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Light triggers expression of philanthotoxin-insensitive Ca\(^{2+}\)-permeable AMPA receptors in the developing rat retina

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Ca\(^{2+}\)-permeable AMPA receptors (AMPARs) are expressed throughout the adult CNS but yet their role in development is poorly understood. In the developing retina, most investigations have focused on Ca\(^{2+}\) influx through NMDARs in promoting synapse maturation and not on AMPARs. However, NMDARs are absent from many retinal cells suggesting that other Ca\(^{2+}\)-permeable glutamate receptors may be important to consider. Here we show that inhibitory horizontal and AII amacrine cells lack NMDARs but express Ca\(^{2+}\)-permeable AMPARs. Before eye-opening, AMPARs were fully blocked by philanthotoxin (PhTX), a selective antagonist of Ca\(^{2+}\)-permeable AMPARs. After eye-opening, however, a subpopulation of Ca\(^{2+}\)-permeable AMPARs were unexpectedly PhTX resistant. Furthermore, Joro spider toxin (JSTX) and IEM-1460 also failed to antagonize, demonstrating that this novel pharmacology is shared by several AMPAR channel blockers. Interestingly, PhTX-insensitive AMPARs failed to express in retinae from dark-reared animals demonstrating that light entering the eye triggers their expression. Eye-opening coincides with the consolidation of inhibitory cell connections suggesting that the developmental switch to a Ca\(^{2+}\)-permeable AMPAR with novel pharmacology may be critical to synapse maturation in the mammalian retina.

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AMPAR ionotrophic glutamate receptors (iGluRs) are prominent excitatory neurotransmitter receptors expressed in the CNS. They assemble as tetramers from four possible subunits (i.e. GluR1–4) that impart distinct functional properties (Dingledine et al. 1999). The GluR2 subunit is critical since it regulates important characteristics such as Ca"^2+" permeability (Burnashev, 1996), external and internal polyamine block (Bowie et al. 1999), the pore’s unitary conductance (Dingledine et al. 1999) as well as subunit assembly and stoichiometry (Greger et al. 2003). GluR2 homomers are Ca"^2+" impermeable, insensitive to polyamine block and exhibit a small unitary conductance (Bowie et al. 1999; Dingledine et al. 1999). In contrast, GluR2-lacking AMPARs are Ca"^2+" permeable, blocked by polyamines and exhibit large single-channel currents (Bowie et al. 1999; Dingledine et al. 1999). Although most synaptic AMPARs are GluR2-containing heteromers (Dingledine et al. 1999), discrimination between Ca"^2+"-permeable and -impermeable isoforms has been difficult to achieve using ion-permeability measurements or imaging studies. As an alternative, polyamines, such as PhTX, have been used as pharmacological markers of Ca"^2+"-permeable AMPARs (Toth & McBain, 1998; Laezza et al. 1999; Liu & Cull-Candy, 2000; Thiagarajan et al. 2005; Plant et al. 2006; Ge et al. 2006). This approach is based on the observation that recombinant AMPAR heteromers are rendered Ca"^2+" impermeable with fewer GluR2 subunits than required to eliminate polyamine block (Washburn et al. 1997; Cull-Candy et al. 2006). Consequently, AMPARs sensitive to external polyamine block are considered, from a conservative standpoint, to be Ca"^2+" permeable whereas the absence of block identifies Ca"^2+"-impermeable isoforms.

Although Ca"^2+"-permeable AMPARs are expressed sparingly, they serve important roles in CNS physiology and pathology. Ca"^2+" influx following AMPAR stimulation is associated with long-term plasticity at neuronal (Laezza et al. 1999; Liu & Cull-Candy, 2000) as well as neuron–glial (Ge et al. 2006) synapses. In contrast, relief from polyamine block (Bowie et al. 1998) provides synaptic AMPARs with

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a novel form of short-term plasticity (Rozov & Burnashev, 1999). In disease states, Ca$^{2+}$-permeable AMPARs have been implicated in ischaemia (Liu et al. 2004), epilepsy (Krestel et al. 2004) and the growth of glioblastomas (Ishiuchi et al. 2002). Despite this understanding of Ca$^{2+}$-permeable AMPARs, little is known of their role in development. In the retina, for example, although glutamatergic transmission is known to drive propagating Ca$^{2+}$ waves during development (Wong & Wong 2000; van Zundert et al. 2004) and the growth of glioblastomas (Ishiuchi et al. 2002). Despite this understanding of Ca$^{2+}$-permeable AMPARs, little is known of their role in development. In the retina, for example, although glutamatergic transmission is known to drive propagating Ca$^{2+}$ waves during development (Wong & Wong 2000; van Zundert et al. 2004), all investigations so far have focused on the role of NMDARs (Wong & Wong, 2000; van Zundert et al. 2004).

Here, we have used a multidisciplinary approach to examine Ca$^{2+}$-permeable AMPARs during postnatal development of the mammalian retina. We show that Ca$^{2+}$-permeable AMPARs are primarily expressed in the rat retina by two inhibitory cell types, horizontal and AII amacrine cells. Prior to eye-opening, Ca$^{2+}$-permeable AMPARs are fully blocked by PhTX as reported elsewhere in the CNS. Unexpectedly however, after eye-opening, Ca$^{2+}$-permeable AMPARs exhibit insensitivity to PhTX. This developmental switch occurs at a time point when neuronal connections are being established with presynaptic bipolar cells suggesting that synapses of distinct Ca$^{2+}$-permeable AMPARs may fulfil important roles in synapse maturation.

**Methods**

**Dissection of the retina and slice preparation**

All experiments were performed on neonatal and adult Sprague–Dawley rats (male or female). Light-adapted animals were maintained on a 12 h light–dark cycle in accordance with the guidelines established by the Canadian Council on Animal Care. For light-deprived animals, pregnant mothers were housed in a separate room devoid of light and therefore pups were dark-reared from birth. In such cases, animals and the preparation of tissue was performed in a dim infrared light source to ensure strict conditions of light deprivation. All rats were decapitated under deep isoflurane anaesthesia, eyes were enucleated and hemisectioned in a cutting solution containing 233 mm sucrose, 7 mm α-(+)-glucose, 7 mm MgCl$_2$, 3.75 mm sodium pyruvate, 2.5 mm KCl, 1.25 mm NaH$_2$PO$_4$, 1 mm ascorbic acid, 0.5 mm CaCl$_2$, pH 7.4. The retina was then carefully detached from the pigment epithelium and sclera using a pair of fine dissecting forceps (No. 4, Dumostar).

