

**LOCALIZATION OF CHEMICAL AND ELECTRICAL SYNAPSES IN THE
RETINA**

by

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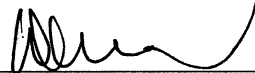
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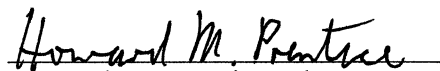
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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Wen Shen, Department of Biomedical Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

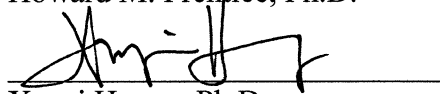
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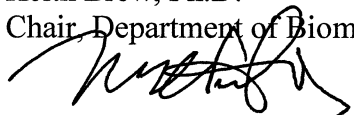
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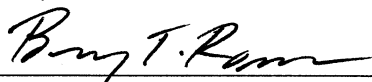
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ABSTRACT

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The amphibian retina is commonly used as a model system for studying function and mechanism of the visual system in electrophysiology, since the neural structure and synaptic mechanism of the amphibian retina are similar to higher vertebrate retinas. I determined the specific subtypes of receptors and channels that are involved in chemical and electrical synapses in the amphibian retina. My study indicates that glycine receptor subunits of GlyR α 1, 3 and 4 and glutamate receptor subunit of GluR4 are present in bipolar and amacrine dendrites and axons to conduct chemical synapses in the retinal circuit. I also found that the gap junction channel, pannexin 1a (panx1a), is present in cone-dominated On-bipolar cells and rod-dominated amacrine processes possibly to connect rod- and cone-pathway in the inner retina. In addition, panx1a may form hemi-channels that pass ATP and Ca²⁺ signals. The findings of my study fill the gap of our knowledge about the subtypes of neurotransmitter receptors and gap junction channels conducting visual information in particular cell types and synaptic areas.

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CHAPTER 1

INTRODUCTION

1.1. Simple Anatomy of the Retina

The vision is the most fundamental of our senses and it is devastating when we loss of our vision due to neurodegeneration and disease. The eye as the primary visual organ has a very specific design with three different layers to capture and analyze light. The external layer is formed by the sclera and cornea. The cornea covers both the pupil and the iris. The sclera forms part of the supporting wall of the eyeball. The intermediate layer is divided into two parts: anterior containing iris and ciliary body and posterior that is choroid. The retina is the internal layer and is the most vital layer of vision compared to the other parts of the eye. The retina is a piece of neural tissue, approximately 100 μ M thickness. Within the retina there are multi-layers of neurons and synaptic zones that are responsible for detecting visual images and transmitting these to the brain (Polyak, 1941; Van Buren, 1963; Kolb, 1991).

All vertebrate retinas have a basic structure that is composed of three layers of nerve cell bodies and two layers of synaptic regions. The outer nuclear layer (ONL) contains cell bodies of photoreceptors. The second nuclear layer called the inner nuclear layer (INL) contains cell bodies of the bipolar, horizontal and amacrine cells and the third nuclear layer contains cell bodies of ganglion cells and displaced amacrine cells. All the retinal neurons communicate at two synaptic layers: the outer plexiform layer (OPL) and

inner plexiform layer (IPL) in the distal and proximal retina (Figure 1). In the center of the retina is the optic nerve that contains the ganglion cell axons running to the brain.

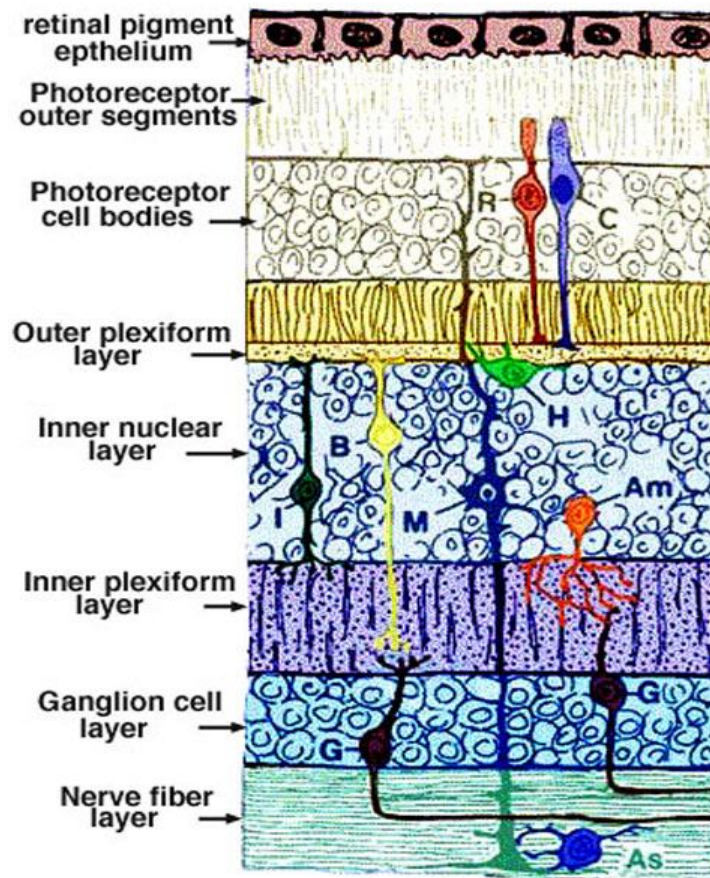


Fig 1. Retinal structure (from <http://webvision.med.utah.edu/>)

1.2. Physiology of the retina

The vision system starts with photo-sensitive neurons called photoreceptors. There are two types of photoreceptors: rods and cones that operate in different ranges of light intensity. Rods are more sensitive in scotopic (low luminance) conditions, but unable to function in a high intensity of light. Cones are sensitive in photopic (high luminance) condition, which means that cones are less sensitive to light, but perform well when light

intensity increases. Also, cones are responsible for detecting color, contrast and motion for our vision. Most mammals have two types of cones, green-sensitive and blue-sensitive cones, however, primates have the third type, red sensitive cones, in addition to the other two types of cones.

Visual signals are processing from photoreceptors to ganglion cells via bipolar cells in the vertical pathway; meanwhile, the lateral neurons, horizontal and amacrine cells provide feedback and forward modulation to the vertical pathways at the outer and inner retinas, respectively. The function of the lateral processing is to form the center-surround receptive field of the light responses in the parallel circuits within the retinas.

At the first synaptic layer, the OPL, rod and cones communicate with bipolar cells and horizontal cells. A rod terminal transmits information to a type of rod bipolar cells. A cone terminal transmits information to ten types of bipolar cells. In dark, rod and cone photoreceptors release glutamate that activates glutamate receptors in bipolar and horizontal cell dendrites. Light ceases glutamate release from photoreceptors. There are two types of bipolar cells: the ON- and OFF-type. In dark, the OFF-bipolar cell responds to glutamate by depolarization; whereas the ON-bipolar cell hyperpolarizes to glutamate due to the different glutamate receptors, ionotropic and metabotropic receptors that are respectively expressed in the dendrites of ON- and OFF-bipolar cells. Glutamate binding to ionotropic receptors opens the channels that allow cation passing, leading to depolarization of OFF-bipolar cells. In contrast, glutamate activates metabotropic receptors in ON-bipolar dendrites and triggers G-protein sensitive cascades, resulting in closing cation-permeable channels and causing the ON-bipolar cell hyperpolarization. When a light stimulus is present, it stops glutamate release from

photoreceptors. Therefore light depolarizes ON-bipolar cells and hyperpolarizes OFF-bipolar cells. Thus a light signal processing through bipolar cells becomes parallel “On” and “Off” signals. Ganglion cells receive the “On” and “Off” signals from bipolar cells and processing the preliminary visual information by firing action potentials sending to the central brain via optical nerves. Ganglion cells are the only output neurons of the vertebrate retina. Indeed, ganglion cells are not only synapses with bipolar cells, but also amacrine cells in the second synaptic layer of the retina, the IPL. In order to keep the On and Off signals through the ganglion cells to the brain separately, the IPL is divided into two sub-layers, called sublaminae a and sublaminae b. (Famigletti and Kolb, 1976). In sublaminae a, OFF bipolar cells connect with OFF ganglion cells. In sublaminae b, ON ganglion cells receive the signals from ON bipolar cells.

The visual information is modified by about 21 types of amacrine cells before being transferred to the brain. Two types of amacrine cells are the key in the rod pathway. One is a small-field cell called AII which use glycine as a neurotransmitter (Kolb and Famigletti, 1974). The other type is A17 using GABA as a neurotransmitter (Pourcho and Goebel, 1983). The AII and A17 amacrine cells are pivotal in the circuitry of rod based, dim-light vision in the mammalian retina. Rod bipolar cell use the AII and A17 amacrine cells as intermediaries to pass signals to ganglion cells. The AII amacrine cells receive glutamate inputs from rod-bipolar cells and pass the signals to ON-cone bipolar cells via electrical synapses, the gap junction channels.

1.3. Neurotransmitters in the Retina

Many scientists who are researching on the retina focus a great deal of attention on neurotransmission between the neurons of the retina. They use various techniques like

autoradiography, immunocytochemistry and molecular biology to identify neurons for neurotransmitters, receptors and transporters of these neurotransmitters. The dendrites and processes of the neuron can be stained with various neurotransmitter antibodies, so neurons can be readily classified by using immunocytochemical techniques. (Davanger, 1991; Crooks and Kolb, 1992)

It is well established that the visual signals pass through the vertical pathways of the retina via glutamatergic synapses. Both rods and cones use the excitatory amino acid glutamate to transmit signals to the second order neuron in the retina. And glutamate is also considered to be the neurotransmitter of all bipolar cells and most ganglion cells in the vertebrate retina. (Marc et al., 1995)

γ -aminobutyric acid (GABA) and glycine are the essential neurotransmitters that modulate glutamate signals in retina, as known that glutamate transductions are simultaneously modified by the lateral neurons, horizontal cells (GABAergic neuron) and amacrine cells (either GABAergic or glycinergic neurons). Moreover, the interplexiform cells provide a long-range feedback loop from the IPL to OPL. The other neuromodulators like dopamine, serotonin, acetylcholine (ACh) and substance P are also found in one or more types of amacrine, interplexiform or ganglion cells in the vertebrate retina. (Kolb et al., 1981; Kolb et al., 1992, Kolb et al., 1995; Keyser et al., 1989; Hughes, 1991; Schütte and Weiler, 1987; Hurd and Eldred, 1993).

