Functional NMDA receptors are expressed by both AII and A17 amacrine cells in the rod pathway of the mammalian retina

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ABSTRACT

At many glutamatergic synapses, non-NMDA and NMDA receptors are co-expressed postsynaptically. In the mammalian retina, glutamatergic rod bipolar cells are presynaptic to two rod amacrine cells (AII and A17) that constitute dyad postsynaptic partners opposite each presynaptic active zone. Whereas there is strong evidence for expression of non-NMDA receptors by both AII and A17 amacrines, the expression of NMDA receptors by the pre- and postsynaptic neurons in this microcircuit has not been resolved. Here, using patch-clamp recording from visually identified cells in rat retinal slices, we investigated the expression and functional properties of NMDA receptors in these cells with a combination of pharmacological and biophysical methods. Pressure application of NMDA did not evoke a response in rod bipolar cells, but for both AII and A17 amacrines, NMDA evoked responses that were blocked by a competitive antagonist (CPP) applied extracellularly and an open channel blocker (MK-801) applied intracellularly. NMDA-evoked responses also displayed strong Mg^{2+}-dependent voltage block and were independent of gap junction coupling. With low-frequency application (60 s intervals), NMDA-evoked responses remained stable for up to 50 min, but with higher-frequency stimulation (10-20 s intervals) NMDA responses were strongly and reversibly suppressed. We observed strong potentiation when NMDA was applied in nominally Ca^{2+}-free extracellular solution, potentially reflecting Ca^{2+}-dependent NMDA receptor inactivation. These results indicate that expression of functional, i.e. conductance-increasing, NMDA receptors is common to both AII and A17 amacrine cells and suggest that these receptors could play an important role for synaptic signaling, integration or plasticity in the rod pathway.

Key words: amacrine cells; rod pathway; NMDA receptors; retina

INTRODUCTION

The vast majority of excitatory synaptic transmission in the central nervous system is mediated by the amino acid glutamate (Hassel and Dingledine 2012). After synaptic release, glutamate diffuses across the synaptic cleft and binds to different types of receptors in the postsynaptic membrane. There are two main types of ionotropic glutamate receptors (iGluRs), termed N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. These two types were originally defined based on their sensitivity to exogenous agonists, and non-NMDA receptors are further classified into AMPA and kainate receptors (reviewed by Hassel and Dingledine 2012; Smart and Paoletti 2012). More recently, molecular investigations have revealed that the pharmacologically defined types of receptors are composed of different subunits. NMDA receptors are heteromeric tetramers composed of two obligatory GluN1 subunits and two accessory GluN2 subunits (GluN2A-D). AMPA receptors are homo- or heteromeric tetramers composed of GluA1-4 subunits and kainate receptors are homo- or heteromeric receptors composed of GluK1-5 subunits. At many glutamatergic synapses, the postsynaptic density contains both non-NMDA and NMDA receptors such that presynaptic release of glutamate activates both types of receptors, giving rise to a dual-component excitatory postsynaptic current (EPSC; Hassel and Dingledine 2012). At some synapses, however, either NMDA or non-NMDA receptors seem to be missing and the EPSCs are mediated solely by one type of receptor, e.g. by NMDA receptors at "silent synapses" (Malinow et al. 2000) and non-NMDA receptors at climbing- and parallel-fiber synapses on Purkinje cells (Perkel et al. 1990). Because of their special functional properties, including high Ca^{2+} permeability, Mg^{2+}-dependent voltage block at negative membrane potentials and slow kinetics (Traynelis et al. 2010), NMDA receptors and their role in synaptic signaling and plasticity have attracted intense investigation (Paoletti et al. 2013).

In the retina, glutamate is used as a neurotransmitter by both photoreceptors and bipolar cells, mediating input to horizontal cells and bipolar cells, and to amacrine cells and ganglion cells, respectively (Massey and Maguire 1995). In the

cone pathway, the synaptic transmission between cone bipolar cells and ganglion cells can involve both non-NMDA and NMDA receptors, in both the ON and OFF pathways (Copenhagen et al. 1993) and NMDA receptors can contribute to contrast coding and temporal processing in ganglion cells (Manookin et al. 2010; Stafford et al. 2014). In the rod pathway, rod bipolar cells do not output their signals directly to ganglion cells (Strettoi et al. 1990, 1992). Instead, they contact AII amacrine cells which are presynaptic to ON-cone bipolar cells via electrical synapses and to OFF-cone bipolar cells and ganglion cells via glycinergic synapses (Strettoi et al. 1992, 1994). Through these connections, the AII amacrine is crucial for retinal signal processing not only under scotopic conditions (reviewed by Bloomfield and Dacheux 2001), but also under mesopic and photopic conditions as well (Manookin et al. 2008; Münch et al. 2009). AMPA-type non-NMDA receptors make a substantial contribution to the EPSC evoked in AII amacrine cells by depolarization of presynaptic rod bipolar cells (Singer and Diamond 2003) and AII amacrine cells were thought not to express NMDA receptors (Boos et al. 1993). There is increasing evidence, however, from both physiological (Hartveit and Veruki 1997; Bloomfield and Xin 2000; Zhou and Dacheux 2004) and immunocytochemical (Kothmann et al. 2012) investigations that AII amacrine cells do express NMDA receptors, suggesting that glutamatergic neurotransmission in these cells is more complex than originally believed. In addition to being presynaptic to AII amacrine cells, rod bipolar cells are also presynaptic to a second type of rod amacrine cell, termed AI or A17 (Kolb and Famiglietti 1974). A17 amacrines are wide-field amacrines that provide inhibitory GABAergic feedback to the rod bipolar cell (Nelson and Kolb 1985; Raviola and Dacheux 1987; Hartveit 1999; Chávez et al. 2006) and it has been suggested that they do not express NMDA receptors (Menger and Wässle 2000). At each specialized presynaptic release site (ribbon) of the axon terminal of a rod bipolar cell, two postsynaptic processes from an AII and an A17 amacrine cell collectively constitute a postsynaptic dyad (Kolb and Famiglietti 1974; Raviola and Dacheux 1987). On this basis, we decided to examine in more detail the properties of NMDA receptors