For cobalt staining, the retina was cut into four pieces and oxygenated with 95% O$_2$–5% CO$_2$ at room temperature (22°C) for 30 min. For electrophysiology, the retina was chilled to 4°C with ice slush prepared from oxygenated cutting solution. The retina was then transferred in cutting solution to the upper surface of a small cube of 4% agar. The retina was drained of excess cutting solution using absorbent tissue and promptly annealed to the agar cube using a drop of 2% low melting point agar prepared at 42°C in 119 mm NaCl, 2.5 mm KCl, 10 mm glucose, 26 mm Hepes, 1.25 mm Na$_2$HPO$_4$, 2 mm sodium pyruvate, 0.5 mm ascorbic acid, 2.5 mm CaCl$_2$, 1.5 mm MgCl$_2$, pH 7.4. The ensemble was then fixed to the platform of a vibratome (Leica, VT1000S) using cyanoacrylate adhesive and rapidly immersed into ice-cold oxygenated cutting solution. Slices of 200 μm were obtained, transferred to ACSF (125 mm NaCl, 2.5 mm KCl, 25 mm NaHCO$_3$, 2.5 mm CaCl$_2$, 1 mm MgCl$_2$, 10 mm glucose, 2 mm sodium pyruvate, 0.5 mm ascorbic acid, pH 7.4 using 5 n NaOH) and oxygenated at room temperature.

**Cobalt staining technique**

Ca$^{2+}$-permeable AMPARs were identified using a modified protocol of the cobalt (Co$^{2+}$) staining technique (Pruss et al. 1991). Experiments were performed in either normal light or infrared condition. In each case, dissected retina were first incubated for 30 min in oxygenated assay buffer (5 mm KCl, 2 mm MgCl$_2$, 12 mm glucose, 20 mm bicarbonate, 139 mm sucrose, 57.5 mm NaCl and 0.75 mm CaCl$_2$) which, when required, provided a sufficient period to allow receptor antagonists to equilibrate with the tissue. The retina pieces were then stimulated each for 15 min in oxygenated assay buffer containing 5 mm CoCl$_2$ with saturating agonist concentration (1-glutamic acid (10 mm l-Glu), N-methyl-d-aspartate (50 μM NMDA) or (1)-(++)-2-amino-4-phosphonobutyric acid (40 μM AP4)) in the presence or absence of GluR antagonists (6-cyano-7-nitroquinoxaline-2,3-dione (20 μM CNQX) (DL)-2-amino-5-phosphonovaleric acid (40 μM APV), 4-(8-methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepine-5-yl)-benzenamine hydrochloride (40 μM GYKI 52466) (RS)-(S)-cyclopropyl-4-phosphonophenylglycine (40 μM CPPG)). Channel blockers, PhTX 343 tris(trifluoroacetic acid, pH 7.4 using 5 n NaOH) and IEM-1460, were purchased from Sigma, and Joro spider toxin-3 (JSTX) was a gift from Dr Heather Durham (Montreal Neurological Institute, McGill University). All other drugs were purchased from Tocris (Missouri, USA), stored frozen in stock solutions at −20°C and were then diluted into ACSF or assay buffer as required. Retina pieces were then rinsed in assay buffer and incubated in 2 mm EDTA for 5 min to remove excess Co$^{2+}$. After an additional rinse, Co$^{2+}$ was precipitated with 0.24% ammonium sulphide in assay buffer and the retina fixed for 4 h in 0.8% glutaraldehyde in phosphate buffer. Each retina piece was cryoprotected overnight at 4°C in phosphate buffer saline (PBS) containing 30% sucrose. The retina was then embedded in OCT (Tissue Tek, CA, USA) and snap-frozen at −50°C with 2-methylbutane. Sections of 20 μm were cut on a cryostat (2700 Frigcut, Riechert-Jung) and rinsed...
with distilled water. Silver enhancement of the cobalt sulphide precipitate was performed using the intenSE kit (Amersham, USA). The enhancement step is both time- and temperature-sensitive. To obtain the optimal reaction time (~30–40 min), we routinely constructed a calibration curve of Co$^{2+}$ staining at different time points at room temperature. After silver enhancement, each retinal section was rinsed and mounted in 10% glycerol in PBS. Photomicrograph images were captured with a Zeiss AxioPlan 2 Imaging microscope equipped with a high resolution colour digital camera and connected to a computer with Zeiss Axiovision 4.1 Software (Zeiss, Canada). Photomicrographs were obtained with a 40× objective and stored in the Zeiss Axiovision format. To avoid variation in staining intensity, the exposure settings of the digital camera for all samples were kept constant. Moreover, staining between control and treated conditions shown in all figures of this study were compared from retina taken from the same animal and that was processed for Co$^{2+}$ staining in parallel. To ensure reproducibility, all Co$^{2+}$ staining experiments shown in this study were repeated on several retinae pieces from at least three different animals.

**Image analysis and quantification**

Images from bright-field microscopy were obtained in black and white format and imported into an MCID Elite Image Analysis system (Imaging Research Inc., St Catharines, Ontario, Canada). In each case, measurements of the relative optical density were performed by the system within the limits of a rectangle of 90 μm × 8.1 μm which was applied to the outer or inner plexiform layers (OPL or IPL) of the retina that included either horizontal cells or sublamina a and b, respectively. A total number of eight images were obtained per retina slice which was repeated for eight adult rats. The data between different treatments were compared using a paired t test and expressed as the mean ± s.e.m.

**Immunohistochemistry**

Retina pieces prepared for Co$^{2+}$ staining were used for immunofluorescence. In this case, however, each retina piece was fixed using 4% paraformaldehyde in 0.2 M phosphate buffer at pH 7.4 for 2 h and cryoprotected in 30% sucrose buffer overnight at 4°C. Retinae were embedded in OCT Tissue Tek and frozen as previously described. Sections of 20 μm were then cut on a cryostat and processed for confocal microscopy as on-slide sections. Briefly, slides were rinsed three times for 5 min in 0.1 M PBS–0.3% Triton X-100 (PBS-T) and blocked in 10% normal goat serum in PBS-T. Sections were incubated overnight with a rabbit anti-calbindin-D 28K antibody (1: 2000, Sigma) a specific marker for horizontal cells (Oguni et al. 1998). Slides were subsequently washed as before in PBS-T and incubated with a goat anti-rabbit IgG Alexa 488 (1: 800, Molecular Probes, USA) secondary antibody. After washing in PBS, slides were mounted using an anti-fading medium (Vectashield, Vector Laboratories) and observed on a Zeiss 510 LSM confocal microscope.

