Discussion

The retina in teleost fish continues to grow throughout life by adding new cells of all types from progenitor cells at the retinal margin. In the present study, I have developed living slice preparations of the peripheral retina of adult goldfish. In the peripheral retina, there is a continuous gradient of developmental stages that extends from multipotent progenitor cells at the retinal margin to the fully mature, central retina. Electrophysiological characteristics of marginal progenitor cells, cells in the intermediate region and ganglion cells in the mature region were examined using whole-cell voltage-clamp recordings. Electrophysiologically examined cells were morphologically identified by injecting LY and/or biocytin. Results are summarized in the Table 4.

All progenitor cells examined were slender in shape and coupled with neighbors by gap junctions. They were electrically inexcitable. In the intermediate region, all cells had round somata and some of them had neurites. Cells found along the vitreal surface expressed the voltage-gated Na^+ current, but cells located in more distal region did not. Some of intermediate cells located in the proximal region developed axon-like processes. They are probably immature ganglion cells because of their location and Na^+ current expression. Intermediate cells did not reveal apparent gap junctional coupling.

1. Gap junctional coupling between marginal progenitor cells

Gap junctions are membrane channels allowing direct intracellular exchange of ions and small molecules (for reviews, see Loewenstein, 1979; Warner, 1988; Bennett et al., 1991; Dermietzel and Spray, 1993; Spray, 1994; Kumar and Gilula, 1996). Cell-to-cell communication via gap junctions is widespread in early vertebrate

embryogenesis, but becomes lost as development proceeds (Potter et al., 1966; for reviews see Loewenstein, 1979; Warner, 1988; Guthrie and Gilula, 1989). In the CNS including the retina, gap junctional communication is pronounced before and during the period of synaptic formation and initial establishment of neural circuits (Dixon and Crony-Dillon, 1972; Warner, 1973; Fujisawa et al., 1976; Spray et al., 1981; Spitzer, 1982; Sakaguchi, et al., 1984; LoTurco and Kriegstein, 1991; Dermietzel and Spray, 1993; Cook and Becker, 1995). Here, I provided morphological, electrophysiological, and pharmacological evidence to show that progenitor cells couple with each other via gap junction.

1-1. Voltage dependence of whole-cell currents

Current across gap junctions is sometimes regulated by the difference between membrane potentials of coupled cells (for reviews, see Bennett and Verselis, 1992; Spray, 1994). Quantitative analyses of the voltage dependence of gap junctional currents have been performed on pairs of cells *in vitro* using a dual voltage-clamp technique, where each cell is initially voltage-clamped to the same holding potential and then a voltage step is imposed on one cell, resulting in a change in the holding current of the other cell. This current change is equivalent to current flowing through gap junctions (Spray et al., 1981; Lasater and Dowling, 1985; DeVries and Schwartz, 1989; McMahon, 1994).

In the present study, I applied a single-cell voltage-clamp technique to the slice preparations rather than the dual voltage-clamp technique under the suppression of nonjunctional currents, because of a technical difficulty. Despite these conditions, progenitor cells had much larger membrane conductance than that of isolated progenitor cells. One possible explanation for this observation may be that marginal progenitor cells are electrically coupled each other through gap junctions. This possibility was supported by the facts that the halothane reduced the membrane conductance, and that biocytin transferred from the current-injected cell to its

neighbors. Halothane affects a variety of other ion channels (Malchow et al., 1994; Nikonorov et al., 1998; Winegar and Yost, 1998). However, in this experiment, currents recorded from progenitor cells were always obtained under the suppression of nonjunctional currents. Therefore, I believe that the halothane suppressed mainly junctional currents under such a condition.

The junctional currents in 13 out of 37 cells exhibited voltage- and time-dependent decrease with increasing test voltages. The voltage difference (given by the difference between the holding potential and test voltage) at which the junctional current started to decline varied widely within these cells, ranging from 80 to 140 mV with a mean value of 118 ± 6 mV. This value was much larger than that obtained in many previous experiments using the dual voltage-clamp technique (Spray et al., 1981; Anumonwo et al., 1992; DeVries and Schwartz, 1992; Wang et al., 1992; Chanson et al., 1993; McMahon, 1994). This difference may be caused by the technical problem of the single cell voltage-clamp that makes it difficult to measure the accurate junctional potential difference.

