Results

1. Anatomy of the eye and structure of the retina

Figure 8A shows a transverse paraffin section of an adult goldfish eye which is stained with toluidine blue. The lens and a part of cornea were removed to facilitate making the section. The eye consists essentially of three layers. The outer most layer is a protective tunic (*sclera*) that is transparent in front (*cornea*). The middle layer is mainly vascular, consisting of the choroid, ciliary body and iris. The innermost layer is the retina, containing the essential nervous elements responsible for vision. A peripheral eye in Figure 8A, marked by a red square, is magnified in Figure 8B. Goldfish retina, like other vertebrate retinas, consisting of three nuclear layers (ONL, INL and GCL) and two plexifrom layers (OPL and IPL) decreased in their thickness toward the peripheral edge of the retina and continuously shifted forward the iris. Figure 9 shows a photomicrograph of the central retina in toluidine blue-stained section. The ONL in which somata of rod (R) and cone (C) photoreceptor cells are located, the INL in which somata of bipolar (B), horizontal (H) and amacrine (A) cells are located, and the GCL in which somata of ganglion cells (G) and possibly a few displaced amacrine cells are located.

1-1. Localization of progenitor cells

Figure 10 presents two views of the peripheral portion of an adult goldfish eye which consists of the translucent retina, the opaque pigmented epithelium, and the posterior choroid. The same field is shown as it appears through phase-contrast optics in Figure 10A, and under epifluorescence illumination in Figure 10B. Each arrow points to the so-called "marginal region" of the retina. To the right of this region, the alternating nuclear and synaptic laminae of the adult retina emerge, fan-like, into the

five layers labeled at the right edge of this micrograph. The cells in the marginal region appear to be slender and spindle-shaped, as shown below by LY fills (Fig. 12C). Also, these cells are selectively stained by an antibody directed against PCNA, a marker found in mitotic cells (Fig. 10B). This is consistent with the notion that new retinal cells are generated in adult animals from progenitor cells at the retinal margin (Johns, 1977; Maier and Wolburg, 1979; Hitchcock and Raymond, 1992).

1-2. Definition of a peripheral retina

Figure 11A shows a micrograph of a living retinal slice preparation under Nomarski optics. The overall anatomical properties of this preparation are identical to those of the frozen section described above. I divided the peripheral retina into three broad regions. The "marginal region" consists of a cluster of multipotent progenitor cells of slender shape. The "mature region" consists of a penta-lamina array, that is, three nuclear layers interconnected by two synaptic layers. The "intermediate region" is a transition area between the marginal region and mature region, and consists of a less well-defined laminar array. These three regions are schematically illustrated in Figure 11B and used as landmarks for the cell identifications below.

2. Morphological and functional differentiation of retinal neurons

Ganglion cells are the first retinal neurons to differentiate during development in almost all vertebrate embryos (Hinds and Hinds, 1974; McLoon and Barnes, 1989; Altshuler et al., 1991; Cepko et al., 1996) and during regeneration of newt retina (Cheon et al., 1998). Therefore, to investigate the earliest steps of differentiation, I focused on the morphological and functional development of ganglion cells. Ganglion cells encode visual information into action potentials and transmit them into the brain via the optic nerve that is composed of ganglion cell axon. Voltage-gated

Na^+ channel is one of the crucial ion channels underlying action potentials and their conduction. Then, I examined appearance of the Na^+ currents and the axon as possible markers of functional and morphological differentiation of ganglion cells.

2-1. No voltage-gated Na^+ currents in marginal progenitor cells

Figure 12 presents whole-cell voltage-clamp currents recorded from a cell in the marginal region and its morphology. Recordings were made in the ruptured-patch mode while suppressing voltage-gated K^+ currents. Cell was depolarized from a holding potential of -80 mV to test potentials between -60 and +15 mV, to test for the presence of voltage-gated inward current. Linear leakage subtraction was not used. Depolarization of progenitor cells from a holding potential of -80 to a test potential of -60 mV produced an outward current (Fig. 12A). When this cell type was further depolarized to membrane potentials between -60 and +15 mV in 5 mV increments, outward currents increased linearly in amplitude with the test potential. No voltage-gated inward currents were detected, implying that this type of cell is inexcitable. The currents in Figure 12A were obtained from the cell visualized in Figure 12C by LY injection through the patch pipette. The profile of the preparation and the location of the labeled cell are schematically illustrated in Figure 12D. The cell was shaped like a slender hourglass. Identical measurements were made in 7 other marginal progenitor cells. Depolarization failed to activate an inward current in all of these cells. Five cells were successfully stained. They were consistently slender in shape and neurite-free.

