# 國立清華大學

# 分子醫學研究所

# 博士論文

## 麩胺酸受器在哺乳動物視網膜發育期間功能性表現之探討

## Localization and functional mapping of glutamate receptors in the mammalian retinal development


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# Abstract

Activation of glutamate receptors is critical for the initiation of synaptic plasticity. Although ontogenic expression of ionotropic glutamate receptors has only been recently characterized in the rat retina, glutamate and glutamate receptors have long been suggested to regulate the development of retinal neurons. The function of NMDA glutamate receptors has been associated with visual experience in the developing rat retina; however, the light-dependent regulation of the subunit composition of NMDA glutamate receptors remains controversial. In this study, I first examined the expression patterns of AMPA receptors, which are the most prominent glutamate receptors in the retina, and functionally mapped glutamatergic drive in the developing rabbit retina. Then, I characterized the functional expression of NMDA receptors in the developing rabbit retina, and examined the impact of light deprivation on regulation of the subunit composition of NMDA receptors. The results revealed that both AMPA and NMDA glutamate receptors were expressed and functional during early stages of the developing rabbit retina. This indicates that ionotropic glutamate receptors are functional in the early stage of synapse development and may contribute to the synaptic maturation in the retinal circuits. However, the expression of functional NMDA receptors and their subunit composition in the developing rabbit retina are independent of visual experience. This suggests that visual experience plays a less significant role on developmental plasticity of NMDA receptor function in the retina than that in the cortex.

# 中文摘要

在視網膜的神經網絡發育過程中，麩胺酸及麩胺酸受器扮演著非常重要的角色。然而，目前對於麩胺酸受器在視網膜上的表現研究僅侷限於大鼠品系。此外，神經塑性之啟動也有賴於麩胺酸受器之活化。在發育的大鼠視網膜中，視覺經驗會影響NMDA麩胺酸受器之功能，但在視網膜中NMDA麩胺酸受器之功能性表現是否受光刺激之影響仍不明確。本研究以兔子視網膜為研究對象，觀察在視網膜發育期間，最主要的視網膜麩胺酸受器– AMPA麩胺酸受器在視網膜上的功能性表現及分布情形；此外，也觀察了在發育期間受到光照剝奪的兔子視網膜中，NMDA麩胺酸受器的功能性表現及分布情形是否會受影響。研究結果發現，在發育中兔子視網膜的神經突觸形成前，AMPA及NMDA麩胺酸受器就已經表現並具有功能，這顯示了麩胺酸受器可能參與了視網膜中突觸的早期發育及神經網絡的成熟。此外，在視網膜發育過程中，NMDA麩胺酸受器之功能及表現並不會受到光照剝奪的視覺經驗所影響，這說明了視覺經驗對視網膜中NMDA麩胺酸受器功能性表現的影響並不像在大腦視覺皮質中那麼明顯。

# Abbreviations

| | |
|---|---|
| AGB | agmatine; 1-amino-4-guanidobutane |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |
| AP5 | 2-amino-5-phosphonopentanoate |
| APB | 2-amino-4-phosphonobutyrate |
| ChAT | choline acetyltransferase |
| CNS | central nervous system |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| dLGN | dorsal lateral geniculate nucleus |
| DR | dark-reared |
| DSGC | direction-selective ganglion cell |
| EPSC | excitatory postsynaptic current |
| GABA | γ-aminobutyric acid |
| GCL | ganglion cell layer |
| iGluR | ionotropic glutamate receptor |
| KA | kainate; kainic acid |
| INL | Inner nuclear layer |
| IPL | Inner plexiform layer |
| mGluR | metabotropic glutamate receptor |
| nAChR | nicotinic acetylcholine receptor |
| NBL | neuroblastic layer |
| NR | normal-reared |
| NMDA | *N*-methyl-$_D$-aspartate |
| ONL | Outer nuclear layer |
| OPL | Outer plexiform layer |
| VZ | ventricular zone |

# Contents

# Chapter 1: Introduction

## 1.1 The functional structure of the retina

Retina is an outgrowth of the neural tube ectoderm, thus it has been considered as a part of the central nervous system (CNS) (Dowling, 1987). Similar to the layered structure of the cortex, the retina also comprises several distinct layers. Among them, there are three nuclear layers where retinal neuron's somata are located, including the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL); and two synaptic layers where the neural processes are distributed, including the outer plexiform layer (OPL) and the inner plexiform layer (IPL). Rod and cone photoreceptors together form the ONL, cell bodies of ganglion cells and some displaced amacrine cells can be found in the GCL, and the INL contains all somata of bipolar cells, horizontal cells, amacrine cells, as well as Müller glial cells. In vertebrate eyes, light must travel across all these retinal layers before it stimulates the rod and cone photoreceptors. Visual signals are thus initiated and propagated vertically from the photoreceptors to the bipolar cells via the OPL, where axon terminals of rods and cones make synaptic connections with the dendrites of bipolar cells; then from the bipolar cells to the ganglion cells via the IPL, where axon terminals of bipolar cells synapse on the dendrites of ganglion cells (Wässle, 2004). The axons of ganglion cells then form the optic nerves and carry the visual signals to their target brain regions, including dorsal lateral geniculate nucleus (dLGN) and superior colliculus, located in the thalamus and the midbrain, respectively. In addition, the lateral inhibitions mediated by horizontal cells in the OPL

and amacrine cells in the IPL shape the spatial and temporal receptive field properties of ganglion cells. In the vertebrate retina, the above mentioned vertical visual signaling pathway is mediated by excitatory glutamatergic transmission, and the lateral inhibition is mainly mediated by γ-aminobutyric acid (GABA) and glycine neurotransmitters (Rodieck, 1998).

## 1.2 Glutamate and glutamate receptors in the retina

In the vertebrate retina, photoreceptors, bipolar cells, and ganglion cells are glutamatergic neurons (Massey, 1990), while horizontal cells and some amacrine cells are GABAergic neurons (Yazulla et al., 1986; Chun and Wässle, 1989; Pourcho and Owczarzak, 1989). Pre-synaptically released glutamate diffuses across the synaptic cleft and binds to various glutamate receptors on the post-synaptic dendrites in both the IPL and OPL. In the outer retina, glutamate released from photoreceptors elicits the excitatory activities on the dendrites of bipolar cells and horizontal cells; whereas in the inner retina, glutamate released from bipolar cells depolarize ganglion cells and amacrine cells. Furthermore, the axons of retinal ganglion cells projecting to the dLGN and superior colliculus also use glutamate as their excitatory neurotransmitter.

Glutamate receptors (GluRs) are categorized into two groups: ionotropic GluRs (iGluRs) and metabotropic GluRs (mGluRs) (Seeburg, 1993; Hollmann and Heinemann, 1994; Pin and Duvoisin, 1995; Dingledine et al., 1999). Most iGluRs are heteromers, which form the ligand-gated cation channel by themselves. On the other hand, the G protein-coupled mGluRs are composed of single polypeptides and exert the neuronal

functions via intracellular second messenger cascades (Pin and Duvoisin, 1995). On the basis of their pharmacological and electrophysiological properties, ionotropic GluRs are subdivided into three main types and are named after their preferred agonists, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate (KA; also known as kainic acid), and *N*-methyl-$_D$-aspartate (NMDA) (Monaghan et al., 1989; Hollmann and Heinemann, 1994; Ozawa et al., 1998). Depending on whether being activated preference by NMDA or not, iGluRs can also be described as NMDA and non-NMDA (i.e., AMPA and KA) receptors.

The non-NMDA receptors are tetrameric receptors (Rosenmund et al., 1998). To date, four types of subunits responsible for the AMPA receptors have been identified: GluR1, GluR2, GluR3, and GluR4 (Nakanishi, 1992), and five types of subunits responsible for the kainate receptors are also characterized: GluR5, GluR6, GluR7, KA1 and KA2 (Hollmann and Heinemann, 1994). Previous studies have shown that the subunit composition affects the functional properties of the iGluRs (Seeburg, 1993; Bochet et al., 1994; Angulo et al., 1997). Glutamate binding to a non-NMDA receptor opens its cation channel which is permeable to both $Na^+$ and $K^+$, but not to $Ca^{2+}$ in most cases (Mayer and Westbrook, 1987). Activation of these receptors at the resting membrane potential typically allows $Na^+$ to enter the postsynaptic neuron, thus resulting in membrane depolarization. Both AMPA and kainate are able to evoke fast excitatory postsynaptic currents (EPSCs) mediated by AMPA receptor. However, the EPSCs elicited by AMPA desensitize rapidly, whereas kainate-elicited ones are non-desensitized (Yamada and Tang, 1993).

The NMDA receptors are also tetrameric cation channels composed of subunits from three families: the NR1, NR2, and NR3 (Monyer et al., 1992; Laube et al., 1998; Cull-Candy et al., 2001). The NR1 subunits are combined with two of the four NR2 subunits (NR2A, NR2B, NR2C, and NR2D) or the NR3 (NR3A and NR3B) subunits to form a functional NMDA receptor (Laube et al., 1998). Their distinct physiological and pharmacological properties depend on the subunit composition (Hollmann et al., 1993; Ishii et al., 1993; Zukin and Bennett, 1995). Specifically, the NR1 subunit is required for channel function, whereas the NR2 or NR3 subunit regulates channel properties (Monyer et al., 1992; Flint et al., 1997). Binding of glutamate to the NMDA receptor induces the opening of its non-selective cation channel, which has a higher permeability for $Ca^{2+}$ than $Na^{+}$ or $K^{+}$ ions (MacDermott et al., 1986; Mayer and Westbrook, 1987). However, the NMDA-gated EPSCs in NMDA receptors have slower kinetics than those EPSCs gated by AMPA or KA in non-NMDA receptors (Lester et al., 1990). The NMDA receptors are characterized of having multiple binding sites for glutamate, glycine, $Mg^{2+}$, $Zn^{2+}$, and recognition of polyamine. The glutamate, glycine, and $Mg^{2+}$ binding sites are critical for NMDA receptor activation (Mayer et al., 1984; Nowak et al., 1984; Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). In contrast, the $Zn^{2+}$ and polyamine recognition sites are not necessary for receptor activation, but affect the efficacy of the ion channels (Westbrook and Mayer, 1987; Ransom and Stec, 1988; Williams et al., 1994).

*In situ* hybridization and immunocytochemical studies have shown that various types of AMPA and kainate receptor subunits are expressed in bipolar cells, horizontal cells, amacrine cells and ganglion cells in mammalian retinas (Hamassaki-Britto et al., 1993; Brandstätter et al., 1994; Peng et al., 1995; Zhang et al., 1996; Gründer et al., 2000; Grünert et al., 2002; Hack et al., 2002; Grünert et al., 2003; Kamphuis et al., 2003).

Studies with patch clamp recordings from these neurons also revealed that application of glutamate agonists, AMPA and kainate, can elicit inward currents (Slaughter and Miller, 1983; Massey and Miller, 1988; Cohen and Miller, 1994). Moreover, the agmatine functional assays (see Chapter 2.4 below) in the mammalian retina indicate that incubating the retinal tissue with AMPA or kainate can activate various populations of retinal neurons, including bipolar cells, horizontal cells, amacrine cells, and ganglion cells (Marc, 1999; Marc, 1999; Sun and Kalloniatis, 2006; Acosta et al., 2007).

In the mammalian retina, *in situ* hybridization studies show that NR1 and NR2A-C are expressed on both amacrine cells and ganglion cells in the inner retina (Brandstätter et al., 1994; Hartveit et al., 1994; Watanabe et al., 1994). Furthermore, immunohistochemistry studies revealed that NR1 and NR2A-D proteins are expressed in both the inner retina and the OPL of the retina (Goebel et al., 1998; Fletcher et al., 2000; Gründer et al., 2000; Sucher et al., 2003; Kalloniatis et al., 2004). Electrophysiological studies have confirmed that functional NMDA receptors are expressed on amacrine cells and ganglion cells in the inner retina (Slaughter and Miller, 1983; Massey and Miller, 1988; Cohen and Miller, 1994; Hartveit and Veruki, 1997). The AGB assay (see Chapter 2.3 below) also demonstrate that the functional NMDA receptors are expressed both amacrine cells and ganglion cells *in vitro* (Marc, 1999; Sun et al., 2003; Kalloniatis et al., 2004; Sun and Kalloniatis, 2006; Acosta et al., 2007).

## 1.3 Retinal development

The retina tissue originates from an outgrowth (called optic vesicle) on the lateral side of the neural tube. The optic vesicle differentiates into a dual-layer structure, the outer layer becomes the pigment epithelium cells and the inner layer develops into retinal neuroepithelium (Sernagor et al., 2006). Retinal cytogenesis begins from cell mitosis and takes place at the outer most part of the neuroepithelium. By using $^{3}$H-thymidine labeling technique, ganglion cell is reported the first cell type that could be identified in the retinal neuroblast (Carter-Dawson and LaVail, 1979). Next to the development ganglion cells are amacrine, horizontal, and cone photoreceptor cells. Both bipolar and Müller glial cells are identified in later developmental stages. In the case of rod photoreceptor cells, they differentiate throughout the retinal cytogenesis period (Carter-Dawson and LaVail, 1979; Young, 1985). In the development of embryonic rabbit retina, the GCL and IPL can be well observed in embryonic day 20 (E20), and the ONL, OPL, and INL can be found only after birth. Note that the rabbit's gestation is around 31 days, and the neonatal rabbit's eye opening is around postnatal day 10 (P10). At birth (postnatal day 0, P0), the IPL is well differentiated, and the appearance of conventional synapse is well documented (McArdle et al., 1977). However, the synaptic vesicles in the conventional synapse are not abundant at P0, but increase drastically from P18 to P30. The ribbon synapses (a pre-synaptic structure at the axonal terminals of photoreceptors and bipolar cells) are not observable in the IPL at P7, but appear to increase from P9 on, at around the time of eye opening. In contrast, the ribbon synapses in the OPL are found at P0 and become matured at P11.

Previous immunocytochemical studies have specified that endogenous glutamate are

expressed in the cells of neuroblastic layer from E20, including future photoreceptors and bipolar cells (Pow et al., 1994). This suggests that glutamate not only mediates synaptic transmission in the mature mammalian retina, but also functions in embryonic stages. Therefore, glutamate and their receptors are likely to participate in the regulation of cell survival and synaptogenesis in retinal development.

In the developing retina, there are bursting activities of action potentials which fire spontaneously and rhythmically in immature ganglion cells, and propagate to neighboring ganglion cells in a wave-like manner, termed as retinal waves (Wong, 1999; Firth et al., 2005). Many studies have shown that these bursting activities are associated with the remodeling of synaptic connection in dLGN and superior colliculus before normal visual function begins (Katz and Shatz, 1996; Firth et al., 2005).

In the developing rabbit retina, the retinal wave can be divided into three stages (Syed et al., 2004). The stage I wave appears at around E22, the time before synaptogenesis in the inner retina, and persists for only 24 hours. These fast propagating activities, mainly mediated by gap junctions and adenosine receptors, sweep across the retina frequently without boundaries. The transition from stage I to stage II is at around E23. The stage II wave begins at E24 and continues to P1, while the conventional synapses are also developing in the IPL. At this stage, the spontaneous activities are driven by cholinergic transmission through the nicotinic acetylcholine receptors (nAChR) containing β2 subunit, and retain strong and propagating features like waves in stage I. It takes about 2 days (P2-3) for the transition from stage II to stage III. The emergence of stage III wave begins at P3 to P4 when ribbon synaptogenesis starts in the IPL, and lasts to around P7 to P8. From P2 to P3 period, there are two coordinated transitions in

neurotransmitter system: the nicotinic receptors are replaced by muscarinic receptors, and glutamate system is substituted for ACh system (Zhou and Zhao, 2000). Recently, it has also been observed that the spontaneous bursting activity occurs in progenitor cells in the neuroblast layer, called ventricular zone (VZ), in developing rabbit retinas (Syed et al., 2004). Interestingly, the progenitor cell activities in the VZ propagate laterally in a rate similar with the inner retina waves. Although the physiological function of VZ waves remains unclear, it is believed that the retinal waves occur in both VZ and inner retina may influence the precise wiring of retinal circuitry during development.

## 1.4 Visual experience in retinal development

Previous studies have shown that visual experience is essential for normal development of visual system (Gordon and Stryker, 1996; Kirkwood et al., 1996). In the developing visual cortex, light deprivation not only alters the formation of direction selectivity in the ferret (Li et al., 2006), but also leads to the loss of NMDA receptor function in the cat (Fox et al., 1991). In the dLGN, naturalistic visual stimuli presented through unopened eyelids activate neurons significantly, and dark-rearing prior to natural eye-opening also alters the ON-OFF segregation in the ferret (Akerman et al., 2002). Therefore, the light stimulation before eye-opening is important in synaptic plasticity and circuit refinement during visual system development in the higher centers. However, it is less certain if the retina itself is also susceptible to visual deprivation during development (Daw, 2005). In the rodent retinas, it has been shown that dark rearing reduces the light-evoked responsiveness of the inner retinal neurons (Tian and Copenhagen, 2001; Giovannelli et al., 2008). Light deprivation also reduces the maturational loss of the

ON-OFF responsive ganglion cells and the pruning of dendrites (Tian and Copenhagen, 2003; Xu and Tian, 2007). Furthermore, an electroretinogram study has shown that the light response of the inner retina in the dark-reared mice is significantly suppressed (Xu and Tian, 2007). In the developing rabbit retina, light deprivation has been shown to delay morphological differentiation of bipolar cells (Wu and Chiao, 2007), but has no significant effect on the maturation of ON-OFF direction selective ganglion cells (Chan and Chiao, 2008). Similar results have also been confirmed in the mouse retina (Elstrott et al., 2008; Chen et al., 2009).

It is known that NMDA receptors play significant roles in experience-dependent plasticity in the developing visual thalamus and cortex (Bear and Colman, 1990; Carmignoto and Vicini, 1992; Kirkwood et al., 1996; Catalano et al., 1997; Daw et al., 1999; Quinlan et al., 1999), but very few studies examine the role of NMDA receptors in plastic events in the developing retina. Using Western blots, Xue and Cooper (2001) showed that the NMDA receptor subunit expression is differentially modulated by visual experience in the developing rat retina. However, Guenther et al. (2004) found that the subunit composition of NMDA receptors is not affected in dark-reared rats, albeit there is altered NMDA receptor function after light deprivation.

## 1.5 Motivation and specific aims

Glutamate has been suggested to regulate the development of retinal neurons, but ontogenic expression of ionotropic glutamate receptors has only recently been characterized in the rat retina. Previous studies have shown that differences in the subunit

composition determine the distinct functional properties of the AMPA and NMDA receptors. Thus, glutamate signaling during development may alter significantly, depending on the subunit composition of these iGluRs. To determine the importance of AMPA and NMDA receptor heterogeneity in the mediation of retinal circuit maturation, it is important to characterize iGluR subunit expression at the various different stages of development.