The other cells examined did not show voltage dependency, although they even exhibited large junctional currents. The mean slope conductance of these cells (5.6 nS) was about two times higher than that of cells exhibiting voltage-dependent behavior (2.4 nS). One possible explanation for the lack of the voltage dependency is that the voltage drop across gap junction channels could not reach the values at which voltage-dependent closing of the channels becomes apparent. The higher slope conductance of the clamping cell may reflect more coupling cell number that can attenuate the junctional potential difference. Alternatively, the presence of voltage-independent gap junction channels as reported in fish horizontal cells (Lu et al., 1999) is another possibility.

1-2. Comparison with gap junctional coupling between other retinal neurons

pH sensitivity of gap junctions has been shown in a variety of preparations

(Spray et al., 1981; for review, see Spray and Bennett, 1985) including retinal neurons (DeVries and Schwartz, 1989; Hampson et al., 1994). The conductance of gap junctions between pairs of isolated horizontal cells in fish retina was reduced by a decrease in intracellular pH (DeVries and Schwartz, 1989). In the rabbit retina, dye coupling of horizontal cells was reduced by low extracellular pH (Hampson et al., 1994). In the present study, the extracellular pH changes also altered whole-cell currents of marginal progenitor cells in a manner similar to the effect of intracellular pH changes on fish horizontal cell gap junctions. It is, therefore, possible to consider that the extracellular pH changes alter the intracellular pH, and then modulate the intracellular junctional conductance. In this case, the intracellular pH changes may be attenuated by the dialysis of a clamped cell with HEPES-containing pipette solution. Alternatively, the conductance of the nonjunctional membrane is directly modulated by the extracellular pH. However, since the experiment was performed under the suppression of the nonjunctional current, the former consideration seems more likely.

Gap junctions can be modulated by several neurotransmitters and intracellular second messengers in the mature retina. Dopamine a modulatory neurotransmitter in adult vertebrate retina has been shown to reduce electrical and dye coupling between horizontal cells in fish (Teranishi et al., 1983; Lasater and Dowling, 1985; DeVries and Schwartz, 1989; McMahon, 1994), turtle (Piccolino et al., 1984) and mouse (Weiler et al., 2000) retinas, and amacrine cells of rabbit retina (Hampson et al., 1992). It has been suggested that dopamine elevates the intracellular cAMP concentration within horizontal cells which in turn may activate a cAMP-dependent protein kinase and then reduce junctional conductance (Lasater and Dowling, 1985; Lasater, 1987). In the present study, however, neither dopamine, cAMP, nor its analog modulated the junctional current between retinal progenitor cells. Hampson et al. (1994) reported that in the rabbit retina dopamine reduced horizontal cell dye-coupling at the extracellular pH 7.2, but not at pH 7.4. Therefore, I tested the effect of dopamine on the gap junctional current at both pH 7.2 and 7.4. However, I could not

observe such a pH-dependent dopamine effect. Dopamine- and cAMP-resistive gap junctional current has been also observed between progenitor cells in early regenerating retina of adult newt (Chiba and Saito, 2000) and between cultured horizontal cells in the retina of adult skate (Qian et al., 1993).

It has been shown that retinoic acid, which is known as a light-released neuroactive substance (McCaffery et al., 1996), modulated gap junctional conductances between horizontal cells of fish (Zhang and McMahon, 2000) and mammalian (Weiler et al., 1999) retinas. This was not the case in marginal progenitor cells of goldfish retina.

Tracer coupling studies have revealed that neurons of the same type or even in different types of adult vertebrate retinas make gap junctional communication (for reviews, see Vaney, 1994; Cook and Becker, 1995). Recently, several efforts have been made to identify the expression of gap junctional proteins (connexins) in the vertebrate retina, and a variety of connexins have been demonstrated (for reviews, see Söhl et al. 2000; Vaney and Weiler, 2000; White and Bruzzone, 2000; Dermietzel et al., 2000). Thus, it is interesting to speculate that cellular communication by means of gap junctions in the retina can be modulated independently by extra- or intra-cellular signal molecules. My colleagues preliminary attempted to identify the types of connexins of marginal progenitor cells using several commercially available connexin (Cx) antibodies, such as Cx43, Cx32 and Cx26, but none of them could detect any labeling in progenitor cells. Future work is needed to determine the types of connexins expressed in marginal progenitor cells of goldfish retina.