expressed by AII amacrine cells and to investigate the potential expression of NMDA receptors by A17 amacrine cells. We also wanted to re-examine the presence of NMDA receptors in rod bipolar cells, for which there is conflicting evidence, suggesting both the presence (Karschin and Wässle 1990; Wenzel et al. 1997; Lo et al. 1998) and absence of NMDA receptors on these cells (Hartveit 1996; Fletcher et al. 2000). Here, we provide direct evidence that not only AII, but also A17 amacrine cells express functional (i.e. conductance-increasing) NMDA receptors, detected by recording whole-cell current responses evoked by application of NMDA. No such responses were observed in rod bipolar cells. Our results suggest that NMDA receptors could play a significant role in synaptic signaling or plasticity in the rod pathway.

METHODS

Retinal slice preparation and visual targeting of neurons

General aspects of the methods have previously been described in detail (Veruki et al. 2003). Female albino rats (Wistar HanTac; 4-7 weeks postnatal) were deeply anaesthetized with isoflurane in oxygen and killed by cervical dislocation (procedure approved under the surveillance of the Norwegian Animal Research Authority). Vertical retinal slices were visualized using an Axioskop 2 FS (Zeiss) with a ×40 water immersion objective and infrared differential interference contrast (IR-DIC) videomicroscopy.

Solutions and drug application

The standard extracellular perfusing solution was continuously bubbled with 95% O_2 - 5% CO_2 and had the following composition (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, pH 7.4. In some experiments, $MgCl_2$ was omitted from the extracellular solution (with no replacement of divalent cations) to relieve the voltage-dependent block of NMDA receptors. For these experiments, cells were held in the Mg^{2+}-free bath solution for at least 10 minutes before applying NMDA to

ensure a complete washout of the divalent cations. In a few experiments, both $MgCl_2$ and $CaCl_2$ were omitted from the extracellular solution (with no replacement of divalent cations). Recording pipettes were filled with (mM): 125 potassium gluconate, 8 KCl, 5 Hepes, 1 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 4 Na_2ATP, and 2 N-(2, 6-dimethylphenylcarbamoylmethyl)triethylammonium bromide (QX-314; Tocris Bioscience, UK) or 125 potassium gluconate, 8 NaCl, 10 Hepes, 1 $CaCl_2$, 5 EGTA, 4 MgATP and 2 QX-314). pH was adjusted to 7.3 with KOH. For the acquisition of current-voltage (*I-V*) relationships, recording pipettes were filled with (mM): 125 $CsCH_3SO_3$, 8 NaCl, 10 Hepes, 1 $CaCl_2$, 5 EGTA, 15 tetraethylammonium chloride (TEA-Cl), 4 MgATP. pH was adjusted to 7.3 with CsOH. For most nucleated patch experiments cells were filled with (mM): 125 CsCl, 8 NaCl, 10 Hepes, 1 $CaCl_2$, 5 EGTA, 15 TEA-Cl, 4 MgATP. pH was adjusted to 7.3 with CsOH. For some nucleated patch experiments recording pipettes were filled with 125 potassium gluconate, 8 NaCl, 10 Hepes, 1 $CaCl_2$, 5 EGTA, 4 MgATP and 2 QX-314. pH was adjusted to 7.3 with KOH.

For visualization of complete cellular morphologies with wide-field fluorescence microscopy after the recording, Lucifer yellow (1 mg/ml; Sigma-Aldrich), Alexa Fluor 488 (50 μM; Invitrogen) or Alexa Fluor 594 (40 μM; Invitrogen) was included in the intracellular solutions.

For pressure application from glass pipettes, drugs were dissolved in a vehicle solution containing (mM): 145 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 Na-Hepes, and 10 glucose. $MgCl_2$ and $CaCl_2$ were eliminated from this solution to match the bath solution as necessary. We used either a singlebarrel puffer pipette (similar in size and shape to the patch pipettes described below) filled with NMDA or a multibarrel puffer pipette with NMDA in one or more barrels and NMDA with the specific NMDA receptor antagonist (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 400 μM) in one or more separate barrels (seven barrels in total; for details, see Hartveit 1996). In all cases, the concentration of NMDA in the pipette was 1 mM. In these recordings, an NMDA receptor co-agonist (Kleckner and