The amplitude of currents recorded from the cell in Figure 12A is plotted against test potential in Figure 12B. The I-V relationship was linear, and a slope conductance of about 24 nS was estimated from the slope of line. For 8 marginal cells in the presence of K^+ current blockers, the slope conductance ranged from 10.6 to 26.4 nS, with a mean value of 19 ± 2.4 nS. This value was much greater than that obtained from the mechanically isolated marginal progenitor cells (Fig. 24). The evidence that

larger slope conductance recorded from intact marginal progenitor cells may be caused by gap junctional coupling will be described in chapter 3.

2-2. Appearance of Na^+ currents in cells of intermediate region

Seventeen intermediate cells along the vitreal side of the retina were examined morphologically and electrophysiologically. They all exhibited a voltage-gated transient inward current. Nine cells were successfully stained with LY. Each of these had a round cell body. The diameter of these somata ranged from 4 to 10 μm, with a mean value of 7 ± 1 μm (SE). Figure 13A shows a family of currents obtained from a cell in the intermediate region. In this experiment, the capacitive and leak currents were reduced by P/5 leak linear subtraction (see Materials and Methods). As in the recordings from marginal cells, this cell was depolarized from a holding potential of -80 mV to test potentials between -60 and +15 mV. Transient inward currents were activated by steps more positive than -35 mV. The inward current reached a maximum value of approximately 370 pA at around -15 mV, and decreased in amplitude at more positive test potentials. The currents in Figure 13A were obtained from the cell visualized in Figure 13B by LY injection through the patch pipette. The profile of the preparation and the location of the labeled cell are schematically illustrated in Figure 13C. The cell had a round cell body and was located a few cells away from the marginal region along the innermost level of retina where ganglion cells usually exist. An axon-like process was not observed in this cell.

Five out of 9 labeled cells in the intermediate region had an axon-like process at the vitreal side of the retina. The length of these processes varied from cell to cell. Figure 14 shows the morphology of an intermediate cell which was closely located to the retinal margin. The same field was visualized under a combination of epifluorescence and incandescent lights in Figure 14A and under epifluorescence alone in Figure 14B. Reconstruction of the LY-labeled cell is shown in Figure 14C. The cell was characterized by a round cell body and a long axon-like process, with

several varicosities, extending along the vitreal surface.

Figure 15 shows three other examples of morphology of the Na^+ channel-expressed cells that are located in transition area between the intermediate and mature regions. A cell in Figure 15A has two processes, one is extending to the marginal region (arrow) and the other run along the vitreal surface to the central retina (arrowhead). A cell in Figure 15B has a process extending in the IPL without an axon-like process (arrowhead). A cell in Figure 15C has a process extending laterally in the IPL (arrowhead).

Transient inward currents expressed in the intermediate cells were reversibly suppressed by the Na^+ channel blocker TTX (Fig. 16). In Figure 16A, a family of currents was recorded before application of 1 μM TTX by using the same voltage-clamp protocol as that in Figure 13A. Figure 16B shows the currents activated by the same voltage protocol, 2 minutes after TTX application. The current was almost fully blocked by TTX at that concentration. The effect on Na^+ currents was reversible after washing with TTX-free saline (Fig. 16C). The results above altogether indicate that the inward current passed through Na^+ channels that are voltage-gated and TTX-sensitive.

The peak amplitude of the inward currents recorded from 17 intermediate cells are plotted against test potential in Figure 17 (closed circles). The activation threshold of the inward current ranged from -35 to -45 mV with a mean value of -40 ± 1 mV. The maximum inward current ranged from 56 to 492 pA, with a mean value of 257 ± 32 pA. These values are listed in the Table 3.

Seven intermediate cells located in more distal region of the retina were electrophysiologically examined. None of these expressed Na^+ currents. An example is shown in Figure 18. In this case, whole-cell voltage-clamp currents were recorded from a cell at the middle level of the intermediate region. The voltage-clamp protocol is the same as that in Figure 13. No voltage-gated inward currents were detected under suppression of K^+ and Ca^{2+} currents (Fig. 18A). LY-labeling allowed the

detailed morphology of the cell to be visualized at different focus planes. The cell had a round soma (Fig. 18B) and a long branching process, with several varicosities, extending into the IPL (Fig. 18C). Reconstruction of the LY-labeled cell and its process are shown in Figure 18D. This cell appeared to be an immature amacrine cell because the location of the cell body and no axon-like process.