2. Functional differentiation of ganglion cells from marginal progenitor cells

2-1. Development of voltage-gated Na^+ currents

The present electrophysiological studies using retinal slice preparations demonstrated that voltage-gated Na^+ currents are first expressed in round cells of intermediate region closely located to a cluster of the marginal progenitor cells, facing the vitreal side of the retina. The amount of Na^+ currents in the intermediate cells varied from cell to cell. However, the current tended to increase in amplitude from cells adjacent to the marginal region towards cells close to the mature region. On average, the maximum Na^+ current amplitude of the intermediate cells (257 pA) was more than 6-fold smaller that of mature ganglion cells (1,621 pA). Moreover, I described that the Na^+ current activation threshold is more negative in mature ganglion cells (ca., -54 mV) than in intermediate cells (ca., -40 mV). To my knowledge, this is the first intraretinal comparison of electrophysiological properties of mature neurons and their normal developmental progenitor cells.

More than half of the Na^+ current-expressed intermediate cells along the vitreal side which were identified by the LY fills had processes. Some of them extended single processes into the optic fiber layer, although the length of the process varied from cell to cell. This provides the best anatomical evidence that recordings were made from retinal ganglion cells. Remaining cells that did not present any processes may have lacked them prior to slice preparation, their processes may have been removed during slice preparation, or my Lucifer fills may have been incomplete. Therefore, I do not exclude the possibility that immature forms of other cell types, such as displaced amacrine cells that also generate Na^+ currents in mature retina when depolarized. However, it should be noted that one cell located at the relatively distal level of the intermediate region (Fig. 18), which probably differentiate into conventional amacrine cells, did not exhibit any Na^+ currents.

It has been reported in many lower vertebrate retinas, ganglion cells always differentiate first, and well before the retina segregates into the distinct synaptic layers, which are followed in rough sequence by photoreceptor cells and retinal interneurons, such as horizontal, bipolar, and amacrine cells (for review, see Altshuler

et al., 1991). These results suggest that the intermediate cells closely located at the vitreal side may start to differentiate into ganglion cells soon after leaving a cluster of progenitor cells. These results also suggest that the differentiation of progenitor cells into ganglion cells may be controlled by environmental or positional cues. It remains unclear what the environmental factors are, or when and how their effects are exerted.

2-2. Na^+ current properties

My two basic observations (increase in current amplitude and shift of activation threshold) agree well with those obtained in studies of ganglion cells isolated from other species at different developmental stages (e.g., cat: Skaliora et al., 1993; mouse: Rorig and Grantyn, 1994; rat: Schmid and Guenther, 1996, 1998). How these changes in current properties occur is not yet known for retinal ganglion cells of any species. The increase in whole-cell current amplitude, and to some extent increased detectability of small currents during small depolarizations, could result from changes in single-channel conductance, channel opening probability, susceptibility to steady-state inactivation, and/or channel density. These possibilities would be best resolved by a combination of single-channel and whole-cell current recordings. This, in turn, would yield a result of general interest, because the presence of Na^+ current in mature, but not progenitor cells, has been reported for a variety of other cell types during development (e.g., Spitzer, 1979; Bader et al., 1983; MacDermott and Westbrook, 1986) and during different stages of regeneration (Kaneko and Saito, 1992). In future experiments, it will also be of interest to determine whether changes in the relative numbers of different Na^+ channel subtypes (O'Dowd et al., 1995; Garcia et al., 1998) or in channel subunit composition (Wollner et al., 1988) contribute to the differences I have found in Na^+ current properties.

