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Characterization of glycinergic synapses in vertebrate retinas

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Summary

Glycine is one of the essential neurotransmitters modulating visual signals in retina. Glycine activates Cl^- permeable receptors that conduct either inhibitory or excitatory actions, depending on the Cl^- electrical–chemical gradient (E_{Cl}) positive or negative to the resting potential in the cells. Interestingly, both glycine-induced inhibitory and excitatory responses are present in adult retinas, and the effects are confined in the inner and outer retinal neurons. Glycine inhibits glutamate synapses in the inner plexiform layer (IPL), resulting in shaping light responses in ganglion cells. In contrast, glycine excites horizontal cells and On-bipolar dendrites in the outer plexiform layer (OPL). The function of glycinergic synapse in the outer retina represents the effect of network feedback from a group of centrifugal neurons, glycinergic interplexiform cells. Moreover, immunocytochemical studies identify glycine receptor subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$ and β) in retinas, forming picrotoxin-sensitive α-homomeric and picrotoxin-insensitive α/β-heteromeric receptors. Glycine receptors are modulated by intracellular Ca^{2+} and protein kinas C and A pathways. Extracellular Zn^{2+} regulates glycine receptors in a concentration-dependent manner, nanomolar Zn^{2+} enhancing glycine responses, and micromolar Zn^{2+} suppressing glycine responses in retinal neurons. These studies describe the function and mechanism of glycinergic synapses in retinas.

Introduction

In vertebrate retinas, visual signals are processed by five basic types of neurons: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. They form synaptic contacts at two active layers, the outer plexiform layer (OPL) and inner plexiform layer (IPL), located in the distal and proximal retina (Figure 1). Glutamate, γ-aminobutyric acid (GABA) and glycine are major neurotransmitters conducting visual signals. The light signal transfers into a glutamate signal within photoreceptors. Glutamate releases from photoreceptors activating bipolar cells, which in turn release glutamate to activate ganglion cells, the retinal output neurons, through the vertical pathway. There are lateral neurons, horizontal cells (GABAergic neurons) and amacrine cells (either GABAergic or glycinergic neurons) synaptic contact with photoreceptors and bipolar cells in the OPL and IPL, respectively. Glutamate transductions are modulated by the inputs from the lateral neurons. Moreover, between two synaptic layers, the newest found group of neurons, interplexiform cells, creates a long-range feedback loop from the IPL to OPL, conducting network information. Glycinergic synapses are provided by glycinergic interplexiform cells and amacrine cells in the outer and inner retina (see Figure 1).

Glycinergic synapse is juxtaposed with GABAergic synapse in many regions of the central nervous system (CNS), including retina. Both glycine and GABA activate Cl^- permeable iono-

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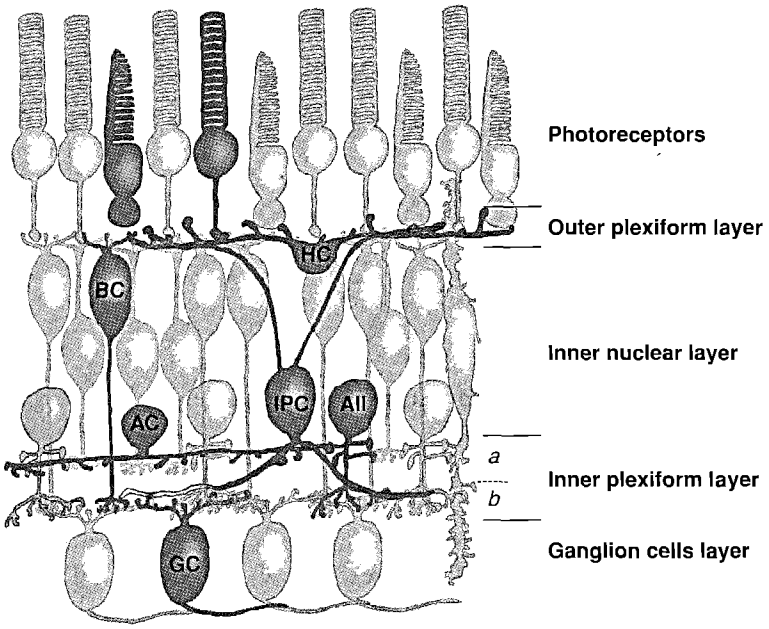