Although subunit localization studies provide spatial and temporal patterns of iGluRs in the developing retina, the functionality of AMPA and NMDA receptors cannot be inferred directly, because whether an iGluR is functional may also depend on heteromeric subunit assembly (Monaghan et al., 1989; Seeburg, 1993; Hartveit et al., 1994; Gründer et al., 2000; Sucher et al., 2003). Agmatine (1-amino-4-guanidobutane; AGB), a cationic guanidinium analogue that permeates open cationic channels, was originally shown to be a useful marker of measuring cation fluxes in frog sympathetic ganglion cells (Yoshikami, 1981). In recent years, it has been established as a reliable tool for the examination of functional iGluRs in the mammalian retina (Marc, 1999; Marc, 1999; Kalloniatis et al., 2002; Sun et al., 2003; Kalloniatis et al., 2004; Sun and Kalloniatis, 2006). The AGB permeation technique provides high spatial resolution of the activated iGluRs in the adult retina (Marc et al., 2005) and has been applied to the study of iGluR functionality in the developing mouse retina (Acosta et al., 2007; Acosta et al., 2008). The rabbit is one of the key species for studies of mammalian retinal organization, and the morphology and physiology of its retinal neurons are particularly well known. Examining functional expression of these iGluR subunits in the developing rabbit is crucial to understanding the significance of the ontogenesis of glutamate receptor in the mammalian retina.

Furthermore, activation of NMDA glutamate receptors is critical for the initiation of synaptic plasticity. In the developing rat retina, the function of NMDA receptors has been associated with visual experience, though the light-dependent regulation of the subunit composition of NMDA receptors is controversial. Thus, it is important to elucidate the role of visual experience on functional expression of NMDA receptors in the developing rabbit retina.

The primary goals of this study are: (1) to characterize the expression of the AMPA receptor subunits (GluR1, GluR2/3 and GluR4) and the NMDA receptor subunits (NR1 and NR2A/B) at various development stages in the rabbit retina, (2) to functionally map the AMPA and NMDA receptors using the AGB assay at various development stages in the rabbit retina, and (3) to compare the functional expression of NMDA receptor between normal- and dark-reared rabbits retina at various development stages in the rabbit retina.

# Chapter 2: Materials and Methods

## 2.1 Retina preparation

Retinas from New Zealand White rabbits at different developmental stages between embryonic day (E) 21 and the adult stages were used. The day of birth was designated as postnatal day (P) 0. Normal-reared neonates (NR) were bred and raised in a normal light/dark cycle, and were purchased from a local breeder. Dark-reared neonates (DR) were obtained by transferring pregnant rabbits to a complete dark room before parturition and the pups were kept with mothers in the darkness until experimentation.

Before dissection, all rabbits were deeply anesthetized using a 1:1 mixture of ketamine (150 mg/kg) and xylazine (30 mg/kg). After enucleation and hemisection, the vitreous humor was removed and the retina was carefully detached from the retinal pigment epithelium. The animal was then euthanized with an overdose of ketamine. All procedures were approved by the institutional animal care and use committee and were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The isolated retina was cut into pieces and then fixed in 4% paraformaldehyde and 0.01% glutaraldehyde in phosphate buffer (PB; 0.1M, pH 7.4) for 20-30 minutes at room temperature. After rinsing, which was followed by cryoprotection in 30% (wt/vol) sucrose in 0.1M PB, the retinas were sectioned vertically in 12 μm slices with a cryostat. For the AGB assay, the retina pieces were incubated with agmatine (see below) before fixation and cryosectioning. It is known that retinal neurons develop at

different rates at different eccentricities (Robinson, 1990). To ensure similar sampling locations, an area between central and mid-peripheral region on the ventral side of the retina was chosen (excluding the far temporal and nasal sides, and typically has the eccentricity of 2-4 mm below the visual streak).

## 2.2 Immunohistochemistry

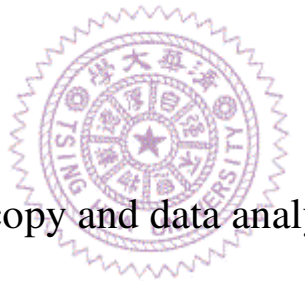
In order to reduce background staining, any non-specific binding sites within the retinal slices were blocked by incubation with 4% normal donkey serum (Jackson ImmunoResearch Laboratory, West Grove, PA) in 0.1M PB and 0.1% Triton X-100 for 1 hour at room temperature. After blocking, the samples were incubated with the primary antibodies for 48 hours at 4°C. The specificity of the antiserums against to the glutamate receptor subunits was tested by Western blots and showed bands of ~110 kDa molecular weight for GluR1, GluR2/3, and GluR4, ~116 kDa for NR1, and ~180 kDa for NR2A/B on Western blot analysis against the rabbit brain tissue (Fig. 1 and Fig. 2). To confirm the retinal layers and to label the AII amacrine cells and some ganglion cells in the inner retina, the retinal slices were co-incubated with calretinin. In a separate experiment, the retinal slices were also co-incubated with choline acetyltransferase (ChAT) to label cholinergic amacrine cells. After rinsing, secondary antibodies conjugated to Cy5 and FITC (1:100; Jackson ImmunoResearch Laboratory) were applied overnight at 4°C to visualize the glutamate receptor subunits and calretinin respectively. The specificity of the immunostaining was evaluated by omitting the primary antibody during the incubation steps (Fig. 3). The retinal slices were finally mounted in the mounting medium containing 90% glycerol and 5% propylgallate for confocal imaging. To ensure a direct comparison

of the intensity of staining for the different glutamate receptor subunits, we performed the immunohistochemistry at the same time on all samples, and all subsequent image acquisitions were taken using the same confocal settings. The problem of signal saturation in the immunocytochemistry applications was avoided by using lower dilution factors of primary antibody and carefully adjusting the confocal setting when taking images. Average 3-6 retinas were used for each studied stage. All primary polyclone antibodies used in this study are summarized in Table 1.

## 2.3 AGB functional assay

For the agmatine functional mapping experiments, retinas were first incubated in a modified physiological buffer (Edwards medium, Edwards et al., 1989). The buffer was bubbled with 95%$O_2$/5%$CO_2$ for an hour before adding 25 mM AGB and low or high concentrations of glutamate agonists (2 μM AMPA, 20 μM AMPA, 100 μM NMDA and 500 μM NMDA) for the activation studies. Each concentrations of AMPA or NMDA have been shown to activate different retinal neurons above the basal level in mice (Sun and Kalloniatis, 2006). All incubations were performed for 6 minutes at 37°C. Retinal pieces were then fixed and cryosectioned as described above. Rabbit polyclonal AGB antibody was used to visualize the activated cells. The rabbit antiserum was selective for AGB glutaraldehyde linked to bovine serum albumin, as determined by dot immunoassays (see Chemicon data sheet). The detailed immunohistochemical procedure used for the AGB activation assay has been described previously (Marc, 1999; Marc, 1999; Kalloniatis et al., 2004; Sun and Kalloniatis, 2006; Acosta et al., 2007; Chang and Chiao, 2008). There was no cross-reactivity shown against arginine, glutamate and other amino acids

(manufacturer's technical information). When the adult rabbit retinas were probed with anti-AGB antibody, there was no endogenous AGB signal, and AGB immunoreactivity was only detected in the retina when the incubation medium contained AGB (Marc, 1999). To ensure selective activation of the AMPA receptors, 100 μM of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; AMPA/ KA antagonist) or 50 μM of AP5 (2-amino-5-phosphonopentanoate; NMDA antagonist) was co-incubated with AGB and 20 μM AMPA or 500 μM NMDA in a control experiment. The retinal slices were then mounted in the mounting medium for confocal imaging. All reagents used in this AGB activation assay including AGB, NMDA, and AP5 were obtained from Sigma-Aldrich Corp. (St. Louis, MO). The number of retinas used at each developmental stage was three or more.



## 2.4 Confocal microscopy and data analysis

All images were acquired using a confocal scanning module (LSM 510, Zeiss, Germany) mounted on a fluorescence microscope (Axioskop 2 Plus Mot, Zeiss). A 40X objective lens (Plan-Neofluar, 0.75 NA, Zeiss) was used and a single optic slice less than 1.5 μm was obtained. Phase contrast images were acquired after confocal scanning to identify the retinal layers.

To quantify the strengths of the immunoreactivity of glutamate receptor subunits (GluR1, GluR2/3, GluR4, NR1, and NR2A/B) at different developmental stages, the intensities of fluorescent signals above a background noise level in the region of interest (e.g., in the IPL) were computed. The differences of glutamate receptor subunits

immunoreactivity across all developmental stages (n = 4-6) were assessed using one-way ANOVA analysis. The difference of NR1 or NR2A/B fluorescence intensities between NR and DR rabbits at each developmental stage (n = 4-6) was assessed using Student's two-tailed *t* test. Differences were considered statistically significant at the $p < 0.05$ level.

# Chapter 3: Localization and functional mapping of AMPA receptor subunits in the developing rabbit retina

The AMPA receptor subunits GluR1, GluR2/3 and GluR4 were present in the rabbit retina from E26 but showed different spatial distributions and temporal expression patterns throughout the postnatal stages analyzed. Most AMPA receptor subunits were found in both the IPL and OPL. Based on the results of the AGB activation assay, the AMPA receptors identified in the immunohistochemical study were confirmed to be functional from E26 in the rabbit retina.

## 3.1 Expression of AMPA receptor subunits in the adult rabbit retina



All AMPA receptor subunits (GluR1, GluR2/3 and GluR4) have been previously shown to express in both the IPL and OPL in the adult rabbit retina (Ghosh et al., 2001; Li et al., 2002; Deng et al., 2006; Jeong et al., 2006; Pan and Massey, 2007). To confirm their expression patterns in the retinal slices, antibodies against the AMPA subunits were used to characterize their localizations in the retinal layers. Figure 4 shows confocal images of double-labeling of the AMPA subunits GluR1, GluR2/3 or GluR4 (red) and calretinin (green). Calretinin is known to label AII amacrine cells and some ganglion cells in the rabbit retina (Völgyi et al., 1997; Jeon and Jeon, 1998; Massey and Mills, 1999). The immunoreactivity for GluR1 was abundant in the IPL and weak in the OPL. In

contrast, the expression of GluR2/3 was apparent in both IPL and OPL and also within some neurons in the GCL. Similarly, GluR4 was strongly expressed in both IPL and OPL. This immunolabeling pattern of the GluRs in the retinal slices of adult rabbit is consistent with previous results using the same antibodies (Jeong et al., 2006).

## 3.2 Expression of AMPA receptor subunits in the developing rabbit retina

Confocal images of GluR1 subunit expression in the rabbit retina at different developmental stages are shown in Figure 5. The GluR1 immunoreactivity was absent at E21 (Fig. 5A), but was detectable in the IPL and the GCL at E26 (Fig. 5B). After birth, GluR1 expression was found to be prominent in the IPL at P0 (Fig. 5C) and expression of GluR1 in the OPL was first detectable at P2 (Fig. 5D). The immunoreactivity of GluR1 was most abundant in both IPL and OPL at P4-P6 (Fig. 5E and 5F). However, GluR1 expression became slightly reduced at P8 (Fig. 5G) and had reached the lower adult level at P10 (Fig. 5H). The strength of GluR1 immunoreactivity was also quantified at all developmental stages (Fig. 6), and the trend was generally consistent with the one shown in Figure 5. This GluR1 expression pattern in the developing rabbit retina is dramatically different from that in the developing rat retina, where GluR1 immunoreactivity was never observed in the OPL in Brown Norway rats (Gründer et al., 2000), although GluR1 expression could be detected at P14 in White Wistar rats (Hack et al., 2002). In the INL, unlike the rat retina, there was no co-localization of GluR1 expression and AII amacrine cells (calretinin immunoreactive neurons) at any of the postnatal stages (data not shown),

which is consistent with previous finding that GluR1 is not present at the synapses between rod bipolar cells and AII amacrine cells (Li et al., 2002).

Confocal images of GluR2/3 subunit expression in the rabbit retina at different developmental stages are shown in Figure 7. The immunoreactivity of GluR2/3 could be barely detected in some neurons of the inner retina at E21 (Fig. 7A) and labeling became moderate in the GCL at E26 (Fig. 7B). GluR2/3 was steadily labeled in the IPL and weakly labeled in the OPL at P0 (Fig. 7C) and the immunoreactivity was consistently labeled in both IPL and OPL at P2 (Fig. 7D). Note that the GluR2/3 immunoreactivity was concentrated in two bands in the IPL at P2, in which the lower band coincides with the processes of the calretinin labeled cells (data not shown). From P4 to P8, GluR2/3 was strongly expressed in both IPL and OPL (Fig. 7E, 7F and 7G). However, GluR2/3 expression was reduced and had reached the adult level at P10 (Fig. 7H). The GluR2/3 immunoreactivity strength was also quantified at all developmental stages (Fig. 8), and it was similar to the one shown in Figure 7. The expression pattern of GluR2/3 in the developing rabbit retina is similar to that in the developing rat retina, though the immunostaining in both the IPL and OPL was detected earlier in rabbits (Gründer et al., 2000; Johansson et al., 2000; Hack et al., 2002). The fact that the immunoreactivity of GluR2/3 shows two bands in the IPL throughout the developmental stages implies that GluR2/3 is expressed early in both cholinergic amacrine cells (Firth et al., 2003; Jeong et al., 2006) and AII amacrine cells (Ghosh et al., 2001; Li et al., 2002), which is similar to the adult rabbit retina.

Confocal images of GluR4 subunit expression in the rabbit retina at different developmental stages are shown in Figure 9. Similar to the GluR2/3 immunoreactivity

found during the embryonic stages, GluR4 expression was barely detected at E21 (Fig. 9A) and moderately labeled in both the IPL and GCL at E26 (Fig. 9B). GluR4 immunoreactivity was also weakly detected in both the IPL and OPL at P0 (Fig. 9C). In contrast to the GluR2/3 immunoreactivity, GluR4 expression in the OPL was strong at P2-P4, while its immunostaining in the IPL remained moderate (Fig. 9D and 9E). The immunoreactivity of GluR4 in the IPL did not reach its highest level until P6-P8, indicating a late expression pattern compared to other AMPA receptor subunits (Fig. 9F and 9G). The expression of GluR4 in both the IPL and OPL reached the adult level at P10 (Fig. 9H). The strength of GluR4 immunoreactivity was also determined at all developmental stages (Fig. 10), and the trend matched to the one shown in Figure 9. Overall, the expression pattern of GluR4 in the developing rabbit retina is similar to that of GluR2/3 and shows equivalent immunoreactivity in both the IPL and OPL in the developing rat retina (Gründer et al., 2000; Hack et al., 2002). Analogous to GluR2/3, there were two bands of GluR4 immunoreactivity discernible in the IPL beginning on P2, which corresponds to GluR4 expression in cholinergic amacrine cells (Firth et al., 2003) and AII amacrine cells (Li et al., 2002) in the adult rabbit retina and this indicates that this specific pattern of expression is already present at early developmental stages.

## 3.3 Functional mapping of AMPA receptors in the developing rabbit retina

Functional AMPA receptors were probed by the AGB assay. Similar to the AGB permeation patterns in the adult rabbit retina (Marc, 1999), we found that no endogenous AGB signal was observed when the retina was incubated in Edwards medium without AGB (Fig. 11A) and there was a basal AGB permeation after incubating the retina with 25 mM AGB in the absence of glutamate receptor agonists (Fig. 11B). AGB permeation in the presence of 2 μM AMPA significantly increased in the neurons of the outer retina (horizontal cells, bipolar cells and cone photoreceptors), as well as in some amacrine cells and ganglion cells of the inner retina (Fig. 11C). With a high concentration of AMPA (20 μM), AGB permeation further increased in those cells in both inner and outer retinas (Fig. 11D). This shows that the AGB signal activated by the AMPA in the adult rabbit retina is dose dependent. It was noted that the AGB immunoreactivity in the presence of AMPA showed two distinct bands in the IPL (Fig. 11C and 11D), which colocalized with the ChAT bands (Fig. 12). This indicates that the AMPA receptors are dominant on the cholinergic amacrine cells of the rabbit retina. To examine if the AGB signal activated by AMPA is also agonist specific, we co-treated 20 μM AMPA with 100 μM CNQX (AMPA/kainate receptor antagonist) and found that AGB signals were drastically reduced to the level of basal AGB permeation (Fig. 11E). However, when we co-treated 20 μM AMPA with 50 μM AP5 (NMDA receptor antagonist), the AGB permeation was similar to that with 20 μM AMPA alone (Fig. 11F).

To characterize basal AGB permeation in the developing rabbit retina, Figure 13 shows AGB immunoreactivity at the various postnatal stages with or without AGB

treatment. Throughout all the developmental stages analyzed, there was no endogenous AGB signal (Fig. 13A-13D). This result is identical to that found in the adult retina (Fig. 11A). When the retina was incubating with 25 mM AGB, a basal level of AGB permeation was seen in the absence of glutamate receptor agonists in both embryonic and postnatal stages (Fig. 13E-13H). This basal AGB permeation pattern in the developing retina is similar to the one observed in the adult retina (Fig. 11B). To ensure the AGB signal activated by AMPA is also agonist specific in the developing retina, we co-treated 20 μM AMPA with 100 μM CNQX and found that AGB signals were drastically reduced to the basal level, but it did not completely abolish the basal AGB uptake (Fig. 14)

The functional mapping experiment revealed that 2 μM AMPA could consistently activate AMPA receptors in amacrine cells and ganglion cells as early as E26, though a few AGB signals could be detected in some neurons at E21 (Fig. 15A and 15B). This indicates that the expression of AMPA receptor subunits found at E26 in this study is indeed functional and this precedes synapse formation in the IPL. It should be noted that the AGB immunoreactivity was evident in a few immature neurons in the outermost part of neuroblastic layer (NBL) when activated with 2 μM AMPA at E26, but these AGB signals reduced significantly after birth. The AGB permeation pattern showed two conspicuous bands in the IPL at P0 (Fig. 15C). The AGB signals also appeared in some amacrine cells and ganglion cells at the same age (see calretinin immunoreactive cells in Fig. 16). The two-bands of AGB signal persisted throughout all postnatal stage (Fig. 15C-15H). By co-labeling ChAT with 2 μM AMPA activated AGB signals in all postnatal stages, we confirmed that functional AMPA receptors were mainly localized in the cholinergic amacrine cells during development (Fig. 12). The AGB immunoreactivity in the OPL appeared to express predominately in horizontal cells from P0 stage onwards

(Fig. 15C-15H), though some bipolar cells were labeled at a later stage (e.g., P10). The overall AGB permeation pattern gradually increased its strength after birth and had reached the adult level at P10 (Fig. 15C-15H).

