Ⅲ General Discussion

The CNS including retina is formed at least by four major steps in the development, as follows

1) neurogenesis – the mitotic division of nonneuronal cells to produce neuroblasts;
2) differentiation – the generation of specific neuronal cell types;
3) migration – the movement of post-mitotic neurons to their destination in tissue; and
4) synaptogenesis – the establishment of synaptic connections between neurons.

Over the past ten years, the temporal ordering of expression of a number of genes governing vertebrate retinogenesis has been described (Perron et al., 1998; for reviews, see Cepko, 1999; Marquardt and Gruss, 2002). Moreover, recent cellular and molecular evidence suggests that each stage is not only regulated solely by gene expression, but also influenced by interaction between cell-to-cell communications (for reviews, see Guthrie and Gilula, 1989; Kandler and Katz, 1995; Lewis, 1996; Dorsky et al., 1997).

Fig. 26A shows a schematic diagram illustrating morphological and functional characteristics of regenerating retinas at different stages. The present results indicate that progenitor cells do not exhibit voltage-gated Na^+ currents, but show electrical and tracer coupling with each other throughout gap junctions. Loss of gap junctions between progenitor cells correlates with the appearance of voltage-gated Na^+ currents in premature ganglion cells. On the one hand, functional AMPA, GABA and glycine receptors are expressed at the time of, or shortly after the appearance of voltage-gated Na^+ currents well before synaptogenesis, while NMDA receptors are expressed around the beginning of synaptogenesis.

1. Cell-to-cell communications associated with neurogenesis and neuronal differentiation

Recent molecular analyses suggest that the cell-cell interactions mediated by the Delta-Notch lateral-inhibitory signaling pathway may be involved in neurogenesis (Fig. 26B). The *Notch* gene encodes a cell-surface receptor protein and its expression is needed for the progenitor cells to remain in the undifferentiate 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 the retina *in vivo* and *in vitro* development, demonstrating that determination to a neural fate in vertebrate retinas may be regulated by the Notch-Delta signaling (Austin et al., 1995; Dorsky et al., 1997; Silva et al., 2003).

Gap junctions are another important pathways mediating cell-to-cell communication. In the adult CNS, gap junctions can function as sites of electrical communication between excitable cells (Lamb, 1976; for reviews, see Spray et al., 1999; Bennett, 2000). Molecular studies suggest that there are a number of different types of gap junctional proteins (connexins) in a variety of neurons and glia (Rozental et al., 2000a). Several lines of evidence suggest that, in various developing tissues that are not excitable, connexins are particularly abundant and that the degree of gap junction or connexin expression in the developing CNS including retina is not a static phenomenon, but changes during development (Dixon and Cronly-Dillon, 1972; Fujisawa et al., 1976; Connors et al., 1983; Sakaguchi et al., 1984; 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; Rozental et al., 2000b). Therefore, it has been speculated that gap junctional communication

may play an important role in many developmental events, such as cell proliferation, cell migration, differentiation, synapse formation, and correlation of the spontaneous electrical activity necessary for the refinement of synaptic connections (for reviews see Dermietzel and Spray, 1993. Fulton, 1995; Cook and Becker, 1995; Roerig and Feller, 2000). In fact, interference of gap junctional communication in early developing chick retina results in a reduction in the eye size (Becker and Mobbs, 1999). The above two possible cell-to-cell communications, the Delta-Notch lateral-inhibitory signaling pathway and gap junctions, are not mutually exclusive mechanisms responsible for neurogenesis and neuronal differentiation (Fig. 26B).

Using immunohistochemical techniques, Umino and Saito (2002) have demonstrated that Cx43 is a major gap junctional protein in progenitor cells of regenerating newt retina and that the amount of Cx43 gradually decreases as retinal regeneration proceeds. Here, I provided morphological and physiological evidence suggesting a good correlation between the loss of gap junctions and the appearance of voltage-gated Na^+ currents. Taken together, I would speculate that ganglion cell differentiation may be controlled by the Cx43-mediated gap junctional communication. An important question is whether early blocking of the gap junctions would lead to accelerate the ganglion cell differentiation. We did not address this question in this study. However, the finding that voltage-gated Na^+ channels were expressed in RPE cells, from which neural retina can regenerate, much earlier in their dissociation culture than *in vivo* regeneration system (Sakai and Saito, 1997) suggests that cell-to-cell communication via gap junctions may regulate the appearance of voltage-activated Na^+ channels in ganglion cells too, and thus regulate ganglion cell differentiation. It is likely that several steps separate the loss of gap junctions and the expression of voltage-gated Na^+ channels, such as cell fate determination, initial differentiation (expression of molecular markers common to almost all neurons), migration to