Figure 1. The cellular organization of vertebrate retina. Glycinergic synapses are provided by glycinergic amacrine cells (AC) and interplexiform cells (IPC) in the IPL and OPL, respectively. AII: AII amacrine cells, BC: bipolar cell, GC: ganglion cells, a: sublamina a; b: sublamina b.

tropic receptors that conduct a fast action. Unlike GABA that also conducts a slow inhibition by activating metabotropic receptors, glycine only activates ionotropic receptors. Yet the G-protein coupled metabotropic glycine receptor has not been discovered. In pharmacology, glycine receptor is distinct from GABA receptor by its agonists and antagonists. Therefore, using pharmacological tools, receptor antagonists, can effectively separate the mixed effects of glycinergic and GABAergic synapses in the neural network. So far, strychnine is the only known potent glycine receptor antagonist that is widely used for studying the functions and properties of glycine receptors.

Glycine receptors are cloned and identified as pentameric receptor channels composed of α and β subunits. The β-subunit is a constitutive subunit binding with the anchoring protein, gephyrin [1]. Alternatively, the homopentamer α-subunit receptors are also identified, and these forms are present in the extrasynaptic areas in the adult neural system [2] as well as in immature neurons [3]. During neuronal development, α/β heteromeric receptors replace α-homomeric receptors [4]. Thus, the α/β composition glycine receptors are predominantly expressed at the postsynaptic sites of glycinergic synapse in the adult CNS. The homomeric or heteromeric assembled receptors show distinct pharmacological properties, for example α/β heteromeric receptors are much less sensitive to picrotoxin than homomeric α-subunit receptors [5]. To date, four distinctive α-subunits ($\alpha1$–$\alpha4$) and one β-subunit are molecularly characterized. $\alpha1$–$\alpha3$ are the most popular subunits existing in the CNS. $\alpha4$-subunit is found in mouse, chick and zebrafish [6–8]. The receptor subunit variations determine the divergence of glycinergic synaptic strength in the CNS.

The effect of glycinergic synapse could be either inhibitory or excitatory based on whether the Cl^- electrical–chemical gradient potential (E_{Cl}) is negative or positive to the cell resting membrane potential. Glycine produces an inhibitory effect in a majority of neurons in the adult CNS since the E_{Cl} is negative to the resting potential in the neurons, and activating glycine receptors cause a Cl^- influx. However, in some neurons, especially in immature neurons, the E_{Cl} is positive to the resting potential, therefore glycine generates an excitatory effect mediated by Cl^- efflux [9, 10]. Indeed, the E_{Cl} related inhibitory and excitatory effects of glycine are present in adult retinal neurons, although the function of the excitatory glycine response is largely unknown. Nevertheless, glycin-

ergic synapse in retinas has been studied over the years. Much of our knowledge on the functions of glycine transduction in retina has been gained by electrophysiological and immunohistochemical studies. Here we summarize the evidence from recent studies about glycinergic synapse in retinas.

