor cells

Without exception, progenitor cells examined in the ‘intermediate-I and -II’ regenerating retinas, like those in the ‘early’ regenerating retina (Chiba and Saito, 2000), did not exhibit voltage-gated Na⁺ currents. Their morphology varied from oval to slender shape. Most of the progenitor cells were electrically coupled with each other through gap junctions. The presence of gap-junction currents between progenitor cells correlated with tracer coupling following intracellular injection of biocytin.

Recently, Umino and Saito (2002) showed that connexin (Cx) 43 is a major gap junctional channel protein between progenitor cells in the regenerating newt retina. Cx43 is also abundantly present at early stages of developing CNS including retina (Dermietzel et al., 1989; Yancey et al., 1992; Nadarajah et al., 1997; Rozental et al., 1998; Becker and Mobbs, 1999). It should be noted, however, that LY could pass through Cx43-mediated gap junctions in osteoblastic cell lines (Steinberg et al., 1994) and in cardiac myocytes (Lal et al., 1993). On the other hand, LY could not pass through gap junctions of progenitor cells, presumably mediated by Cx43, in the regenerating newt retina. I do not yet know whether this discrepancy is due to the presence of Cx43 isoforms in the different preparations or the difference of the amount of Cx43 expression. This must be clarified in future studies.

I recorded two different types of gap-junction currents, a voltage-dependent and a voltage-independent current. One possible explanation is that progenitor cells possess two types of Cxs. Different Cxs types are known to have different voltage sensitivities (Spray et al., 1999). Using immunohistochemistry, Umino and Saito found that Cx43, but not Cx26 and Cx32, is abundant in progenitor cells of regenerating retinas (Umino & Saito, 2002). An alternative explanation

is that these cells may only express a voltage-sensitive channel, but the voltage drop across gap junction channels was not sufficient to modulate them. Because of technical difficulties I did not analyze isolated pairs of cells with a dual voltage-clamp and thus could not control the potential across a gap junction. Rather, I studied all cells in my slice preparation with a single-cell voltage-clamp technique and with nonjunctional current suppression. However, the mean slope conductance of cells showing voltage-independent behavior (38.9 ± 6.3 nS, $n=18$) was higher than that of cells exhibiting voltage-dependent behavior (22.4 ± 5.8 nS, $n=7$). Umino and Saito (2002) also found that one of the gap junctional channel proteins, Cx43, is abundant between progenitor cells in the regenerating newt retina. Taken together, these results suggest that one type of channel may be responsible for both voltage-dependent and independent gap-junction currents.

4.2. Loss of gap junctions between progenitor cells during cellular differentiation

In the present study, I observed a large range of gap junctional conductances between progenitor cells. This may be partly due to the unavoidable experimental errors such as severing coupling among cells during slice preparation. Nevertheless, the mean conductance value (34.3 nS, $n=25$) of progenitor cells in the ‘intermediate-I’ regenerating retina was much larger than that (18.8 nS, $n=16$) of progenitor cells in the ‘intermediate-II’ regenerating retina. Therefore, I suspect that the apparent decrease in the conductance of progenitor cells during regeneration is partly related to cellular differentiation. LoTurco and Kriegstein (1991) found that neuroblasts in early developing neocortex are physiologically coupled by gap junctions into cluster of 15 to 90 cells and that the number of cells within a cluster decreases with the increase in membrane resistance as development progresses.

Several lines of evidence suggest that the number of gap junctions, the degree of gap-junction currents or expression of gap junctional channel proteins in the developing CNS including the retina is not a static phenomenon, but changes during development (Dixon and Cronly-Dillon, 1972; Fujisawa et al., 1976; Connors et al., 1983; Dermietzel et al., 1989; LoTurco and Kriegstein, 1991; Peinado et al., 1993a,b; Penn et al., 1994; Cook and Becker, 1995; Nadarajah et al., 1997; Rozental et al., 1998; Becker et al., 2002). It has been therefore proposed that gap junctional communication may play an important role in a number of developmental events such as cell proliferation, cell migration, differentiation and synapse formation (for reviews, see Dermietzel and Spray, 1993; Fulton, 1995; Cook and Becker, 1995; Roerig and Feller, 2000).

I used the appearance of Na⁺ currents as a possible marker of ganglion cell differentiation. Many cells with a round soma located in the most proximal layer of the 'intermediate-II' regenerating retina expressed voltage-gated Na⁺ currents and were immunoreactive to Na⁺ channel antibody (Cheon et al., 1998). These facts suggest that they are premature ganglion cells. I further speculate that a few rounded cells, which did not express Na⁺ currents, are also premature ganglion cells that had migrated into their final destination. In this case, the fact that none of these premature ganglion cells showed apparent electrical and tracer coupling, indicates that the uncoupling may have an important role in ganglion cell differentiation during regeneration. This notion may be supported by the fact that the loss of Cx43 occurs first at the most proximal level of the 'intermediate-II' regenerating newt retina where ganglion cells differentiate (Umino and Saito, 2002). It has been reported that a similar decay of gap junctions associated with ganglion cell differentiation at the CMZ in peripheral retina of goldfish (Tamalu et al., 2001).