**Electrophysiology recordings**

Acutely isolated retinal slices were visualized using an infrared (IR) video camera (Dage-MTI, Michigan City, IN, USA) attached to an upright microscope (Olympus, Japan) that was equipped with 60× differential interference contrast (DIC) objective. All amacrine cells were visually identified by their thick dendritic processes and triangular-shaped cell bodies that were positioned at the junction of the inner nuclear (INL) and plexiform layers (IPL) (Fig. 2A). In contrast, cell bodies of horizontal cells were oval-shaped, much larger in size and were positioned horizontally in the proximal region of the INL (Fig. 2B). The identity of each cell type was confirmed post hoc using Lucifer Yellow to provide complete visualization of dendritic morphology. All whole-cell recordings were performed on light-adapted retina using borosilicate glass pipettes (7–10 MΩ) and a MultiClamp 700A amplifier (Axon Instruments, Union City, CA, USA) in voltage-clamp mode. Membrane currents were digitized at 5 kHz using the Digidata 1322A (Axon Instruments) and filtered at 2.5 kHz using a low-pass Bessel filter (Frequency Devices, MA, USA). Cells with series resistances < 25 MΩ that were compensated up to 80% were used for analysis. In all cases, recordings were monitored to ensure stability in series resistance and the event amplitude. All amacrine cells and horizontal cells had cell capacitances of 13.7 ± 5.2 pF (n = 30) and 42.4 ± 15.9 pF (n = 8), respectively. All recordings were performed at room temperature (22°C). In experiments performed on dark-reared animals, the entire electrophysiological experiment was performed in dim infrared conditions.

Oxygenated ACSF used to bathe retinal slices routinely contained bicuculline (10 μM) and strychnine (1 μM) to block GABAergic and glycinegic synaptic activity. Although presynaptic GABA<sub>C</sub> receptors expressed by rod bipolar cells are not blocked by bicuculline (Łukasiewicz et al. 2004), their activation regulates the frequency of glutamatergic activity onto AII cells which was not examined in this study. In experiments where miniature synaptic events were recorded, tetrodotoxin (TTX, 0.5 μM) was also added to the ACSF to eliminate spontaneous activity. The internal solution contained 125 mM CsCH<sub>3</sub>SO<sub>3</sub>, 5 mM Hepes, 4 mM Na<sub>2</sub>ATP, 5 mM Cs<sub>2</sub>BAPTA, 1 mM CaCl<sub>2</sub>, 2 mM QX-314 bromide to block...
sodium channels and 0.5% biocytin HCl with 0.5 mg ml$^{-1}$ Lucifer Yellow to visualize cell morphology. pH was adjusted to 7.4 with 5 n CsOH. Local agonist/antagonist applications were performed using a homemade flowpipe from theta tubing that had been pulled to a final tip diameter of 300–400 μm. The tip of the theta tubing was routinely placed within 1 mm of the cell chosen for study.

Data analysis

Data acquisition and analysis was performed using pCLAMP9 (Molecular Devices) software and illustrated using Origin 7 (Microcal, Northampton, MA, USA). Extended structures of channel blockers (Fig. 6A) were drawn using ChemDraw 8 (CambridgeSoft, Cambridge, MA, USA). Miniature and spontaneous synaptic activity was analysed using WinEDR (Windows Electrophysiology Disk Recorder) that was kindly provided by Dr John Dempster (Strathclyde University, UK). Synaptic events were primarily detected by an amplitude threshold algorithm set to identify events that were four times the standard deviation of baseline noise. This was followed by visual confirmation to eliminate false positives due to variable baseline noise levels or multiphasic events. The threshold method did not affect the outcome of analysis described in this study since similar results were achieved using a template-based algorithm for event detection (Clements & Bekkers, 1997). Data were rejected for analysis if it showed signs of time-dependent decline in amplitude or of the kinetic parameters. Amplitude distributions were fitted by the sum of two to three Gaussian functions of the singular form:

$$y = \frac{\text{Area}}{(\text{Variance} \times \nu(\pi/2))} \times \exp(-2((x - \text{Peak})/\text{Variance})^2)$$

where y refers to the number of observed events and x to the peak amplitude which has units of picoamps. For Gaussian fits of pooled data (e.g. Figs 7 and 10), biasing was avoided by selecting a similar number of events from each cell. The number of Gaussian functions required to fit amplitude distributions is model independent and therefore is not used in this study to indicate distinct populations of AMPAR synapses. Likewise, the distribution of decay kinetics was fitted by the sum of two to three exponential functions that were used in this study in a model-independent manner.

Biocytin labelling

After completion of each electrophysiological experiment, retina slices were fixed in 4% paraformaldehyde for 30 min at room temperature and stored at 4°C until processed for biocytin staining. Retina slices were then washed in sterile PBS and incubated for 10 min in PBS containing 10% methanol and 1% H$_2$O$_2$. They were subsequently washed and transferred for 1 h to 2% Triton dissolved in PBS. A standard reaction of diaminobenzidine was then performed using the ABC kit from Vectastain (Burlingame, CA, USA). After washes, slices were processed for 30 min using 3,3′-diaminobenzidine (DAB) prepared with 1% CoCl$_2$ and 1% NiCl$_2$, rinsed and mounted in 10% glycerol in PBS.

Results

Ca$^{2+}$-permeable AMPARs are expressed primarily by inhibitory cells

The distribution of Ca$^{2+}$-permeable AMPARs was initially examined in the adult rat retina using Co$^{2+}$ staining. The advantage of this technique is that divalent-permeable AMPARs will transport other divalent ions, such as Co$^{2+}$, in addition to Ca$^{2+}$. The transported Co$^{2+}$ can then be revealed as a dark brown precipitate identifying the cell expressing Ca$^{2+}$-permeable AMPARs. All Co$^{2+}$ staining experiments shown in this study were repeated on several retinae pieces from animals taken from at least three different rat litters. Figure 1 (left panel) shows a photomicrograph of a transverse section of the adult retina (i.e. 3 months old) stained with Co$^{2+}$ following stimulation with 10 mM l-glutamate (l-Glu). Two populations of cell bodies were primarily stained with Co$^{2+}$ with their processes visible either as lateral or vertical extensions labelled in the outer or inner plexiform layers (OPL or IPL), respectively (Fig. 1, left panel). Cells stained at the OPL–INL interface are consistent with inhibitory horizontal cells based on their morphology and immunopositive staining with the selective marker, calbindin (Oguni et al. 1998) (Fig. 1, upper middle panel). Cells stained at the INL–IPL interface are consistent with AII (A2) amacrine cells, another inhibitory cell in the mammalian retina (Famiglietti & Kolb, 1975). In agreement with this, injection of biocytin into visually identified AII cells in acutely isolated retinal slices revealed that the position of the cell body and that of the proximal dendrites closely matched that of Co$^{2+}$-stained cells (Fig. 1, lower middle panels).