Dingledine 1988; Traynelis et al. 2010), either 10 μM glycine in the presence of 10 μM strychnine and 100 μM picrotoxin or 200 μM D-serine, was always included in the application pipette. For the nucleated patch experiments, individual barrels of the multibarrel pipettes were filled with 1 mM NMDA (with co-agonist), 1 mM GABA or 1 mM glycine. For some experiments, 1 or 2 mM (5*S*,10*R*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801; Tocris Bioscience) was added to the intracellular solution. Other neurotransmitter receptor antagonists and ion channel blockers were added directly to the extracellular solution at the following concentrations (supplied by Tocris Bioscience, unless otherwise indicated): 1 μM strychnine (Research Biochemicals, Natick, MA, USA) to block glycine receptors; 10 μM (-)-bicuculline methochloride to block $GABA_A$ receptors; 50 μM (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) to block $GABA_C$ receptors, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block non-NMDA receptors; 0.3 μM tetrodotoxin (TTX) to block voltage-gated Na^+ channels; 100 μM 2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid sodium salt (meclofenamic acid [MFA] sodium salt; Sigma-Aldrich). Solutions were either made up freshly for each experiment or were prepared from aliquots stored at -20°C and diluted to the final concentration on the day of the experiment.

Electrophysiological recording and data acquisition

Patch pipettes were pulled from thick-walled borosilicate glass (outer diameter, 1.5 mm; inner diameter, 0.86 mm) to obtain an open-tip resistance that ranged from 5 to 7 MΩ when filled with intracellular solution. Whole-cell voltage-clamp recordings were performed with an EPC9-dual amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) and controlled with PatchMaster software (HEKA Elektronik). After establishing a GΩ-seal, currents caused by the recording electrode capacitance (fast capacitative current; "C_{fast}" function in PatchMaster) were automatically measured and neutralized by the amplifier. After breaking into the cell, currents caused by the cell membrane capacitance (slow capacitative current; "C_{slow}" function in

PatchMaster) were partially neutralized by the amplifier. For whole cell recordings, series resistance (R_s) was monitored throughout the recording, at intervals of 1 minute for most experiments, by applying a series of 20 mV hyperpolarizing voltage pulses (16 ms duration) after transiently disabling the C_{slow} neutralization circuitry of the amplifier. The charging transients were analyzed off-line by FitMaster software (HEKA Elektronik) by fitting the decay phase during the voltage pulse with a double exponential function and R_s was calculated from the amplitude of the voltage pulse and the peak current amplitude extrapolated to the onset of the pulse. R_s was not compensated. Cells with $R_s > 40$ MΩ were not included in the final material.

To establish a nucleated patch recording, the pipette was slowly withdrawn after establishing the whole-cell recording configuration, while continuous light suction (~50 mbar) was applied to the pipette. When a nucleated patch was successfully isolated, the reduced membrane capacitance resulted in capacitative current transients of the opposite polarity that were cancelled by re-adjustment of the C_{slow} neutralization circuitry.

The sampling interval was set to either 50 or 100 μs and before sampling, signals were low-pass filtered (analog 3- and 4-pole Bessel filters in series) with a corner frequency (-3 dB) of 4 kHz. All recordings were carried out at room temperature (22 - 25°C). The data acquisition software (PatchMaster) corrected all holding potentials for liquid junction potentials on-line. Theoretical liquid junction potentials were calculated with JPCalcW (Axon Instruments / Molecular Devices).

Wide-field fluorescence microscopy

All cells were inspected with wide-field fluorescence microscopy after recording. By visual observation through the microscope oculars, we inspected the morphology of each dye-filled neuron and its processes and the relationship between the branching pattern and the strata of the inner plexiform layer. For documentation, every dye-filled neuron was sketched by hand. In addition, for some cells we acquired digital image stacks using a TILLvisION system (TILL Photonics). Image acquisition and

post-acquisition deconvolution to remove noise and increase resolution was performed as described previously (Castilho et al. 2015a).

General data analysis

Data were analyzed with FitMaster (HEKA Elektronik), IGOR Pro (WaveMetrics, Lake Oswego, OR, USA), and Excel (Microsoft, Redmond, WA, USA). The peak amplitude of NMDA-evoked currents was measured as the mean amplitude between two vertical cursors positioned close to the peak response as identified by eye. For acquisition of I-V relationships, the holding potential was incremented by 10 mV intervals. Data points of *I-V* relationships were connected by straight lines and reversal potentials (E_{rev}) were determined by the intersection with the abscissa.

Data are presented as means ± SE (n = number of cells). Statistical analyses with comparisons between or within groups were performed using Student's two-tailed t test (paired or unpaired, as indicated). Differences were considered statistically significant at the $P < 0.05$ level. For illustration purposes, most raw data records were low-pass filtered (digital Gaussian filter, -3 dB at 500 Hz - 2 kHz).

Fig. 1 near here

RESULTS

Targeting and identification of neurons in the rod bipolar-AII-A17 microcircuit in rat retinal slices

The cell bodies of AII and A17 amacrine cells each have a characteristic shape and location at the border between the inner nuclear layer and the inner plexiform layer and as such, they can be readily targeted in an acute retinal slice preparation (Fig. 1*A*). AII amacrine cells have cell bodies that span the border between the inner nuclear layer and the inner plexiform layer and are bi-stratified narrow-field amacrine cells whereas A17 amacrine cells have dome-shaped cell bodies with long, thin processes that carry distinct varicosities and terminate near the ganglion cell layer of the retina. Rod bipolar cells tend to have their cell bodies in the distal part of

the inner nuclear layer, in close apposition to the outer plexiform layer (Fig. 1*A*). The intracellular solutions contained fluorescent dyes that diffused into the cells during recording and allowed unequivocal identification of the complete morphology after recording (Fig. 1*B*). For all three types of retinal neurons, NMDA and other drugs were applied from singlebarrel or multibarrel pipettes positioned close to the location of the neuron in the inner plexiform or the inner nuclear layer (Fig. 1*A*). Nucleated patches were isolated from AII and A17 amacrine cells by pulling the cell body out of the slice after establishing the whole-cell configuration and were used with drug application from multibarrel or singlebarrel puffer pipettes (Fig. 1*C*). For this study, we recorded from a total of 70 AII amacrine cells, 63 A17 amacrine cells, and 6 rod bipolar cells.