At the higher concentration, 20 μM AMPA could activate a few more cells in the NBL and in the inner retina at E21 (Fig. 17A). However, more prominent AGB signals and the two band pattern in the IPL were reliably detected at E26 (Fig. 17B). This dose dependent AMPA activation indicates that glutamate receptors with different compositions of AMPA subunits may have different sensitivities towards AGB permeation. After birth, AGB immunoreactivity was localized in the horizontal cells of the OPL, in the two bands of the IPL, as well as in some amacrine cells and ganglion cells after treatment with 20 μM AMPA (Fig. 17C, also see calretinin immunoreactive cells in Fig. 18). Similar to the low concentration of AMPA treatment, the AGB signals increased in both IPL and OPL from the P0 stage onwards (Fig. 17C-17H). At P10, the AGB immunoreactivity pattern had reached the adult level (Fig. 17H), as compared to Fig. 11D.

# Chapter 4: The expression of functional NMDA receptors is independent of visual experience in the developing rabbit retina

The NMDA receptor subunits NR1 and NR2A/B were present in the rabbit retina from the late embryonic stages onwards, but showed different spatial distributions and temporal expression patterns throughout the postnatal stages analyzed. After birth, light deprivation had no significant effect on the distribution and expression patterns of NR1 and NR2A/B. Using the AGB activation assay, functional NMDA receptors were found as early as E21 in the developing rabbit retina, and their postnatal development did not appear to depend upon visual experience.

## 4.1 Expression of NR1 subunits in the normal- and dark-reared rabbit retinas

Confocal images of NR1 subunit expression in the normal-reared rabbit retina at different developmental stages are shown in Figure 19. The NR1 immunoreactivity was barely detectable at E21 (data not shown) and was only sparse in the inner retina at E26 (Fig. 19A). After birth, NR1 expression was prominent in the IPL and was also detectable in the OPL at P0 (Fig. 19B). The immunoreactivity of NR1 in the OPL was steadily maintained throughout all postnatal stages, and the expression in the IPL gradually increased and reached a maximum at P10 (Fig. 19C-19H). This NR1 expression pattern in the developing rabbit retina is similar to that found in the developing rat retina (Gründer et al., 2000), where NR1 immunoreactivity was present from E20/21 on and could be

observed at all developmental stages, although only a splice variant of the NR1 subunit (NR1C2') was found in the OPL of adult rat retinas by a different group (Fletcher et al., 2000).

The confocal images of NR1 subunit expression in the dark-reared rabbit retina at different postnatal stages are shown in Figure 20. Note that the result for E26 (Fig. 20A) is identical to the one shown in Figure 19A. Compared with the spatial distribution and temporal expression pattern of NR1 subunit observed in the normal-reared rabbits, the NR1 immunoreactivity showed a very similar trend in the dark-reared ones. This lack of influence of visual deprivation on NR1 subunit expression, however, is distinctly different from the findings in the rat retina, where dark-rearing for one week caused an increase in the relative amount of NR1 at P12 by Western blot analysis (Xue and Cooper, 2001).

To quantitatively compare the strengths of NR1 subunit expression between normal- and dark-reared rabbit retinas at each developmental stage, the average immunofluorescence intensities were computed for both the OPL and IPL (Fig. 21). Using the Student two-tailed *t* test, we found that there were no significant differences in NR1 immunoreactivity between normal- and dark-reared rabbits at all postnatal stages analyzed. This result is consistent with an earlier study in the rat retina that light dependent regulation of physiological properties of NMDA receptor is not mediated by changes in NR subunit composition (Guenther et al., 2004).

## 4.2 Expression of NR2A/B subunits in the normal- and dark-reared rabbit retinas

Confocal images of NR2A/B subunit expression in the rabbit retina at different developmental stages are shown in Figure 22. The immunoreactivity of NR2A/B was just detectable in the inner retina as early as E21 (data not shown) and at E26 (Fig. 22A). At the P0 stage, NR2A/B labeling was clearly observed in the inner part of the INL and the GCL (Fig. 22B). From the P2 stage on, more neurons in the INL and GCL were labeled by NR2A/B and their immunoreactivity steadily increased (Fig. 22C-22G). The expression of NR2A/B in the OPL and IPL was more prominent from P4 on (Fig. 22D-22G), although there was moderate NR2A/B labeling at P0 and P2 in some cases. Interestingly, in the mature retina (P25), the immunoreactivity of NR2A/B in all regions showed a slight decrease (Fig. 22H). The expression results for NR2A/B are also similar to those reported for the developing rat retina, where NR2A/B immunoreactivity could be detected from E20/21 on (Gründer et al., 2000). However, in a different study, the NR2A labeling was found to be absent in the OPL in the adult rabbit retina, and punctate labeling was not observed before P9 in the IPL in the rat retina (Hartveit et al., 1994).

The confocal images of NR2A/B subunit expression in the dark-reared rabbit retina at different postnatal stages are shown in Figure 23. Compared with the spatial distribution and temporal expression pattern of NR2A/B observed in the normal-reared rabbits, the NR2A/B immunoreactivity also shows a very similar trend in the dark-reared ones. This visual experience independence of NR2A/B expression in the developing rabbit retina is different from what was found in the developing rat retina, where dark-rearing for one week caused a significant decrease in the level of NR2A and no

change in the level of NR2B expression at P12 by Western blot analysis (Xue and Cooper, 2001).

To quantitatively compare the strengths of NR2A/B expression between normal- and dark-reared rabbit retinas at each developmental stage, the average immunofluorescence intensities were computed separately for the OPL, INL, IPL, and GCL (Fig. 24). Using Student two-tailed *t* test, we found that there were no significant differences in NR2A/B immunoreactivity between the normal- and dark-reared rabbits at all postnatal stages analyzed. This result further supports the observation that both NR1 and NR2A/B expression in the developing rabbit retina is not visual experience dependent.

## 4.3 Functional mapping of NMDA receptors in the normal- and dark-reared rabbit retinas

The functional NMDA receptors were probed by the AGB assay. Similar to the AGB permeation patterns in the adult rabbit retina (Marc, 1999), when the retina was incubated in Edwards medium without AGB, no endogenous AGB signal was detected in the developing rabbit retina (Fig. 11). However, there was a basal AGB permeation after incubation with 25 mM AGB in the absence of glutamate receptor agonists in embryonic retinas (Fig. 25B and 25F) and at various postnatal stages (Fig. 26, left column). This basal AGB permeation pattern in the postnatal retinas was also similar to the one observed in the dark-reared rabbit retina (data not shown). AGB permeation in the presence of 100 μM NMDA significantly increased in a subset of inner retina neurons in

both E21 and E26 retinas (Fig. 25C and 25G). With a high concentration of NMDA (500 μM), AGB permeation further increased in the cells from the inner retina (Fig. 25D and 25H). This shows that functional NMDA receptors can be detected as early as E21 in the developing rabbit retina, and that AGB signal activation by NMDA is dose dependent. This observation also indicates that the NMDA receptor subunits detected by immunohistochemistry analysis at E21 were indeed functional and this preceded synapse formation in the IPL. To examine if the AGB signal activated by NMDA is agonist specific, we co-treated with 500 μM NMDA and 50 μM AP5 (a NMDA receptor antagonist) using embryonic and postnatal retinas, and found that the AGB signal was drastically reduced to the basal level of AGB permeation (Fig. 27). In contrast, when we co-treated with 500 μM NMDA and 100 μM CNQX (AMPA/kainate receptor antagonist), the AGB permeation was similar to that with 500 μM NMDA alone.

The functional mapping experiment revealed that 100 μM NMDA was able to consistently activate NMDA receptors in the postnatal retinas of the normal-reared rabbits (Fig. 26, the second column from the left). At the higher concentration, 500 μM NMDA could activate more cells in the inner retina (Fig. 26, the third column from the left). The AGB immunoreactivity was observed as two bands in the IPL, as well as in a subset of amacrine cells and ganglion cells from P0 to P8 when the retinas were activated by 100 and 500 μM NMDA (Fig. 26A-26E). Interestingly, the AGB permeation pattern in the IPL showed three bands from P10 to P25 (Fig. 26F-26G). By co-labeling ChAT with 100 μM NMDA activated AGB signals in all postnatal stages, we found that the functional NMDA receptors were not localized exactly with the cholinergic amacrine cells during development (Fig. 28); however, the AGB immunoreactivity in the inner retina did show partial co-localization with calretinin immunoreactive cells at all developmental stages

examined (Fig. 29). This AGB permeation pattern is similar to the one observed in the developing mouse retina, though their earliest AGB signal was detected in amacrine cells at the P1 stage (Acosta et al., 2007).

In the dark-reared rabbits, it appeared that both 100 and 500 μM NMDA activated a similar trend of AGB permeability at all postnatal stages (Fig. 26, right column; data not shown for 100 μM NMDA activation). This result indicates that functional NMDA receptor expression in the developing rabbit retina is not affected by light deprivation after birth. This is somewhat different from the observation in the developing rat retina where the modulation of NMDA receptor function is light dependent, despite the fact that subunit composition is not affected by the visual experience (Guenther et al., 2004).

# Chapter 5: Discussion

## 5.1 Expression of AMPA receptor subunits in the developing retina

It has been shown that glutamate is present in the early stages of mammalian retinal development (Redburn et al., 1992; Pow et al., 1994; Redburn and Rowe-Rendleman, 1996; Fletcher and Kalloniatis, 1997) and that glutamate signaling plays a crucial role in establishing specific circuits during retinal development (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995). Earlier studies using *in situ* hybridization reveal the expression pattern of AMPA receptor subunits in the mammalian retina (Hughes et al., 1992; Müller et al., 1992; Hamassaki-Britto et al., 1993). Over the past decade, the localization of AMPA glutamate receptors in adult retinas have been intensively studied by immunohistochemistry (Peng et al., 1995; Qin and Pourcho, 1996; Hof et al., 1998; Hack et al., 1999; Morigiwa and Vardi, 1999; Qin and Pourcho, 1999; Qin and Pourcho, 1999; Hack et al., 2001; Haverkamp et al., 2001; Grünert et al., 2002). In contrast to the wealth of data available for adult retinas, only a few studies have been carried out to characterize the distribution of AMPA receptors in the developing retina and all of them have used rat retinas (Gründer et al., 2000; Johansson et al., 2000; Hack et al., 2002), except one study in the developing chick retina (Silveira dos Santos Bredariol and Hamassaki-Britto, 2001). Here we reported the first evidence showing the expression of AMPA receptor subunits in the developing rabbit retina.

### 5.1.1 AMPA receptor subunits in the outer retina

While the GluR1 subunit is predominately expressed in the dendrites of the OFF-cone bipolar cells in cat and rodent retina (Qin and Pourcho, 1999; Brandstätter and Hack, 2001), previous studies have indicated that GluR1 expresses weakly in the OPL of the adult rabbit retina (Jeong et al., 2006) and this which is consistent with our immunostaining results (Fig. 4B). Interestingly, the immunoreactivity of GluR1 in the OPL was moderate at P2 (Fig. 5D) and reached its highest level at P6 (Fig. 5F). This transient increase in GluR1 expression in the OPL during the first week of the postnatal stage implies that synapse formation between photoreceptors, the horizontal cells and the OFF cone bipolar cells may require substantial functional AMPA receptors whose subunit composition involves GluR1. Although the expression of GluR2/3 and GluR4 was stronger than that of GluR1 in the OPL of adult retinas (Fig. 4F and 4J) (Jeong et al., 2006), the phenomenon of a transient increase in GluR2/3 and GluR4 expression in the OPL from P2 to P8 (Figs. 7 and 9) is similar to the expression pattern for GluR1 in the developing retina. It has been known that the beginning of ribbon synapse formation in the outer retina occurs at P2-P6 in the developing rabbit retina (McArdle et al., 1977). Our finding of a transient increase in the AMPA subunits in the OPL during the first postnatal week supports the hypothesis that AMPA glutamate receptors may be involved in the synaptogenesis between photoreceptors, horizontal cells and bipolar cells during early retinal development. Taken together, this evidence indicates that the AMPA receptors and glutamate neurotransmission play an important role in the maturation of outer retina circuitry during development (Brandstätter and Hack, 2001; Reese et al., 2005).

### 5.1.2 AMPA receptor subunits in the inner retina

AMPA receptor subunits have been shown to express widely in different types of ganglion cells and amacrine cells in mouse retinas (Jakobs et al., 2007). In adult rabbit retinas, previous studies have indicated that GluR2/3 and GluR4 mainly express in AII amacrine cells (Ghosh et al., 2001; Li et al., 2002), cholinergic amacrine cells (Firth et al., 2003) and directive selective ganglion cells (Jeong et al., 2006). The immunoreactivity of GluR2/3 and GluR4 in the developing rabbit retina also showed corresponding bands in the IPL (Figs. 7 and 9). In a similar way to the transient increase of AMPA subunit expression in the developing OPL, all AMPA subunits showed increased immunoreactivity during the first week of the postnatal stage and reached an adult level after P10. Glutamate transmission has been indicated to be involved in dendritic remodeling of ganglion cells during development (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995; Wong et al., 2000; Wong and Wong, 2001). Our evidence that AMPA subunit expression increases in the IPL during the early postnatal days supports the idea that glutamate transmission via AMPA receptors is required to refine the inner retina circuitry (Wong and Ghosh, 2002).

## 5.2 Functionality of AMPA receptors in retinal development

Functional mapping of glutamate receptors by observing AGB entry secondary to agonist activation has been widely used to study the functionality of glutamate receptors in mammalian retinas (Marc, 1999; Marc, 1999; Kalloniatis et al., 2002; Marc and Jones, 2002; Kalloniatis et al., 2004; Marc et al., 2005; Sun and Kalloniatis, 2006). The AGB

permeation pattern activated by different glutamate agonists is comparable to the results obtained from electrophysiological experiments and neurotransmitter release studies (Marc, 1999; Marc, 1999). Moreover, the immunocytochemically identified neurons and AGB gating patterns in the retina show a good correspondence to glutamate receptor distribution patterns (Sun and Kalloniatis, 2006). Despite this great advantage, only one recent study has used this method to investigate glutamate receptor functionality in the developing mouse retina (Acosta et al., 2007). Here we reported the first evidence of functional activation of AMPA glutamate receptors in the developing rabbit retina.

### 5.2.1 Localization of functional AMPA receptors

The appearance of functional AMPA receptors at E26 (Figs. 15B and 17B) is consistent with the earlier finding that diffuse glutamate labeling in the rabbit retina was detectable during various embryonic stages (Redburn et al., 1992; Pow et al., 1994; Redburn and Rowe-Rendleman, 1996). This also indicates that AMPA glutamate receptors are functional well before synaptogenesis in the developing retina and may contribute to the regulation of the neuronal cytoarchitecture and to cell migration (Mattson, 1988; Wong and Godinho, 2003). In the mouse retina, it has been shown that AMPA receptors were expressed in embryonic retinal progenitor cells, and glutamate activation can regulate cell proliferation and cell fate specification (Martins et al., 2006). Thus, the early expression of functional AMPA receptors found in the embryonic rabbit retina may be important for generating the correct proportion of retinal cell types during development. After birth, the AGB permeation pattern showed two conspicuous bands in the IPL when activated by both low and high concentrations of AMPA (Figs. 15 and 17)

and this result is similar to that of the adult retina (Fig. 11). We have shown that these two bands correspond to the ChAT bands (dendritic processes of cholinergic amacrine cells) in the adult rabbit retina (Fig. 12). We also confirmed that the two bands of AGB signals observed in the IPL also correspond to the ChAT bands during all postnatal stages of the developing rabbit retina (Fig. 12). This implies that AMPA glutamate receptors are functionally dominated in the cholinergic amacrine cells throughout these developmental stages, although the expression pattern of all AMPA subunits (GluR1, GluR2/3 and GluR4) in the IPL of the developing rabbit retina does not form these two bands as distinctly (Figs. 5, 7, and 9).

### 5.2.2 Ontogenesis of glutamate receptors and retinal degeneration

The expression of functional glutamate receptors is important for normal retinal development (Chalupa and Günhan, 2004). In recent years, it has been well recognized that the functional expression of glutamate receptors is significantly altered in rodent models of retinal degeneration (Marc et al., 2007). In the *rd* mice, the photoreceptor degeneration mice, it is known that the expression levels of AMPA receptor subunit GluRs increased during development (Namekata et al., 2006). It has also been reported that photoreceptor degeneration in the *rd1* mouse retina was correlated to the glutamate-mediated excitotoxic mechanisms (Delyfer et al., 2005). Furthermore, the increase expression of GluRs was also associated with neuronal degeneration observed in the retina of experimental glaucomatous rats (Wang et al., 2005). Although the rabbit does not have the equivalent animal model of retinal degeneration, results of glutamate receptor ontogenesis described in the present study provide relevant evidences for

examining the role of AMPA receptors in rodent models of retinal degeneration.

## 5.3 Expression of NMDA receptor subunits in the developing retina

Previous studies using *in situ* hybridization and Western blotting have revealed the expression pattern of NMDA receptor subunits in various mammalian retina (Brandstätter et al., 1994; Watanabe et al., 1994; Kreutz et al., 1998; Goebel and Poosch, 1999; Xue and Cooper, 2001; Xue et al., 2002; Jakobs et al., 2007), and the localization of NMDA glutamate receptors in adult retinas have also been intensively studied by immunohistochemistry (Hartveit et al., 1994; Wenzel et al., 1997; Goebel et al., 1998; Koulen et al., 1998; Lo et al., 1998; Araki and Hamassaki-Britto, 2000; Fletcher et al., 2000; Pourcho et al., 2001; Grünert et al., 2002; Grünert et al., 2003; Kalloniatis et al., 2004). In contrast to the wealth of data available for adult retinas (review: Brandstätter et al., 1998; Brandstätter and Hack, 2001), only a few studies have attempted to characterize the distribution of NMDA receptors in the developing retina and all of them have used rodent retinas (Hartveit et al., 1994; Gründer et al., 2000; Sucher et al., 2003). We report here the first evidence showing the expression of NMDA receptor subunits NR1 and NR2A/B in the developing rabbit retina.

Several studies in the adult cat and rat retinas have shown that the NR1 and NR2A/B subunits are expressed weakly in the OPL, suggesting a limited role for the NMDA receptors in the outer retina (Koulen et al., 1998; Fletcher et al., 2000; Pourcho et al.,

2001; Kalloniatis et al., 2004). Similarly, in the developing rat and rabbit retinas, NR1 and NR2A/B expression can be detected in the OPL soon after birth (Figs. 1 and 4; Gründer et al., 2000). However, the immunoreactivity of NR1 and NR2A/B in the OPL has been attributed to the horizontal cells in the developing rat retina, which is different from our findings where there is localization to the cone pedicle and rod spherule in the developing rabbit retina. This observation is further supported by the functional mapping shown in Figures 25 and 26 (also see Kalloniatis et al., 2004), and by an electron microscopic study of NR1C2' in the rat retina (Fletcher et al., 2000). Thus, these results suggest that these NMDA receptors at the photoreceptor synaptic terminals may function as glutamate autoreceptors (Brandstätter and Hack, 2001), and play an important role in the maturation of the outer retina circuitry during development.