Co$^{2+}$ staining with l-Glu is due to Ca$^{2+}$-permeable AMPARs and not other GluRs subtypes for several reasons. First, Co$^{2+}$ staining was blocked by GYKI 52466 (40 μM), a selective AMPA-receptor antagonist (Dingledine et al. 1999) (Fig. 1) and not APV (20 μM) or CPPG (40 μM) which block NMDARs or metabotropic GluRs (mGluRs), respectively (Fig. 1, right). Second, selective agonists NMDA (50 μM) or AP4 (40 μM) failed to stain the retina (Fig. 1, right) confirming that Co$^{2+}$ staining by l-Glu does not involve NMDARs or mGluRs. Third, Co$^{2+}$ does not enter cells via voltage-gated Ca$^{2+}$ channels since depolarization with 50 mM KCl failed to elicit staining.
(data not shown) consistent with the known blocking effect of Co\textsuperscript{2+} on Ca\textsuperscript{2+} channels. As described below, complementary electrophysiological experiments further demonstrate that, unlike other retinal cells, the primary route of Ca\textsuperscript{2+} entry into horizontal and AII amacrine cells during glutamatergic transmission is via AMPA and not NMDA iGluRs.

**Inhibitory cells express synaptic AMPARs but not NMDARs**

The contribution of AMPA and NMDARs to glutamatergic transmission was assessed by the ability of subtype-selective iGluR antagonists to block excitatory postsynaptic currents (EPSCs) (Fig. 2). To do this, AII and horizontal cells were first visually identified in retinal slices by morphology (Fig. 2A and B) and later confirmed by intracellular filling with Lucifer yellow. As expected from Co\textsuperscript{2+} experiments, individual AII and horizontal cells responded robustly to local application of non-NMDAR agonists such as kainate (50 \textmu M KA, Fig. 2C) and L-Glu (10 mM, not shown). Moreover, EPSCs and responses to exogenous agonists on AII cells were equally sensitive to CNQX (Fig. 2C, \( n = 11 \)) further validating the complementary nature of Co\textsuperscript{2+} labelling and electrophysiological data. In contrast, individual EPSCs were poorly resolved in horizontal cells due to the membrane noise (\( n = 8 \)) and, in some cases, an appreciable holding current between -20 to -100 pA was present (\( n = 3 \), Fig. 2D). In each cell type, however, synaptic activity was insensitive to the NMDAR antagonist APV (40 \textmu M, Fig. 2E and F) but completely abolished by GYKI52466 (40 \textmu M, \( n = 4 \) for each cell type) (Fig. 2E and F) demonstrating that horizontal and AII amacrine cell synapses express AMPARs with little or no contribution from NMDARs. Interestingly, Hartveit & Veruki (1997) have described responses elicited by AII cells following bath application of NMDA suggesting that, although this inhibitory cell population expresses NMDARs, they are excluded from the postsynaptic density.

![Figure 1](https://example.com/image1.png)

**Figure 1.** Ca\textsuperscript{2+}-permeable AMPARs are expressed by inhibitory cells of the mammalian retina

Left panels, photomicrograph of Co\textsuperscript{2+} staining routinely observed with 10 mM L-Glu in a 3-month-old adult rat retina (slide no. 290036-1L). Staining was restricted to processes of the outer and inner plexiform layers as well as cell bodies of horizontal and AII amacrine cells. Co\textsuperscript{2+} staining elicited by 10 mM L-Glu was abolished by pretreatment with 40 \textmu M GYKI52466, an AMPAR selective antagonist. Middle upper panel, photomicrograph of calbindin immunofluorescence identifying the cell bodies and processes of horizontal cells. Middle panel, lower right and left, photomicrographs of visually identified AII amacrine cells filled with biocytin to reveal the location of the cell body in the INL and extension of dendritic processes. Right panels, Co\textsuperscript{2+} staining elicited by 10 mM L-Glu was unaffected by pretreatment with NMDA or the mGluR antagonists, APV (40 \textmu M) (slide no. 070406-3L) and CPPG (300 \textmu M) (slide no. 070406-9L), respectively. NMDA and group III mGluR agonists, NMDA (50 \textmu M) (slide no. 070406-5R) and L-AP4 (100 \textmu M) (slide no. 070406-9L), respectively, also failed to produce appreciable staining suggesting that inhibitory cells accumulate Co\textsuperscript{2+} due to the activation of AMPARs. Unless otherwise stated, scale bars represent 20 \textmu m.
Expression of Ca\(^{2+}\)-permeable AMPARs prior to eye-opening

To examine if both horizontal and AII amacrine cells express Ca\(^{2+}\)-permeable AMPARs throughout retinal development, we studied their distribution in the postnatal rat retina before (P1 to P11, Fig. 3) and after eye-opening (P14 to 6 months, Fig. 4). All retinal cell types are present in the rat retina prior to eye-opening (i.e. < P10) (Rapaport et al. 2004) whereas synaptic contacts undergo further refinement before and after eye-opening (i.e. P1–P21) (Mumm et al. 2005; Morgan et al. 2006). In view of this, eye-opening is an important marker of distinct developmental phases in the rodent retina. It is not that eye opening per se is causative but, as discussed below, that the visual experience of light entering the eye triggers important activity-dependent events. At birth, the rat retina has immature morphology with a prominent neuroblastic layer though the outer segment of the photoreceptor layer is absent (Fig. 3, top outerleft). In comparison, a few days prior to eye-opening (e.g. P11), the retina exhibits adult-like morphology with a clear separation

Figure 2. Horizontal and all amacrine cells express synaptic AMPARs but not NMDARs

A, videomicrograph showing the position of the cell body close to the IPL (see arrowhead) and prominent dendrite characteristic of AII amacrine cells (see triangles) in acute retinal slice preparations. B, videomicrograph identifying the large triangular cell body of a typical horizontal cell (see arrowhead) and lateral dendritic processes (see triangles). C, whole-cell recording (\(V_h = -60\) mV) from an all amacrine cell showing spontaneous postsynaptic events (○) and inward current elicited by local application of 50 \(\mu\)M KA (cell no. 051208c3). Bath application of the non-NMDAR antagonist, CNQX (20 \(\mu\)M), blocked both spontaneous synaptic events and membrane current elicited by KA (●). D, whole-cell recording (\(V_h = -60\) mV) from a horizontal cell showing that the spontaneous activity and holding current were insensitive to APV (40 \(\mu\)M), but blocked by GYKI 52466 (40 \(\mu\)M) (cell no. 060620c1). E, spontaneous events recorded from another AII amacrine cell (cell no. 060516c3, \(V_h = -60\) mV) were insensitive to APV but blocked by GYKI 52466. F, spontaneous events recorded from the same horizontal cell as in D showing in more detail that synaptic activity was insensitive to APV but blocked by GYKI 52466.
of plexiform and nuclear layers (Fig. 3, top innerleft). Consistent with this maturation pattern, Co\(^{2+}\) staining from P1 to P11 retinae showed signs of cell migration as well as morphological refinement (Fig. 3). For example, although immature horizontal cells have clearly migrated to their outer position in the retina shortly after birth (e.g. P1), further dendritic refinement is apparent at stages P4 and P7 (Fig. 3, bottom left). These cells can be identified as horizontal cells since they were immunopositive to calbindin (data not shown). Cells labelled in the inner region of the retina between P1 and P7 are most likely immature amacrine cells which have more distinctive staining by P11. Interestingly, like the adult retina (Fig. 1), Co\(^{2+}\) staining of the ganglion cell layer (GCL) and INL suggests that AII cells are not the only cell type in this region of the retina labelled at P11 (Fig. 3, see below). As expected of Ca\(^{2+}\)-permeable AMPARs (Washburn et al. 1997; Toth & McBain, 1998), Co\(^{2+}\) staining was completely abolished from P1 to P7 by PhTX (50 \(\mu\)M) (Fig. 3), a polyamine channel blocker of Ca\(^{2+}\)-permeable non-NMDARs (Bowie et al. 1999). At P11, however, Co\(^{2+}\) staining was not fully blocked in both horizontal and amacrine cells (Fig. 3). The differential effect of PhTX cannot be explained by differences in the total number of AMPARs expressed by individual cells since the intensity of staining in all cases was comparable. Consequently, pharmacologically distinct Ca\(^{2+}\)-permeable AMPARs are apparently expressed in the developing retina.