Fig. 2 near here

Both AII and A17 amacrine cells, but not rod bipolar cells, in rat retinal slices respond to application of NMDA

AII and A17 amacrine cells form the dyad postsynaptic targets of ribbon synapses of glutamatergic rod bipolar cell axon terminals (Fig. 2*A*). There is evidence that NMDA receptors in glutamatergic synapses can have not only a conventional postsynaptic (including extrasynaptic) localization (Sheng and Kim 2012) to mediate synaptic transmission and control postsynaptic excitability, but also a presynaptic localization, potentially to control transmitter release (Engelman and MacDermott 2004). Accordingly, NMDA receptors could be expressed by any or all of the three neuronal elements of this synaptic microcircuit. In principle, NMDA receptors could also be localized postsynaptically at rod bipolar cell dendrites in the outer plexiform layer where they receive glutamatergic input from rod photoreceptors (Massey and Maguire 1995).

We tested for the presence of functional, i.e. conductance-increasing, NMDA receptors in AII and A17 amacrine cells and in rod bipolar cells by pressure application of NMDA (1 mM, 1 - 2 s duration) from a puffer pipette with nominally

Mg^{2+}-free solution in both the bath and the pipette (hereafter referred to as Mg^{2+}-free solution). In both AII and A17 amacrine cells, NMDA evoked large inward currents at holding potentials of -60 mV and -70 mV, respectively (close to the resting potentials of the two cell types; Fig. 2*B, C*). The inward currents displayed relatively fast rise and decay times. All the AII and A17 amacrine cells tested under these conditions responded to NMDA. For AII amacrine cells, the average peak response was 64 ± 7 pA (n = 17, range 28 - 152 pA) and for A17 amacrine cells the average peak response was 85 ± 8 pA (n = 22, range 36 - 182 pA).

In contrast to the two types of rod amacrine cells, NMDA evoked no response in any of the rod bipolar cells tested (n = 6 cells). The traces illustrated for a rod bipolar cell in Fig. 2*D*, demonstrate how we examined two positions of the puffer pipette, with application directed either towards the axon terminal in the inner plexiform layer or towards the dendrites in the outer plexiform layer. In a typical recording, the first application of NMDA was performed within 1 - 3 min after breaking into the cell and establishing the whole-cell recording configuration. To minimize the likelihood that fast rundown of NMDA receptor channels (Horn and Korn 1992) could take place before the first application, we tested three rod bipolar cells with pressure application of NMDA within 20 s after breaking into the cells, but still did not observe any responses. In some recordings, we observed small sustained shifts in the current that were tightly synchronized to the duration of drug application. These shifts were not accompanied by changes in noise, as expected for channel gating (cf. Fig. 2*B, C*). When the pressure application of NMDA was preceded and followed by application of the vehicle solution (without agonist) from another barrel in the multibarrel complex (Fig. 2*D*), no shift in current was observed, suggesting that it was caused by a small difference in liquid junction potential between the bath solution and the solution in the puffer pipette. These results strongly suggest that rod bipolar cells do not express functional (conductance-increasing) NMDA receptor channels.

Fig. 3 near here

Functional properties of NMDA receptors in AII and A17 amacrine cells

To examine whether the responses evoked by NMDA in AII and A17 amacrine cells were indeed mediated by NMDA receptors, we applied NMDA together with the specific NMDA receptor antagonist CPP (400 μM), with Mg^{2+}-free solution in both the bath and the puffer pipette. For these experiments, we used a multibarrel pipette and first applied NMDA alone from one barrel, followed by co-application of NMDA and CPP from another barrel (the antagonist was included in the same barrel as NMDA). By directing the tip of the multibarrel pipette towards photoreceptors at the edge of the slice, we used pressure-evoked movements of photoreceptor outer segments as a visual control to verify that fluid was adequately ejected from all barrels used during the pharmacological testing. In the presence of CPP, the NMDA responses were completely blocked for both AII (n = 8 cells) and A17 (n = 5 cells) amacrine cells (Fig. 3*A, B*) and for both cell types the responses to NMDA recovered quickly following washout of CPP (Fig. 3*A, B*).