their final position and the terminal differentiation of neurons into distinct cell types (expression of voltage-gated ion channels and neurotransmitter receptors). Therefore, the present data can not directly link the loss of gap junctions with the expression of voltage-gated Na^+ channels.

Why does the appearance of Na^+ currents correspond with the disappearance of gap junctional coupling? Since progenitor cells are not excitable, gap junction channels have been considered to act as mediators of molecular rather than electrical (ionic) signals. Therefore, progenitor cells may exchange certain molecular signals throughout gap junctions to (1) maintain them in an undifferentiated state, or (2) interfere with their cellular differentiation. In the developing CNS, it has been considered that many extrinsic factors, including growth factors and neurotransmitters, activate intracellular second messenger systems that can lead to change in gene expression and cytoskeletal reorganization (Mattson, 1988; Lauder, 1993; Cameron et al., 1998; Roerig and Feller, 2000). Calcium, IP_3 (inositol 1,4,5-trisphosphate) and cAMP (adenosine 3',5'-cyclic monophosphate) are important second messengers and have a role in a variety of neuronal functions, including transmitter release, regulation of neurite outgrowth and gene activities. These substances appear to be small enough to diffuse throughout gap junctions in many biological systems (Lawrence et al., 1978; Sáez et al., 1989; Meyer, 1991; Yuste et al., 1995; Kandler & Katz, 1998; for review, see Roerig and Feller, 2000). I have not yet tried to examine what kinds of substances can diffuse throughout gap junctions of progenitor cells in the regenerating newt retina. It will be a challenge to find out the molecules that can pass through gap junctions and regulate neuronal differentiation in the near future.

2. Neurotransmitter receptors associated with neuronal differentiation and synaptogenesis

In the adult CNS, neurotransmitters and their receptors are concentrated at synaptic sites for functioning of fast signal transmission between neurons. However, accumulating data indicate that they may also regulate a variety of developmental events, such as proliferation and differentiation of neural precursor cells (Cameron et al., 1998), and outgrowth (for reviews, see Mattson, 1988; Lipton and Kater, 1989; McDonald and Johnston, 1990; Lauder, 1993), plasticity (Cline and Constantine-Paton, 1990; Huang and Redburn, 1996), survival (Nichol et al., 1995) and cell death (Choi and Rothman, 1990; Rörig and Grantyn, 1993) of immature neurons in the developing CNS. Therefore, a detailed knowledge of the order of appearance of neurotransmitters and their receptors in the developing and regenerating CNS including retina (Dupont et al., 1987; LoTurco and Kriegstein, 1991; Spitzer, 1991; Blanton and Kriegstein, 1992; Skaliora et al., 1993; Rörig and Grantyn, 1994; Chiba and Saito, 1995b; Liets and Chalupa, 2001) could provide helpful insights into basic questions such as neurogenesis, neural differentiation, synaptogenesis and initiation of synaptic transmission.

In the present study, I demonstrated that the appearance of excitatory and inhibitory amino acid receptors mainly occurs after neuronal migration has been completed. In addition, I also demonstrated that these receptors appear at the time of, or shortly after, the appearance of voltage-gated Na^+ currents. Among these receptors, NMDA receptors appear later than AMPA, GABA and glycine receptors. These results, together with the findings that glutamate and GABA are synthesized at the time of the formation of synaptic layer (Chiba et al., 1997; Chiba, 1998), suggest that transmitter receptors are expressed earlier than the onset of the transmitters themselves. Additional evidence in support this comes from the order of the appearance of cholinergic system components,

ACh and ACh receptors, in developing and regenerating newt retina (Cheon and Saito, 1999; Cheon et al., 2001). The developmental lag between neurotransmitters and their receptors is very similar to the development of these parameters in chick (Sheffield and Fischman, 1970; Hughes and LaVelle, 1974; Yamashita and Fukuda, 1993) and mammalian retinas (Wong, 1995), suggesting that mechanisms that control neuronal differentiation during retinal development and regeneration are similar.