Why does the cellular differentiation correlate with the disappearance of gap

junctional coupling? Progenitor cells may exchange certain molecular signals through gap junctions to (1) maintain them in a mitotic state, or (2) interfere with their cellular differentiation. Daughter cells, after uncoupling from progenitor cells, may respond to environmental factors during cell migration into their final destination and differentiate into certain types of neurons.

4.3. Reappearance of gap junctions between matured ganglion cells

Tracer coupling studies on adult vertebrate retinas have revealed that neurons of the same type or even in different types are coupled via gap junctions (for review, see Vaney, 1994; Xin and Bloomfield, 1997; Mills and Massey, 2000). Regenerated amacrine cells in the retina of the goldfish restored tracer coupling with their homologous counterparts both inside and outside the patch of regenerated retina (Hitchcock, 1997). The gradual development of tracer coupling between ganglion cells has been described in the developing ferret retina (Penn et al., 1994). In contrast, in the developing chick retina, tracer coupling is weak between newly-born ganglion cells at embryonic stage 5 (E5), increases with time until E11 and markedly decreases at E14 after the onset of synaptogenesis (Becker et al., 2002).

In the regenerating newt retina, I expected that premature ganglion cells in the ‘intermediate-II’ regenerating retina might gradually reestablish gap junctions with each other during subsequent regeneration. However, I did not observe significant tracer coupling between regenerating ganglion cells, although they developed dendritic processes in the course of regeneration. Furthermore, only one third of biocytin-labeled ganglion cells exhibited tracer coupling even in the control retina. This may be in part explained by an unavoidable experimental limitation of the slice preparation; that is, the number of coupled ganglion cells is attenuated by slicing the retina. Other possible explanations are that the injection of biocytin was not enough to label multiple

cells through either dendro-somatic or dendro-dendritic processes, that gap junction channels were closed under the condition that tracer was injected, or that histochemical procedures used to identify the tracer were not sufficiently sensitive. Also the density of gap junctions between ganglion cells may be low in the newt retina. It is also worth while mentioning that OFF-center ganglion cells are coupled, but not ON-center cells in rabbit retina (Hu and Bloomfield, 2003). Further studies are necessary to determine how and when regenerated neurons recreate new gap junctions and what type(s) of the Cx are responsible for the coupling.

4.4. Appearance and maturation of Na⁺ currents during regeneration

The activation voltage for Na⁺ currents was more positive (-45 mV) in the newly-born ganglion cells of the 'intermediate-II' regenerating retina than in mature ganglion cells (-56 mV). The amplitude of Na⁺ currents in the intermediate cells varied from cell to cell, however, it consistently increased during regeneration. On average, the maximum Na⁺ current amplitude of premature ganglion cells (432 pA) was smaller than that of mature ganglion cells (953 pA). Similar changes in Na⁺ current amplitude and activation threshold have been observed in studies of dissociated retinal ganglion cells of embryonic cat (Skaliora et al., 1993) and embryonic mouse (Rörig and Grantyn, 1994), in ganglion cells in slices of rat retinas (Schmid and Guenther, 1996, 1998), and in ganglion cells in peripheral regions of slices of the adult goldfish retina (Tamalu et al., 2001; Tamalu et al., 2000).

I observed a few rounded cells, which did not express Na⁺ currents, in the most proximal layer of the 'intermediate-II' regenerating retina. I further observed a large variation of the Na⁺ current value of the 'intermediate-II' regenerating cells. These results may reflect the difference in the degree of development between individual cells in the same regenerating stage. The

previous study of my laboratory on the development of ganglion cells during regeneration using an Na⁺ channel antibody indicated that the degree of the Na⁺ channel-immunoreactive staining varied from cell to cell (Cheon et al., 1998). In the 'intermediate-III' and 'late' regenerating retinas, current variation may reflect not only the difference in the degree of cellular development but also sampling of amacrine cells that are beginning to differentiate (Cheon and Saito, 1999). Current variation may also result from poor control of space clamp voltage in cells having developed dendritic processes. It has been reported that more than half of cells in the GCL are displaced amacrine cells (Ball and Dickson, 1983). The impairment of space clamping by dendritic processes may account for the difference in Na⁺ channel properties observed between immature ganglion cells and mature ganglion cells. An alternative explanation is that the electrophysiological properties of Na⁺ channels are different in cell bodies and axons. Further studies are required to examine the density and kinetic properties of Na⁺ channels in process-free cell bodies that have been mechanically isolated at the different stage of regeneration.