CHAPTER 2

MATERIAL AND METHODS

2.1 Animals

Larval tiger salamanders were purchased from the Kons Scientific(Germantown, WI). The animals were kept in an aquarium with water temperature at 12 °C and were adapted by a 12-h dark/light cycle. All procedures were performed in accordance with National Institutes of Health guidelines and were approved by the Committee on Animal Research, Florida Atlantic University (IACUC).

2.2 Immunocytochemistry

The immunocytochemical data presented in this study were obtained in retinal sections and tissues from 12 salamanders. Fluorescence labeling in retinal sections or flat-mounted retinal tissues was detected by a Zeiss LSM 700 confocal microscope system. Single scanning and multiple scanning along Z-direction were applied with the Zen software. The images were collected and processed with the software.

2.2.1 Antibodies

The primary antibodies, the sources, concentrations and references used in this study are listed in Table 1. The secondary antibodies were a FITC-conjugated goat anti-mouse antibody excited at 492 nm and emitted at 520 nm and a Cy3-conjugated goat anti-rabbit or a donkey anti-goat antibody with excitation at 550 nm and emission at 570 nm (Jackson ImmunoResearch, West Grove, PA).

Table 1. Antibodies

Antibody	Host	Source	Dilution	References
Pannexin	Rabbit(polyclonal)	Dr. Dvorianchikova	1:500	Dvorianchikova, et al., 2006
Calretinin	Goat(polyclonal)	Chemicon	1:8000	Deng et al., 2001
Go α	Mouse(monoclonal)	Chemicon	1:2000	Zhang & Wu, 2003
sEAAT2b	Rabbit(polyclonal)	Dr. Amara	1:1000	Eliasof et al., 1998
SV2	Mouse(monoclonal)	Dr. Buckley	1:20	Buckley & Kelly, 1985
GluR4	Rabbit(polyclonal)	Chemicon	1:1000	Vardi, N, et al., 1998
GlyR- α 3	Rabbit(polyclonal)	Chemicon	1:1000	Kuhse, J, et al, 1990
GABA	Rabbit(polyclonal)	Immunosolution	1:1000	Morgado, et al., 2008
Glycine	Rabbit(polyclonal)	Dr. David Pow	1:4000	Kalloniatis, & Fletcher, 1993
GlyR- α 1	Rabbit(polyclonal)	Chemicon	1:1000	Kuhse, J, et al, 1990
GlyR- α 4	Rabbit(polyclonal)	Chemicon	1:1000	Heinze L, et al., 2007

2.2.2. Frozen air-dried retinal vertical section preparation

Freshly enucleated eyes were fixed in 4% paraformaldehyde in PBS solution for 25 minutes followed by dissection of the retina from the eyecup. The isolated retinas were gradually dehydrated in 10%, 15%, 20%, and 30% sucrose solution in 0.1 M phosphate buffer (PB). Following an overnight rinse in 30% sucrose, the retinal tissue was embed in OCT compound (Ted Pella, Redding, CA), frozen and dissected at 12-20 μ m thickness.

The frozen sections were collected on silane coated slides, air dried, and stored at -80°C until use.

2.2.3 Single labeling

Retinal sections were rinsed with PBS, then treated with the antibody blocking reagent “cocktail,” consisting of 5% normal goat or donkey serum and 1% BSA in 0.3% Triton X-100 with 0.1% Tween in PBS (PBST), for 30 min. the retinal sections were incubated with a primary antibody, such as anti-GABA, anti-Pannexin 1a, or anti-Glycine, with 3% goat or donkey serum in the PBST solution overnight at 4°C. After rinsing three times, the sections were incubated with FITC-conjugated secondary antibody (1:400) or Cy3-conjugated secondary antibody (1:600) for 40 min in the dark at room temperature. The retinal sections were rinsed with PBS and mounted in Vectorshield (Vector Laboratories, Burlingame, CA).

2.3.4 Double labeling

Retinal sections were rinsed with PBS and treated with the blocking reagent cocktail for 30 min, then incubated in the solution with a mixture of two primary antibodies from different hosts for 2 h at room temperature. The dilutions for the primary antibodies were the same as that for the single antibody labeling. After being treated with primary antibodies, the sections were rinsed three times in PBS and incubated in a solution with FITC-conjugated secondary antibody(1:50) and Cy3-conjugated secondary antibody(1:600) for 40 min in the dark at room temperature, followed by rinsing three times and mounting. If two primary antibodies were from a same host the primary antibodies were applied one for each time, followed by a secondary antibody treatment.

2.3 Western blotting assay

Retinal tissue samples were homogenized in 200 ml buffer solution (mM) 250 sucrose, 10 Tris, 10 HEPES, and 1 EDTA, pH 7.4) containing a protease inhibitor kit (Roche, Mannheim, Germany) with 1%-2% SDS. The homogenate was centrifuged at 600g for 10 min in 4°C. The supernatant was collected and stored at -80°C until use. The proteins were resolved by SDS-PAGE using a 4% to 12% Bis-Tris system. Proteins were electrophoretically transferred from unstained gel to polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was blocked by the block cocktail solution (5% non-fat dry milk in the cocktail, pH, 7.4) for one hour at room temperature, followed by incubation in the block solution with suggested concentration for anti-pannexin 1a (1:500), anti-Glycine receptor $\alpha 3$ (1:1000) for 2 h at room temperature. After three 15 minutes washes in the block solution, the PVDF membranes were incubated with a secondary antibody, horseradish peroxidase-conjugated goat anti rabbit IgG (1:2000) for 2 hours at room temperature followed by three 15 minutes washes in the block solution and a 10 minute wash in PBST. The antibody treated membranes were incubated in the solution with the enhanced chemoluminescent (ECL) reagent (Amersham Biosciences, Piscataway, NJ). The immunoreactivity bands were visualized after a photographic process.

2.4 Measurement of extracellular ATP levels

The retinas were collected from animals that had been kept at least six hours in the dark. Briefly, the animals were decapitated, double-pitched and the eyes were enucleated.

The retinal tissues were removed from the eyecup in Ringer's solution consisting of (mM): 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES and 10 Dextrose, adjusted to

pH = 7.7. A retinal tissue was incubated for 20 min in 1 mL of Ringer's solution at room temperature. The bathing Ringer's solution was discarded and the retinal tissue was re-incubated in a fresh Ringer's solution (0.15mL) in a vial for 5 min and the medium was collected to obtain a basal ATP level. Then, 0.15mL of 50mM KCl Ringer's solution was added into the vial to stimulate the retinal tissue. After 5minute incubation the high KCl medium was collected for ATP assays. To block ATP diffusion from pannexin hemichannels, carbenoxolone (100 μ M) was applied before and during the high K^+ Ringer's solution.

ATP levels in the mediums were measured by the luciferin-luciferase assay using an ATP determination kit (Promega). Briefly, ATP standards (10nM–1000nM) and test samples were added to a 96 well clear plate containing the luciferin–luciferase mixture. The plates were placed on the stage of a microplate luminometer (Molecular Devices, Sunnyvale, CA). The luminescence was converted to ATP concentration using the ATP standard curve. High KCl and carbenoxolone (CBX) showed no effect on the luciferase assay.

Data in figures were expressed as normalized [ATP] that represents the stimulated levels of extracellular ATP divided by the basal levels of extracellular nucleotide (Reigada,2008).

2.5 Cell dissociation

The retinas were collected from animals that had been kept at least six hours in the dark. Briefly, the animals were decapitated, double-pitched and the eyes were enucleated. The retinal tissue was removed from the eyecup in Ringer's solution. Retinal tissue was then dissociated in freshly prepared enzymatic tissue dissociation solution containing

50µl papain (12 U/mL), 400µl of Ringer's solution containing (mM) 5 L-Cysteine and 1 EDTA (adjusted to pH 7.4) for 20-35 minutes at room temperature. The enzymatically treated retina was washed before being mechanically dissociated, by gentle shaking, in Ringer's solution. The dissociated cells were seeded on 18mm glass cover slips freshly coated with lectin and allowed to set for 20 minutes before use. All experimentation was done at room temperature and within a few hours of preparation. (Bulley & Shen. 2010)

2.6 Calcium imaging

Isolated retinal cells seeded on lectin-coated cover slips were incubated for 20-25 minutes in the cell-permeable calcium sensitive Fluo-4 AM dye (3µM in Ringer's solution). The cover slips were washed in Ringer's solution before being placed in the recording chamber. Cells were excited at 480nm with the emission collected at 520nm. A Rolera-MGi Plus camera (Q-imaging) was used to view and collect fluorescence signals. Frame images were taken every 3 seconds with cells exposed for 50 milliseconds at each interval. A Lambda 10-2 (Sutter Instrument Co) controlled by IP Lab 4.0 software was used to open and close the filter shutter at each interval. The neurons were identified by their morphology. Usually, dissociated ganglion cells appeared to have a long axon process extending from the cell somas; the dissociated amacrine cells have larger somas than that of bipolar cells. (Bulley & Shen. 2010)

CHAPTER 3

CHEMICAL SYNAPSES IN THE RETINA

3.1. Background

In the nervous system, a synapse is a microstructure that permits the pre-synaptic neuron to transit an electrical or chemical signal to the post-synaptic neuron. There are two fundamentally different types of synapse: chemical synapse and electrical synapse. In the chemical synapse, the pre-synaptic neuron releases neurotransmitter that binds to receptors located in the postsynaptic neuron membrane. Binding of the neurotransmitter to a receptor can affect the post-synaptic cell in a wide variety of ways.