Fig. 4 near here

These experiments strongly suggested that the responses to NMDA were mediated by NMDA receptors, but they do not by themselves demonstrate conclusively that the responses were mediated by receptors located on the cells that we recorded from. To rule out the possibility that the NMDA-evoked responses were mediated by transsynaptic network effects, we performed three sets of experiments. In the first set we applied an antagonist intracellularly to block NMDA-evoked responses, in the second set we verified the characteristic *I-V* relationship expected for NMDA receptor-mediated currents, and in the third set we tested for the presence of NMDA receptor-mediated responses after blocking gap junction-mediated coupling pharmacologically. We first repeated the recordings with application of NMDA (in Mg^{2+}-free extracellular solution) after including the NMDA receptor open-channel blocker MK-801 in the recording pipette solution (2 mM). In an attempt to use the cells as their own controls, we applied NMDA repeatedly

317 (approximately every 60 s), starting as soon as possible after establishing the whole-
318 cell recording condition. For AII amacrine cells (n = 8 cells), there was no response to
319 NMDA, even during the very first application of NMDA which, for the cell
320 illustrated in Fig. 4*A*, was obtained within 1 min after breaking into the cell. This is
321 most likely explained by the small cell size and a relatively short diffusion distance
322 from the tip of the pipette and cell body to the location of the NMDA receptors. As a
323 positive control, AII amacrine cells in the same slices recorded without MK-801
324 added to the intracellular solution displayed the expected inward currents evoked by
325 application of NMDA (data not shown).
326 In contrast to AII amacrine cells, we typically observed a small, but clear,
327 response in A17 amacrine cells recorded with MK-801 intracellularly when NMDA
328 was applied within 1-2 min after establishing the whole-cell recording configuration.
329 This is consistent with the larger length of the processes of these cells and the
330 presumably correspondingly longer diffusion distance from the tip of the recording
331 pipette and cell body to the location of the NMDA receptors. For the cell illustrated
332 in Fig. 4*B*, the first NMDA-evoked response obtained after breaking into the cell
333 displayed a peak amplitude of approximately 20 pA. With repeated application of
334 NMDA (once every 60 s), we observed a gradual reduction of the response
335 amplitude such that at approximately 4 min of recording, the response was almost
336 completely abolished (Fig. 4*B*). For A17 amacrine cells recorded with MK-801 in the
337 pipette solution, the average NMDA-evoked response was an inward current of 3 ±
338 0.4 pA (n = 4 cells, range 2.6 to 4.2 pA) after approximately 4 min of recording.
339 *Fig. 5 near here*
340
341 **Voltage-dependent block of NMDA receptors in AII and A17 amacrine cells**
342 NMDA receptors display a characteristic Mg^{2+}-dependent voltage block (Nowak et
343 al. 1984). To investigate this property for the NMDA receptors expressed by AII and
344 A17 amacrine cells, we measured the *I-V* relationships of the NMDA-evoked
345 responses in the presence and absence of Mg^{2+} in the extracellular solution. We

recorded NMDA-evoked currents at a series of holding potentials between -80 mV and +60 mV. To block K^+ conductances in the cells, including voltage-dependent conductances, the recording pipette solution contained Cs^+ and TEA^+ (see Methods). In the presence of extracellular Mg^{2+}, the *I-V* curves for AII amacrines displayed a characteristic J-shape, with a negative slope conductance between -70 and -30 mV (Fig. 5*A, C*; *filled circles*), as is expected for NMDA receptors. When Mg^{2+} was omitted from the bath and puffer pipette solution, the NMDA-evoked responses were markedly enhanced at negative holding potentials (Fig. 5*B*) and the corresponding *I-V* curves became considerably more linear (Fig. 5*B*, *C*; *open circles*). In the presence of Mg^{2+}, the E_{rev} for AII amacrine cells was 5.9 ± 3.6 mV (range -2.0 to 16.1 mV; $n = 6$ cells) and in the absence of Mg^{2+} it was 4.8 ± 4.0 mV (range -6.1 to 18.0 mV; $n = 5$ cells). There was no statistically significant difference between these values ($P = 0.8377$; unpaired t test).

When A17 amacrine cells were tested with NMDA at different holding potentials, we noticed that for some cells (with normal extracellular Mg^{2+}), the rise time was longer for responses at negative potentials between -80 and -30 mV (Fig. 5*D*). Even with good voltage and space clamp control, such as when recording from round cells without processes, the inward current through NMDA receptor channels is not zero at these negative potentials. Because of the negative slope conductance, this current could rise to a regenerative depolarization with gradual release from the Mg^{2+}-dependent voltage block when recording NMDA-evoked currents in A17 amacrine cells with long thin processes and suboptimal conditions for good space clamp. This could contribute to the slow increase in the inward current at negative potentials observed for some A17 amacrines (Fig. 5*D*).

Because of these problems with A17 amacrine cells, we measured the current responses at the negative holding potentials at the same point in time where the peak responses at other holding potentials occurred. The *I-V* curve displayed a slight J-shape with strong outward rectification (Fig. 5*D, F*; *filled circles*). Similar to the result in AII amacrine cells, when we measured NMDA-evoked responses in the absence of

Mg^{2+} in the extracellular solution, the responses were markedly enhanced at negative holding potentials (Fig. 5*E*), consistent with alleviation of a Mg^{2+}-dependent voltage block. In this condition, the *I-V* curves were considerably more linear (Fig. 5*F*; *open circles*). In the presence of Mg^{2+}, the E_{rev} for A17 amacrine cells was 4.5 ± 6.2 mV (range -22 to 22 mV, $n = 7$ cells) and in the absence of Mg^{2+} it was 8.0 ± 4.8 mV (range -5.6 to 27 mV; $n = 6$ cells). The difference between these values was not statistically significant ($P = 0.67749$; unpaired *t* test).