In the inner retina, all previous immunohistochemistry studies have unequivocally shown that NR1 and NR2A/B are heavily expressed, suggesting that NMDA receptors mainly mediate signal transmission in the IPL (Hartveit et al., 1994; Koulen et al., 1998; Fletcher et al., 2000; Pourcho et al., 2001; Grünert et al., 2002; Kalloniatis et al., 2004). In the developing rat and rabbit retinas, NR1 and NR2A/B expression can be detected in the IPL/GCL as early as E21, and this has been shown to steady increase after birth (Figs. 1 and 4; Gründer et al., 2000). This temporal expression profile indicates that NMDA receptors may participate in various early developmental events and the synaptogenesis of the inner retina. The spatial distributions of NR1 and NR2A/B, however, are somewhat different in developing rabbit retina. While NR1 is strictly confined to the IPL, NR2A/B is found in some neurons of the INL and GCL in addition to the IPL (Figs. 19 and 22). In developing rat and cat retinas, both NR1 and NR2A/B expression can be observed in the INL and GCL (Goebel et al., 1998; Gründer et al., 2000; Pourcho et al., 2001), although

other studies using different antibodies have shown restricted expression patterns and a presence in the IPL only (Hartveit et al., 1994; Fletcher et al., 2000). Nevertheless, this extensively labeling of NR1 and NR2A/B in the inner retina suggests that many amacrine and ganglion cells express NMDA receptors early during development. This finding also supports a previous study where it was found that all ganglion cells in the developing rat retina showed NMDA-evoked currents despite different subunit compositions (Guenther et al., 2004). This implies that NMDA receptors are likely to be involved in the establishment of the distinct synaptic connections in the inner retina.

## 5.4 Functionality of NMDA receptors in retinal development

The appearance of functional NMDA glutamate receptors as early as E21 (Fig. 25) is consistent with previous findings showing that diffuse glutamate labeling in the rabbit retina was detectable during various embryonic stages (Redburn et al., 1992; Pow et al., 1994; Redburn and Rowe-Rendleman, 1996), and it also suggests that NMDA receptors are functioning well before synaptogenesis in the developing retina. It is thought that this may contribute to the regulation of the neuronal cytoarchitecture and to cell migration (Mattson, 1988; Wong and Godinho, 2003). In contrast, the first functional NMDA receptors that can be detected by the AGB assay are found at P1 in the developing mouse retina (Acosta et al., 2007). In the developing rat retina, it has been reported that activation of NMDA receptors induces a BDNF-dependent neuroprotective effect in the differentiating retinal cells, and that NMDA receptor activation may control the programmed cell death of developing retinal neurons (Martins et al., 2005; Hernández et al., 2007). Thus, the early expression of functional NMDA receptors found in the

embryonic rabbit retina may be important for generating the correct proportion of retinal cell types during development.

After birth, functional NMDA receptors are mainly found in amacrine and ganglion cells (Fig. 26), which is consistent with previous electrophysiological studies of the adult rabbit retina (Massey and Miller, 1990; Linn and Massey, 1991). More importantly, the AGB permeation pattern shows two distinct bands in the IPL when activated by both low and high concentrations of NMDA from P0 to P8 and then is found as three conspicuous bands from P10 to adulthood (Fig. 26). This suggests that the dendrites of the NMDA receptor expressing cells in the inner retinal may undergo significant remodeling at around the time of eye-opening at P8-10. Interestingly, we have shown that these two bands do not exactly correspond to the ChAT bands (dendritic processes of cholinergic amacrine cells) of the P4 retina (Fig. 28), which is unlike the results of our previous study on AMPA activation, where the AGB signaling exactly co-localized with the ChAT labeling in the IPL throughout all postnatal stages (Chang and Chiao, 2008). This indicates that major glutamate transmission in cholinergic amacrine cells is not mediated by NMDA receptors. Furthermore, we found that the AGB immunoreactivity in the inner retina was co-localized with a subset of calretinin immunoreactive cells at all developmental stages (Fig. 29), which is similar to previous findings for the developing mouse retina (Acosta et al., 2007).

## 5.5 Visual experience and functional expression of NMDA receptors

In the developing visual cortex, it has been demonstrated that the subunit composition of the NMDA receptors changes during development. Particularly, NR2A and NR2B subunits undergo a well-characterized developmental shift in the cortex, and this shift is retarded by visual deprivation (Yashiro and Philpot, 2008). Although our use of a NR2A/B antibody prevents us from examining the NR2A/NR2B ratio change in the developing rabbit retina, we did not observe significant subunit composition change of the NMDA receptors in dark-reared rabbits. This finding supports an earlier study where it was found that the experience-dependent regulation of NMDA receptor function in the rat retina is not correlated to alterations in NMDA receptor subunit composition (Guenther et al., 2004). However, in a separate study, Xue and Cooper (2001) showed that relative to animals raised in a normal light-dark cycle, a period of 1 week of dark-rearing caused an increase in the relative amount of NR1 protein, a decrease in the level of NR2A, and no change in the level of NR2B subunit expression in P12 rats. The discrepancy between the results of rats and rabbits may be due to the fact they are different species and/or to the use of a different methodology. It is also possible that the total protein level of the NMDA receptor subunits in the rat retinal extracts might not reflect directly the functionality of NMDA receptors. Our immunohistochemical approach as well as the AGB assay shows consistent results whereby the functional expression of the NMDA receptors is independent of visual experience in the developing rabbit retina. Alternatively, it has been argued that light deprivation modulation of NMDA receptor function might be different in the various retinal neurons, and the developmental effects observed at a population level do not necessarily reflect any alterations observed at the single cell level

(Guenther et al., 2004). Although we are unable to preclude the possibility that individual retinal cells in the developing rabbit retina may alter their NMDA receptor functionality when deprived of light, our ganglion cell recordings from dark-reared rabbits have shown that the maturation of direction selectivity is not dependent on visual experience (Chan and Chiao, 2008). Whether the developmental regulation of NMDA receptor function in other rabbit retinal neurons is visual experience dependent awaits further electrophysiological experiments.

In summary, the results presented here demonstrate not only that NMDA receptors participate in the synaptic maturation of the retinal circuits during the early stages of the development, but also that the functional NMDA receptors in the developing rabbit retina, as well as their subunit composition, are independent of visual experience. This finding suggests that visual experience plays a less significant role in the developmental plasticity of NMDA receptor function in the retina than in the cortex.

## 5.6 The importance of ionotropic glutamate receptors in retinal development

Glutamate and glutamate receptors have been suggested to regulate the development of retinal neurons. The functional ionotropic AMPA and KA glutamate receptors can be observed during terminal cell division and early neuronal differentiation in the embryonic rat neuroepithelium (Maric et al., 2000). In our study, the ontogenic expression of ionotropic AMPA and NMDA glutamate receptors are characterized in the developing

rabbit retinas. We found that both AMPA and NMDA receptors are expressed well before birth (E21). The functional AMPA receptors are present in the GCL and NBL neurons, whereas NMDA receptors are functional in the ganglion and amacrine cells only. Similarly to our findings, the functional AMPA receptors are expressed in the embryonic mouse retina, although the functional NMDA receptors are present later in amacrine cells at the P1 stage (Acosta et al., 2007). In addition, the increase of cytosolic free calcium concentration were mediated by both non-NMDA and NMDA receptors from E21 to birth in the developing rabbit retina (Wong, 1995). Moreover, expression of the functional ionotropic glutamate receptors in the developing rabbit retina is accompanied by increased receptor subunit immunolabelings during the first postnatal week. This indicates that functional ionotropic glutamate receptors exist in the early stage of retinal synapse development. Glutamate and its ionotropic receptors are also involved in the regulation of dendritic refinement and neuronal migration in the developing retina before eye opening (Mattson, 1988; Komuro and Rakic, 1993; Bodnarenko et al., 1995). Taken together, our findings suggest that ionotropic glutamate receptors contribute to the synaptic maturation in the retinal circuit formation.

### 5.6.1 Ionotropic glutamate receptors and cell death

It has been shown that high levels of glutamate in the neonatal retina are critical for the regulation of the differential activation and remodeling of developing neurons (Redburn et al., 1992; Haberecht and Redburn, 1996). In retinal development, about half the population of ganglion cells dies by maturity (Robinson, 1990; Wong and Godinho, 2003). In the developing rat retina, amacrine cells and horizontal cells increase their death

rate by 20% between P2 and P10 (Alexiades and Cepko, 1997). It has been hypothesized that cell death through non-NMDA receptors plays a significant role in retinal maturation, possibly by selective $Ca^{2+}$ permeation via the GluR2 subunits (Allcorn et al., 1996; Cellerino et al., 2000; Liu and Zukin, 2007; Osswald et al., 2007). Our results of the functional expression of AMPA glutamate receptors in the neonatal rabbit retina support the idea that glutamate signaling through these early activated AMPA receptors is essential for the regulation of cell death and retinal circuit maturation, although programmed cell death modulated by NMDA receptor activation may be another dominant factor (Martins et al., 2005; Hernández et al., 2007).

NMDA glutamate receptors are well known to participate in conventional synaptic transmission in adult mammalian retinas. On the other hand, studies in the developing ferret retina have pointed out that an excitotoxic response to glutamate was observed in cholinergic amacrine cells in P3 (Johnson et al., 2001; Reese et al., 2001; Farajian et al., 2004). In the present study, functional NMDA receptors are shown to express as early as E21 stage in the rabbit retina. This indicates that the temporal appearance of functional NMDA receptors may play roles in the cell death regulation of differentiating retinal neurons, and blockade of NMDA receptors may induce an increase of cell death during retinal development (Martins et al., 2005).

### 5.6.2 Glutamate activation and retinal wave

Previous studies have indicated that synchronized spontaneous activity in the developing retina (spontaneous retinal waves) is essential for many developmental events

(Wong, 1999; Sernagor et al., 2001). In the rabbit retina, the spontaneous waves show a dramatic and coordinated transition in the excitatory drive from a fast cholinergic to a fast glutamatergic input around P1-P3 (Zhou and Zhao, 2000; Syed et al., 2004). After P3, the local excitation of the retinal waves is mainly mediated by glutamatergic transmission (Zhou and Zhao, 2000). Our findings show that both the AMPA and NMDA receptor subunits expression is transiently increased at P2-P8 and that the high levels of AGB permeation secondary to AMPA or NMDA activation after birth coincides well with this period of spontaneous retinal waves (stage III) (Syed et al., 2004). Thus, our results support that the ionotropic AMPA and NMDA glutamate receptors may play important roles in mediating this synchronized spontaneous activity during the postnatal stages.

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# Table

**Table 1** The primary polyclone antibodies and their dilution factors used in this study.

| Antibody | Dilution | Catalog # | Company |
|---|---|---|---|
| Rabbit anti-GluR1 | 1:200 | AB1504 | Chemicon, Temecula, CA |
| Rabbit anti-GluR2/3 | 1:200 | AB1506 | Chemicon, Temecula, CA |
| Rabbit anti-GluR4 | 1:200 | AB1508 | Chemicon, Temecula, CA |
| Rabbit anti-NR1 | 1:200 | AB1516 | Chemicon, Temecula, CA |
| Rabbit anti-NR2A/B | 1:200 | AB1548 | Chemicon, Temecula, CA |
| Rabbit anti-agmatine | 1:400 | AB1568 | Chemicon, Temecula, CA |
| Goat anti-calretinin | 1:400 | AB1550 | Chemicon, Temecula, CA |
| Goat anti-ChAT | 1:200 | AB144 | Chemicon, Temecula, CA |

# Figures

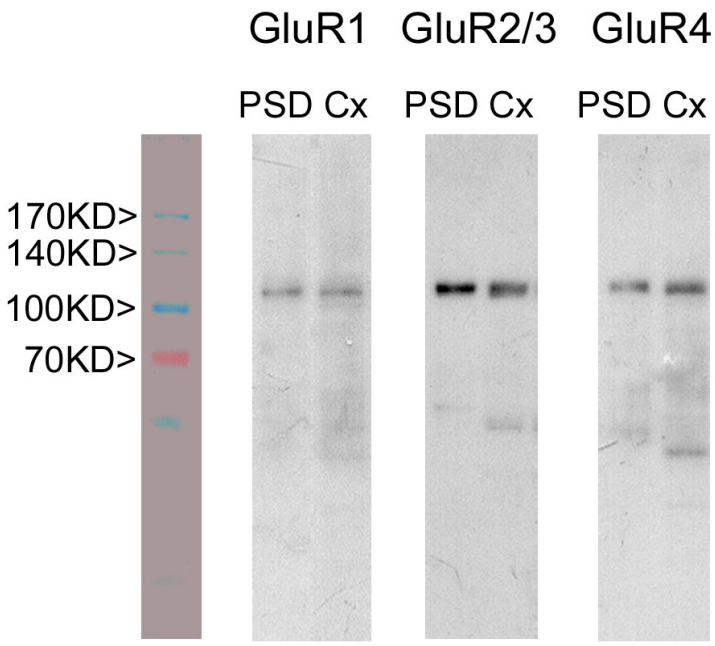


**Figure 1** Specificity of antibodies against AMPA receptors subunits GluR1, GluR2/3 and GluR4. Western blot analysis was done in the postsynaptic density of the pig brain cortex (PSD) and the crude extract of the rabbit cortex (Cx) probed with GluR1, GluR2/3, and GluR4 antiserums used in this study. These antibodies against GluR1, GluR2/3, and GluR4 recognized proteins with molecular weights of ~110 kDa in the brain tissues of both pig (Chang et al., 1996) and rabbit.

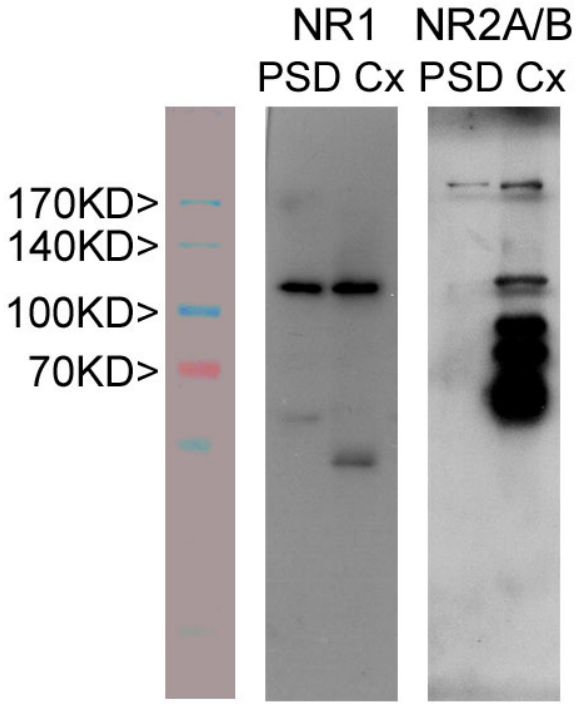


**Figure 2** Specificity of antibodies against NMDA receptor subunits NR1 and NR2A/B. Western blot analysis was done in the postsynaptic density of the pig brain cortex (PSD) and the crude extract of the rabbit cortex (Cx) probed with NR1 and NR2A/B antiserums used in this study. These antibodies against NR1 and NR2A/B recognized proteins with molecular weights of 116 kDa and 180 kDa respectively in the brain tissues of both pig (Chang et al., 1996) and rabbit.

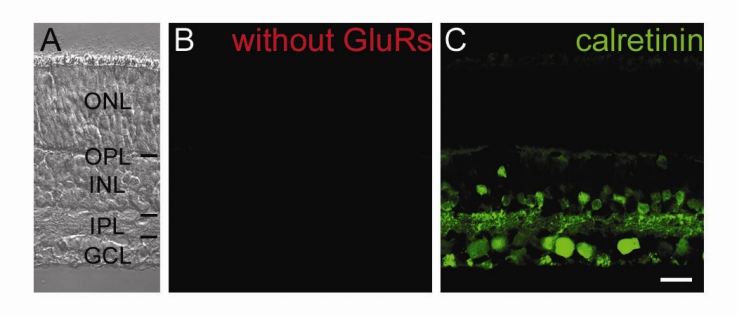


**Figure 3** Specificity of GluRs immunoreactivity in the P10 rabbit retina. The retinal slice was incubated with the primary antibody against calretinin, but omitted the primary antibody against GluRs. After rinsing, secondary antibodies against GluRs (Cy5 conjugated) and calretinin (FITC conjugated) were applied. (A)-(C) Confocal images show double-labeling of the expression of the AMPA receptor subunit GluRs (red) and calretinin (green). The phase contrast image is shown in panel A to identify the retinal layers. No GluRs immunoreactivity was detected in this preparation (B) when calretinin signals were clearly shown (C). This negative control experiment demonstrates that the immunohistochemistry used in the present study was specific. Scale bar, 20 μm.