2-3. Na^+ currents in ganglion cells in mature region

Whole-cell currents were recorded from 10 cells along the vitreal side of the mature region. These are probably ganglion cells, because of their position, although the existence of a few displaced amacrine cells can not be unequivocally excluded. Five cells were successfully stained with LY. They had a round cell body with an axon-like process. The diameter of cells ranged from 8 to 11 μm with a mean value of 10 ± 1 μm (SE). Figure 19A shows a family of transient Na^+ currents obtained from a mature ganglion cell. The voltage-clamp protocol is the same as that in Figure 13. Depolarizing voltage pulses from a holding potential of -80 mV to -50 mV activated a transient inward current. As the test potential became more positive, the large transient current activated and decayed faster, and was followed by a small persistent inward current component. Both current components were totally blocked by 1 μM TTX application or by replacing NaCl with Tris-HCl (data not shown), as found in isolated retinal ganglion cells (Ishida, 1991; Hidaka and Ishida, 1998). Figure 19B shows an LY-labeled cell after the current recordings shown in Figure 19A. The cell had a round soma with a short axon-like protrusion (arrow in Fig. 19B inset) and was located in the most proximal region of the retina. The remaining 4 cells also had axon, although its length varied from cell to cell. Two examples are shown in Figure 20. A cell in Figure 20A had a long axon (arrows) and sent a process (arrowhead) into the proximal part of the IPL where depolarizing bipolar cell axons terminate, suggesting that it is an ON-type ganglion cell. The micrograph is focused on the processes. On the other hand, a cell in Figure 20B sent a process into the distal part of the IPL

where hyperpolarizing bipolar cell axon terminates, suggesting that it is an OFF-type ganglion cell.

The peak amplitude of Na^+ currents obtained from 10 ganglion cells in mature region were plotted against test potential (Fig. 17, closed squares) to compare these with the current-voltage plots obtained from cells in intermediate region. The activation threshold of the inward current ranged from -50 to -60 mV, with a mean value of -54 ± 1 mV. The peak inward current ranged from 817 to 4,313 pA with a mean value of 1,621 ± 263 pA. These values are listed in the Table 3 together with those of the intermediate cells.

3. Gap junctional coupling between marginal progenitor cells

In chapter 2, I have described that intact progenitor cells at the retinal margin responded to both depolarization and hyperpolarization with much larger currents than those expected in isolated marginal progenitor cells. Therefore, I suspected that these currents are mainly the gap junctional currents, which are driven by potential differences between a clamped cell and its neighbors. In this chapter, I will present the evidence that marginal progenitor cells couple with each other via gap junctions.

3-1. Electrical coupling

Thirty-seven cells at the retinal margin, where mitotic cells are present in a cluster (Fig. 10), were successfully examined by whole-cell voltage-clamp recording. None of them exhibited voltage-gated Na^+ currents, suggesting that they are all electrically inexcitable (Fig. 12). Figure 21 shows whole-cell currents recorded from a progenitor cell under the suppression of the nonjunctional currents flowing through ion channels, such as voltage-gated K^+ and Ca^{2+} channels, in the cell membrane. The cell was initially held at -35 mV, close to the resting potential, and voltage-clamped

to test voltages between -195 and +125 mV in 40 mV increments. The current traces resulting from a series of voltage steps of 350 msec duration are superimposed in Figure 21A. Voltage steps of either polarity produced instantaneous currents (I_{inst}) followed by steady state currents (I_{ss}). Figure 21B shows the I_{ss} amplitude (measured at 345 msec after a step) against test voltages (V). The current was proportional to voltage steps over a wide range of negative and positive voltages. A conductance of about 1.7 nS was estimated from the slope of the I-V curve. Twenty-four out of 37 cells exhibited such a current profile, and their slope conductance varied from 1.6 to 14 nS with a mean value of 5.6 ± 0.72 nS. In chapter 2, I estimated a mean conductance value of 19 ± 2.4 nS for 8 marginal progenitor cells. The major difference between two experiments was in the presence of Co^{2+} ion. So far, it is not clear that Co^{2+} ion influences gap junctional currents directly or indirectly.