Fig. 6 near here

Blocking gap junction-mediated coupling with MFA does not block NMDA-evoked responses in AII or A17 amacrine cells

We have previously demonstrated that MFA completely blocks the electrical synapses between AII amacrine cells and between AII amacrine cells and ON-cone bipolar cells (Veruki and Hartveit 2009). To exclude the possibility that the NMDA-evoked responses observed in either AII or A17 amacrine cells were mediated indirectly by gap junction coupling to other cells, we repeated application of NMDA while we blocked gap junction coupling pharmacologically with MFA. NMDA (1 mM, 1 s duration) was applied once every 60 s, starting immediately after establishing the whole-cell recording configuration when we also added MFA (100 μM) to the extracellular solution to block electrical coupling. Because it takes approximately 30 min before the electrical coupling is completely blocked (Veruki and Hartveit 2009), the application of NMDA was continued for 40 - 45 min in the maintained presence of MFA. For the AII amacrine cell illustrated in Fig. 6A and the A17 amacrine cell illustrated in Fig. 6B, there was virtually no change in the responses over time. Similar results were seen for two other AII amacrine cells. These results strongly suggest that NMDA-evoked responses in AII or A17 amacrine cells were mediated by receptors on the cells themselves and not indirectly by gap junction-coupling to other neurons.

Fig. 7 near here

Neurotransmitter receptors in nucleated patches from AII and A17 amacrine cells

For a more detailed biophysical analysis of the functional properties of the NMDA responses expressed by AII and A17 amacrine cells, whole-cell recording and drug application from puffer pipettes is not an ideal method. The voltage control, including space-clamp, is suboptimal in recordings from both AII and A17 amacrine cells, both because of the presence of homologous and heterologous electrical coupling mediated by gap junctions (reviewed by Hartveit and Veruki 2012) and because of the presence of long and thin processes, particularly on the A17s (Fig. 1*B*). In addition, the speed of drug application obtainable with puffer pipettes and *in vitro* slices is too slow and variable for adequately measuring the kinetic properties of neurotransmitter receptors. On this background, we attempted a more detailed kinetic and biophysical investigation of the *I-V* properties of NMDA receptors expressed by AII and A17 amacrine cells by recording NMDA-evoked responses from nucleated patches. These are comparable to small round cells that offer a much better voltage and space clamp control and can be tested with faster drug application. In our laboratory, we have previously observed responses of conventional outside-out or nucleated patches from AII amacrine cells to application of several neurotransmitters and receptor agonists, including glutamate (Veruki et al. 2003), kainate (Mørkve et al. 2002), AMPA (Mørkve et al. 2002; Castilho et al. 2015b), and glycine (Gill et al. 2006).

When we tested nucleated patches from AII and A17 amacrine cells, we applied NMDA (1 mM), GABA (1 mM) and glycine (1 mM) from separate barrels of a multibarrel pipette. For AII amacrine cells, 11/13 nucleated patches responded to GABA with robust currents and 13/13 patches responded to glycine with robust currents (Fig. 7*A*). We did not perform a detailed investigation of the *I-V* properties of the GABA and glycine receptors, but observed inward and outward currents at holding potentials of -60 and +20 mV, respectively (Fig. 7*A*), as expected for chloride-permeable channels and E_{Cl} ~0 mV. When the same 13 nucleated patches were tested

433 with NMDA (at holding potential of -60 and +20 mV), 10/13 patches displayed no
434 response and 3/13 patches responded with minimal currents (≤ 3 pA) and a small
435 increase in membrane noise (Fig. 7*A*).
436 *Fig. 8 near here*
437 For A17 amacrine cells, 15 nucleated patches were examined with drug
438 application. All 15 patches were tested with GABA and responded with robust
439 currents (Fig. 8*A*). Of the same 15 patches, 11 were tested with glycine and all
440 responded with robust currents (Fig. 8*A*). When we tested these same patches with
441 NMDA (in Mg^{2+}-free extracellular solution and a holding potential of -60 mV), 12/15
442 patches displayed no response and 3/15 patches responded with minimal currents (≤
443 3 pA) and an increase in membrane noise (Fig. 8*A*).
444 These results suggested that the general lack of NMDA-evoked responses in
445 nucleated patches from AII and A17 amacrine cells reflects a lack of receptors and is
446 not due to technical problems. As an additional control, we performed experiments
447 where we first verified the presence of an NMDA-evoked response in the whole-cell
448 configuration, isolated a nucleated patch from the same cell and tested it with
449 application of NMDA. For AII amacrine cells tested in this way (n = 3), all displayed
450 robust responses to NMDA in the whole-cell configuration, but only one cell
451 responded after isolating a nucleated patch and the amplitude was < 3 pA (Fig. 7*B*).
452 Similarly, when we tested A17 amacrine cells (n = 3), all displayed robust whole-cell
453 responses to NMDA. When the corresponding nucleated patches were tested, all
454 displayed a visible response to NMDA, but the responses were small (3.8 ± 0.8 pA,
455 range 2.6 - 5.4 pA; Fig. 8*B*). Taken together, these experiments strongly suggested
456 that there are very few NMDA receptors located in the cell membrane of the cell
457 bodies of both AII and A17 amacrine cells, unfortunately precluding a rigorous
458 kinetic and biophysical analysis.
459 *Fig. 9 near here*
460

Agonist-dependent suppression of NMDA receptor-mediated responses in AII and A17 amacrine cells