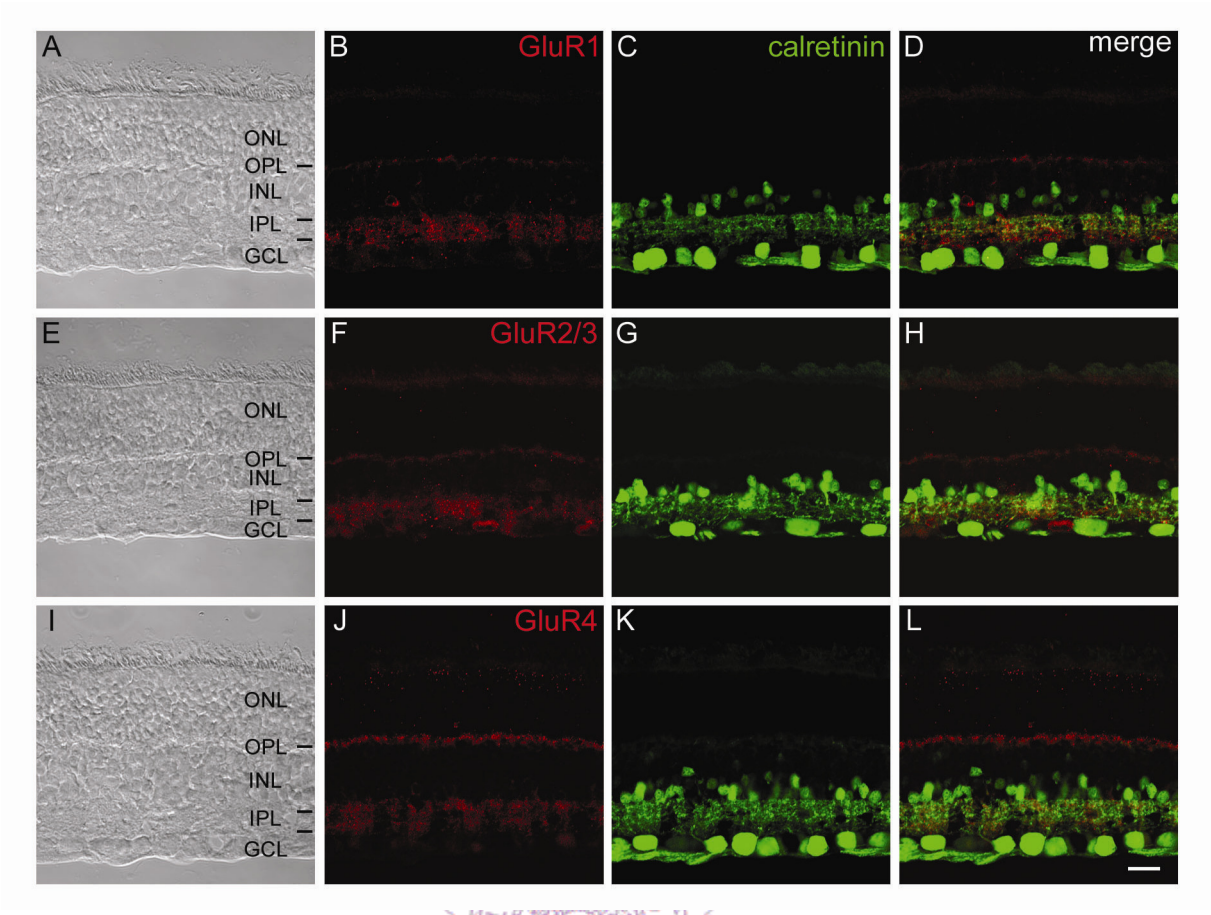


**Figure 4** Expression patterns of AMPA receptor subunits GluR1, GluR2/3 and GluR4 in the adult rabbit retina. (A)-(D) Confocal images show double-labeling of the expression of the AMPA receptor subunit GluR1 (red) and calretinin (green). The phase contrast image is shown in panel A to identify the retinal layers and the merge image in panel D illustrates the co-localization of calretinin positive cells (AII amacrine cells and some ganglion cells) and GluR1 expression. GluR1 immunoreactivity was abundant in the IPL and weak in the OPL. (E)-(H) Similar to GluR1 image series, the expression of GluR2/3 was apparent in both IPL and OPL and also in some neurons in the GCL. (I)-(L) Similar to images of the GluR1 series, GluR4 was strongly expressed in both the IPL and OPL. Scale bar, 20 μm.

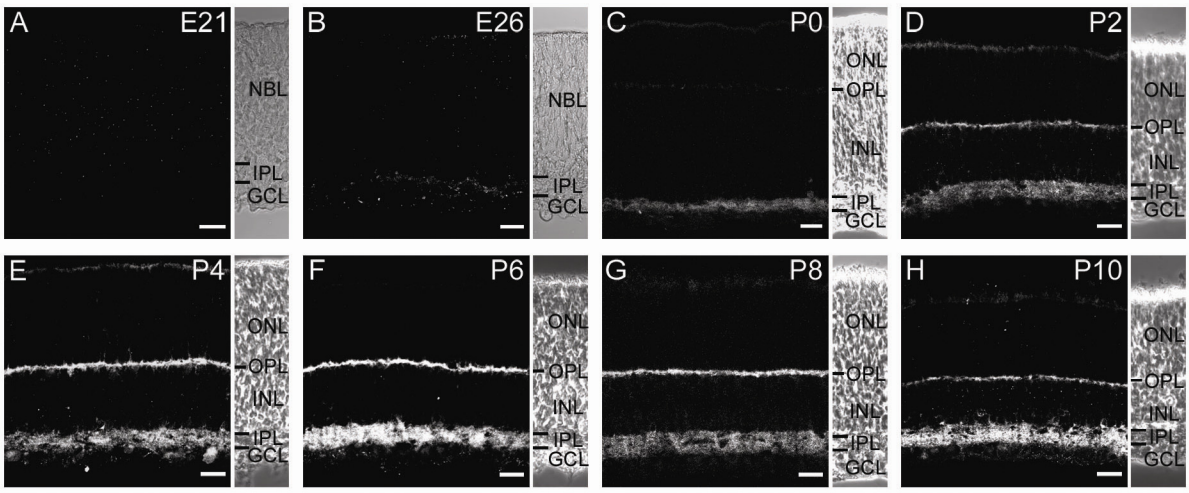


**Figure 5** Localization of AMPA receptor subunit GluR1 in the rabbit retina at different developmental stages. (A) No GluR1 immunoreactivity was present at E21. (B) The expression of GluR1 was first identified in the inner retina at E26. (C) GluR1 expressed strongly after P0 in the IPL. (D) The expression of GluR1 in the OPL was first labeled at P2. (E)-(F) The immunoreactivity of GluR1 was most abundant in both IPL and OPL at P4-P6. (G)-(H) The expression of GluR1 was slightly reduced at P8-P10. Scale bar, 20 μm.

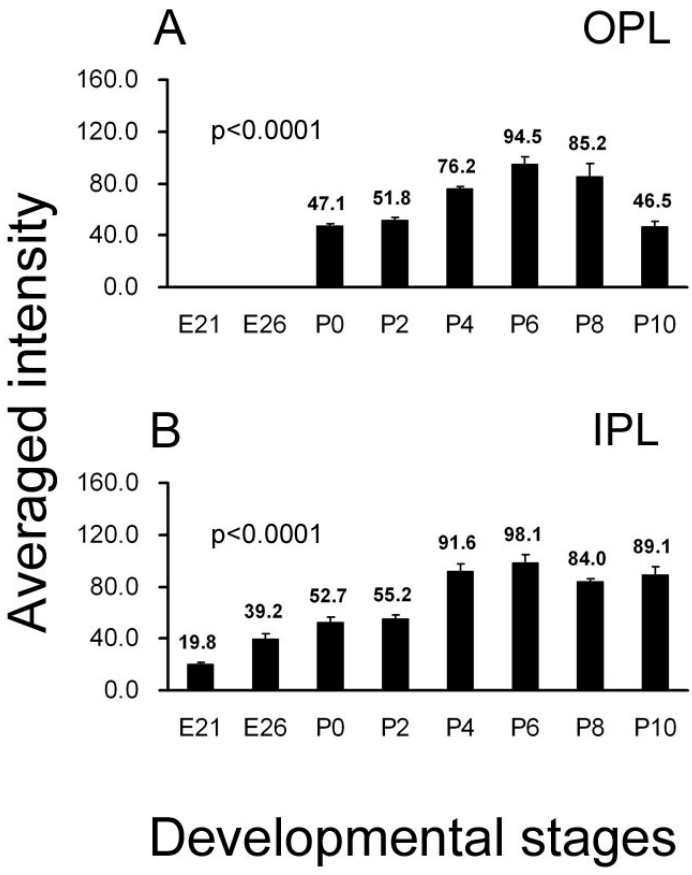


**Figure 6** Quantifications of GluR1 immunoreactivity in the developing rabbit retina. The strengths of AMPA subunits GluR1 immunoreactivity at various developmental stages were determined by averaging the intensities of fluorescent signals above a background noise level in the region of interest (either the OPL or the IPL). (A) Averaged fluorescence intensities of GluR1 immunoreactivity in the OPL of rabbit retinas (n = 3 or 4) at different developmental stages. (B) Averaged fluorescence intensities of GluR1 immunoreactivity in the IPL of rabbit retinas (n = 3 or 4) at different developmental stages. Error bars represent SEM. Using the one-way ANOVA analysis, differences of GluR1 immunoreactivity across all developmental stages were found statistically significant ($p < 0.0001$) in both the OPL and the IPL.

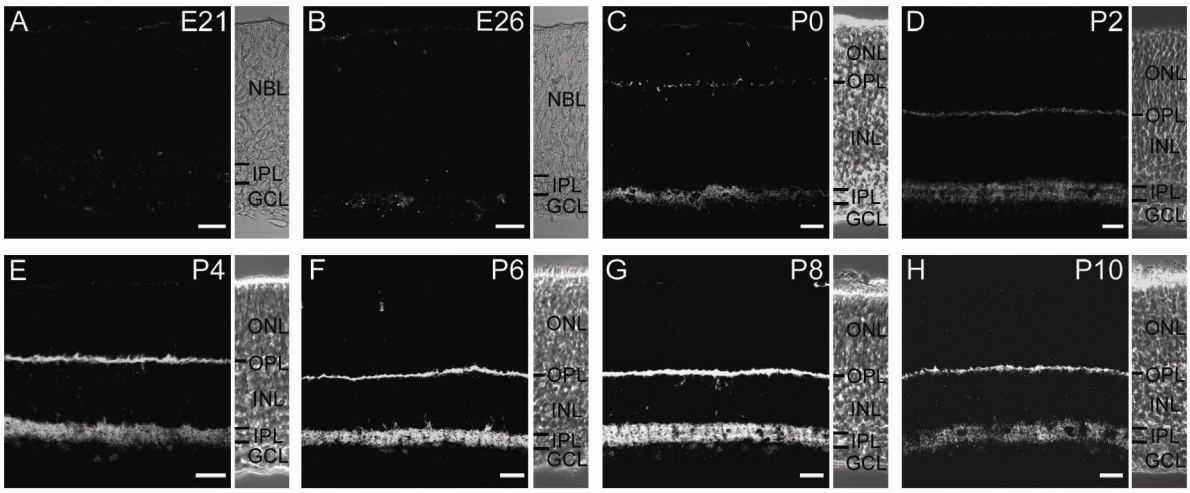


**Figure 7** Localization of AMPA receptor subunit GluR2/3 in the rabbit retina at different developmental stages. (A) The expression of GluR2/3 was faintly labeled in some neurons of the inner retina at E21. (B) The immunoreactivity of GluR2/3 was moderate in the GCL at E26. (C) GluR2/3 was steadily labeled in the IPL and weakly labeled in the OPL at P0. (D) GluR2/3 was consistently labeled in both IPL and OPL at P2. (E)-(G) GluR2/3 immunoreactivity was most abundant in both the IPL and OPL at P4-P8. (H) The expression of GluR2/3 was slightly reduced and reached an adult level at P10. Scale bar, 20 μm.

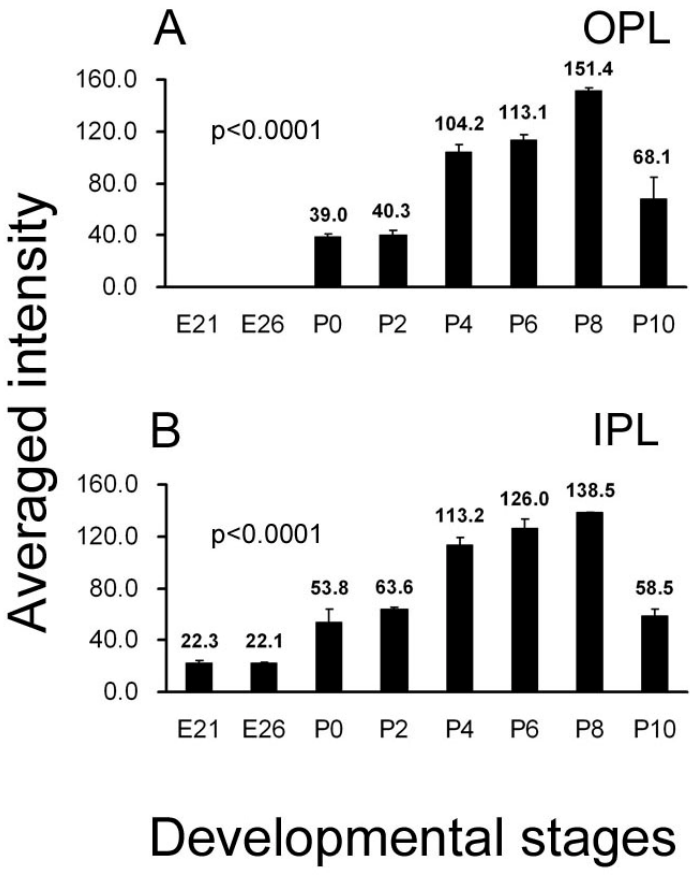


**Figure 8** Quantifications of GluR2/3 immunoreactivity in the developing rabbit retina. The strengths of AMPA subunits GluR2/3 immunoreactivity at various developmental stages were determined by averaging the intensities of fluorescent signals above a background noise level in the region of interest (either the OPL or the IPL). (A) Averaged fluorescence intensities of GluR2/3 immunoreactivity in the OPL of rabbit retinas (n = 3 or 4) at different developmental stages. (B) Averaged fluorescence intensities of GluR2/3 immunoreactivity in the IPL of rabbit retinas (n = 3 or 4) at different developmental stages. Error bars represent SEM. Using the one-way ANOVA analysis, differences of GluR2/3 immunoreactivity across all developmental stages were found statistically significant ($p < 0.0001$) in both the OPL and the IPL.

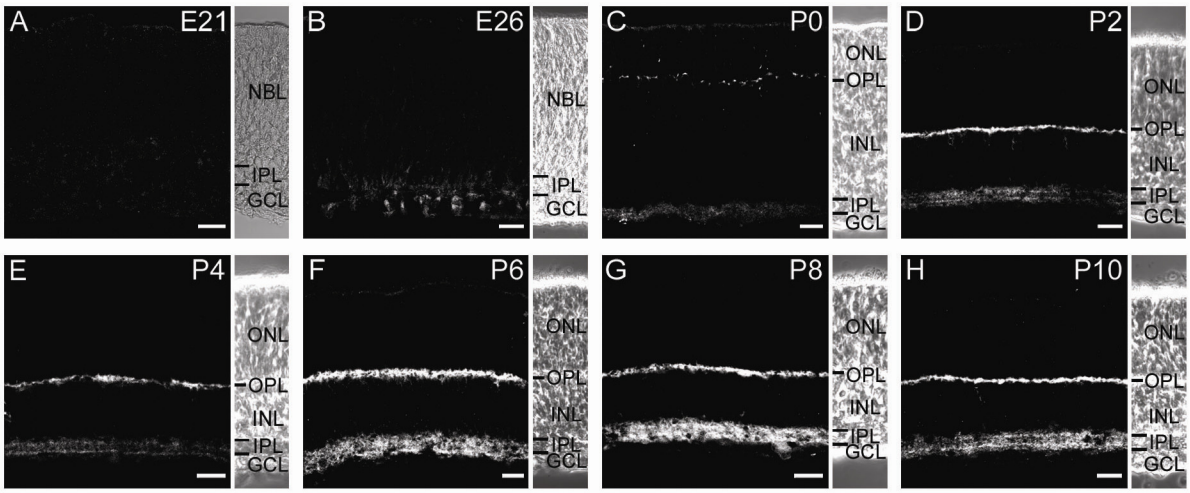


**Figure 9** Localization of AMPA receptor subunit GluR4 in the rabbit retina at different developmental stages. (A) Very weak immunoreactivity could be found in the inner retina at E21. (B) The expression of GluR4 was moderate in both IPL and GCL at E26. (C) GluR4 was steadily labeled in the IPL and weakly labeled in the OPL at P0. (D)-(E) GluR4 immunoreactivity was first strongly detected in the OPL at P2, but remained moderate in the IPL at P2-P4. (F)-(H) The expression of GluR4 in both the IPL and OPL was strong throughout P6-P8 and reached an adult level at P10. Scale bar, 20 μm.

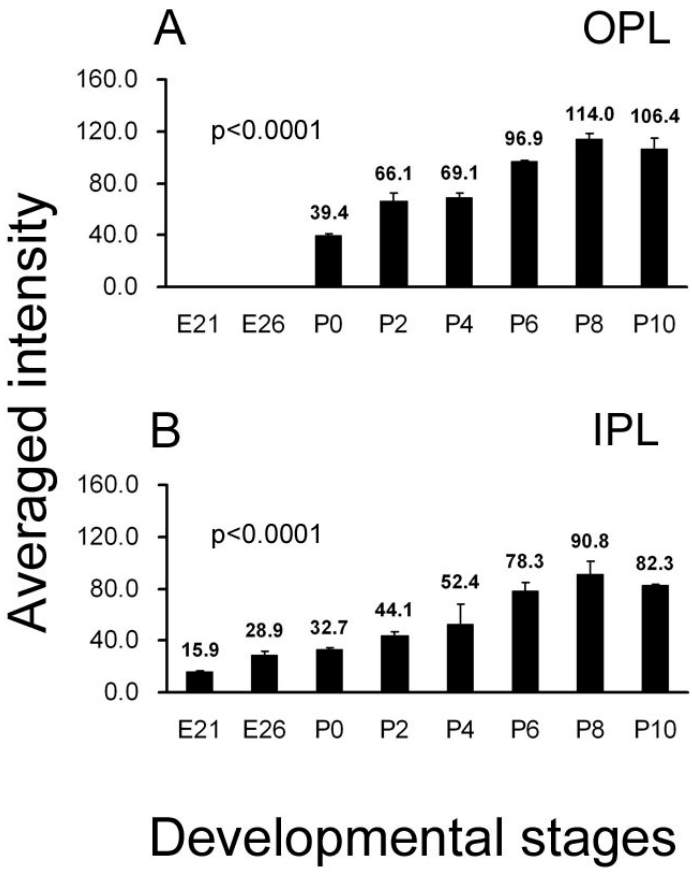


**Figure 10** Quantifications of GluR4 immunoreactivity in the developing rabbit retina. The strengths of AMPA subunits GluR4 immunoreactivity at various developmental stages were determined by averaging the intensities of fluorescent signals above a background noise level in the region of interest (either the OPL or the IPL). (A) Averaged fluorescence intensities of GluR4 immunoreactivity in the OPL of rabbit retinas (n = 3 or 4) at different developmental stages. (B) Averaged fluorescence intensities of GluR4 immunoreactivity in the IPL of rabbit retinas (n = 3 or 4) at different developmental stages. Error bars represent SEM. Using the one-way ANOVA analysis, differences of GluR4 immunoreactivity across all developmental stages were found statistically significant ($p < 0.0001$) in both the OPL and the IPL.