Glycine is a major inhibitory neurotransmitter in the retinas as well as GABA. It exerts its actions by binding to the glycine receptors (GlyRs) that express in the postsynaptic neuron membrane. GlyRs are ligand-gated chloride channels that conduct a fast action, GlyRs are composed of four ligand binding α -subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$) and a structural β -subunit (Lynch, 2004). The β -subunit is a constitutive subunit that interacts with the anchoring protein, gephyrin (Langosch et al., 1988). GlyR α -subunits are required for agonist binding (Schmieden et al., 1989; Sontheimer et al., 1989). Therefore, GlyRs are likely to be heteromeric receptors composed of both α and β subunits. These receptor subunits are differentially distributed in the post-synaptic area of interplexiform cells and glycinergic amacrine cells in vertebrate retinas. GlyR $\alpha 1$, $\alpha 2$ and $\alpha 3$ are the most popular subunits existing in the CNS. GlyR $\alpha 4$ is found in mouse,

chick and zebrafish (Devignot, et al., 2003; Harvey, et al., 2000; Matzenbach et al., 1994). In bullfrog, GlyRs are also expressed on Müller cells as well as neurons. (Balse et al., 2006)

The mainly excitatory neurotransmitter glutamate acts via two classes of receptors, ionotropic receptor and metabotropic receptor. The ionotropic glutamate receptors are subdivided into three groups: α -amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid (AMPA), Kainate (KA) and *N*-methyl-d-aspartate (NMDA) receptors (Bleakman and Lodge, 1998). These ionotropic receptors are ligand-gated ion channels which allow flow of charged ions such as K^+ , Na^+ and Ca^{2+} in response to the ligand binding. This flow of ions results in a depolarization of the cell membrane and then generates an electrical signal that is propagated down the processes of the neuron to the second neuron. AMPA and KA receptors are permeable to both Na^+ and K^+ in some case it may not permeable to Ca^{2+} . AMPA receptors consist of four subunits: GluR1, 2, 3, 4. KA receptors are assembled by subunits KA1 and/or KA2 with GluR5, 6 and 7. In addition to the ionotropic GluRs, there are metabotropic GluRs (mGluR), which are G-protein coupled receptor (Conn and Pin, 1997). mGluRs consist of mGluR1 to mGluR8 subunits.

Ionotropic GluR2/3 is found in photoreceptor terminals in goldfish and rabbit (Peng et al.1995). GluR2/3, GluR4 and GluR6/7 subunits are located in somata and primary dendrites of horizontal cells in cat, rat goldfish and mondey (Morigiwa and Vardi, 1999;Peng et al., 1995; Schultz et al., 1997; Brandstätter et al., 1997; Haverkamp et al., 2001).

The differential distributions of glycine receptor subtypes and glutamate receptor subtypes in retinal neurons may indicate the distinct function of glycine feedback in the pre and post synapses of glutamate pathway. In the present study, I characterized glycinergic synapses in tiger salamander retina by using the polyclonal antibodies against different Glycine receptor, meanwhile, the specific antibody for GluR4 was used as a marker colocalizing with glycine receptor antibodies.

3.2 Results

3.2.1 Expression and localization of glycine receptor subunits in tiger salamander retina

The polyclonal GlyR α 1, α 3 and α 4 antibody detect single band with the molecular weight around 55Kd, 50kD and 70kD from a mouse retinal sample, respectively. The similar protein bands are also detected from salamander retinal samples (Fig.2). This indicates that the specific GlyRs antibody for mouse retina is also restricted for salamander retina. GlyRs in bipolar cell axon terminals receive inputs from glycinergic amacrine cells, whereas in the dendrites of bipolar cells receive inputs from glycinergic interplexiform cells that are the newly found feedback neurons in retina (Yazulla, Studholme, 2004). It is largely unknown which subtype of glycine receptors are expressed in bipolar cells. To detect the subunits of glycine receptors, I used specific antibodies for α -subunits labeling the retinal sections. As show in Fig.3A, the red punctate appearance of this immunoreactivity indicates that GlyR α 1 are expressed in amacrine cells membrane and also located in IPL. Anti-GlyR α 3 is strongly labeled

bipolar cells somata and their dendrites (Fig. 3B), staining for GlyR α 4 is concentrated in ganglion cells membrane (Fig. 3C).

Among those antibodies, double-labeling of GlyR α 3 and the salamander-specific excitatory amino acid transporter (sEAAT2b) shows similar staining patterns (Figs. 4A and 4B) and they overlapped each other shown in the superimposed image (Fig. 4C). Since sEAAT2b is an OFF-bipolar cell marker, the overlap of GlyR α 3 with sEAAT2b suggests that GlyR α 3 composite glycine receptors might synapse with glycinergic amacrine cells and interplexiform cells. The further results show that GlyR α 3 might also present in the ON-bipolar cell dendrites, but with less extent. Double-labeling of GlyR α 3 and G $_{\alpha}$ showed moderately overlapping of these two antibodies in a retina (Figs. 4D to 4E)

Since glycine release from interplexiform cells will activate the receptors not only in bipolar dendrites, but in horizontal cells and photoreceptor terminals. Double-labeling with horizontal cell marker, Calretinin (Deng et al., 2001), and photoreceptor terminal marker, Synaptic vesicle protein II (SV2) show that GlyR α 3 are not glycine receptor subunits for these cells. As shown in Figure 5, GlyR α 3 labeled with either SV2 or Calretinin in single-channel optical scanning images and the merged image demonstrate no overlapping in photoreceptor terminals and horizontal cells.

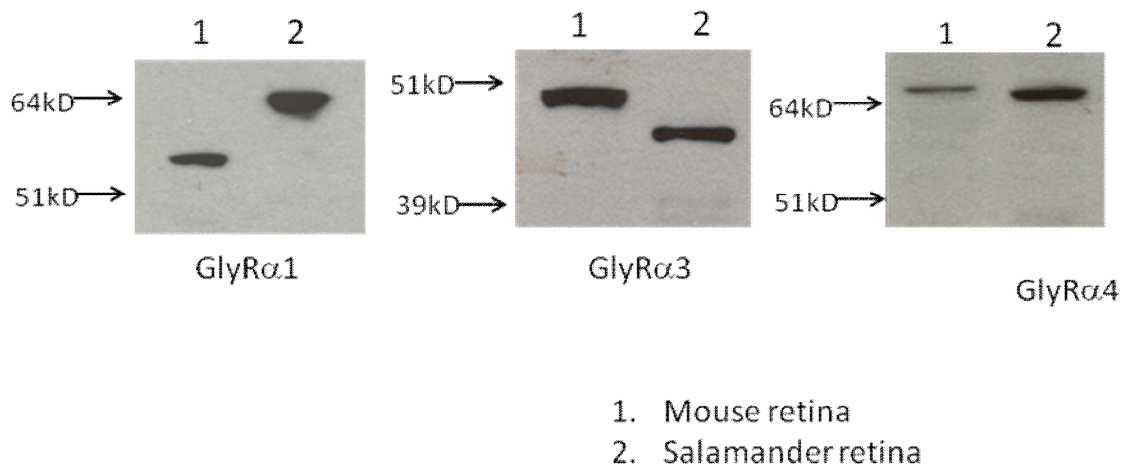


Fig2. Western blotting analysis of Glycine receptor subunit α 1, 3 and 4 protein in the tiger salamander and mouse retinas. (A) The glycine receptor subunit α 1 antibody recognizes single bands located at MW 55kD around in the mouse retinas and 60kD in the salamander retinas. (B) The glycine receptor subunit α 3 antibody recognizes single bands located at MW around 50kD in the mouse retinas and MW around 45kD in the salamander retinas. (C) The glycine receptor subunit α 4 antibody recognizes single bands located at MW around 70kD in the mouse retinas and the salamander retinas.

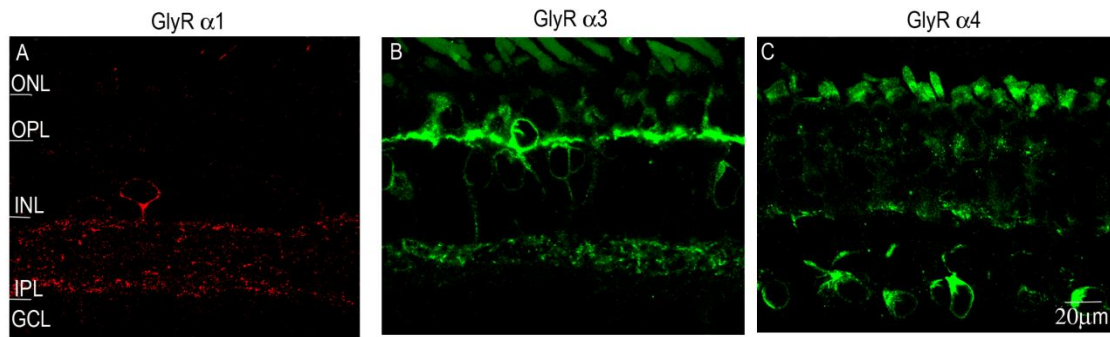


Fig3. Localization of Glycine receptor subunit $\alpha 1,3$ and 4 in tiger salamander retinas. (A) Single-labeling for GlyR $\alpha 1$. Anti-GlyR $\alpha 1$ mainly labels OPL and amacrine cells in INL. (B) GlyR $\alpha 3$ staining in the retina detected by a single-channel optical section. (C) Single-labeling for GlyR $\alpha 4$.