In a previous study of NMDA receptors in AII amacrine cells, we observed a relatively fast time- and agonist-dependent reduction of NMDA-evoked responses, with few cells maintaining responses after being stimulated with repeated application of NMDA for 10 - 15 minutes (Hartveit and Veruki 1997). Because the phenomenon seemed to depend on the duration of whole-cell recording, it was suggested that it was a consequence of receptor rundown, potentially reflecting the perturbation of neuromodulatory control. Such control is often mediated by phosphorylation of specific amino acids in receptor proteins, and there is strong evidence that NMDA receptors in different types of neurons are subject to such control (reviewed by Salter et al. 2009). There is also evidence, however, that NMDA receptors are influenced by more than one mechanism of desensitization (reviewed by Gibb 2010). On this background, we hypothesized that if the observed response reduction corresponded to classical rundown, it should be irreversible over time, whereas if it was caused e.g. by desensitization following agonist-dependent receptor activation, it should reverse, after reducing the frequency or intensity of stimulation.

We tested both AII and A17 amacrine cells by applying NMDA (1 s duration) at intervals of approximately 60 s for recording periods of 20 - 50 minutes (Fig. 9). The recordings were performed in Mg^{2+}-free extracellular solution at negative holding potentials, near the resting membrane potentials of the cells. With this paradigm, we sometimes observed a sequential increase or decrease in response to the first 3 - 4 NMDA-evoked responses, but after that initial change in response, we observed no consistent change of the amplitude of NMDA-evoked responses in either AII (Fig. 9*A*) or A17 (Fig. 9*B*) amacrine cells within our recording periods.

Fig. 10 near here

To investigate the conditions for the response reduction previously observed in our laboratory (Hartveit and Veruki 1997), we repeated the application of NMDA

490 at more frequent intervals. For all cells, we first verified a stable baseline for
491 approximately 5 min with an application interval of 60 s, before switching to more
492 frequent applications of 10 - 15 s intervals. For the AII amacrine cell illustrated in Fig.
493 10*A*, the response to low-frequency application of NMDA was relatively stable at
494 around 250 pA. When the application interval was reduced from 60 s to 10 s, this led
495 to an immediate reduction in the response amplitude that plateaued at around 170
496 pA within 1 - 2 min, corresponding to a suppression of about 34%. In all cases, we
497 made sure that the stimulation interval was sufficiently long for the membrane
498 current to have recovered to the baseline level between subsequent applications of
499 NMDA. After 5 min, the application interval was increased again to 60 s and the
500 response amplitude increased over approximately 2 min, with an almost complete
501 reversal of the suppression seen during the 10 s-interval application period (Fig.
502 10*A*). During a second period of 10 s-interval applications, the suppression was
503 slightly stronger (43%). When the application interval was increased to 60 s again,
504 the responses recovered almost fully to the previous level (Fig. 10*A*). For the whole
505 recording period, the series resistance varied slightly, but the change was gradual
506 and unrelated to the temporally distinct suppression seen during the periods with
507 increased stimulus frequency (Fig. 10*A*). For seven AII amacrine cells tested in this
508 way, the NMDA-evoked response decreased in all cells, from 112 ± 29 pA (range 42 -
509 254 pA) with an application interval of 60 s to 72 ± 24 pA (range 19 - 168 pA) with an
510 application interval of 10 - 15 s, corresponding to an average decrease of 43 ± 9% (Fig.
511 10*B*; $P = 0.0067$, paired t test). For five of the cells we were able to maintain the
512 recordings sufficiently long that recovery could be observed (Fig. 10*B*).
513 The results for A17 amacrine cells were very similar to those for AII amacrine
514 cells. For the A17 amacrine cell illustrated in Fig. 10*C*, the NMDA-evoked response
515 was suppressed by about 60% when the application interval was reduced from 60 s
516 to 12 s. The suppression was reversible and could be repeated (Fig. 10*C*). The series
517 resistance increased slightly during the recording period, but was unrelated to the
518 suppression evoked by increased stimulus frequency (Fig. 10*C*). For eight A17

amacrine cells tested in this way, the NMDA-evoked response decreased in all cells, from 106 ± 19 pA (range 27 - 182 pA) with an application interval of 60 s to 57 ± 13 pA (range 12 - 107 pA) with an application interval of 10 - 15 s, corresponding to an average decrease of 49 ± 3% (Fig. 10*D*; $P = 0.00042$, paired *t* test). The suppression reversed almost completely when the application interval was increased again (Fig. 10*D*; one cell was lost before recovery was observed).

Fig. 11 near here

Reducing extracellular Ca^{2+} increases NMDA responses in AII and A17 amacrine cells