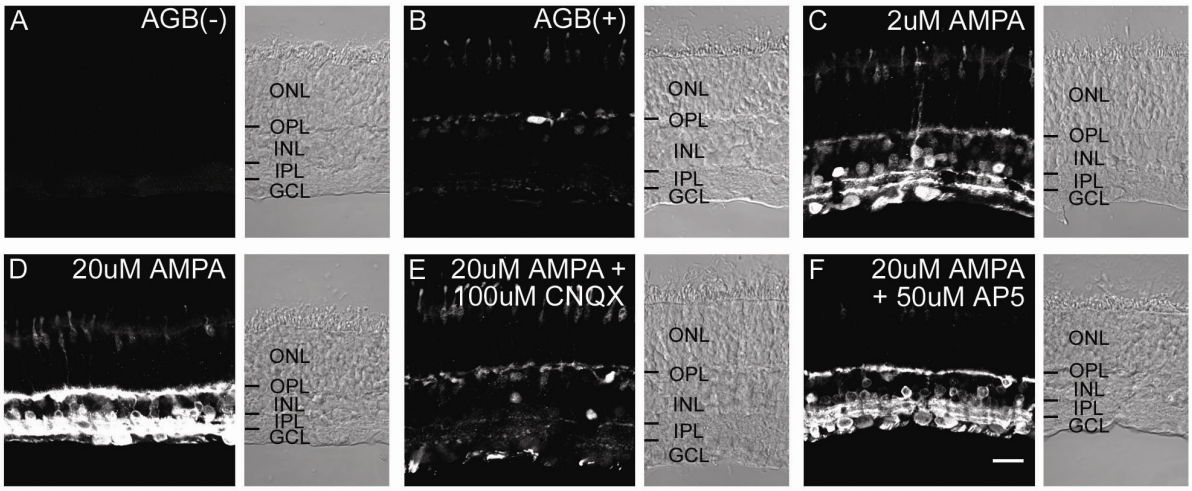


**Figure 11** AGB signals in the adult rabbit retina activated by AMPA is dose dependent and agonist specific. (A) No endogenous AGB signal was observed when the retina was incubated in Edwards medium without AGB. (B) Basal AGB permeation after incubating the retina with 25 mM AGB in the absence of glutamate receptor agonists. There were endogenous AGB signals in some horizontal cells and bipolar cells, as well as cone photoreceptor cells in the outer retina and ganglion cells in the inner retina. (C) AGB signals in the presence of 2 μM AMPA. The low concentration of AMPA increased AGB permeation in horizontal cells, bipolar cells and cone photoreceptors of the outer retina. More importantly, 2 μM AMPA significantly increased AGB signals in some amacrine cells and ganglion cells in the inner retina. (D) AGB signals in the presence of 20 μM AMPA. The high concentration of AMPA further increased AGB permeation in horizontal cells and bipolar cells, as well as amacrine cells and ganglion cells. (E) Co-treatment with 20 μM AMPA and 100 μM CNQX (the AMPA/kainate receptor antagonist) drastically reduced AGB signals to the basal AGB permeation. (F) Co-treatment with 20 μM AMPA and 50 μM AP5 (the NMDA receptor antagonist) had no effect on AGB permeation. Scale bar, 20 μm.

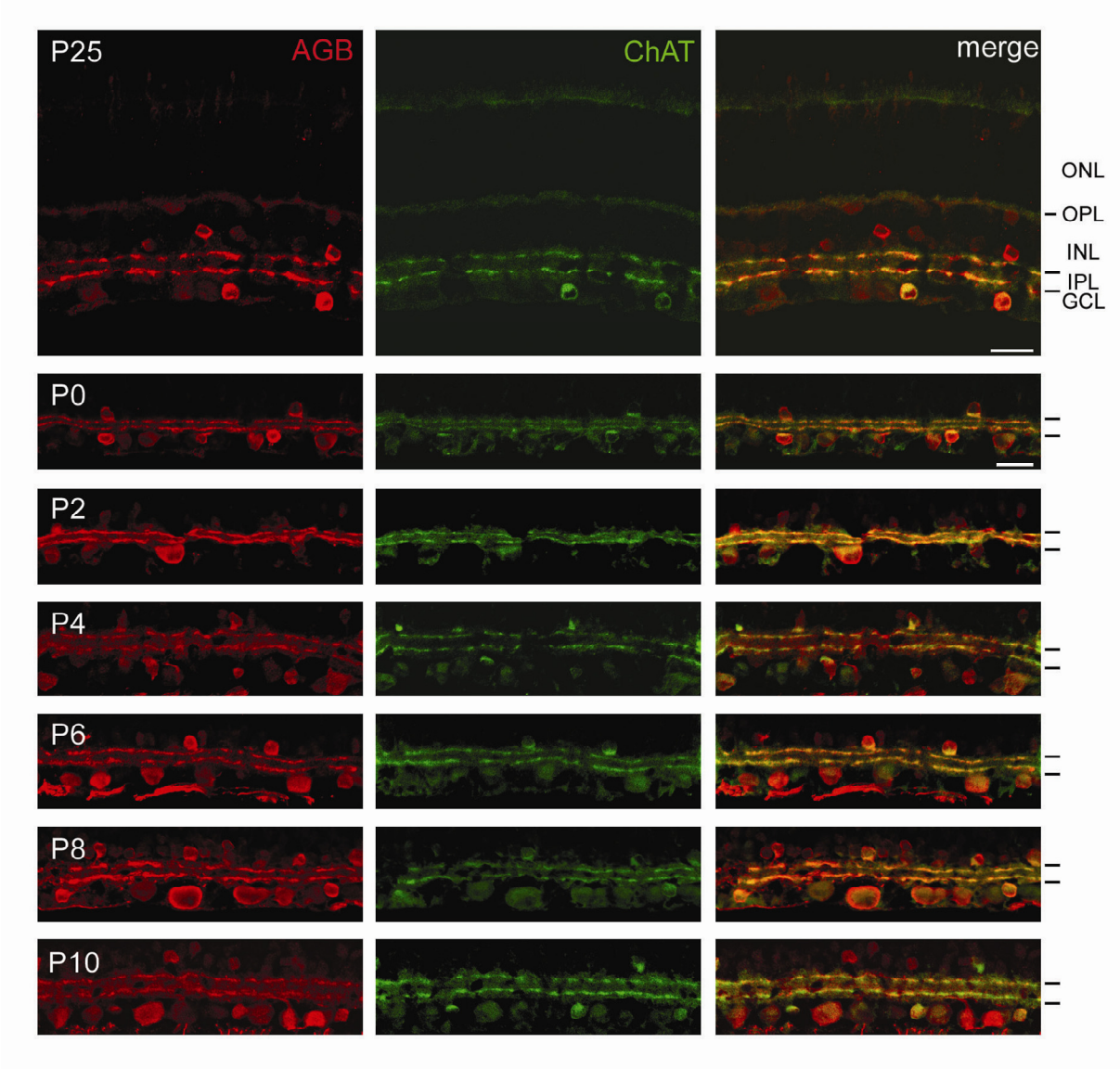


**Figure 12** AGB signals activated by 2 μM AMPA and immunoreactivity of ChAT in the adult rabbit retina (P25) and retinas from various postnatal stages (P0-P10). All retinas were incubated with 25 mM AGB in the presence of 2 μM AMPA. After incubation, the retinal slices were immunolabeled to reveal AGB and ChAT signals. Confocal images show double-labeling of the AGB (red) and ChAT (green) in the inner retina. The merge images are shown in the right column to illustrate the co-localization of cholinergic amacrine cells and AMPA activated cells at different developmental stages (labeled at each panel in the left column). Scale bar, 20 μm.

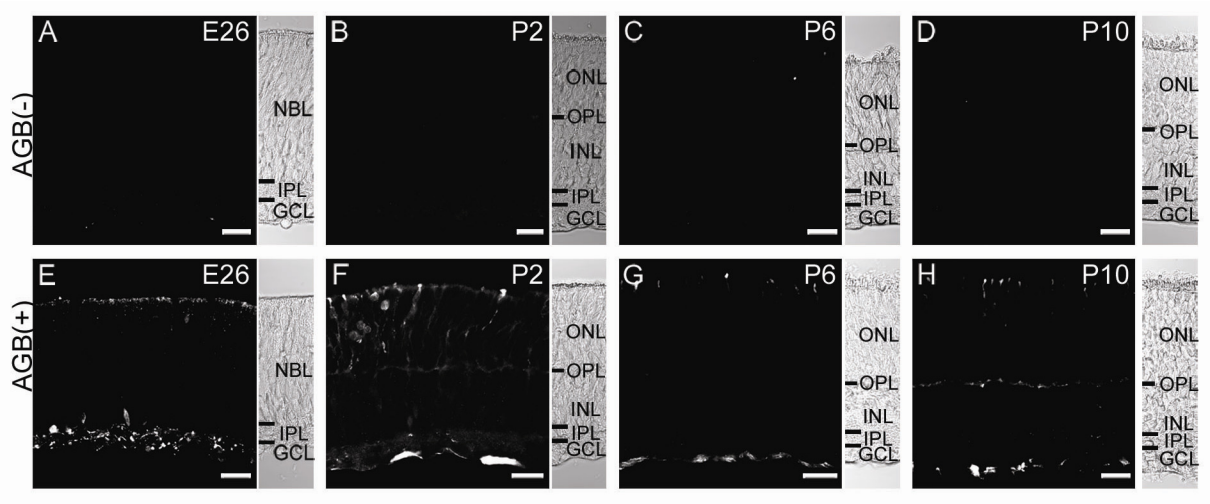


**Figure 13** Basal AGB permeation in the rabbit retina at different developmental stages. (A)-(D) No endogenous AGB signal was observed when retinas from E26 to P10 were incubated in Edwards medium without AGB. (E)-(H) Basal AGB permeation after incubating the retinas from E26 to P10 with 25 mM AGB in the absence of glutamate receptor agonists. Endogenous AGB signals were detected in the inner retina and the outer margin of NBL at E26. At P2, there was a slight increase in AGB permeation in the ONL, but endogenous AGB was decreased at P6 and reached an adult level at P10. Scale bar, 20 μm.

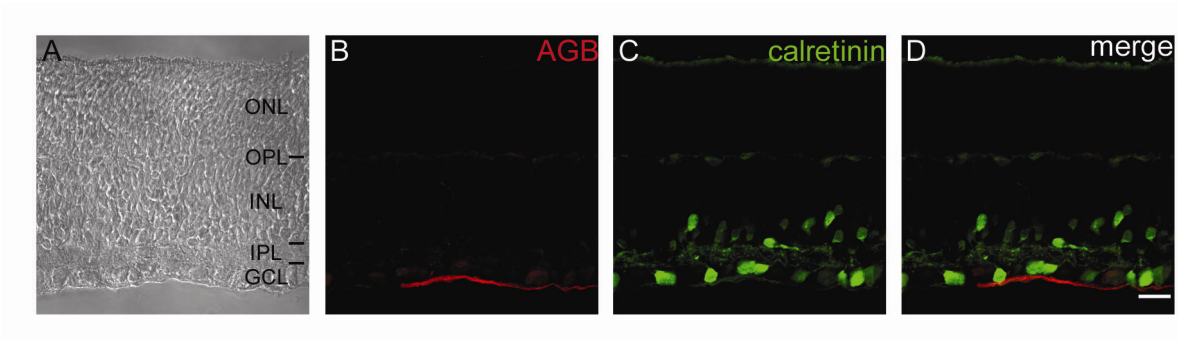


**Figure 14** AGB signals in the P2 rabbit retina activated by AMPA is agonist specific. (A)-(D) Confocal images show basal AGB permeation after incubating the retina with 25 mM AGB in the presence of 20 μM AMPA and 100 μM CNQX (the AMPA/kainate receptor antagonist). The phase contrast image is shown in panel A to identify the retinal layers. Immunoreactivity of calretinin is shown in panel C for comparison. The merge image in panel D illustrates the co-localization of calretinin positive cells and basal AGB signals. Scale bar, 20 μm.

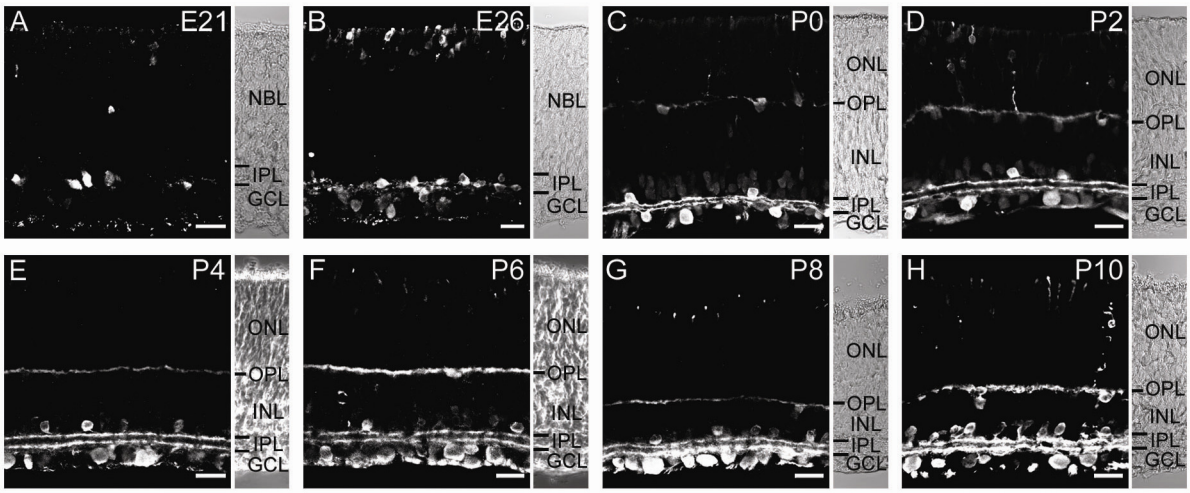


**Figure 15** AGB signals activated by 2 μM AMPA in the rabbit retina at different developmental stages. The retinas were incubated with 25 mM AGB in the presence of 2 μM AMPA. (A) Some AGB signals were detectable in the inner retina at E21. (B) AGB permeation was found in some amacrine cells and ganglion cells, as well as the outer margin of NBL at E26. (C)-(H) After birth (P0-P10), the AGB signals were clearly detectable and steadily increased in some amacrine cells and ganglion cells in the inner retina and exhibited two distinct bands in the IPL. AGB permeation was weakly labeled in the OPL at P0-P4, but showed a significant increase at P6 and reached an adult level at P10. Scale bar, 20 μm.

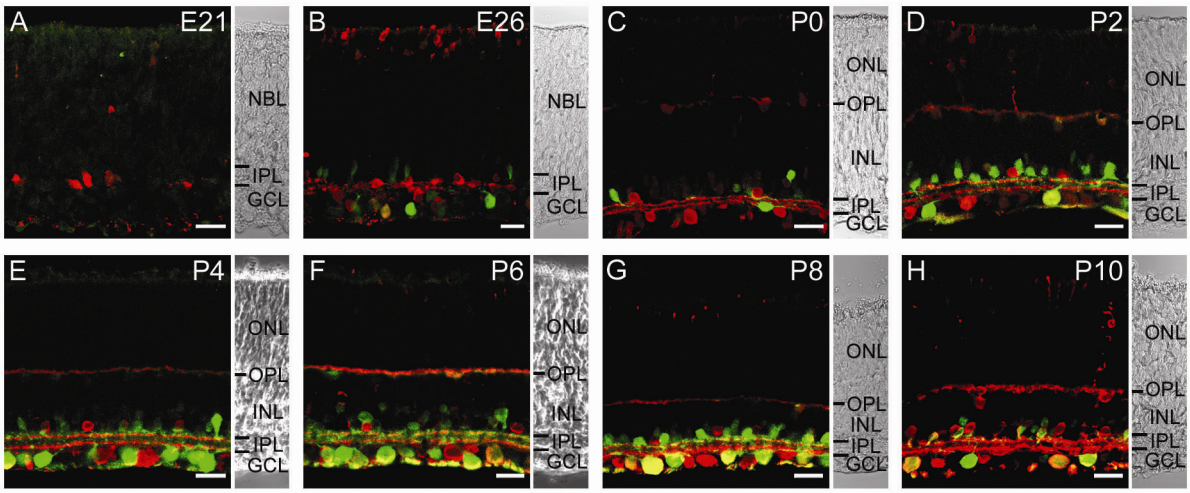


**Figure 16** AGB signals activated by 2 μM AMPA and calretinin immunoreactivity in the rabbit retina at different developmental stages. Notice that AGB signals (red) in this figure are identical to the ones shown in Figure 15. (A)-(H) A few calretinin positive cells (green) were shown to co-localize with AGB permeation throughout various developmental stages (E21-P10). Scale bar, 20 μm.

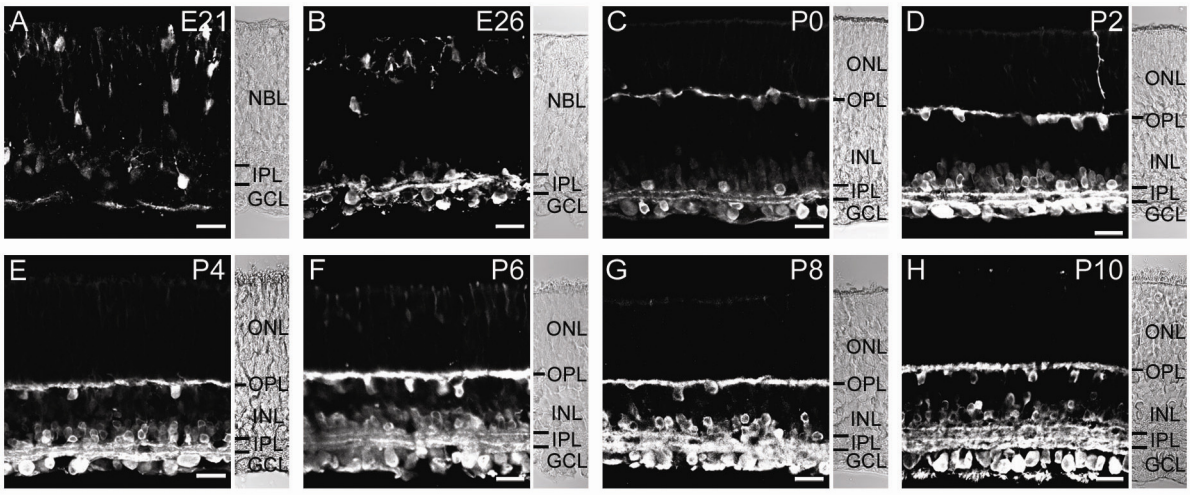


**Figure 17** AGB signals activated by 20 μM AMPA in the rabbit retina at different developmental stages. The retinas were incubated with 25 mM AGB in the presence of 20 μM AMPA. (A) At high AMPA concentration, AGB signals were detectable in both the inner retina and the NBL at E21. (B) AGB permeation was strongly labeled in some amacrine cells and ganglion cells, as well as the outer margin of NBL at E26, with activation of high AMPA concentration. (C)-(H) After birth (P0-P10), AGB signals with 20 μM AMPA were much stronger than with 2 μM AMPA in amacrine cells and ganglion cells. Similarly, AGB permeation also showed a significant increase in the OPL at P6 when activated with a high concentration of AMPA. AGB signals were adult like after P8. Scale bar, 20 μm.