Both external polyamine block and Ca\(^{2+}\) permeability are determined by the copy number of GluR2 subunits in mature AMPAR tetramers (Washburn et al. 1997; Bowie et al. 1999; Dingledine et al. 1999). Loss of polyamine block is proposed to require the inclusion of more GluR2 receptor subunits per tetramer than for the loss of divalent permeability (Washburn et al. 1997). Consequently, it is not possible to assemble mature AMPARs that exhibit divalent permeability whilst having little or no sensitivity to polyamine block (Washburn et al. 1997; Bowie et al. 1999). In view of this, our observation that Ca\(^{2+}\)-permeable AMPARs are insensitive to polyamine block is difficult to reconcile with current understanding of recombinant AMPARs.

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**Figure 3. Expression of Ca\(^{2+}\)-permeable AMPARs before eye-opening**

Top left, outer and inner panels, cresyl violet staining of retina reveals an immature retina morphology at P1 (slide no. 090405-3L) with a prominent neuroblastic layer compared with the well-defined cell layers seen at P11 (slide no. 190405-5L). Middle left, outer and inner panels, Co\(^{2+}\) staining at P1 (slide no. 090405-7L) was blocked by 50 \(\mu\)M PhTX revealing the presence of Ca\(^{2+}\)-permeable AMPARs at birth. Bottom left, outer and inner panels, Co\(^{2+}\) staining with 10 \(\mu\)M L-Glu in P4 (slide no. 120405-6L) and P7 (slide no. 150405-10L) retinae showing the migration and maturation of horizontal and amacrine cells. Middle and right panels, Co\(^{2+}\) staining elicited by L-Glu at P11 (slide no. 190405-2R) failed to be completely blocked by 50 \(\mu\)M PhTX suggesting that Ca\(^{2+}\)-permeable AMPARs develop insensitivity to polyamine block a few days before eye-opening. Scale bars represent 10 \(\mu\)m in all cases.
Figure 4. Inhibitory cells express PhTX-insensitive Ca\(^{2+}\)-permeable AMPARs after eye-opening

Left panels, after eye-opening horizontal and AII amacrine cells were strongly labelled by Co\(^{2+}\) at all developmental stages tested (P14–18, slide no. 220405-7R; P21–28, slide no. 250405-8R; P33–6 months: slide no. 260105-11L). Expression patterns in the INL are further refined during development with the transient expression of Ca\(^{2+}\)-permeable AMPARs in another amacrine cell population(s) that may be A17 cells. Note there is a greater intensity of staining in sublamina b of the IPL (see arrowhead). Right panels, PhTX failed to fully block Co\(^{2+}\) staining after eye-opening at all developmental stages. Horizontal cell dendrites in particular were completely insensitive to block by PhTX. For all cells, Co\(^{2+}\) staining of cell bodies was initially PhTX insensitive (P14–18, slide no. 220405-7R; P21–28, slide no. 250405-8R, respectively). However, at later stages, the staining of cell bodies was abolished by PhTX. In contrast, staining of the IPL was resistant to PhTX block at all stages.

**PhTX-insensitive Ca\(^{2+}\)-permeable AMPARs are expressed after eye-opening**

After eye-opening, different time points were examined for Co\(^{2+}\) staining (i.e. P14, 18, 21, 26, 28, 33, 39 as well as 3 and 6 months). Based on the labelling pattern, we identified three distinct stages: P14–18, P21–28 and P33–6 months. In each case, Co\(^{2+}\) strongly labelled the cell bodies and dendrites of horizontal cells, demonstrating that the expression of Ca\(^{2+}\)-permeable AMPARs continued into adulthood (Fig. 4, left panels). Likewise, cell bodies of AII amacrine cells were stained by Co\(^{2+}\) throughout development; however, their dendrites were visible only in the first few days after eye-opening (e.g. P14–18). At later stages, processes arising from individual AII cells were indistinguishable from the appreciable staining of the entire IPL (e.g. P21–28). The IPL is a meshwork of bipolar cell axons and dendrites from amacrine and ganglionic cells that each terminate into functionally distinct sublayers called sublamina a or b (Mumm et al. 2005) (Fig. 4, P21–28). The narrow dendritic arbor of AII amacrine cells, for example, traverses the entire IPL as revealed by filling individual cells with biocytin (Fig. 5A, left). It is interesting therefore that at all developmental stages examined, we did not observe a uniform intensity of staining in the IPL but that stronger labelling occurred in sublamina b (see arrow in Fig. 4, left, and also Fig. 5B, left). Indeed, statistical evaluation (P < 0.05, Student’s two tailed, paired t test) of Co\(^{2+}\) labelling in the adult IPL revealed that the optical density of sublamina b (0.40 ± 0.03 arbitrary units (a.u.)) was almost 2-fold greater than sublamina a (0.25 ± 0.02 a.u.) (Fig. 5B, right).

It is unlikely that staining of the IPL arises solely from AII cell dendrites but that processes of other cell types also contribute. In support of this, the cell bodies of another population(s) of putative amacrine cells in the INL were clearly labelled during the first week following eye-opening and remained until P21–28. We did not attempt to determine their identity though, based on the position of the cell body (Menger & Wassle, 2000), the staining is consistent with A17 cells which are known to express Ca\(^{2+}\)-permeable AMPARs (Chavez et al. 2006). Similarly, we observed labelling of a population of cells in the GCL (e.g. P14 –P18) that disappeared during retinal maturation. As before, we did not examine this population

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in detail though our observations are consistent with a previous study of Ca\(^{2+}\)-permeable AMPARs expressed by displaced amacrine cells and/or ganglion cells (Zhang et al. 1995).