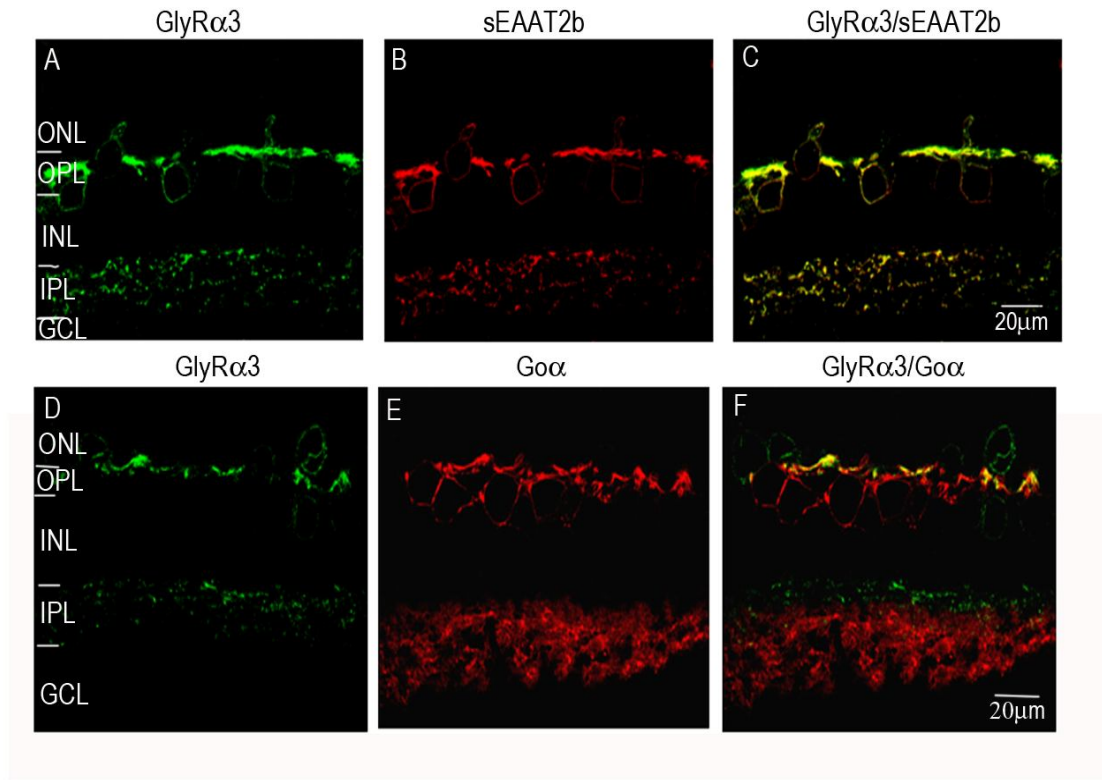


Fig4. Localization of Glycine receptor subunit $\alpha 3$ in ON- and OFF-bipolar cells in retinal vertical sections. (A, B, and C) Double-labeling for GlyR $\alpha 3$ and sEAAT2b, an OFF-bipolar cell marker, in a retinal section. The labeling for GlyR $\alpha 3$ (green) and sEAAT2b (red) was detected in confocal imaging with single-channel optical scanning and superimposed image shows that the two antibodies overlap at the dendritic areas. (D, E, and F) Double-labeling of GlyR $\alpha 3$ and $G_{\alpha\alpha}$, an ON-bipolar cell marker in a retinal section. The labeling for GlyR $\alpha 3$ (green) and $G_{\alpha\alpha}$ (red) is individually shown in single-channel optical sections, as well as the superimposition.

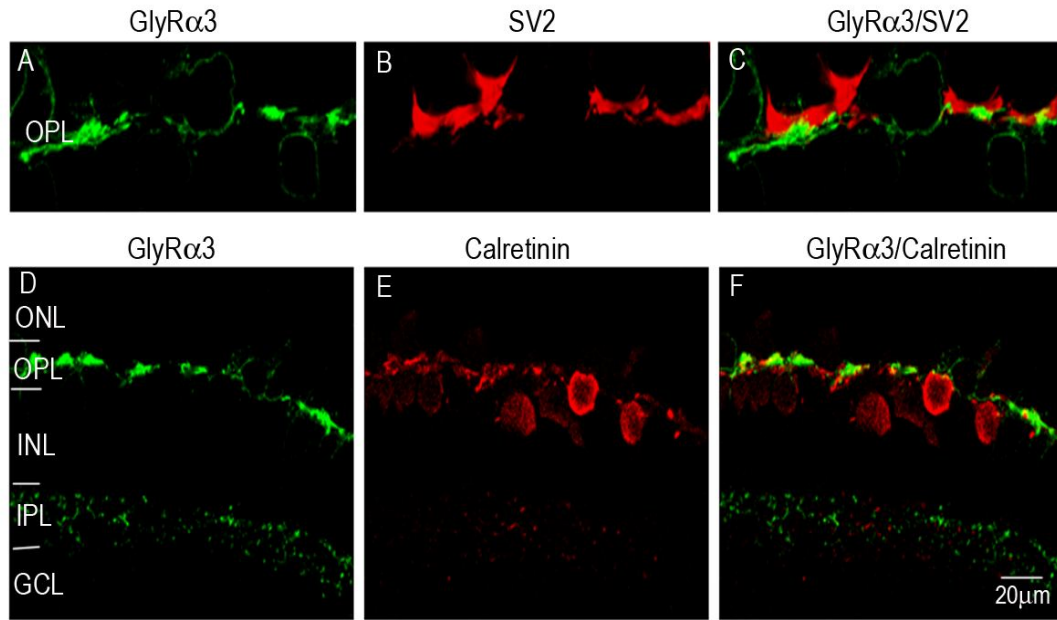


Fig5. Detection of Glycine receptor $\alpha 3$ subunit in photoreceptor terminals and horizontal cells in tiger salamander retinas. (A, B, and C) Double-labeling for GlyR $\alpha 3$ and SV2 marking photoreceptor terminals in a retinal section at the OPL. The labeling for GlyR $\alpha 3$ (green) and SV2 (red) is individually shown in single-channel optical sections. (D, E, and F) Double-labeling for GlyR $\alpha 3$ and Calretinin, a horizontal cell marker, in a retinal section. The labeling for GlyR $\alpha 3$ (green) and Calretinin (red) is individually shown in single-channel optical sections.

3.2.2 Localization of glutamate receptor subunit, GluR4 in the distal retina

Bipolar cells have different receptor channels for glutamate that are either of the metabotropic type or ionotropic type at their dendrites in the OPL. Glutamate binding to ionotropic receptors cause OFF-bipolar cell depolarization; in contrast, glutamate activates metabotropic receptors in ON-bipolar dendrites cause the ON-bipolar cell

hyperpolarization. These receptor types contribute to segregate visual information to ON- and OFF signals within the retinas.

I performed double-labeling for GluR4, an ionotropic glutamate receptor subunits, with $G_{o\alpha}$ and sEAAT2b respectively on vertical sections and flat-mount tissue. Figs 6A to 6F showed that GluR4 was co-localized with sEAAT2b in the OPL, possibly the synaptic dendrites of bipolar cells. This result could be observed in both retinal sections and flat-mounted tissues. Double labeling with antibodies to GluR4 and $G_{o\alpha}$ showed no overlap of these two both in the retinal section and flat-mounted tissue (Figs.6G to 6L). Since GluR4 is an AMPA receptor subunit, the results of GluR4 antibody labeling suggest that AMPA receptor might be expressed in the OFF-bipolar dendrites. I also examined expression of GluR4 in photoreceptor terminals marked with SV2. The results show that there is no overlapping of GluR4 and SV2 antibody labeling in photoreceptor terminals showing both retinal sections and flat-mounted retinas (Fig.7).

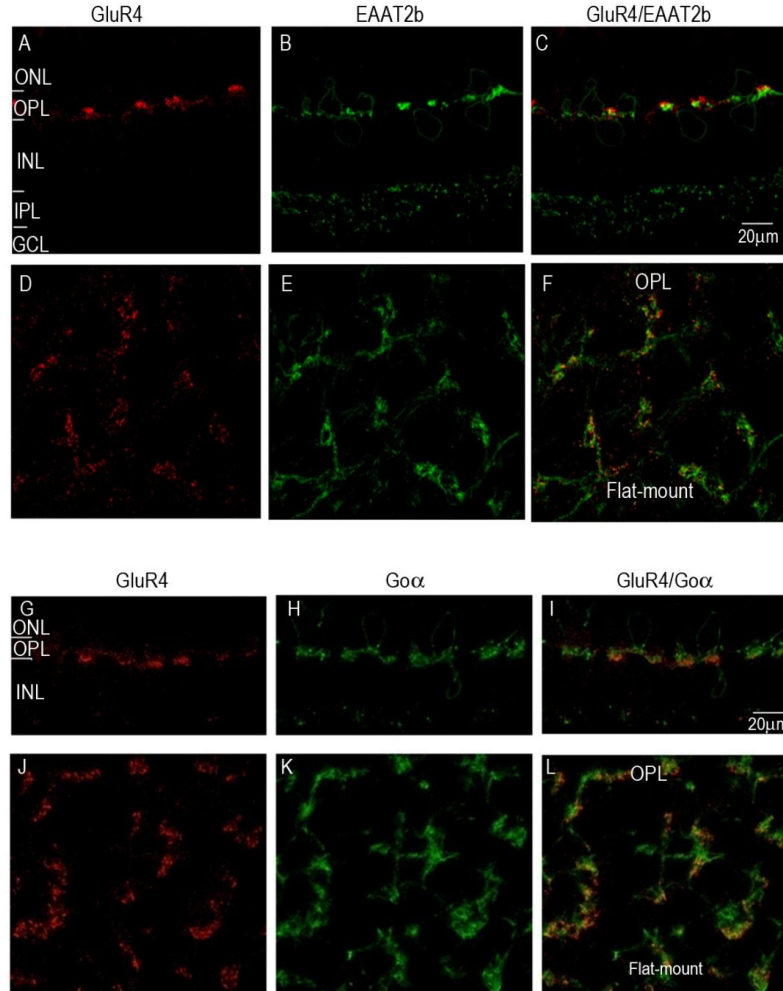


Fig6. Localization of GluR4 in bipolar cell dendrites in tiger salamander retinas. (A, B, and C) GluR4 colocalizes with sEAAT2b, an OFF-bipolar cell marker in the OPL in retinal vertical sections and at the OPL of whole-mounted retina. The red and green images show the individual protein labeled with GluR4 and sEAAT2b and the merged image of two singles. Double-labeling of GluR4 and $G_{o\alpha}$, an ON-bipolar cell marker, in retinal vertical sections; the two antibodies are separately present in the OPL (G, H, and I). (J, K, and L) Double-labeling for GluR4 and $G_{o\alpha}$ in a flat-mounted retina focused at the OPL. There is no overlapping detected in the OPL.

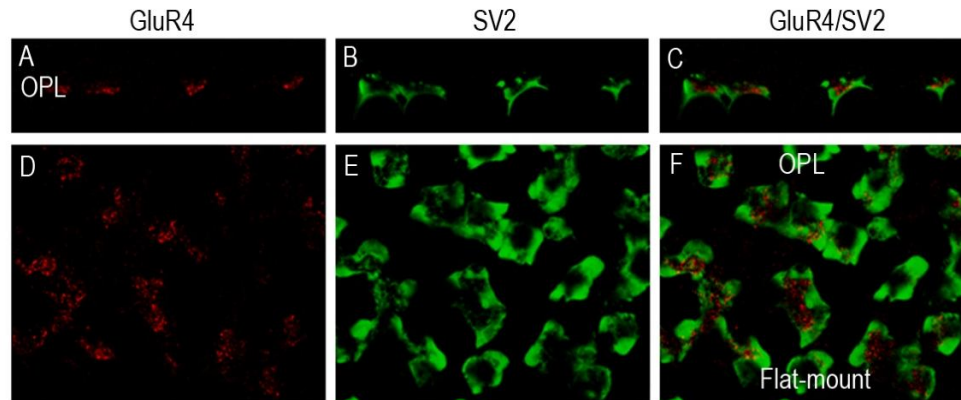


Fig7. Double labeling for GluR4 with SV2 in tiger salamander retinas. (A, B, and C) GluR4 and SV2 antibody labeling at OPL in a retinal vertical section; the superimposed image indicates on colocalization in the OPL. GluR4 and SV2 labeling at the OPL in a flat-mounted retina.