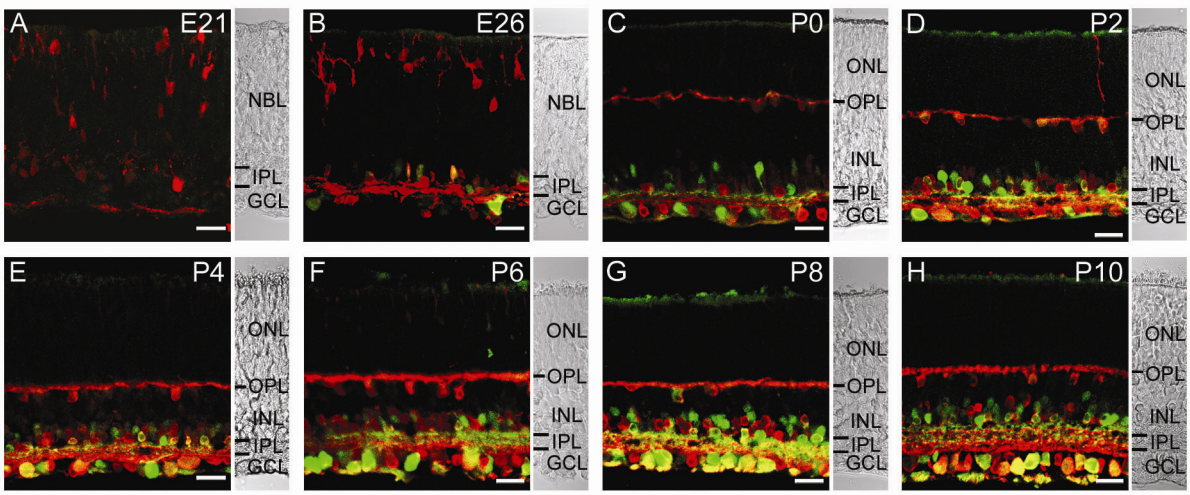


**Figure 18** AGB signals activated by 20 μM AMPA and calretinin immunoreactivity in the rabbit retina at different developmental stages. Notice that AGB signals (red) in this figure are identical to the ones shown in Figure 17. (A)-(H) Some calretinin positive cells (green) were shown to co-localize with AGB permeation throughout various developmental stages (E21-P10). Scale bar, 20 μm.

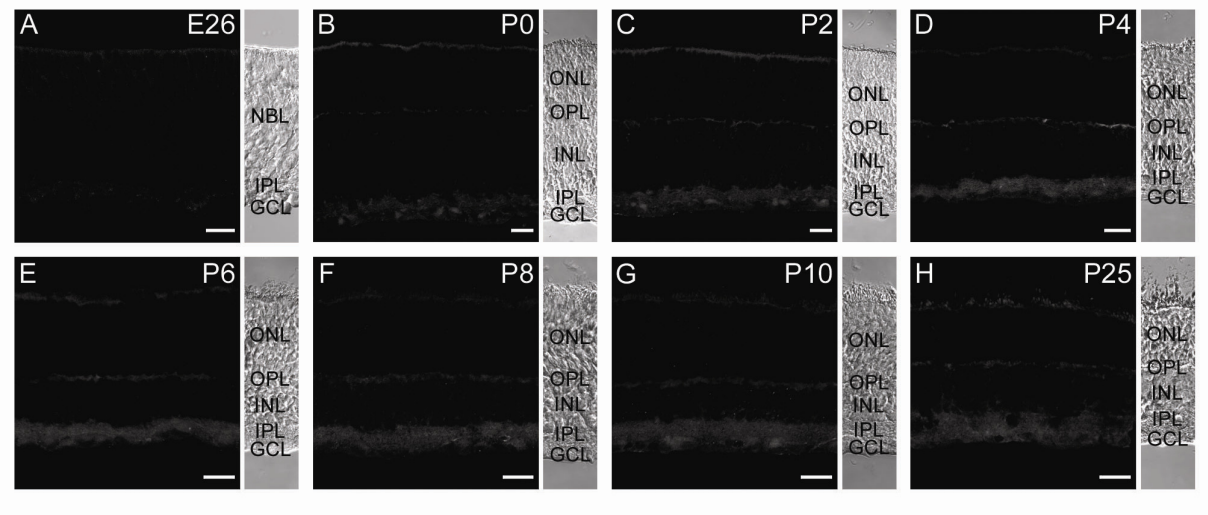


**Figure 19** Localization of NMDA receptor subunit NR1 in the normal-reared rabbit retina at different developmental stages. (A) NR1 immunoreactivity was barely detected at E26. A phase contrast image is shown to identify the retinal layers. (B)-(C) The expression of NR1 was identified in the IPL at P0 and P2. (D)-(G) The expression of NR1 was slightly increased in the inner retina at P4-P10. (H) The expression of NR1 was slightly reduced at P25. Scale bar, 20 μm.

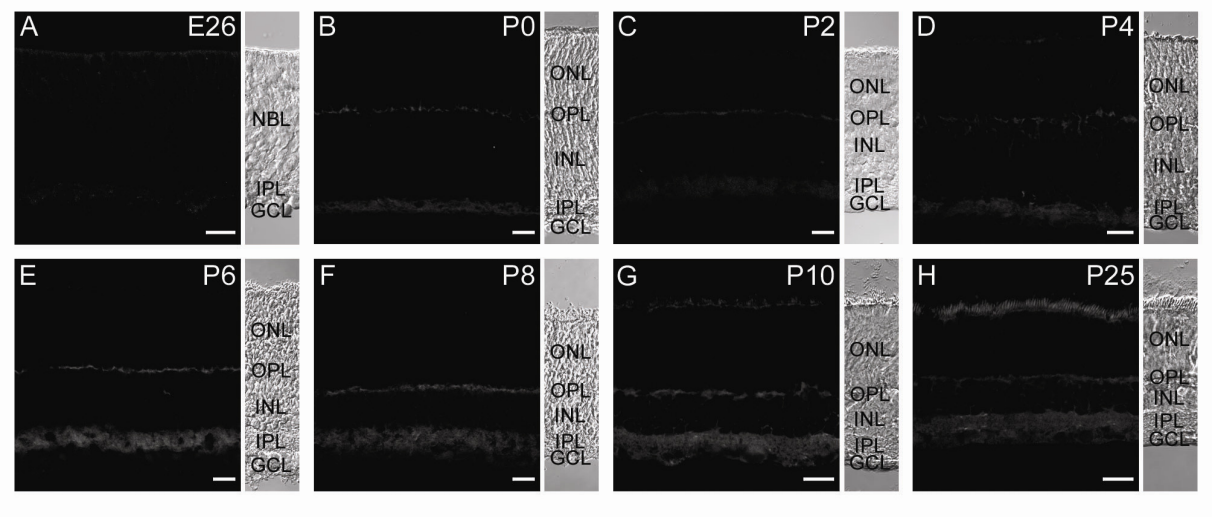


**Figure 20** Localization of NMDA receptor subunit NR1 in the dark-reared rabbit retina at different developmental stages. (A) Identical to Figure 19A, the immunoreactivity of NR1 was weakly detected at E26. (B)-(D) The expression of NR1 was identified in the IPL at P0 and P4. (E)-(G) The expression of NR1 was slightly increased in the inner retina at P6-P10. (H) The expression of NR1 was slightly reduced at P25. Scale bar, 20 μm.

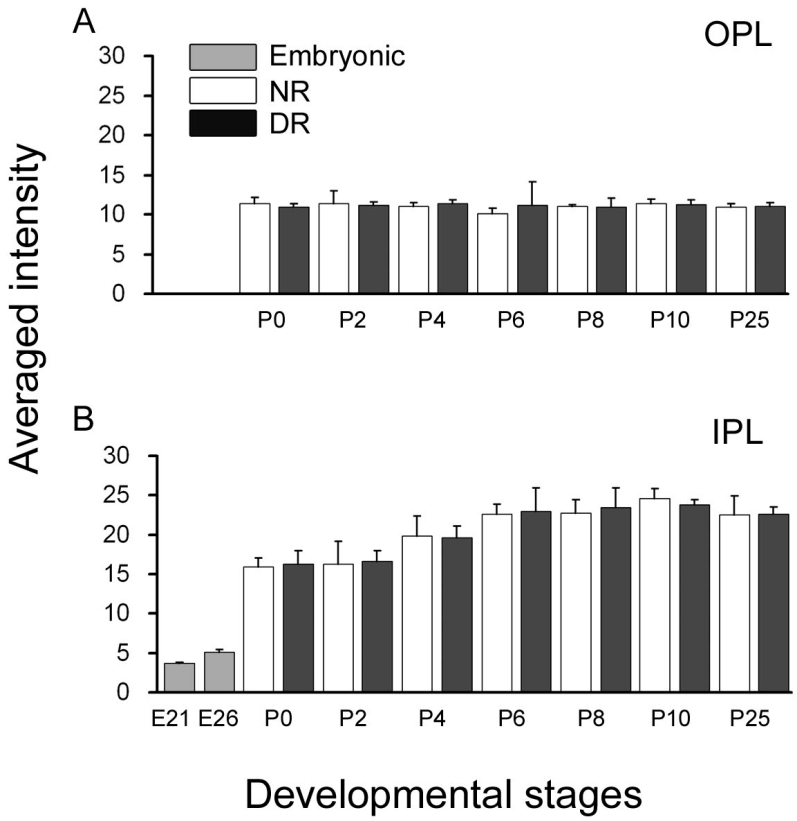


**Figure 21** Quantifications of the immunoreactivity of NR1 in the developing rabbit retina. The strength of NR1 immunoreactivity was computed at various developmental stages by averaging the intensities of fluorescent signals above the background noise level in the region of interest (either the OPL or the IPL). (A) The averaged fluorescence intensities of NR1 immunoreactivity (n = 4-6) in the OPL of the NR (raised in normal diurnal light/dark cycle) and DR (raised in complete darkness) rabbit retinas at different developmental stages. (B) The averaged fluorescence intensities of NR1 immunoreactivity (n = 4-6) in the IPL of NR and DR rabbit retinas at different developmental stages. Error bars represent SEM. Using the Student two-tailed *t* test, no difference in NR1 immunoreactivity between the NR and DR rabbit retinas in both the OPL and the IPL was found across all developmental stages.

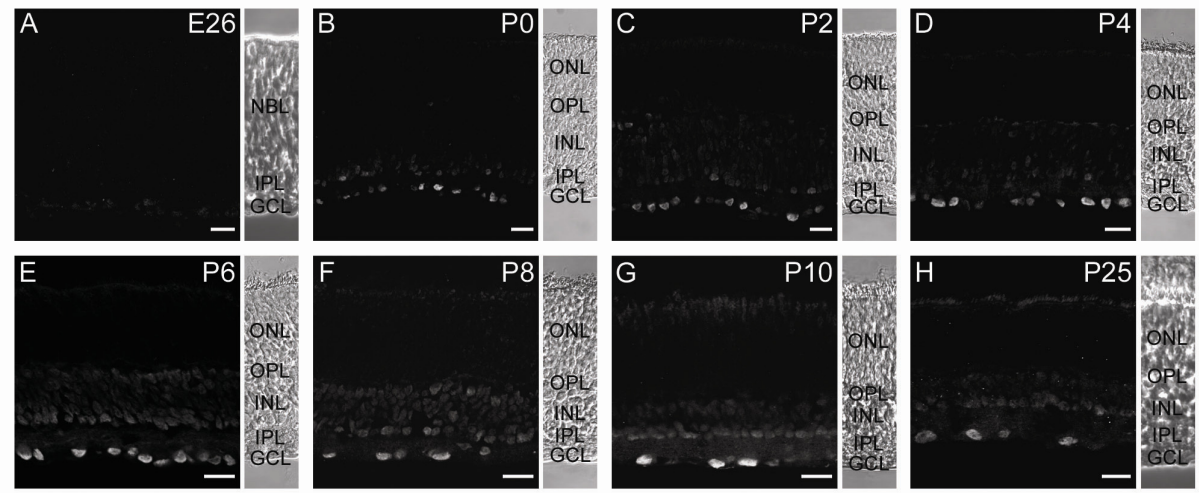


**Figure 22** Localization of NMDA receptor subunit NR2A/B in the normal-reared rabbit retina at different developmental stages. (A) The expression of NR2A/B showed faint labeling in some neurons of the inner retina at E26. (B) The staining of NR2A/B was moderately expressed in both the inner part of the INL and the GCL at P0. (C)-(G) The immunoreactivity of NR2A/B slightly increased after P0 in the INL and strongly labeled in the GCL at P4-P10. (H) The expression of NR2A/B in the INL and GCL was reduced slightly at P25. Scale bar, 20 μm.

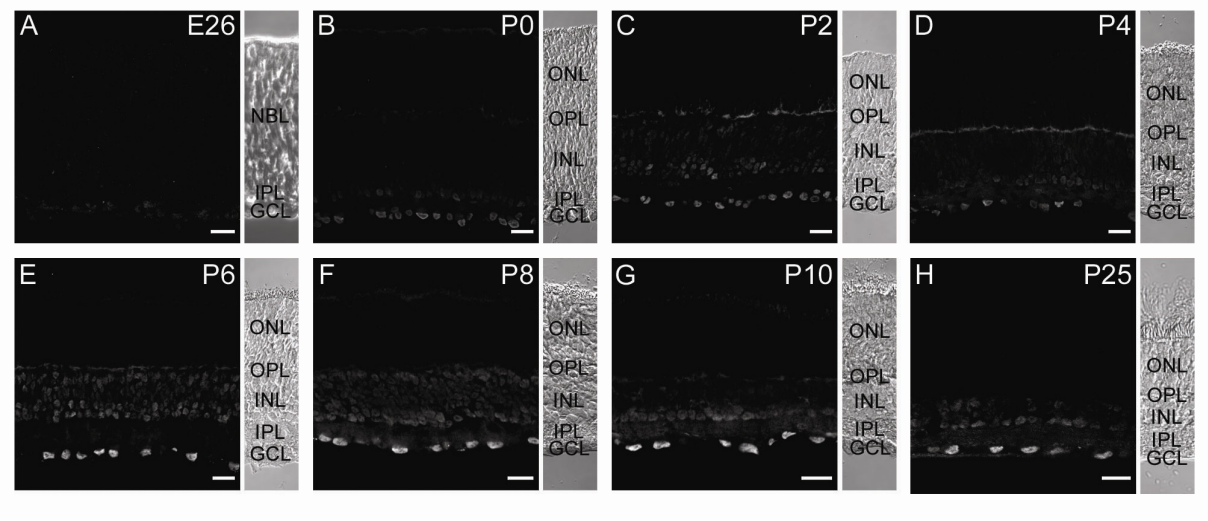


**Figure 23** Localization of NMDA receptor subunit NR2A/B in the dark-reared rabbit retina at different developmental stages. (A) Identical to Figure 22A, the expression of NR2A/B showed faint labeling in some neurons of the inner retina at E26. (B) The staining of NR2A/B was moderately expressed in both the inner part of the INL and the GCL at P0. (C)-(G) The labeling of NR2A/B slightly increased after P0 in the INL and strongly expressed in the GCL at P4-P10. (H) The expression of NR2A/B in the INL and GCL was reduced slightly at P25. Scale bar, 20 μm.

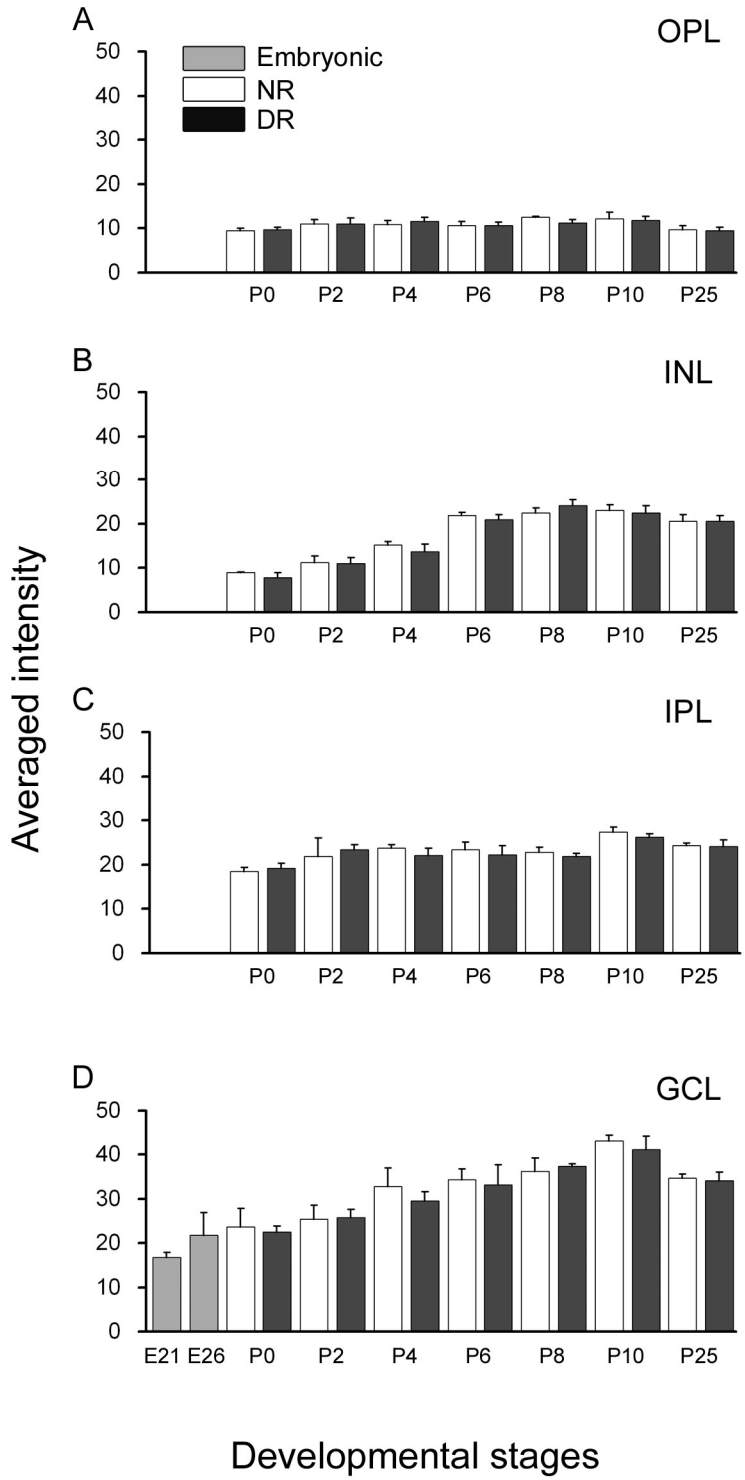


**Figure 24** Quantifications of the immunoreactivity of NR2A/B in the developing rabbit retina. The strength of NR2A/B immunoreactivity in the NR and DR rabbit retinas was computed at various developmental stages by averaging the intensities of fluorescent

signals above a background noise level in the region of interest (including the OPL, INL, IPL and GCL). (A) The averaged fluorescence intensities of NR2A/B immunoreactivity (n = 3-6) in the OPL of rabbit retinas at different developmental stages. (B) The averaged fluorescence intensities of NR2A/B immunoreactivity (n = 3-6) in the INL. (C) The averaged fluorescence intensities of NR2A/B immunoreactivity (n = 3-6) in the IPL. (D) The averaged fluorescence intensities of NR2A/B immunoreactivity (n = 3-6) in the GCL. Error bars represent SEM. Using the Student two-tailed *t* test, no difference in NR2A/B immunoreactivity between NR and DR rabbit retinas in all studied regions was found across all developmental stages.