Glycine receptors in retinas

Glycine receptor α1-, α2- and α3-subunits and a β-subunit are found in vertebrate retinas. Antibody immunoreactivities show that the receptor subunits are differentially distributed in the postsynaptic sites of glycinergic amacrine cells and interplexiform cells [11–18]. In mouse retina, rod-bipolar cell terminals possess heteromeric glycine receptors consisting of α1 and β [19] whereas α2-subunit is found in AII amacrine cells, but rare on ganglion cells in mouse retina [14]. Also the segregation of α2- and α1-subunits in On- and Off-bipolar cell terminals has been found in macaque retinas [15]. These suggest that α1- and α2-subunits are involved in glycinergic amacrine cell synapse to bipolar cells in mammalian retinas. In addition, heteromeric glycine receptors are found in a subgroup of porcine cones [20], suggesting that a glycinergic synapse may occur between interplexiform cell terminals and photoreceptors. Glycine receptors are expressed not only in neurons, but also in Müller cells. Glycine receptors are detected in bullfrog Müller cells [21, 22]. These findings extend our knowledge on the function of glycine receptors to non-excitable cells. The differential distributions of glycine receptor subtypes in retinal neurons may implicate the distinct function of glycinergic synapses in the pre- and post-synapses of glutamatergic and GABAergic pathways. Moreover, recent studies find that α2-subunit is important for rod development in neonatal and early stages of immature mouse retinas. Expression of glycine receptor α2-subunit promotes rod mitosis and increases rod formation in retinal development. The effect is mediated by taurine that is present at a high level in the developing retina [23]. Taurine activates the glycine receptors like a competitive agonist. It is found that the E_{Cl} is high in photoreceptors in the early stages of development. The taurine-mediated excitatory Cl^- response through α2-subunit receptors is critical for rod development.

Pharmacological studies of glycine receptors in retinal neurons reveal that glycine is a potent agonist. Glycine receptors are also sensitive to other agonists, such as taurine, alanine and serine with a sensitivity order: glycine $\gg$ β - alanine $>$ taurine $\gg$ L - alanine $>$ serine[24]. It has been known that the affinities of the agonists, antagonists and receptor desensitization are highly related to the subunit variations. The studies from expressed perch glycine receptor subunits show that the receptor desensitization rate is slower in α1- than α3-formed receptor channels [25]. Picrotoxin has been shown to block mammalian α-homomeric glycine receptors, but it is almost insensitive to α/β heteromeric receptors [5]. Glycine responses in mammalian photoreceptors and bipolar cells are picrotoxin insensitive [19, 20], suggesting the heteromeric glycine receptors in these neurons. However, both picrotoxin sensitive and insensitive glycine responses are observed in amphibian retinas [26, 27]. Possibly mixed homomeric and heteromeric glycine receptors are expressed in low vertebrate retinal neurons. In addition, glycine responses can be separated into a fast and a slow component. About 5–500 nM strychnine blocks a fast glycine response in tiger salamander retinal ganglion cells. The remaining slow glycine response is blocked by 5,7-dichlorokynurenic acid (DCKA) [28]. Possibly, the fast and slow responses indicate that different subtypes of glycine receptors with different kinetics exist in the retinal neurons. These results shine light on pharmacologically differentiating glycine receptors. Recently, Wang and Slaughter [26] find that $GABA_A$ receptor antagonists, bicuculline and SR95531, are also inhibiting glycine receptors in tiger salamander retinal amacrine cells and ganglion cells. Their further studies suggest that these $GABA_A$ antagonists competitively block constructed glycine receptor α1 and α2 homomers in HEK cells.

The apparent affinity of glycine receptors can be modulated by internal Ca^{2+} and Ca^{2+} dependent cascades. Glycine receptors in horizontal cells are found to be modulated by intracellular Ca^{2+} elevation through activation of Ca^{2+} permeable glutamate receptors and voltage-gated Ca^{2+} [29]. Modulations of PKC and PKA on glycine receptors have been found in intact frog retinas [30] and salamander retinal amacrine cells [31]. It has been known that Zn^{2+} can regulate glycine receptor

apparent affinity by a concentration-dependent manner. Extracellular nanomolar Zn^{2+} potentiates an apparent affinity of glycine receptor, but in micromolar concentrations, Zn^{2+} reduces the affinity of glycine receptor in retinal neurons [32, 33].