To determine if Ca\(^{2+}\)-permeable AMPARs are blocked by extracellular polyamines after eye-opening, staining by 10 mM L-Glu was compared in the presence and absence of 50 \(\mu\)M PhTX (Fig. 4, right panels). At all postnatal stages tested, PhTX failed to completely block Ca\(^{2+}\) staining of the cell bodies and dendrites of horizontal cells. In support of this, a comparison between the optical density of staining in the OPL in control (0.40 ± 0.02 a.u.) and PhTX-treated adult retinae (0.43 ± 0.05 a.u.) was statistically indistinguishable (Fig. 5B, right). For all amacrine cells, the effect of PhTX was more complex. At stages P14–18 and P21–28, a subpopulation of AII cells was transiently PhTX insensitive. However, from postnatal period P33 onwards, staining of cell bodies was completely eliminated by PhTX (Fig. 4, lower right). In contrast, however, PhTX failed to completely block Ca\(^{2+}\) staining in the IPL. In sublamina a, the intensity of staining remained unchanged between control (0.25 ± 0.01 a.u.) and PhTX-treated (0.28 ± 0.03 a.u.) retina (Figs 4 and 5B, right). Interestingly, staining in sublamina b was reduced by almost 50% between control (0.40 ± 0.03 a.u.) and PhTX-treated (0.25 ± 0.03 a.u.) retinae (Fig. 5B, right). AII amacrine cells are innervated by two distinct presynaptic cell types each of which terminate in different sublamina of the IPL. Rod bipolar cells form ribbon synapses on the arboreal dendrites of sublamina b whereas OFF-cone bipolar cells form ribbon synapses on the arboreal dendrites of sublamina a (Kolb & Famiglietti, 1974; Strettoi et al. 1992; Chun et al. 1993; Veruki et al. 2003). As discussed below, the reduction of Ca\(^{2+}\) staining in sublamina b and not sublamina a argues against the expression of a single population of Ca\(^{2+}\)-permeable AMPARs on AII cell dendrites, but rather, supports the segregation of pharmacologically distinct receptors into different sublaminae of the IPL (Fig. 5A, right).

Ca\(^{2+}\)-permeable AMPARs are resistant to block by other channel blockers

To examine if PhTX-insensitive AMPARs were resistant to other channel blockers, we compared Ca\(^{2+}\) labelling elicited by 10 mM L-Glu in the presence and absence of JSTX (10 \(\mu\)M) or IEM-1460 (100 \(\mu\)M) (Fig. 6). Compared with the structure of PhTX, both JSTX and IEM-1460 differ in terms of their polyamine chain length and the chemical nature of the bulky headgroup (Fig. 6A). Consequently, if Ca\(^{2+}\) staining due to Ca\(^{2+}\)-permeable AMPARs remained in the presence of all three blockers, our conclusions would not rely solely on the prescribed action of PhTX. In agreement with this, photomicrographs shown in Fig. 6B reveal that Ca\(^{2+}\) staining elicited by L-Glu was principally unaffected by either JSTX (10 \(\mu\)M) or IEM-1460 (100 \(\mu\)M), similar to our findings with PhTX (50 \(\mu\)M). The IC\(_{50}\) value for block of recombinant AMPARs by IEM-1460 (at −80 mV) and JSTX (at −100 mV) is 1.6 \(\mu\)M (Magazanik et al. 1997) and 30 nm (Blaschke et al. 1993), respectively. In view of this, the blocker concentrations used in this experiment are supramaximal in nature, further strengthening our conclusion that the retina expresses Ca\(^{2+}\)-permeable AMPARs with novel pharmacological properties.

Figure 5. All amacrine cell dendrites express two AMPAR populations

A, left, photomicrograph of an All amacrine cell filled with biocytin reveals the extent of its dendritic arborization in the IPL. Right, schematic diagram showing the possible spatial arrangement of different AMPARs on dendrites of individual All cells. B, left, photomicrographs comparing the effect of 50 \(\mu\)M PhTX (slide no. 260105-11R) on the staining elicited by 10 mM Glu (slide no. 260105-11R) in the IPL of a P36 retina. The dotted line demarcates between sublaminae a and b and the scale bar represents 20 \(\mu\)m. Right, images from bright-field microscope were obtained in black and white format and measurements of the relative optical density were determined for the OPL and sublaminae a and b of the IPL using an MCID Elite Image Analysis system (see Methods). A total number of eight images were obtained per treatment which was repeated for retinae taken from 8 adult rats. The data between different treatments were compared using a paired \(t\) test and expressed as the mean ± S.E.M.
All amacrine cell synapses express PhTX-insensitive Ca$^{2+}$-permeable AMPARs

To test if PhTX-insensitive AMPARs are expressed at synapses, electrophysiological recordings of all amacrine cells were performed on acutely isolated retinal slices. Figure 7A and B shows typical miniature EPSCs ($V_h = -70$ mV) recorded from the same amacrine cell in the absence and presence of 50 μM PhTX. In each case, synaptic activity was mediated by AMPARs since, as shown previously, all events were abolished by CNQX or GYKI 52466 (Fig. 2). In control conditions, mEPSC amplitude was best fitted by the sum of three Gaussian functions (Fig. 7C, red line) with values of $-14.1 \pm 0.1$ pA and $-12.0 \pm 0.1$ pA (Fig. 7B). Similar findings were observed in the absence of TTX suggesting that PhTX mainly acts postsynaptically and has little effect on neurotransmitter release. Finally, in agreement with our Co$^{2+}$-staining experiments, spontaneous ($n = 4$) and miniature ($n = 7$) EPSCs resistant to PhTX block were observed in all Amacrine cells tested irrespective of postnatal age (P19–P41, Fig. 7C).