2-3. Na^+ current function

Perhaps the most interesting question that emerges from the present results is

whether an increase in Na^+ current amplitude occurs before or after intermediate cells assume their adult position in the retina, and whether this increase contributes to the maturation of retinal ganglion cell morphology and function within the retina. I have not been able to test this, as I have no method yet to examine single cells before and after blocking retinal ganglion cell Na^+ channels. However, it is well known that TTX hinders the formation of normal central projections in fish (Meyer, 1982; Edwards and Grafstein, 1983), amphibians (Cline, 1991), and mammals (Stryker and Harris, 1986). TTX (i.e., spike blockade) can thus influence the structure of ganglion cell axon terminals. TTX also blocks the normal formation of retinal ganglion cell mosaics via cell loss (Jeyarasasingam et al., 1998). By contrast, TTX does not prevent retinal ganglion cells from forming sublamina-specific dendritic arbors in the inner plexiform layer (Dubin et al., 1986; Wong et al., 1991). Moreover, at least in cerebellum, TTX does not alter migration of immature neurons (Komuro and Rakic, 1992). If the mechanisms that influence fish retinal ganglion cell migration and anatomical maturation are similar to those found in these other systems, TTX might affect the maturation or survival of some fraction of the intermediate cell population, or it might affect the intraretinal position these cells take on just as they become mature ganglion cells.

3. Gap junctional uncoupling and neuronal differentiation

Cell-to-cell communication through gap junctions is common in early embryogenesis, but becomes selectively lost as development proceeds (Potter et al., 1966; for reviews, see Loewenstein, 1979; Warner, 1988; Guthrie and Gilula, 1989). Interference with junctional communication in the early amphibian embryo results in

specific developmental defects (Warner et al., 1984). Therefore, it has been proposed that gap junctions during embryogenesis may provide intracellular pathways for morphogens and other developmentally relevant factors, including Ca^{2+} and a range of additional second-messenger molecules that have a profound influence on a wide variety of cell functions (for reviews, see Ghosh and Greenberg, 1995; Kandler and Katz, 1995; Finkbeiner and Greenberg, 1996; Michikawa et al., 1996).

In the ventricular zone of developing neocortex, cell coupling via gap junctions is regulated by the cell cycle (Bittman et al., 1997). Precursor cells couple into clusters in S phase, remain coupled through G_2 phase, uncouple in M phase, and recouple through G_1. When newly formed postmitotic cells leave the cell cycle and start to migrate their appropriate positions as differentiated cortical neurons, they do not appear to be coupled through gap junctions to either other neurons or glial cells, whereas they regain gap junctional couplings in later stages of differentiation (Yuste et al., 1992; Kandler and Katz, 1998). In spinal Rohon-Beard neurons of *Xenopus laevis*, electrical uncoupling correlates with the appearance of Na^+ spikes (Spitzer, 1982). The expression of neuronal ion channels in cleavage-arrested ascidian blastomeres requires uncoupling from neighboring cells (Saitoe et al., 1996).

In the present study, I observed that marginal progenitor cells are coupled by gap junctions and that immature neurons closely located to the marginal region, some of them are probably immature ganglion cells because of their location and Na^+ channel expression, did not exhibit either electrical or tracer coupling. It would be interesting to investigate in future experiments whether cellular differentiation is premised on the uncoupling from a cluster of progenitor cells, and if so, whether there are molecules that can pass through gap junctions to either subserve or interfere with cellular differentiation.

In recent molecular analysis of retinal development, it has been demonstrated that cell-cell interaction via Notch-Delta signaling system may play a critical component of neuronal induction (Artavanis-Tsakonas et al., 1995; Austin et al.,

1995; Dorsky et al., 1997). The *Notch* gene encodes a cell-surface receptor protein and its expression is needed for the progenitor cells to remain in the undifferentiated state during development. Another transmembrane protein, the product of the *Delta* gene, whose expression pattern overlaps that of the *Notch* gene, is the ligand for the Notch receptor. Their expression eventually diminishes when the cells have differentiated. The inhibition of *Notch* and/or *Delta* expression increases the number of first-born (ganglion) cells *in vivo* and *in vitro* development. It would be interesting to investigate in future experiments whether cellular differentiation is regulated by gap junction, Notch-Delta signaling or a combination of both. Furthermore, since electrical coupling through gap junctions is found in all cell classes that comprise the mature retina, it is also interesting to investigate in future experiments how premature neurons uncoupled from progenitor cells re-create the cell specific gap junctional coupling toward the central retina.