Glycine transporters in retina

Glycine release in glycinergic synapse is up-taken by glycine transporters in glycinergic neurons and glial cells. Two types of glycine transporters, GlyT1 and GlyT2, are identified in the CNS. GlyT1 is localized in glial cells in most areas of the central brain as a major glial cell transporter, whereas GlyT2 is exclusively expressed in neurons as a neuronal transporter in the central brain. The functions of the transporters are described as uptake of glycine from synaptic and extrasynaptic areas. Interestingly, GlyT1 has been found present in amacrine cells and interplexiform cells in mammalian and chicken retinas, as a neuronal transporter. GlyT1 is also found present in bullfrog Müller cells (retinal glial cells) possibly for uptaking glycine in extrasynaptic areas [21]. However, it is unknown that GlyT2 is expressed in these retina tissues. Recent studies show new evidence that GlyT2 might be present in amphibian retinas. The pharmacological studies indicate that amoxipine, a GlyT2 inhibitor, blocks 60% of glycine uptake in frog retinas [34]. Our studies indicate that blocking of GlyT2 by amoxipine increases local glycine level in the OPL, and the effect is mimicked by a low concentration of glycine in tiger salamander retinas [35]. Accordingly, both types of glycine transporters might be present in retinas with a species-dependent manner. The functions of glycine transporters are considered to rapidly terminate glycinergic synapse in interplexiform cells and amacrine cells.

Glycinergic amacrine cells

About 50% of amacrine cells are glycine-containing neurons. However, a lesser number of glycine-containing amacrine cells are expressed glycine transporters that are located in glycinergic neurons [18, 36]. 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 [22, 37, 38]. However, in amphibians, the receptive field of glycinergic amacrine cells is larger than that of GABAergic amacrine cells [39, 40]. In mammalian retinas, AII amacrine cells are narrow field glycinergic neurons that have been extensively studied on the connection of rod bipolar cells to cone bipolar cells in the IPL [41]. AII amacrine cells are bistratified in the IPL and receive glutamate input from rod bipolar cells in the sublamina b and release glycine to cone bipolar cells in the sublamina a (see Figure 1) [42]. In addition to AII amacrine cells, there are another group of glycinergic amacrine cells, such as 4,6,diamidino-2-phenylindole (DAPI), a nuclear dye, positive amacrine cells in rabbit and grand squirrel retinas [43, 44], which send an inhibitory information to the direction-selective neurons in mammalian retinas.

Function of glycinergic synapse in the inner retinal signaling

Electrophysiological studies indicate that glycine inhibits light responses in ganglion cells. This effect is confined in both pre- and post-synaptic neurons in the IPL. Glycinergic feedback from amacrine cells suppresses glutamate release in the bipolar cells [45]. Glycine also suppresses amacrine cell synapses, leading to an inhibition on GABAergic synapse in the inner retina. The reciprocal inhibition of glycinergic and GABAergic synapses has been described in tiger salamander retinal amacrine cells [46] and ganglion cells [47]. Strychnine is commonly used for isolating glycine response in retinal network. Recent studies indicate that there is a strychnine-insensitive glycine response present in tiger salamander amacrine and ganglion cells [48, 49]. The strychnine-insensitive glycine response regulates dark spontaneous excitatory post-synaptic currents (sEPSCs), as well as light stimulated EPSCs in the ganglion cells.

Studies from mouse retinas show that strychnine blocks surround input in ganglion cells driven by a circle stimulus, indicating that glycine feedback and forward contribute to center-surround responses in ganglion cells [50]. Glycine input is preferential to a certain group of cone bipolar cells that are post-synaptic to AII amacrine cells in rabbit retinas [51]. The effect of glycinergic synapse

onto cone bipolar cells seems to be a major factor on transferring On-bipolar cell signal to Off-bipolar cells in rabbit retina, but to a less extent in mouse retinas [52, 53]. Glycine release in AII amacrine cells are driven by L-type voltage-dependent Ca^{2+} channels in mammalian [54], but both L- and N-type Ca^{2+} are involved in glycinergic release in tiger salamander amacrine cells [55]. Possibly, glycine release from wide-field amacrine cells in the amphibian retina needs both L- and N-type Ca^{2+} channels that are spatially present in the proximal and distal processes of the neurons.