3.2.3 Verification of excitatory amino acid transporter II b (EAAT2b) is present in OFF-bipolar cell dendrites

EAATs are the main glutamate transporters in the retinas for uptake of glutamate in the synaptic clefts. In salamander retina 5 types of EAATs have been cloned and sEAAT2b is a subtype of glutamate transporter that is cloned from tiger salamanders. The antibody for sEAAT2b was obtained from Dr. Susan Amoro's laboratory. The antibody seems mainly labeling OFF-bipolar cells. However, it is not know if ON-bipolar cells, horizontal cells and photoreceptors are sEAAT2b positive in the distal retina. Therefore, I examined the sEAAT2b double-labeling with $G_{\alpha\alpha}$, calretinin or SV2 in retinal vertical sections. As shown in Figure 8, the labeling of sEAAT2b seems not only present in the bipolar cell somas, but also present the dendrites colocalized with $G_{\alpha\alpha}$,

suggesting ON-bipolar cell dendrite might also use sEAAT2b as a glutamate transporter. However, sEAAT2b antibody does not colocalize with SV2 and calretinin in photoreceptor terminals and horizontal cells, respectively, indicating these neurons use glutamate transporter other than sEAAT2b.

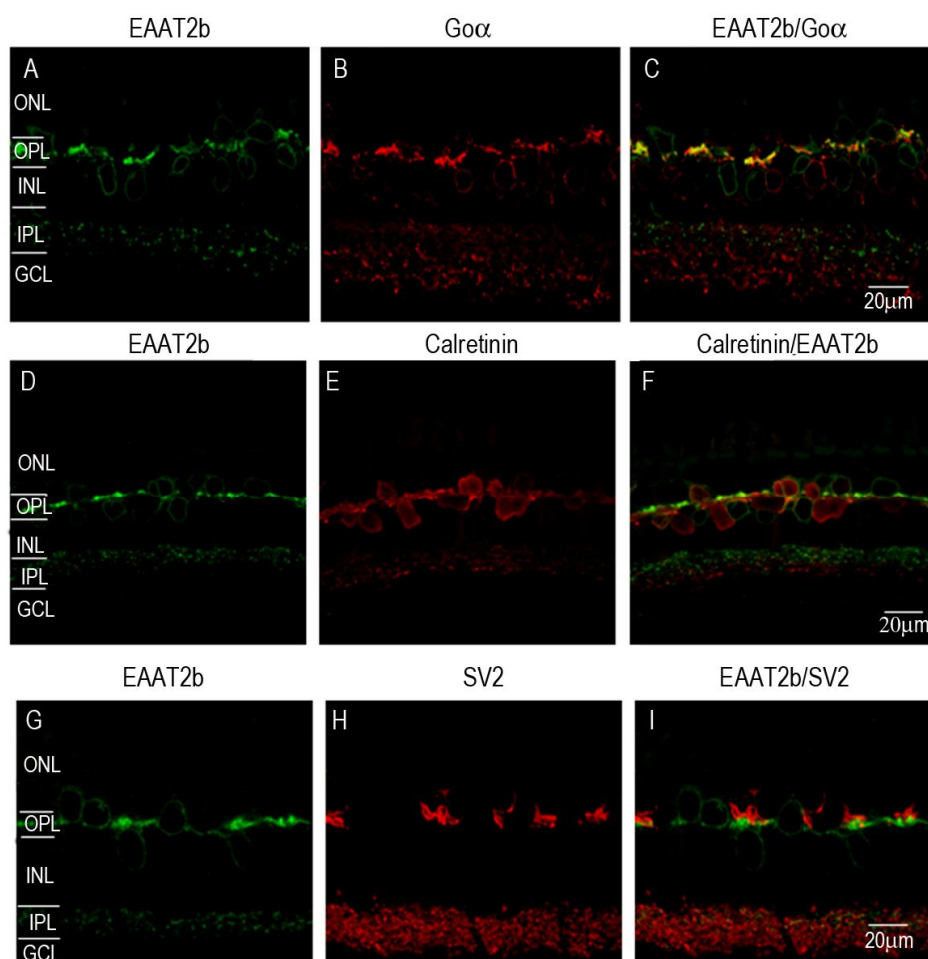


Fig8. localization of sEAAT2b in the distal retinal neurons. (A, B, and C) Double-labeling for sEAAT2b and $G_{o\alpha}$ in a retinal vertical section. The labeling for sEAAT2b (green) and $G_{o\alpha}$ (red) is individually shown in single-channel optical sections. (D, E, and F) Double-labeling for sEAAT2b and Calretinin in a retinal section. The labeling for sEAAT2b (green) and Calretinin (red) is individually shown in single-channel optical sections. (G, H, and I) Double-labeling sEAAT2b and SV2 in a retinal section. The labeling for sEAAT2b (green) and SV2 (red) is individually shown in single-channel optical sections.

CHAPTER 4

ELECTRICAL SYNAPSES WITH THE GAP-JUNCTION CHANNELS AND HEMICHANNELS IN THE RETINA

4.1 Background

Although both chemical and electrical synapses have been defined in vertebrate retinas, the detail information about which subtypes of gap junction proteins are involved in electrical transmission is still needed to be further determined. Particularly, those synaptic proteins in amphibian retinas are largely unknown, despite the low vertebrate retina has been widely used as a model system for study retinal function and mechanism for several decades. As known that each subtype of receptors and gap junction channels has its own kinetics and physiological properties, identifying these protein subtypes in the retina would provide valuable information for interpreting how visual signals are processed in the retinal circuit. Furthermore, understanding the localization and function of gap junction channels in normal physiological conditions would provide a clue for abnormal distribution and expression of these proteins in pathological conditions of the retina.

Gap junctions exist among the most widely distributed cell structures involved in cell-to-cell communication, as well as intra- and extra-cellular commutation. In general, gap junctions are the channel-forming structures in contacting plasma membranes that allow direct metabolic and electrical inter-cellular information delivery. Electrical activity

through gap junctions underlies direct and rapid neuronal communication in the central nervous system. The diversity of functional roles that electrical synapses have is perhaps best exemplified in the vertebrate retina, in which gap junctions are formed in the most neuron types. In recent years research into gap junction-mediated signal transmission and modulation in retinas has focused on AII amacrine-ON cone bipolar cell coupling that feeds signals from rod pathway into the cone pathway. At present, new candidates encoding for gap junction proteins have recently emerged. Pannexin is one of these gap junction proteins that we are concerning about its function in electrical synapses within retinas.

My study of electrical synapses is focused on identifying a particular group of gap junction channels, pannexin channels, in the retinas. To do so, I used the specific antibody for pannexin type I channels to study the distribution of this gap junction proteins, meanwhile, I performed Ca^{2+} imaging and ATP-assay to study the function of these gap junctions in retinal network. Also these studies were carried out on tiger salamander retinas.

4.2 Results

4.2.1 Pannexin 1a gap junction protein expressed in tiger salamander retina

The specific antibody for pannexin-1a (anti-panx1a) was used to localize the protein distributions in tiger salamander retina. The antibody specificity for the amphibian retina was verified in Western blotting assays by comparing the antibody recognized protein bands from the control sample, mouse retina, and amphibian retina sample. Figure 9 shows the results of the polyclonal Panx1a antibody detected protein bands that have two isoforms at the molecular weight around 43kD and 58kD. The proteins with

molecular weight at 58kD were matched from a mouse retinal and salamander retinal sample. A heavy protein band at 43kD was detected by anti-panx1a from mouse retinal sample, but from salamander retinal sample a light protein band was detected with molecular weight less than 43kD. In fact, a heavy protein band was present at the 58kD in salamander retinal sample. This suggests that the 58kD-isoform pannexin might be the major pannexin channel proteins in salamander retina. The larger 58 kD is likely post-translational modification of the protein (Dvorianchikova et al., 2006). Anti-panx1a labeling was performed on both salamander retinal sections and flat-mounted retinal tissues. Figs 9A show that anti-panx1a, strongly labeled a group of bipolar cells and the processes in the IPL in retinal vertical sections in double-labeling preparation, suggesting these neurons might use pannexin gap junctions to conduct intra- and extra-cellular signals and to communicate with neighboring neurons in the retina. To further identify which type of bipolar cells is Panx1a-positive, I used antibodies to specifically label subtypes of bipolar cells. The G_{oa} antibody has been used as a marker for labeling ON-bipolar cells in tiger salamander retinas (Zhang & Wu, 2003). Figs 10A to 10C display confocal imaging of double-labeling of anti-panx1a (red) and anti-G α (green) in a retinal section. The single scanning imaging indicates the anti-panx1a and anti-G α labeling pattern and the superimposed image shows that the two antibodies are overlapped in a group of ON-bipolar cells (see yellow color). The two antibodies were also colocalized in the distal sublaminae b of the IPL in which the axon terminals of cone-dominated ON-bipolar cells are located. Those ON-bipolar cells predominately receive cone inputs. As reported that G_{oa} does not label rod-dominated On-bipolar cells (Zhang & Wu, 2004), the location of the anti-panx1a labeling in the IPL

suggests that these neurons might be cone-dominated ON-bipolar cells.

The same double-labeling experiments were carried on flat-mounted retinal tissues. Fig. 10D to 10F show that the bipolar cells at the distal nuclear layer that was labeled by anti-panx1a and $G_{o\alpha}$ in a flat-mounted retina and a merged image shows that these two antibodies were colocalized in some anti- $G_{o\alpha}$ -positive ON-bipolar cells. The density of the panx1a-positive ON-bipolar cells in the retinal tissue was determined in the flat-mounted retina preparation, depicting that about 1/3 of On-bipolar cells are anti-panx1a positive. Possibly, these neurons are ON-bipolar cells in which pannexin channels are preferentially expressed.