The remaining 13 cells showed a voltage- and time-dependent current decay, as they were voltage-clamped above the certain voltage of either polarity, suggesting that the current across gap junctions is modulated by the voltage difference between a clamped cell and its neighbors. An example is shown in Figure 22. Cell was initially voltage-clamped at a holding potential of -18 mV, close to the resting potential, and then the membrane potential was stepped from -168 to +132 mV in 30 mV increments. The current traces resulting from a series of voltage steps are superimposed in Figure 22A. The current amplitude at the end of the voltage step increased proportionally to voltages of either polarity in the -110 to +75 mV range, but began to decrease with further increasing in the voltage steps. The I_{inst} and I_{ss}, measured at 20 msec and 345 msec of each pulse, were plotted against the test voltages (V) in Figure 22B. The I_{inst}-V curve, illustrated by the open circles, was about linear over a wide range of the test voltage. A conductance of about 2.8 nS was estimated from the slope of the straight line passing through the points. The I_{ss}-V curve, illustrated by the closed circles, was also linear in the range between -110 and +75 mV and approximately identical to the I_{inst}-V curve, but deviated from linearity

with further increasing voltages of either polarity. For 13 cells, the slope conductance estimated from the straight line of the I_{ss}-V curve ranged from 0.57 to 5.2 nS with a mean value of 2.4 ± 0.36 nS.

3-2. Tracer coupling

Thirty-seven progenitor cells that were electrophysiologically examined were morphologically identified by injection of LY through the recording pipette. Unlike retinal horizontal and amacrine cells, they showed no dye coupling, and single cells were always labeled by LY. Their morphology varied from cell to cell on the basis of a slender shape. Eleven out of 37 cells were injected by both LY and biocytin. A typical example is shown in Figure 23. Figure 23A shows a epifluorescence micrograph of the cell (arrow) that was electrophysiologically examined in Figure 21. A single cell was shaped like a crescent in this case. Subsequent histochemical processing localized biocytin that revealed extensive labeling of a cluster of cells in the marginal region (arrow in Fig. 23B). In ten other cases, tracer couplings were also restricted to the area corresponding with a region where a cluster of PCNA-immunopositive cells are present (Fig. 10). The tracer coupling observed here represents true connectivity between cells rather than an artifact of the injection techniques, because LY was seen to label only the injected cell, indicating that the dye leakage due to damage or nonspecific uptake of the dye did not occur. In addition, the coupling could not be obtained by the injection of biocytin into the extracellular space. Therefore, I suggest that marginal progenitor cells are coupled by gap junctions.

3-3. Mechanical uncoupling of marginal progenitor cells

To prove additional evidence that intact progenitor cells are coupled by gap junctions, I detached individual progenitor cells from the marginal region by gentle passage in and out of a glass pipette (tip diameter: approximately 30 μm).

Mechanically isolated cells that became round in shape were voltage-clamped. An example is shown in Figure 24. As would be expected, depolarization of isolated progenitor cell from a holding potential of -80 mV to test potentials between -60 and +15 mV in 5 mV increments did not produce large amounts of passively flowing currents under the suppression of nonjunctional currents (Fig. 24A). The amplitude of currents recorded from the cell in Figure 24A is plotted against test potential in Figure 24B. The I-V relationship was linear, and a slope conductance of about 0.28 nS was estimated from the slope of line. The slope conductance for 7 isolated cells ranged from 0.28 to 0.67 nS, with a mean value of 0.49 ± 0.07 nS. This value was much lower than that value (5.6 nS) obtained from intact progenitor cells. Electrically examined cell was morphologically identified by LY injection through the patch pipette (Fig. 24C). The profile of the preparation and the location of the labeled cell is schematically illustrated in Figure 24D. The cell was round-shaped and neurite-free.

3-4. Effect of uncoupling agents

3-4-1. Effect of halothane

Figure 25 shows the effect of halothane, a volatile anesthetic, that is a well-characterized gap junctional blocker (Wojtczak, 1985). Cell was initially voltage-clamped to a resting potential of -22 mV, and then stepped to test voltages between -182 and +138 mV in 20 mV increments. Each set of records in the figure consists of five superimposed current traces sampled from a series of voltage steps. Before application of the drug (Fig. 25A), a conductance of about 6.8 nS was estimated from the slope of the I-V curve. Application of halothane (5 mM) strongly reduced currents within 2 min and the slope conductance became 0.29 nS (Fig. 25B). This effect was almost completely recovered to the control level in 5 min after washout (Fig. 25C). Similar inhibitory effects of halothane on the current were observed in all cells tested (n = 4).