In addition, glycine also activates *N*-methyl-D-aspartate (NMDA) glutamate receptors acting like a co-transmitter binding on the receptors. NMDA glutamate receptors are permeable not only to Na^{+} and K^{+} but also to Ca^{2+}, conducting an excitatory response in neurons. NMDA receptors are expressed in the inner retinal neurons together with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite receptors to conduct glutamate signals. NMDA receptors are not commonly seen in the outer retina in a majority of examined species [56], except in catfish horizontal cells that possess an NMDA receptor response [57]. Glycine prolongs a sustained light response in the inner retinal neurons [58]. This effect is excitatory through NMDA receptors. Glycine acting on NMDA receptors causes a sustained excitatory current in amacrine and ganglion cells and increases an intracellular Ca^{2+} level in these neurons.

Glycinergic interplexiform cells

Interplexiform cells are centrifugal neurons conducting a retinal neural signal from the IPL to the OPL, creating a long-range feedback loop. There are different types of interplexiform cells found in vertebrate retinas, and they are often classified by a putative neurotransmitter content. These transmitters include dopamine, glycine, GABA and somatostatin. In amphibian retinas, there is a relatively large population of interplexiform cells that exhibit glycine-like and somatostatin-like immunoreactivity [36, 59–61]. The dendritic processes of glycinergic interplexiform cells are bistratified in the IPL at both sublamina a and b (see figure). The axon processes of the neurons are ramified to the OPL, where they spread laterally for 100–200 μm. However, it is still unclear how the glycinergic interplexiform cells make contact with neurons in the distal retina [61]. Immunocytochemical studies indicate that glycine receptors are abundant in horizontal cells, bipolar cell dendrites and some photoreceptors [45]. According to electrophysiological studies glycine and strychnine produce strong effects on the outer retinal neurons, suggesting that synaptic glycine is part of the outer retinal signals. Possibly, glycinergic interplexiform cells are the exclusive source for glycinergic synapse in the OPL since neither photoreceptors nor horizontal cells release glycine.

The action of glycine in the distal retina

Cells in the distal retina possess glycine receptors, yet there are no glycinergic neurons that reside in the distal retina. The actions of glycine and its antagonist, strychnine, have been studied in photoreceptors and the second order neurons. Best studied has been the effect of glycine on horizontal cells. Several laboratories have shown that glycine depolarizes horizontal cells, and this is also observed in isolated horizontal cells [29, 62–66] Interestingly, Borges and Wilson [62] found that low concentrations of glycine hyperpolarized horizontal cells while high concentrations depolarized horizontal cells although this observation has not been pursued and no further explanation for the biphasic response. The depolarizing action of glycine is unusual in the nervous system and is due to the relatively high concentration of chloride in horizontal cells [67, 68].

There are far fewer studies on the effects of glycine in photoreceptors and bipolar cells in the OPL. Maple and Wu (1998) have shown that strong glycine responses are present in bipolar cell dendrites, indicating that glycine receptors are abundant in the dendrites, which are presumably activated by interplexiform cells. The same results are also observed in carp fish retinas, a large glycine current is present in bipolar cells when glycine is locally applied on the OPL [69]. Although exogenous glycine suppresses the response of bipolar cells, this may reflect the function of amacrine cells and not interplexiform cells. The analysis of glycinergic interplexiform cell input to bipolar cells is complicated by a large input to the axon terminal of bipolar cells from

glycinergic amacrine cells. Recent studies indicate that bipolar cells possess a gradient chloride concentration [70]. A high chloride concentration is found in the dendrites of On-bipolar cells and a low chloride is found in the axons and terminal regions. GABA potentially excites On-bipolar cells in the OPL and inhibits the cells in the IPL [71]. The possible mechanism for the biphasic effect is due to the presence of different cation chloride cotransporters, Na^+–K^+–$2Cl^-$ cotransporter (NKCC) and K^+–Cl^- cotransporter (KCC). Immunolabeling experiments indicate that a specific antibody for NKCC1, a chloride accumulator, labels the dendrites of On-bipolar cells, whereas On-bipolar cells axon terminals are exclusively labeled by KCC, a chloride extractor, in mammalian retinas [70, 72]. These results are also observed in amphibian retinas. Our recent whole-cell recording on salamander retinal slices shows that local application of 100 μM glycine in the OPL excites On-bipolar cells. The immunolabeling experiments show that NKCC is present in the dendritic areas of the On-bipolar cells co-localized with a specific antibody for G_{oa}, a marker for salamander On-bipolar cells (unpublished data). The effect of glycine in Off-pathway in the outer retina is largely unknown.