As shown in the previous studies that the antibody for sEAAT2b is exclusively labels OFF-bipolar cells in salamander retinas (Eliasof et al., 1998, Rowan et al., 2010), the anti-sEAAT2b serves as the specific marker for labeling OFF-bipolar cells in the amphibian retina. In contrast, double-labeling of anti-panx1a and sEAAT2b showed no overlap of these two antibodies in retinal sections. Fig. 11A to 11C demonstrates that a typical anti-panx1a labeled ON-bipolar cells and the anti-sEAAT2b labeled OFF-bipolar cells in a retinal section. Note that anti-sEAAT2b labeled OFF-bipolar cells were present in both the inner nuclear layer and outer nuclear layer where the displaced OFF-bipolar cells are located. The two antibodies were present in separated groups of bipolar cells as shown in the superimposition. The insets of Fig. 11A to 11C show enlarged the axon terminals labeled by anti-panx1a and sEAAT2b in the IPL layer. Apparently, the two antibodies were labeled two different groups of axon terminals at the sub-layers for the ON- and OFF-bipolar cell axon terminals. The immunocytochemical study suggests that OFF-bipolar cells in salamander retinas may not use pannexin as they

gap junction channels.

One possibility is that pannexin gap junction channels are paired expressed in pre- and post-synaptic neurons, the ON-bipolar cell terminals and dendritic processes of amacrine cells. Therefore it becomes necessary to examine whether amacrine cells are also pannexin positive as well-known that AII amacrine cells are gap junction coupled to cone-ON-bipolar cells in mammalian retinas (Xia XB & Mills SL, 2004). The previous study indicates that amacrine cells in salamander retina are containing either GABA or glycine (Yazulla and Yang, 1988). To determine whether pannexin is present in the amacrine dendrites in the OPL, I performed double-labeling of anti-panx1a with either anti-GABA or anti-glycine. Figs. 12A to 12C show an example of a double-labeled retinal section, showing that anti-panx1a and anti-GABA were separately present in two groups of neurons with no overlapping in the IPL where bipolar and amacrine cells are synapses (Figs. 12D, 12E, and 12F). On the other hand, double labeling for anti-panx1a and anti-glycine confirmed that a subset of glycinergic amacrine cell processes in the IPL were anti-panx1a positive (Figs. 13A to 13F). The results suggest that there might be an electrical synapse between cone-dominated ON-bipolar cells and glycinergic amacrine cells in salamander retina, similar to that found in higher vertebrate retinas in which glycinergic AII amacrine cells bridge rod-bipolar cells to cone-bipolar cells with gap junction channels. This is a novel finding and it might answer the intriguing question posted many year ago that is whether the lower vertebrate retina has the same neural network as mammalian retinas using gap junction channels to connect rod pathway to cone's.

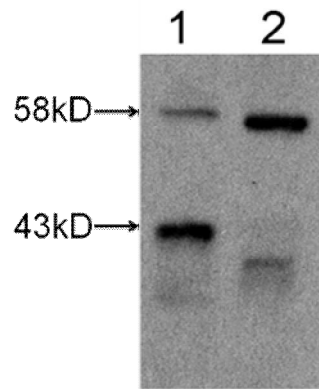


Fig9. The polyclonal pan-1 antibody detects two isoforms of pannexin proteins with the molecular weight around 43kD and 58kD from a mouse retinal sample, as control. The similar protein bands are also detected from salamander retinal samples. This indicates that the specific pan-1a antibody for mouse retina is also restricted for salamander retina.

1. Mouse retina 2. Salamander retina

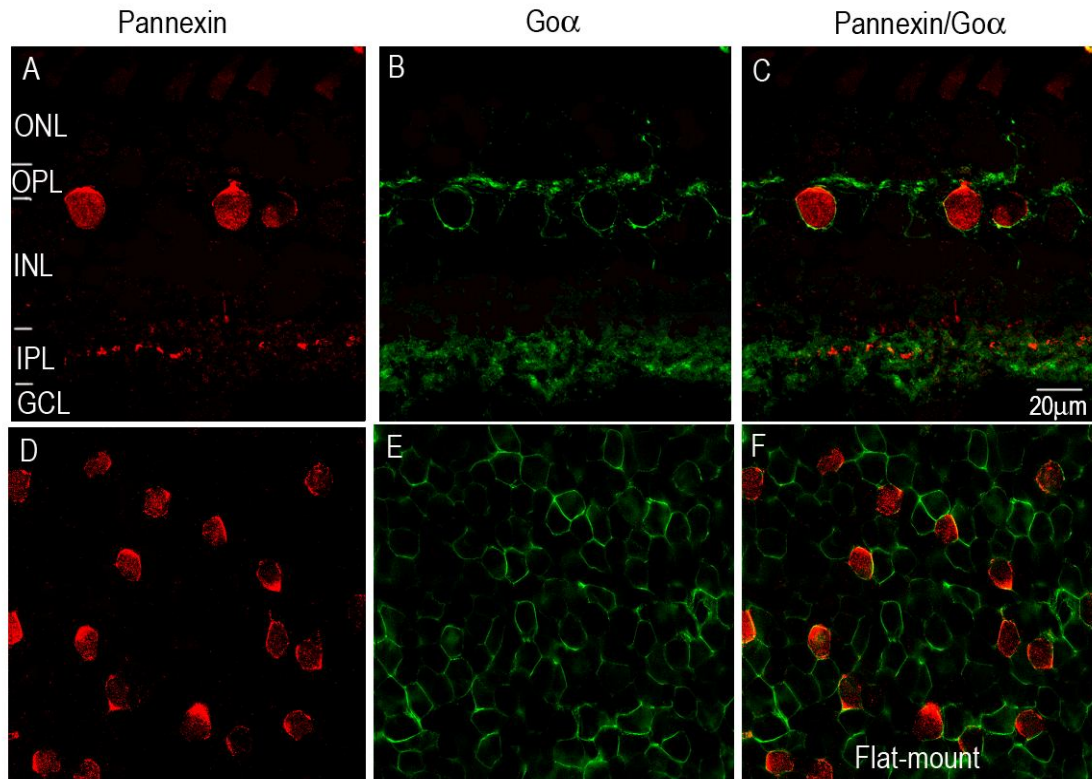


Fig10. Confocal imaging results of antibody labeling of Pannexin with $G_{o\alpha}$ and sEAAT2b, the cellular markers for On-bipolar cells and Off-bipolar cells in tiger salamander retinas. (A, B, and C) Double-labeling for Pannexin and $G_{o\alpha}$ in a retinal section. The labeling for Pannexin (red) and $G_{o\alpha}$ (green) are showed in separate of each single-channel optical scanning and in the superimposition. (D, E, and F) Double-labeling for Pannexin and $G_{o\alpha}$ in bipolar cell somas, scanned at the distal inner nuclear layer in a flat-mounted retina.

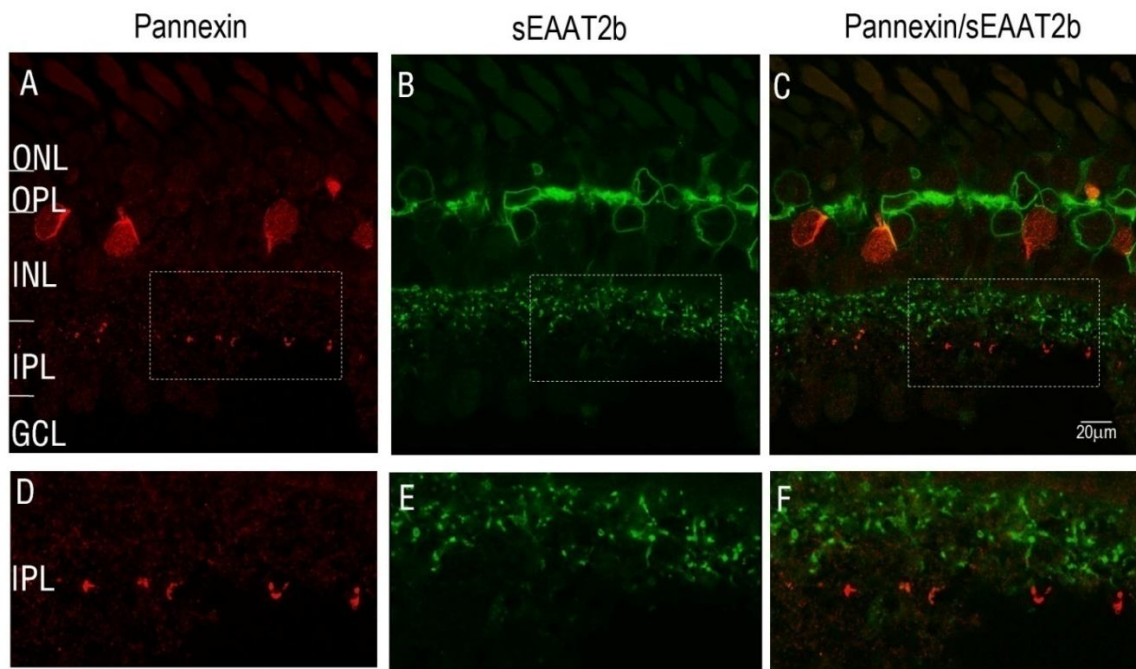


Fig.11 The anti-sEAAT2b antibody specifically labels Off-bipolar cells in salamander retina. Double-labeling for anti-Pannexin and anti-sEAAT2b show that the two antibodies separately labeled two different types of bipolar cells (A, B, and C). The two antibodies have no overlapping in the IPL where bipolar cell terminals and amacrine cell processes are located (D, E, and F).