PhTX slows decay kinetics of synaptic AMPARs

To examine if the kinetic properties of PhTX-insensitive AMPARs are distinct, we initially compared averaged waveforms of mEPSCs in the presence and absence of PhTX. Figure 8A shows averaged waveforms from a typical recording that reveals a slowing in mEPSC decay kinetics in the presence of PhTX. A more detailed analysis of the decay kinetics of all events prior to the application of PhTX were best fitted by the sum of three exponential functions with time constants of $0.90 \pm 0.01$ ms, $1.25 \pm 0.19$ ms and $1.85 \pm 0.60$ ms (Fig. 8B). The kinetics of synaptic events observed in the presence of PhTX were best fitted by the sum of two and not three exponentials (Fig. 8C). The time constants estimated from the fit were $1.26 \pm 0.03$ ms and $1.53 \pm 0.07$ ms (Fig. 8C), each of which corresponded to the slower time constants observed in the control (Fig. 8B). This observation suggests that PhTX may differentiate between two kinetically distinguishable AMPAR synapses. That is, larger synaptic events have faster decay kinetics and are sensitive to PhTX block. In contrast, smaller events have slower decay kinetics and are insensitive to PhTX block. We reasoned that if this was the case, both fast and slow decaying synaptic events should be observed in control conditions. Consequently, control synaptic events of small amplitude ($< -20$ pA) should have slow decay kinetics identical to events observed in PhTX. However, analysis of small amplitude events revealed that their kinetic properties were dissimilar from events observed in PhTX (Fig. 8D). The time constants estimated from the fit of the data were $0.97 \pm 0.01$ ms and $1.37 \pm 0.10$ ms, which closely matches the two main kinetic components observed for events of all amplitude (Fig. 8B). This observation shows that PhTX does not distinguish between kinetically distinct AMPAR populations. The mechanism by which PhTX slows channel kinetics is not clear but may involve channel-trapping of external polyamines that, in turn, affects burst length (Bähring & Mayer, 1998).

In summary, these observations demonstrate that all postsynaptic AMPARs expressed by All amacrine cells bind PhTX but differ in their sensitivity to block.

Light entering the eye triggers expression of novel Ca$^{2+}$-permeable AMPARs

To directly test if light entering the eye triggers expression of PhTX-insensitive AMPARs, we repeated Co$^{2+}$ staining...
(Fig. 9) and electrophysiology experiments (Fig. 10) in retinae taken from dark-reared animals (see Methods). With Co²⁺ labelling, the staining elicited by l-Glu alone was comparable to light-adapted retinae (Fig. 9, left). In contrast, however, PhTX completely abolished staining of the cell bodies of AII cells as well as staining in the IPL (Fig. 9, right). For horizontal cells, Co²⁺ staining, though weak, was significantly reduced in intensity indicating that light may not be the only factor regulating the expression of PhTX-insensitive AMPARs (Fig. 9, right).

In agreement with Co²⁺ staining experiments, AMPAR synaptic activity recorded from AII amacrine cells in dark-reared animals (Fig. 10A and C), was fully abolished by 50 μM PhTX (Fig. 10B and D). In the light-deprived retina, the amplitude of synaptic events exhibited similar properties to responses in the light-adapted retina (Fig. 7) suggesting that dark-rearing apparently does not affect the number of AMPARs at postsynaptic densities. In agreement with this, the peak amplitude distribution was best fitted by the sum of three Gaussian functions (Fig. 10C, red line) with amplitudes of $-31.4 \pm 1.9 \text{ pA}$.

![Figure 7. Synaptic AMPARs expressed by AII cells are resistant to PhTX block](image-url)

A, upper, synaptic activity ($V_h = -70 \text{ mV}$) recorded from an AII amacrine cell (cell no. 050131C2) in control conditions. Lower, summary plot from 7 cells showing that the amplitude of miniature synaptic events was fitted well by the sum of three Gaussian functions (red line). Individual Gaussian fits are shown as continuous black lines. Similar results were observed in the absence of TTX (data not shown). B, upper, in the same cell, bath application of 50 μM PhTX did not fully block synaptic activity but instead reduced peak response amplitude. Lower, in this case, the summary plot of peak synaptic current amplitude was fitted well by the sum of two Gaussian functions (red line). Individual Gaussian fits are shown as continuous white lines. C, plot summarizing the effect of PhTX on the amplitude of spontaneous ($n = 4$) and miniature ($n = 7$) synaptic events from 11 cells. In all cases, analysis was performed only in cells where synaptic events were recorded in presence and absence of 50 μM PhTX. Data have been grouped on whether recordings were performed on postnatal retina of 3 weeks ($n = 6$) or more ($n = 5$). Filled (control) and open (+ 50 μM PhTX) symbols (triangle, circle and square) in each column represent the value of the peak amplitude obtained from individual Gaussian fits. Note PhTX failed to fully block the synaptic activity in all AII amacrine cell recordings.
−19.9 ± 0.4 pA and −15.1 ± 0.1 pA (n = 8). Following bath perfusion of 50 μM PhTX, synaptic events were fully abolished (Fig. 10B and D), contrary to our observations in the light-adapted retina (Fig. 7). Taken together therefore the Co2+ staining and electrophysiology experiments from dark-reared animals demonstrate unequivocally that light entering the eye is critical for the expression of PhTX-insensitive Ca2+-permeable AMPARs.

**Discussion**

Here we describe the expression of Ca2+-permeable AMPARs with novel pharmacology in inhibitory retinal cells that lack synaptic NMDARs. At eye-opening, there is a developmental switch in receptor phenotype where Ca2+-permeable AMPARs develop insensitivity to several well-known channel blockers: PhTX, IEM-1460 and JSTX. AMPARs exhibiting this novel pharmacology are absent from dark-reared animals demonstrating that light entering the eye is critical for their expression. Eye-opening and the expression of PhTX-insensitive AMPARs occurs at a time point when immature axons from bipolar cells are targeting specific sublamina of the IPL. The convergence of these events may identify the synapses of Ca2+-permeable AMPARs as discriminatory targets for incoming axons of developing bipolar cells.
Developmental expression of Ca\textsuperscript{2+}-permeable AMPARs

Ca\textsuperscript{2+}-permeable AMPARs have been reported in either horizontal or AII amacrine cells of the retina of several vertebrates including rat (Morkve et al. 2002; Singer & Diamond, 2003; Veruki et al. 2003), salamander (Gilbertson et al. 1991), skate (Kreitzer et al. 2003) and cat (Pourcho et al. 2002); however, our study provides the first systematic description during postnatal development. Figure 11 summarizes our main finding that the vertebrate retina possesses distinct populations of Ca\textsuperscript{2+}-permeable AMPARs whose expression is regulated by light entering the eye (i.e. visual experience). Although we observed PhTX-insensitive AMPARs before eye-opening (i.e. P11, Figs 3 and 11, see arrows), experiments in dark-reared animals demonstrate that light entering through closed eyelids probably accounts for their expression. In horizontal cells, PhTX-sensitive AMPARs are present at birth until eye-opening. A developmental switch in their functional properties occurs, generating AMPARs with almost no sensitivity to polyamine block (Fig. 11A). Like horizontal cells, light entering the eye triggers expression of PhTX-insensitive AMPARs by AII amacrine cells (Fig. 11B). In this case, PhTX-insensitive receptors are transiently expressed on cell bodies but disappear as development proceeds (Fig. 11B). In the IPL, both PhTX-sensitive and -insensitive AMPARs are present following eye-opening (Fig. 11B, hatched bar). Interestingly, Co\textsuperscript{2+}, staining experiments suggest that PhTX-sensitive AMPARs may be restricted to sublamina b (Figs 4 and 5B).