3-4-2. Effect of pH

pH sensitivity of gap junctions has been reported in the CNS neurons including retina (DeVries and Schwartz, 1989; Hampson et al., 1994) as well as in a variety of nonneuronal cells (Spray et al., 1981; for review, see Spray and Bennett, 1985). Figure 26 shows the effect of extracellular pH on whole-cell currents recorded from a progenitor cell. Cell was voltage-clamped to a resting potential of -24 mV, and then the membrane potential was stepped from -184 to +136 mV in 20 mV increments. Each set of records in Figure 26 consists of five superimposed current traces sampled from a series of voltage steps. At pH 7.4 (Fig. 26B), a conductance of about 2.9 nS was estimated from the slope of the I-V curve. When pH dropped to 6.4 (Fig. 26A), the conductance decreased into 2.6 nS, while when pH rose to 8.4 (Fig. 26C), it increased into 3.4 nS. The effect of pH either 6.4 or 8.4 on whole-cell currents was completely recovered after a 5 min washout. Similar effects of pH on whole-cell currents were observed in 4 other cells. The mean conductance change for 5 cells was 1.1 ± 0.38 nS between 6.4 and 8.4 pH.

Figure 27 shows the effect of extracellular pH on voltage- and time-dependent components of gap junctional currents. Cell was voltage-clamped to a resting potential of -29 mV, and then the membrane potential was stepped from -189 to +131 mV in 40 mV increments. The current traces resulting from series of voltage steps are superimposed. The current amplitude at the end of the voltage step increased proportionally to voltages of either polarity in the -110 to +50 mV range, but began to decrease with further increasing in voltage steps. The I_{ss} measured at 50 msec of each pulse was plotted against the test voltages (V) in Figure 27D. At pH 7.4 (Fig. 27B), a conductance of about 2.3 nS was estimated from the linear range of the I-V curve. When pH dropped to 6.4 (Fig. 27A), the conductance decreased into 1.8 nS, while when pH rose to 8.4 (Fig. 27C), it increased into 2.6 nS. The I-V relation deviated from the linear line below -110 mV and above +50 mV. A degree of voltage-dependent decline in current tended to decrease with increasing in the pH.

3-4-3. Effects of dopamine and retinoic acid

Dopamine, an intrinsic retinal neurotransmitter, has been shown to reduce electrical and dye coupling between horizontal cells in fish by increasing intracellular cAMP levels (Teranishi et al., 1983; Lasater and Dowling, 1985; DeVries and Schwartz, 1992; McMahon, 1994). Figure 28 shows a current profile before (A) and after (B) application of dopamine (100 μM). Cell was initially voltage-clamped to a resting potential of -20 mV, and then stepped to test voltages between -180 and +140 mV in 20 mV increments. Each set of records in the figure consists of five superimposed current traces sampled from a series of voltage steps. Before application of the dopamine, a conductance was about 2.4 nS. Application of dopamine did not alter the conductance (n = 6). Furthermore, neither external application of the membrane-permeable cAMP analog, 8-bromo-cAMP (200 μM), nor internal application of cAMP (5 mM) through patch pipette could block coupling currents.

Recently, it has been shown that retinoic acid modulates the gap junctional conductance between horizontal cells in the mammalian (Weiler et al., 1999) and fish retinas (Zhang and McMahon, 2000). I also tested the effect of retinoic acid (300 μM) on whole-cell currents of marginal progenitor cells. Before application of the retinoic acid, a conductance was about 1.7 nS (Fig. 28C). Application of all-trans retinoic acid did not alter the conductance (Fig. 28D).

4. No gap junctional coupling between immature neurons

Ten cells closely located to the marginal region were examined electrophysiologically and morphologically. Six cells faced to the vitreal side of the retina, and 4 cells were located in more distal region. Figure 29 shows an example of

whole-cell currents recorded from a cell along the vitreal side and its morphology. Cell was initially held at -80 mV and stepped to test voltages between -60 and +15 mV in 5 mV increments. Three current traces resulting from a series of voltage steps are superimposed in Figure 29D. Inward currents were activated by voltage steps more than -45 mV. The inward current was completely suppressed by the Na^+ channel blocker TTX (data not shown). The cell that was electrophysiologically examined in Figure 29D was morphologically identified by injection of both LY and biocytin through the recording pipette. A single cell with a round soma was identified under an epifluorescence microscope (arrow in Fig. 29A). Subsequent histochemical processing to localize biocytin also revealed a single cell with a round soma of about 8 μm in diameter (arrow in Fig. 29B) and no other labeled cells were observed nearby. There were several cells in the neighborhood of a labeled cell with different foci. None of them, however, were labeled by biocytin. The outline of the peripheral retina and the localization of the labeled cell were schematically illustrated in Figure 29C. Identical measurements were made in 5 other cells facing the vitreal side. They all exhibited voltage-gated Na^+ currents, but did not exhibit tracer coupling. Four cells located in more distal region of the retina exhibited neither voltage-gated Na^+ currents nor tracer coupling.