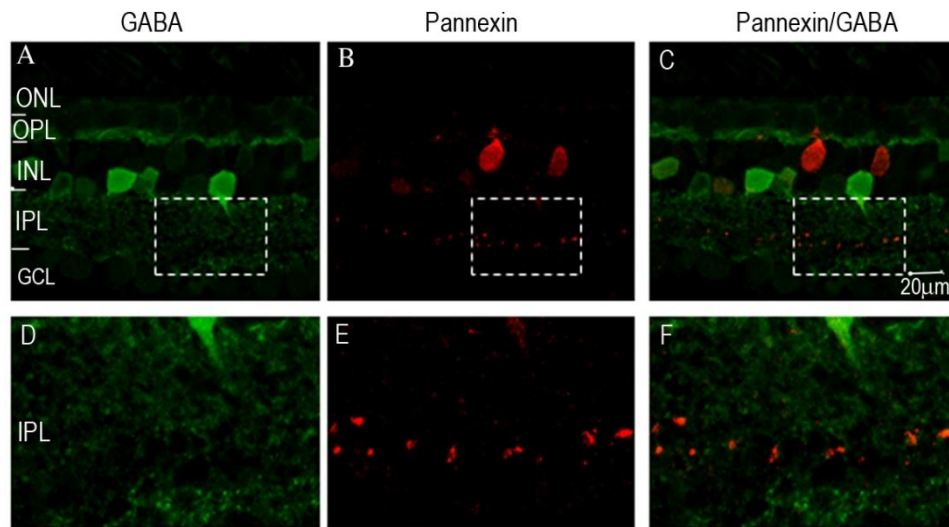


Fig12. Localization of Pannexin and GABA in tiger salamander retinas. Double-labeling for Pannexin and GABA in a retinal section (A, B, and C) and at the IPL (D, E, and F, the inset of A, B, and C). The labeling for Pannexin (red) and GABA (green) was detected in confocal imaging with single- channel optical scanning. The images were merged with both red and green with computer software

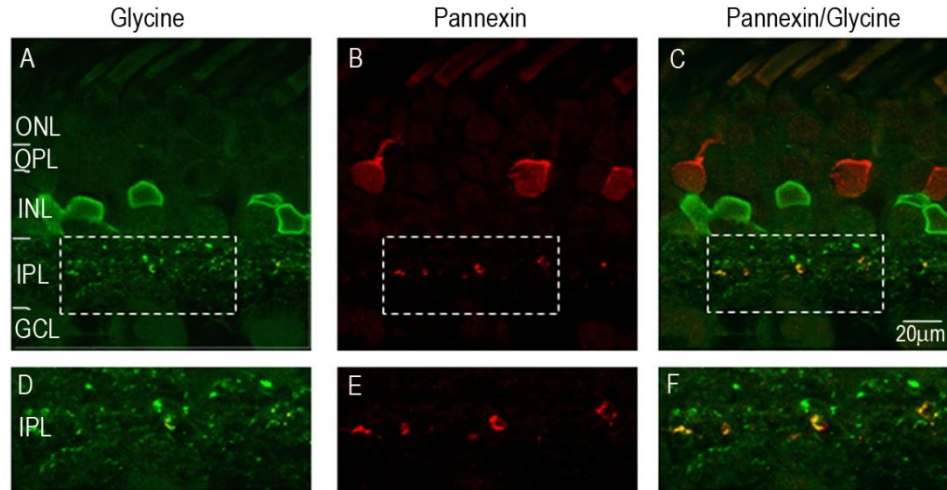


Fig13. Colocalization of pannexin with glycinergic amacrine cells. Double-labeling of Pannexin and Glycine in a retinal vertical section (A, B, and C) and at the IPL (D, E, and F). The labeling for Pannexin (red) and Glycine (green) is individually shown in single-channel optical sections.

4.2.2 Carbenoxolone (CBX) inhibits retinal ATP release via pannexin gap junction channels

Recent studies suggest that pannexin channels can form hemi-channels at the cell surface (Thompson et al., 2008; Iglesias et al., 2009). Once the channels are activated intracellular Ca^{2+} , ATP and glutamate are released. To test the possibility that pannexin channels in salamander retina are an ATP release source, I treated retinal tissues with a high K^+ (40mM) Ringer's solution to depolarize cells and stimulated intracellular ATP release. Extracellular ATP was detected and measured by luciferin/luciferase bioluminescence ATP assay Kit (Sigma-Aldrich). The total ATP levels were detected from the extracellular mediums of control Ringer, high K^+ , and high K^+ with CBX (50µM) to block gap junction channels. Fig. 13 shows the statistic results of the

external ATP level changes in control conditions and activated conditions with and without the gap junction channel blocker, demonstrating that blockage of gap junction channels has significant reduced ATP release to extracellular solution. CBX at concentration of 50 μ M blocks pannexin channels more effectively than connexin channels (Cruikshank et al., 2004; Bruzzone et al., 2005; Iglesias et al., 2008). The reduction of ATP release by CBX in the retinas might be due to blockage pannexin channels, since 50 μ M CBX has no significant effect on connexin channels in whole-cell recording (data not shown).

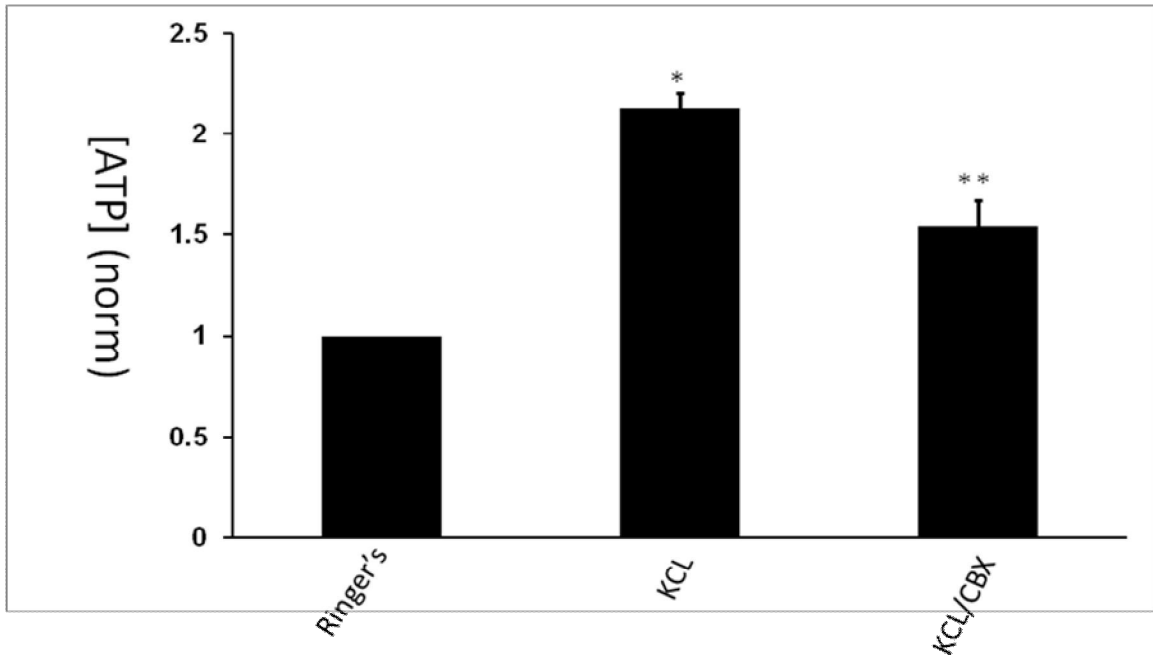


Fig14. CBX, 100 μ M blocked the release of ATP in eyecups exposed to high K^+ (n=5) compared to cells exposed to high K^+ alone (n=5). The ATP levels in the high K^+ of CBX and high K^+ were significantly different from Ringer's controls (n=5). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. high K^+ alone.

4.2.3 CBX suppressed glutamate-induced Ca^{2+} influx through pannexin gap junction hemichannels

To determine if pannexin channels in the ON-bipolar cells are Ca^{2+} permeable, I used Ca^{2+} imaging techniques to detect internal Ca^{2+} changes in isolated retinal neurons. Retinal tissues were treated in papain enzyme solution for 30-45minutes followed by mechanical dissociation. The isolated cells were seeded on lectin-coated glass cover-slips and set for 15 minutes before loading with Fluo-4 AM, a membrane permeable Ca^{2+} indicator. The internalized Fluo-4 molecules bind with intracellular free Ca^{2+} ions, which cause an increase of the fluorescence intensity of Fluo-4. Intracellular Ca^{2+} changes can be detected by monitoring fluorescence intensity changes in cell cytosolic regions. Again, I used a high K^+ (50mM) to excite the Fluo-4 loaded isolated cells in Ca^{2+} imaging recording. Internal Ca^{2+} increased when the high K^+ was applied. The increment of internal Ca^{2+} should be due to cell depolarization in high K^+ , leading to activation of voltage-gated Ca^{2+} channels. To separate the effect of Ca^{2+} influx via voltage-gated Ca^{2+} channels or pannexin channel, Cd^{2+} was used to block Ca^{2+} channels. The remaining Ca^{2+} increase by high K^+ was sensitive to 50 μM CBX (Fig. 16). The CBX blocks pannexin channels, therefore the effect of CBX on the high K^+ induced internal Ca^{2+} increase evidenced pannexin channels in the retinal neurons are Ca^{2+} permeable. Although it is difficult to determine the cell types in the isolated neurons, the CBX sensitive internal Ca^{2+} changes were detected from only 25% of the isolated cell recorded and these cells with relative small soma, presumably these were cone-dominated On-bipolar cells.