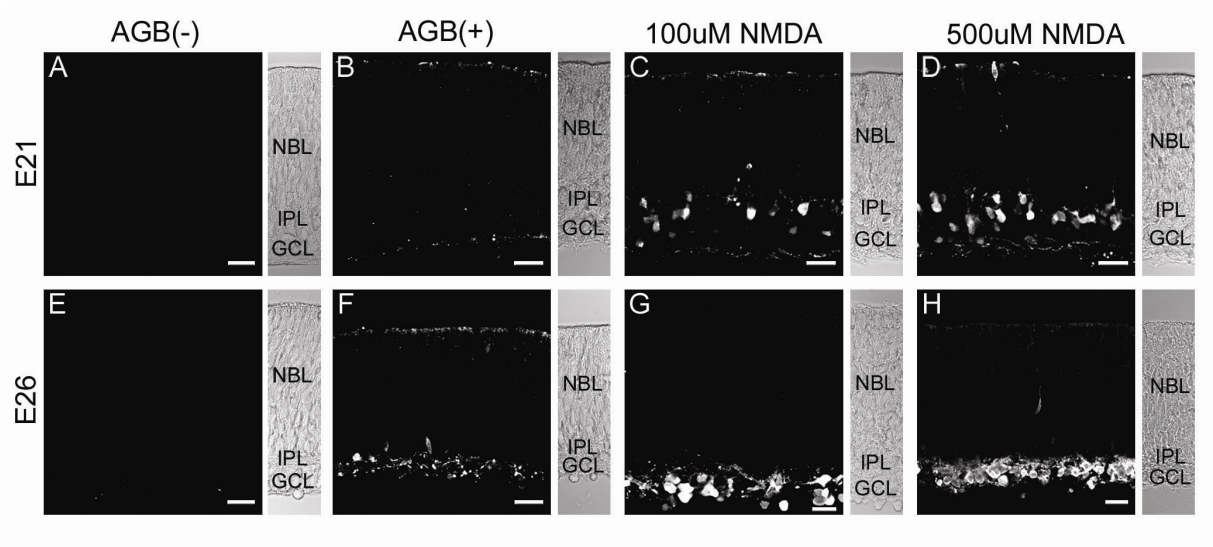


**Figure 25** AGB signals activated by NMDA in the embryonic rabbit retinas are dose dependent and agonist specific. (A)&(E) No endogenous AGB signal was observed when the retina was incubated in Edwards medium without AGB at E21 and E26. (B)&(F) Basal AGB permeation after incubating the retina with 25 mM AGB in the absence of glutamate receptor agonists. There was weak basal AGB permeation signal in the IPL at E21, and in some inner retina neurons at E26. (C)&(G) In the presence of 100 μM NMDA, AGB permeation increased in the inner retina neurons both at E21 and E26. (D)&(H) In the presence of 500 μM NMDA, the AGB signals further increased in the inner retina neurons both at E21 and E26. Scale bar, 20 μm.

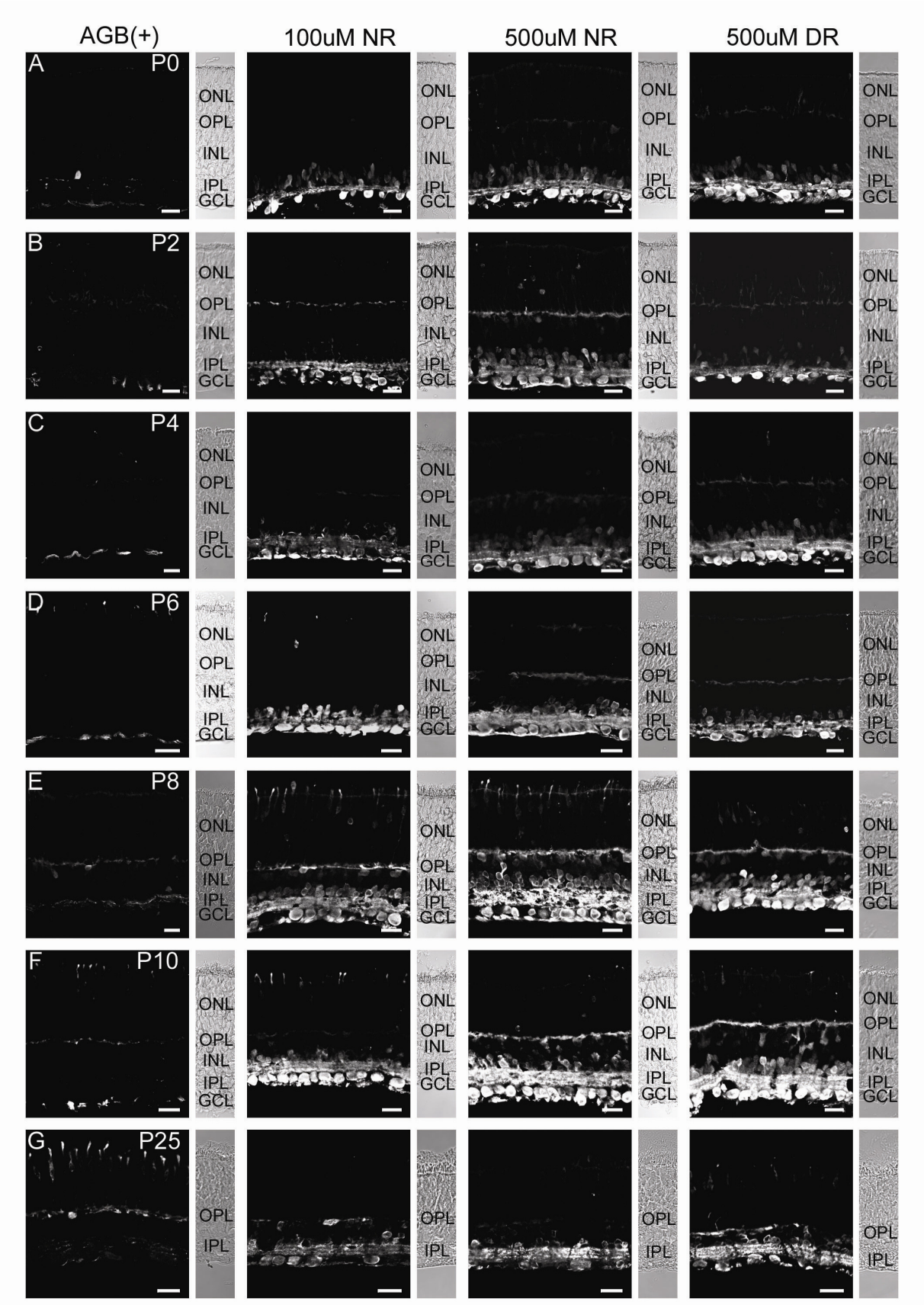
AGB(+)
100uM NR
500uM NR
500uM DR
A P0
B P2
C P4
D P6
E P8
F P10
G P25
ONL
OPL
INL
IPL
GCL

**Figure 26** AGB signals activated by NMDA in both normal- and dark-reared rabbit retinas at different postnatal stages. The basal AGB permeation after incubating the retina with 25 mM AGB in the absence of glutamate receptor agonists showed the presence of weak endogenous AGB signal. When the retinas were incubated with 25 mM AGB in the presence of NMDA at either 100 or 500 μM, AGB permeation increased in the inner retina neurons in a dose-dependent manner. The AGB signals activated by 500 μM NMDA in the dark-reared rabbit retinas showed a similar trend to the normal-reared rabbit retinas. Scale bar, 20 μm.

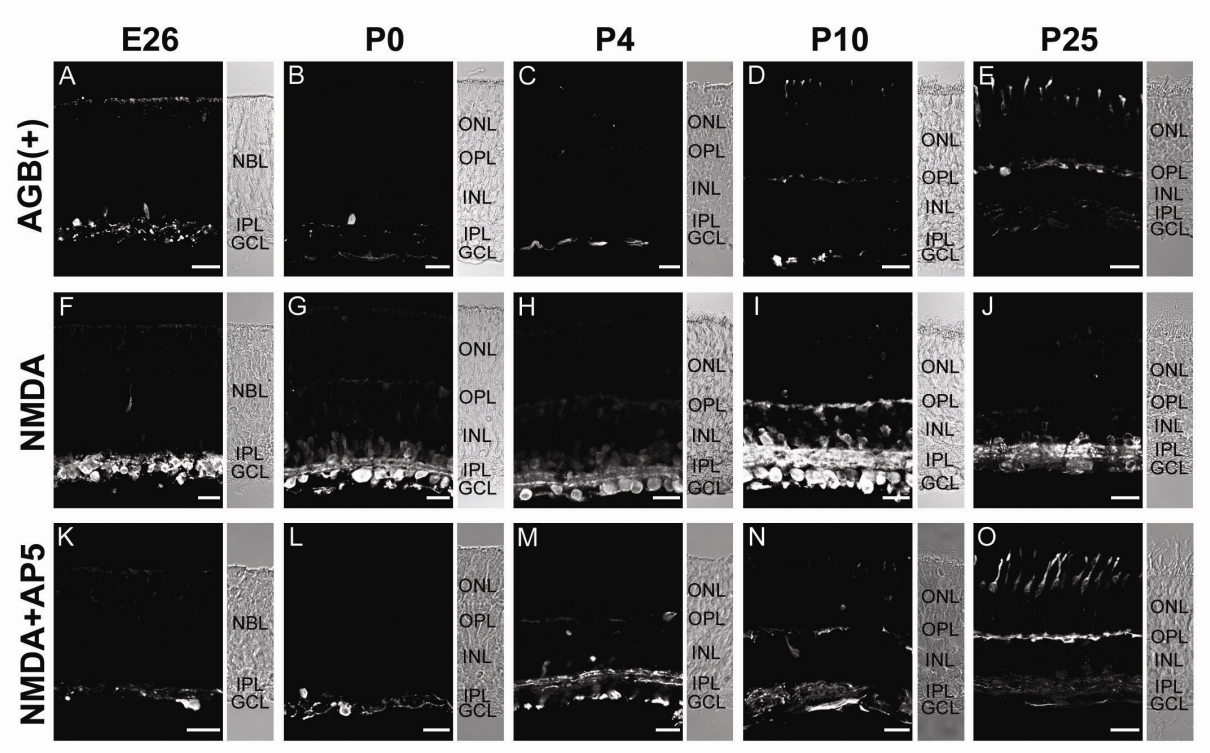


**Figure 27** AGB signals in the presence of 500 μM NMDA is agonist specific in the developing rabbit retina. Co-treatment with 500 μM NMDA and 50 μM AP5 (a NMDA receptor antagonist) drastically reduced the level of AGB signal during basal AGB permeation at each stage of the developing rabbit retina. Scale bar, 20 μm.

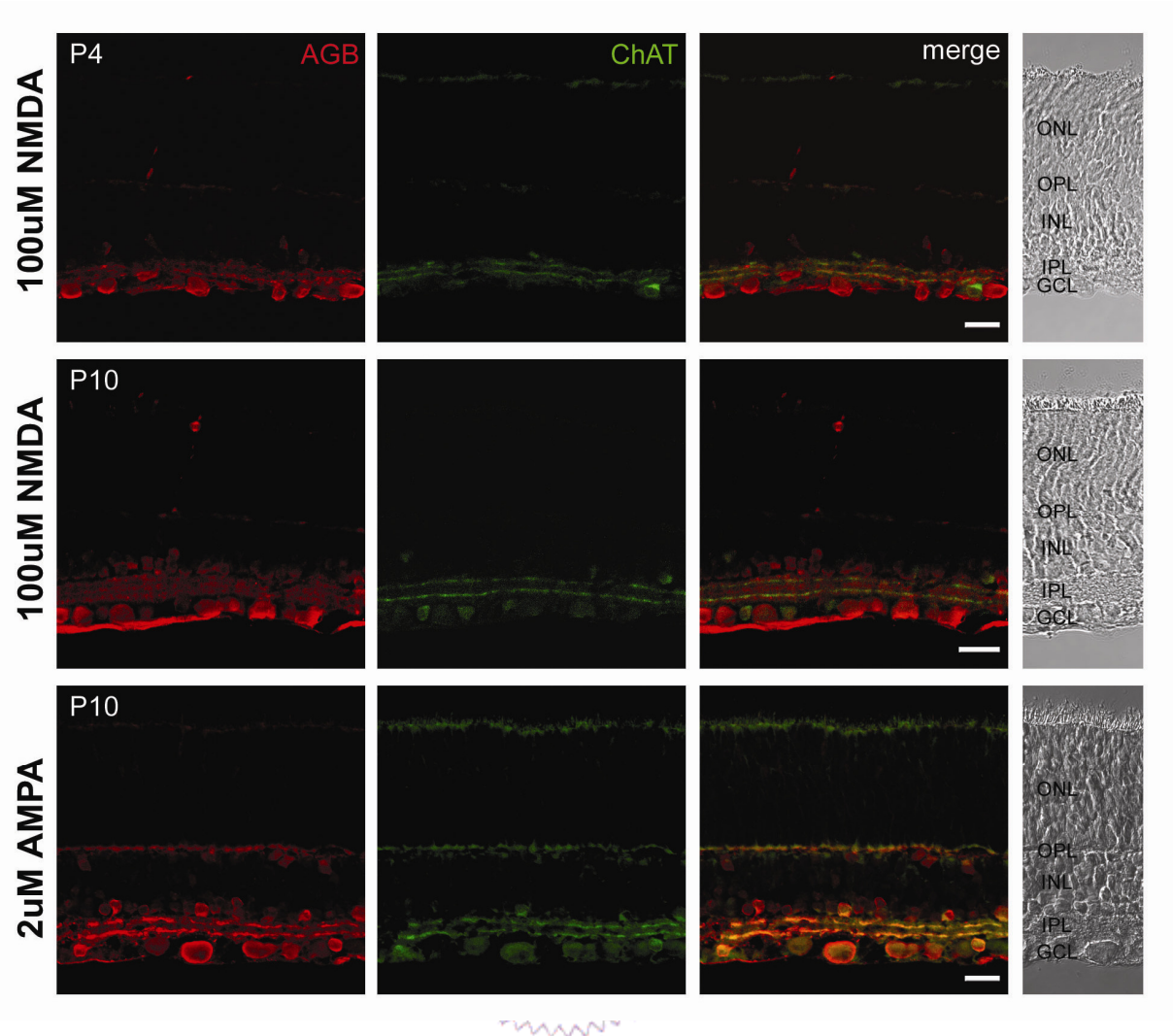


**Figure 28** The correlation between the AGB signals activated by glutamate agonists (100 μM NMDA or 2 μM AMPA) and the immunoreactivity of ChAT in the developing rabbit retina. All retinas were incubated with 25 mM AGB in the presence of glutamate agonists. After incubation, the retinal slices were immunostained to reveal the AGB and ChAT signals. The confocal images show double-labeling of AGB (red) and ChAT (green) in the inner retina. The merged images are shown in the right column to illustrate the co-localization of the cholinergic amacrine cells and various different glutamate agonist activated cells. Scale bar, 20 μm.

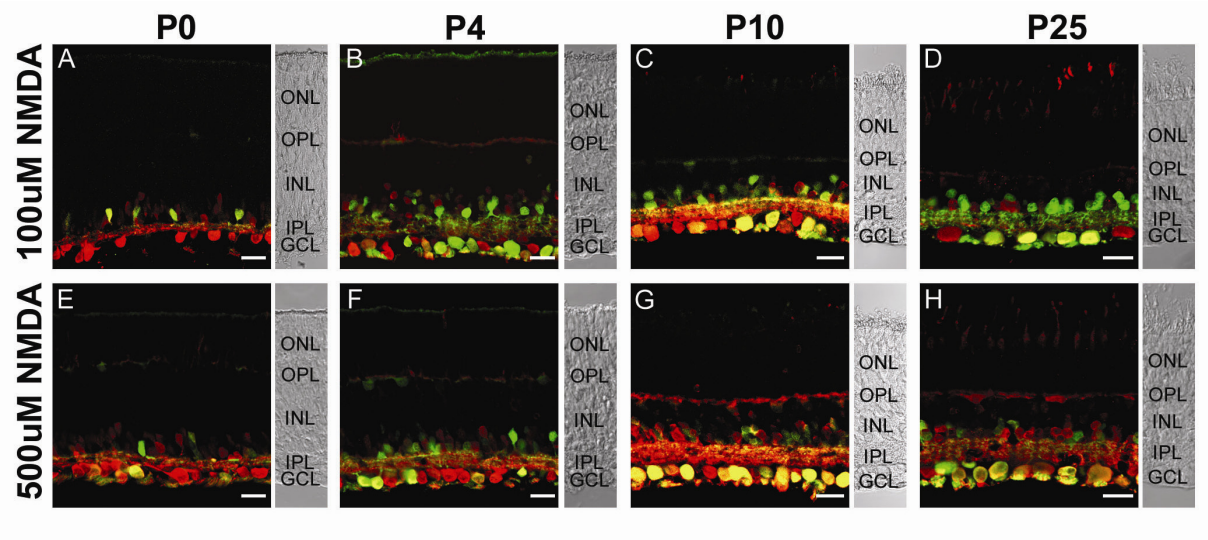


**Figure 29** The correlation between AGB signals activated by different concentrations of NMDA and calretinin immunoreactivity in the rabbit retina at different developmental stages. (A)-(D) AGB permeation (red) activated by 100 μM NMDA. (E)-(H) AGB signals activated by 500 μM NMDA. A few calretinin positive cells (green) in the INL appeared to co-localize with AGB permeation throughout all developmental stages (P0-P25), and some calretinin positive cells in the GCL co-localize with AGB signals only during various late developmental stages (P10-P25). Scale bar, 20 μm.