In the developing CNS, the activation of neurotransmitter systems often accompanies changes in intracellular concentration of Ca^{2+} ions ($[Ca^{2+}]_i$). Now several line of research suggest that Ca^{2+} ions may play a central role in communicating the extrinsic neurotransmitter signals to intrinsic signaling cascades and gene expressions controlling neuronal development (Bixby and Spitzer, 1984; Kater et al., 1988; Spitzer, 1994; Finkbeiner and Greenberg, 1998; Berridge, 1998; Hardingham et al., 1999).

NMDA receptors are permeable to Ca^{2+} ions in immature as well as mature neurons (for reviews, see Ascher and Nowak, 1987; Ozawa et al., 1998). Activation of the receptor is required for promotion of neurite sprouting (Mattson, 1988), induction of long term potentiation in hippocampus associated with learning and memory (for review, see Bliss and Collingridge, 1993; Malenka and Nicoll, 1999) and the critical developmental period of ocular dominance plasticity within the visual center in several different species (Tsumoto et al., 1987; Fox et al., 1989; Constantine-Paton, et al., 1990). Non-NMDA receptors, AMPA/KA receptors, are predominately permeable to both Na^+ and K^+ ions in mature neurons, but become permeable to Ca^{2+} ions in immature neurons. The shift of this ion selectivity of AMPA receptors between mature and immature neurons is determined by composition of subunits taken from a set of four proteins, GluR1-4. GluR2-containing AMPA receptors show little permeability for Ca^{2+} ions, while AMPA receptors without the GluR2

subunit show high permeability for Ca^{2+} ions in the CNS (for review, see Ozawa et al., 1998). Ca^{2+}-permeable AMPA receptors are also present in developing chick (Catsicas, et al., 2001) and rat retinal neurons (Rörig and Grantyn, 1993), and appear before NMDA receptors. Activation of Ca^{2+}-permeable AMPA receptors reduces neurite outgrowth in developing chick (Catsicas, et al., 2001) or induces cell death in rat ganglion cells which may contribute regulation of cell numbers in the postnatal retina (Rörig and Grantyn, 1993). GABA has been considered to be the major inhibitory neurotransmitter in the adult CNS. It activates $GABA_A$ receptors and inhibits neuronal firing by increasing a Cl^- conductance. However, GABA-mediating signals has been also implicated in the regulation of many developmental events, from cell proliferation to circuit reformation (for reviews, see Ben-Ari, 2002, Owens and Kriegstein, 2002). For examples, GABA can decrease proliferation of cortical progenitor cells (LoTurco et al., 1995), promote neurite outgrowth in the rat superior cervical ganglia (Wolff et al., 1978) or reduces neurite outgrowth in rat hippocampal pyramidal neuron (Mattson and Kater, 1989). Several lines of evidence indicate that GABA produces a $[Ca^{2+}]_i$ rise through $GABA_A$-receptor-mediated membrane depolarization and activation of voltage-gated Ca^{2+} channels (Yamashita and Fukuda, 1993; Berninger et al., 1995; Leinekugel et al., 1995; Maric et al., 2001).

In the present study, I could not find any significant difference of pharmacological properties and reversal potential values of AMPA, NMDA, GABA and glycine-induced responses between premature and mature ganglion cells, suggesting that ion permeability of excitatory and inhibitory amino acid receptors may not change during regeneration. However, I also found that rectification properties of current-voltage relations for AMPA and NMDA responses were more voltage dependent in premature ganglion cells than mature ganglion cells. Furthermore, recent studies using Ca^{2+} imaging techniques have

shown that activation of glutamate and GABA receptors produces a significant $[Ca^{2+}]_i$ rise in mitotic progenitor cells and premature ganglion cells in the early regenerating newt retinas (Ohmasa and Saito, 1999; Ohmasa, 2000). Therefore, the near future studies using combination of whole-cell patch clamp and Ca^{2+} imaging techniques should provide better understanding of the roles of transmitter and their receptors during regeneration.