There have been very few studies showing glycine response in photoreceptors. Our immunocytochemical studies show that anti-glycine receptor β-subunit is co-localized with anti-synaptic vesicle protein 2 (SV2) on the plasma membrane limited areas, suggesting that glycine receptors might be present in tiger salamander photoreceptor terminals (unpublished data). In addition, whole cell recording in isolated cones from perch retinas shows that the isolated cones are responding to glycine puff, although the response is only observed in 25% of the isolated cones [20]. Antibody labeling experiments also demonstrate that photoreceptor terminals are KCC-negative in mammalian retinas [72]. Whole cell recording on tiger salamander retinal photoreceptors indicates that the E_{Cl} in rods is around –20 mV, and presumably –45 to –35 mV in cones [73, 74], suggesting that the effect of glycine on photoreceptors might be excitatory due to a high E_{Cl}. Because the E_{Cl} is positive to the resting potential in the outer retinal neurons and neuronal regions, the effect of glycinergic synapse in the distal retina is most likely excitatory which is uncommon in the CNS.

Conclusion and prospects

Glycinergic synapses from interplexiform cells and amacrine cells control retinal signals by exciting outer retinal neurons and inhibitory inner retinal neurons in adult animals. These effects are through activating Cl^- permeable receptors. Because of the presence of the cation Cl^- cotransporters that play opposite roles in controlling intracellular Cl^- level. NKCC is predominantly present in the outer retina uptaking Cl^-, whereas KCC is mainly expressed in the inner retina for extracting Cl^-. Consequently, the E_{Cl} is high in the outer retinal neurons and low in the inner retina. That results in a dual function of glycine in retinas. Immunoantibody labeling provides strong evidence that different α-subunits and a β-subunit are present in vertebrate retinas, forming homomeric and heteromeric glycine receptors. Electrophysiological and pharmacological studies indicate that picrotoxin-insensitive glycine response is present in mammalian retinal neurons, indicating the presence of α/β heteromeric receptor channels. In addition to α/β heteromeric receptor channels, amphibian retinas are also expressed as α-hemomeric receptor channels. The diversity of glycine receptor subtypes determines the variability of glycinergic synapse strength in retinal signal transduction and adaptation.

Although the spatial localizations of glycine receptor subtypes are extensively studied on vertebrate retinas, the physiological role of the diversity of glycine receptors is unrevealed. It is important to understand the relationship between subunit combinations and functional properties of the receptors. Discovery and development of new agonists and antagonists for glycine receptors would be an achievable approach to distinguish the special function of glycine receptor in glycinergic synapse in the function of retina.

To further approach the strychnine-insensitive glycine receptor response is also a prospect towards functional study of glycinergic synapses. According to recent studies, strychnine-insensitive glycine receptor responses are present in amphibian amacrine and ganglion cells, controlling glutamate signal in the inner retina. These results open a window for exploring a new type of glycine receptors. Further pharmacological and electrophysiological identifications are necessary for characterizing the properties of strychnine

-insensitive glycine receptor response in retinal neurons, as well as in genetically expressed cell system.

How glycinergic synapses contribute to retinal signal integration would be an interesting topic in the further studies. Glycinergic synapse and GABAergic synapse are present in the same location in the OPL and IPL. GABAergic synapse has been considered to be related to special functions in retina, such as, center-surround and feedback inhibition. However, the contribution of glycinergic synapse in these functions is still ambiguous. The functional distinctions of glycinergic synapse and GABAergic synapse needs to be addressed in future studies.

In summary, glycinergic synapse is important for neural transduction and modulation in the CNS. Although recent progress in understanding the role of glycinergic synapse has been rapid, the receptor pharmacology and tissue specific function still need to be further addressed. Retinal tissues are the best model system for studying neurotransmitter function and mechanism. To understand glycinergic synapse in retina will serve better understanding the function of glycine in the CNS.

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