Comparison with other studies

We conclude that horizontal and AII cells express novel Ca\textsuperscript{2+}-permeable AMPARs based on their unexpected pharmacology. Specifically, we show insensitivity to three known channel blockers that are conventionally used in other studies at much lower concentrations. For example, Chavez et al. (2006) achieved full block of Ca\textsuperscript{2+}-permeable AMPARs in A17 retinal cells using as little as 1 \mu M PhTX, representing a concentration 50 times less than used in this study. Similarly, the IC\textsubscript{50} value (−80 mV, IC\textsubscript{50} = 1.6 \mu M) for block of recombinant AMPARs (Magazanik et al. 1997) by IEM-1460 and block of native receptors by 500 nm JSTX (Bellone & Luscher, 2006) are also at concentrations 20–50 times lower than used in this study.

Numerous studies have routinely used PhTX as a pharmacological marker of Ca\textsuperscript{2+}-permeable AMPARs (Washburn & Dingledine, 1996; Washburn et al. 1997; Toth & McBain, 1998; Toth et al. 2000; Plant et al. 2006). In such instances, PhTX’s use was based on the assumption that divalent permeability and cytoplasmic polyamine block have identical molecular determinants. In support of this, mutation of the Q/R site in the AMPAR pore affects both divalent permeability and channel block (Bowie et al. 1999). However, mutation of another residue in the pore region affects only polyamine block (Dingledine et al. 1992), suggesting that it is possible to assemble AMPARs that...
exhibit Ca\(^{2+}\) permeability whilst lacking polyamine block. As discussed below, there are several possible molecular mechanisms that may account for this phenotype.

**Does the retina express novel AMPARs?**

The expression of Ca\(^{2+}\)-permeable AMPARs with novel pharmacology can be explained in several ways. One possibility is that PhTX insensitivity reflects the up-regulation of a scaffolding protein or phosphorylation event that changes the pharmacological properties of AMPARs. Although both mechanisms regulate AMPAR behaviour (Dingledine *et al.* 1999), accessory proteins or the phosphorylation state of the AMPAR have not been shown to affect pharmacology or ion permeation (see Mayer, 2005 for review). An alternative possibility is that the retina expresses a novel AMPAR subunit or that a known subunit undergoes alternate processing to affect PhTX sensitivity. There are several reasons to support either of these possibilities.

First, cloning studies during the 1990s focused their attention on cDNA libraries from CNS tissue that, for practical reasons, excluded the retina and spinal cord (Hollmann, 1999). To our knowledge, only one study has cloned AMPARs from the retina (Ueda, 1997). In this case, the author used a goldfish cDNA library and reported considerable diversity in mRNA phenotypes consistent with significant processing of primary transcripts. Second, cloning studies of other proteins involved in glutamate signalling, such as glutamate transporters (Eliasof *et al.* 1998) or metabotropic receptors (Nakajima *et al.* 1993), exhibit properties unique to the retina. Indeed, the retina is recognized as a CNS structure that often expresses novel signalling proteins, such as GABA\(_C\) receptors (Feigenspan *et al.* 1993) that are expressed at much lower levels elsewhere in the CNS. Third and finally, several electrophysiological studies have identified non-NMDARs in the retina with atypical pharmacological properties (Ishida & Neyton, 1985; Aizenman *et al.* 1989; Gilbertson *et al.* 1991; Connaughton & Nelson, 2000). For example, Ishida & Neyton (1985) reported an unusual non-competitive antagonism of retinal AMPARs by receptor agonists that is not found in native (Patneau & Mayer, 1991) (e.g. hippocampus) and recombinant (Dingledine *et al.* 1999) AMPARs.

In summary, although the molecular basis by which AMPARs develop PhTX insensitivity remains to be established, our study highlights the identity of an AMPAR with novel pharmacological properties.

**Physiological role of Ca\(^{2+}\)-permeable AMPARs**

The physiological basis for the phenotypic switch and segregation of Ca\(^{2+}\)-permeable AMPARs is not clear. Elsewhere in the CNS, such as the brain stem (Rubio & Wenthold, 1997), hippocampus (Toth & McBain, 1998) and cerebellum (Landsend *et al.* 1997), iGluRs are targeted to specific postsynaptic sites in the same neuron suggesting that discrete expression of Ca\(^{2+}\)-permeable AMPARs may be an important facet of the role they fulfil in neurotransmission. In the retina, two mechanisms may be considered to account for our observations.

Firstly, light entering the eye may trigger expression of AMPARs with higher Ca\(^{2+}\) permeability to facilitate
synapse maturation. In the cerebellum, divalent permeability of AMPARs is dynamically regulated (Liu & Cull-Candy, 2000), which, based on our understanding of retinal ganglion cells (Wong & Wong, 2000), would help drive Ca\(^{2+}\)-dependent events that are undoubtedly required for inhibitory cell synapse maturation. An important caveat, however, is that unlike AMPARs in the cerebellum, both inhibitory cells we have studied express Ca\(^{2+}\)-permeable AMPARs throughout retinal development. Moreover, qualitative assessment of Co\(^{2+}\) staining intensity suggests that divalent permeability of inhibitory cell types is unchanged during development (cf. Fig. 3 and 4). Consequently, if a switch in divalent permeability occurs, it is likely to be modest in nature.

An alternative possibility is that the phenotypic change in AMPARs serves not to alter Ca\(^{2+}\) permeability but facilitate the establishment of lamina-specific connections. In support of this, we have shown that dendrites of AII cells express PhTX-sensitive AMPARs that are apparently segregated to sublamina b of the IPL (Figs 3 and 5B). On the presynaptic side, AII amacrine cells are innervated by ON bipolar cells that form synapses with their arboreal dendrites located in sublamina b of the adult (Kolb & Famiglietti, 1974; Veruki et al. 2003). However, during development, axons of ON bipolar cells extend and retract processes into both sublaminae a and b before the mature axonal arbor is finally established (Morgan et al. 2006). Interestingly, this process is not complete until P19–21 (Morgan et al. 2006) suggesting that PhTX-sensitive AMPARs are restricted to sublamina b in advance of axonal maturation. As yet, the molecular identity of lamination cues are not known; however, they are thought to be provided by amacrine cells and in place before bipolar cell stratification occurs (Morgan et al. 2006). In view of this, it would be interesting to test in future studies if distinct AMPARs, perhaps in concert with adhesion molecules (Yamagata et al. 2002), act as molecular guideposts to ensure faithful subcellular wiring of the IPL.

References


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Light triggers expression of philanthotoxin-insensitive Ca$^{2+}$-permeable AMPA receptors in the developing rat retina
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