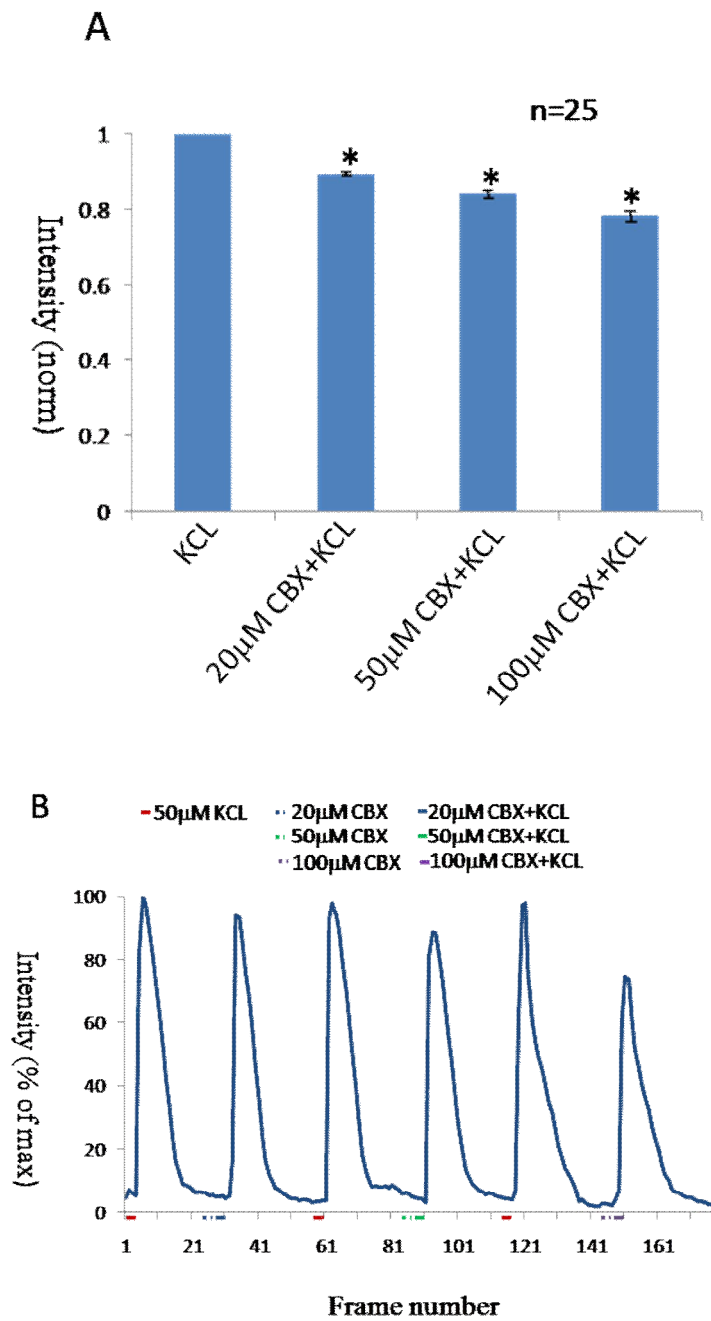


Fig15. Statistical results of CBX regulation of high K^+ -induce $[Ca^{2+}]_i$ in isolated retina neurons. CBX suppresses the $[Ca^{2+}]_i$ in the second-order neurons in a dose- dependent manner. * $p < 0.05$ vs. high K^+ alone.

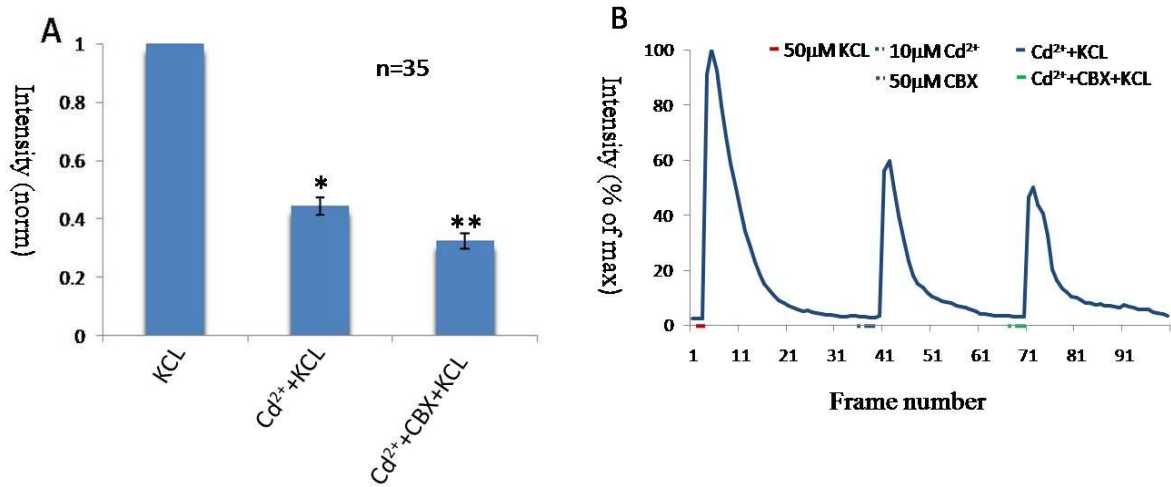


Fig16. CBX suppress high K⁺ induced Ca²⁺ influx through pannexin hemichannel. Cd²⁺ blocks voltage-gated Ca²⁺ channels, which reduces 56% of high K⁺-induced [Ca²⁺]_i in the neurons; with Cd²⁺ CBX still suppresses high K⁺-induced [Ca²⁺]_i. * p<0.05 vs. high K⁺ alone; **p <0.05 vs. Cd²⁺ & high K⁺.

CHAPTER 5

DISCUSSION

Glycinergic neurons are involved in mediating the antagonistic surrounds of retinal receptive fields, feedback control of synaptic signal and network adaptation. About half of amacrine cells are glycinergic neurons. However, a lesser number of glycine-containing amacrine cells are expressed glycine transporters (Shen and Jiang, 2006) , suggesting that both glycine-containing and glycinergic amacrine cells might co-exist in the retinas. In mammalian retinas, the distinct structure for glycinergic amacrine cells is narrow-field processes, differing from GABAergic amacrine cells that have wide-field lateral processes. However, in amphibian retinas, the receptive field of glycinergic amacrine cells is larger than that of GABAergic amacrine cells (Shen and Jiang, 2006). Bipolar cells, amacrine cells and ganglion cells receive glycine input from glycinergic amacrine cells in proximal retina. However it's not clear which glycine receptor subunits are responsible for the signal transmission. Glycine receptor subunits are cloned and well defined in the previous studies. Also the antibodies for these subunits are commercially available. My result imply that amacrine cells may receive glycine input through GlyR α 1, GlyR α 4 possibly involved in glycine feedback signals transiting from glycinergic amacrine cells to ganglion cell in tiger salamander retinas.

There are at least 12 subtypes of bipolar cells in the tiger salamander retinas. These bipolar cells are generally classified into cone-dominant ON- and OFF-bipolar cells and

rod-dominant ON- and OFF-bipolar cells. They receive glutamate input from photoreceptor and glycine input from the feedback neurons. The receptor subunits and localizations are studied in both ON- and OFF-type bipolar cell dendrites. This reveals a synaptic pattern of glycine feedback in distal retinal glutamate synapses. To understand glycinergic synapses and glutamatergic synapses in retina will extend our knowledge on the function of glycine and glutamate in CNS.

G_{oα} antibody will be used to mark ON-bipolar cell dendrites in both retinal vertical sections and whole-mounted retinas. sEAAT2b will be used to mark OFF-bipolar cell dendrites in both retinal vertical sections and whole-mounted retinas. The result of double-labeling of GlyRα3 with G_{oα} shows there is detectable overlap of two antibodies in the OPL. To localize glycine receptor subunits in ON-bipolar cells dendrites will provide information about if glycine feedback from network produces same or different response in the ON- and OFF-bipolar cells in the distal retinas.

Glutamate is considered to be the most important excitatory neurotransmitter in the retina (Ozawa et al., 1998). Glutamate diffuses across the synaptic cleft and binds to glutamate receptors that express on the dendrites of the post-synaptic neurons after releasing from pre-synaptic neuron (Yang, 2004). Earlier studies showed that post-synaptic responses of OFF-bipolar cells are mediated by AMPA receptors in the lower vertebrate retina. However which subunits of AMPA receptors involve in the transmission between photoreceptors and second order neurons is unclear. By using single- and double- immunostaining techniques, I have demonstrated that AMPA receptor subunit GluR4 is abundantly expressed in the OPL. GluR4 subunits tend to cluster underneath the cone pedicles. It indicates GluR4 is involved more in cone pathway than

in rod pathway. My immunocytochemistry data imply that both GlyR α 3 and GluR4 are possibly present in OFF-bipolar cells. Since GluR4 is a subunit of AMPA receptors that have been known as a major synaptic receptors in OFF-bipolar cells, the colocalization pattern of glycine receptor subunit with the glutamate receptor subunit will be useful to interpret glycine feedback to the OFF-bipolar cells in the distal retina.

The pannexin genes have been cloned and they are homologous to the invertebrate gap junction proteins, innexins, but heterologous to the vertebrate gap junction proteins, connexins (Yen MR & Saier MH, 2007). There are currently three pannexins in human and rodent genomes (Pannx1, Pannx2 and Pannx3) and orthologous sequences have been identified in zebrafish (Bruzzone R et al., 2003; Sasakura Y et al., 2003; Baranova A et al., 2004). Pannexins-formed channels have been considered differing from connexin channels since pannexin channels are mostly like forming hemichannels in native cells, only in expressed cells pannexins are formed inter cell gap-junction connections (Bruzzone R et al., 2003) whereas most connexin proteins form paired gap-junction connections, as well as hemi-channels in the CNS. The channel structure study indicates that pannexins appear to form large-pore channels in the plasma membrane with large unitary conductance that could readily pass large molecules (<1kDa), such as ATP, IP3 and amino acid. Another distinction between the pannexin and connexin channels is Ca^{2+} and Mg^{2+} regulation. Connexin hemichannels are sensitive to extracellular Ca^{2+} and Mg^{2+} concentrations. High concentrations of these ions in the external solution could close connexin channels, however, pannexin channels are unaffected by the external Ca^{2+} and Mg^{2+} levels (Bruzzone R et al., 2005).

In the brain cells, ATP release via pannexin 1 channels activates pre-synaptic

purinergic A1 receptors that in turn inhibit neurotransmitter release in the pre-synaptic terminals. In salamander retina, pannexin 1 channels are seemed expressed in the On-type cone dominated bipolar cells. If ATP release from these neurons via pannexin hemi-channels, apparently it could directly regulate glutamate release from photoreceptors by activation of purinergic receptors. My results suggest that a possible function of pannexin channels might relate to a feedback regulation of ON-bipolar cells to cone photoreceptors. Further study is necessary to determine the conditions for ATP release from pannexin channels and feedback regulation of photoreceptors.

Antibody double labeling results indicate that pannexin1 may also present in the process of glycinergic amacrine cells. This is corresponding to the connexin gap junction coupling pathway between cone-dominated On-bipolar cells and AII amacrine cells in the higher vertebrates. However, the function of pannexin 1 expressed in both bipolar terminals and amacrine processes in the amphibian retina is need to be further determined.

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