One possible mechanism that can explain the response suppression observed when the frequency of application was increased is a negative feedback mediated by Ca^{2+} influx through the NMDA receptor channel itself (Rosenmund and Westbrook 1993). Influx of Ca^{2+}, acting via calmodulin, constitutes a system for negative feedback of the Ca^{2+}-permeable NMDA receptor channels (Ehlers et al. 1996; Zhang et al. 1998). To examine the potential Ca^{2+}-sensitivity of NMDA receptors in AII and A17 amacrine cells, we applied NMDA (1 s duration) at 60 s intervals and after a stable baseline had been established in Mg^{2+}-free extracellular solution, we switched to a Mg^{2+}- and Ca^{2+}-free extracellular solution. For the AII amacrine cell illustrated in Fig. 11*A*, the reduced extracellular Ca^{2+} immediately led to an increased response amplitude from a baseline response of approximately 250 pA to a level of approximately 430 pA, corresponding to an increase of 72%. When we switched back to the original extracellular solution and Ca^{2+} was washed back in, the amplitude of the NMDA-evoked responses returned to approximately 275 pA (Fig. 11*A*). For five AII amacrine cells tested in this way, the NMDA-evoked response increased in all cells, from 142 ± 33 pA (range 67 - 252 pA) in control (Mg^{2+}-free) bath solution to 250 ± 52 pA (range 118 - 433 pA) in Mg^{2+}- and Ca^{2+}-free bath solution, corresponding to an average increase of 81 ± 13% (Fig. 11*B*; $P = 0.0067$, paired *t* test). For three cells, we

observed a reversal of the increase when we switched back to normal Ca^{2+}, for the other two cells, the recording was lost before we could observe recovery.

When A17 amacrine cells were tested in the same way, the results were very similar to those observed for AII amacrine cells. For the A17 amacrine cell illustrated in Fig. 11*C*, the response increased by 110%. For five A17 amacrine cells, the NMDA-evoked response increased in all cells, from 74 ± 22 pA (range 17 - 133) in control (Mg^{2+}-free) bath solution to 118 ± 32 pA (range 36 - 201) in Mg^{2+}- and Ca^{2+}-free bath solution, corresponding to an average increase of 71 ± 11% (Fig. 11*D*; $P = 0.0108$, paired *t* test). Reversal was observed for three cells, with two cells lost before recovery could be observed.

DISCUSSION

In this study we have used a combination of physiological, pharmacological and biophysical methods to investigate the potential expression of functional (i.e. conductance-increasing) NMDA receptors by AII and A17 rod amacrine cells, as well as rod bipolar cells, in the rat retina. This information is important for understanding the neurotransmission between glutamatergic bipolar cells and rod amacrine cells, including possible mechanisms of synaptic integration and plasticity. The present results provide strong evidence that NMDA receptors are expressed by both AII and A17 amacrine cells, suggesting that these receptors can have a more important role in mediating glutamatergic signals to both types of amacrines than previously suspected. In the following, we will discuss the basis for our conclusion, discuss possible implications with respect to functional roles for NMDA receptors in these cells, and suggest how these questions might be investigated in future work.

For rod bipolar cells, we found no evidence for expression of functional (conductance-increasing) NMDA receptors. This is consistent with a previous report from our laboratory (Hartveit 1996), but we refined our investigation in the current study by increasing the spatial area over which the puffer pipette was positioned to ensure that we would not miss any region of the cell and we made sure that fast

rundown of receptor channels could not explain the absence of responses by minimizing the interval between breaking into the cells and applying NMDA. Earlier physiological evidence for functional NMDA receptors on rod bipolar cells is relatively weak, with no investigation of *I-V* properties and antagonist specificity (Karschin and Wässle 1990). The current results cannot by themselves explain previous evidence for immunoreactivity of rod bipolar cells for GluN1 (Lo et al. 1998) and GluN2D (Wenzel et al. 1997) subunits. Assuming that the immunolabeling cannot be explained by antibody cross-reactivity, the physiological relevance of the expression is questioned by the absence of any evidence for a conductance that can be activated by NMDA.

Evidence for expression of NMDA receptors by AII and A17 amacrine cells

Whether a specific type of neuron expresses a specific type of neurotransmitter receptor is apparently a simple and well-defined question, but strong and unequivocal evidence can often be difficult to obtain. First, the type of neuron needs to be well-defined and it is necessary to be able to uniquely identify and target individual neurons of the right type. In our case, both AII and A17 amacrine cells are well-defined types of amacrine cells with unique cellular morphologies (reviewed by Masland 2001) and reasonably well-characterized synaptic inputs and outputs (reviewed by Hartveit and Veruki 2012). In addition, as demonstrated in this study and several previous studies, both from our laboratory (Veruki and Hartveit 2002a, 2002b, 2009; Mørkve et al. 2002; Veruki et al. 2003; Castilho et al. 2015a) as well as from others (e.g. Boos et al. 1993; Menger and Wässle 2000; Singer and Diamond 2003; Eggers and Lukasiewicz 2006; Grimes et al. 2010, 2014), both AII and A17 amacrine cells can be reliably identified and targeted in the acute retinal slice preparation (Fig. 1*A*). The morphology observed with IR video microscopy and contrast enhancement techniques (e.g. DIC) is not sufficient, however, for a secure identification. Therefore, all cells we recorded from were also examined with fluorescence microscopy after filling with a fluorescent dye in the intracellular

605 solution, allowing us to visually inspect the complete neuronal morphology as
606 preserved in the acute retinal slice preparation (Fig. 1*B*).
607 In our physiological experiments, we stimulated NMDA receptors by puffer
608 pipette application of the specific agonist NMDA. Binding of agonist to the receptor
609 triggers channel opening and the resulting current can be recorded as an inward
610 current. When measuring such currents, a series of control experiments are necessary
611 before one can conclude that specific receptors are expressed by the neuron being
612 recorded. First, it is necessary to demonstrate that the response is mediated by
613 specific receptors. Second, it is necessary to verify that the activated receptors display
614 functional properties that are characteristic for the specific receptor type. Third, it is
615 important to provide evidence that the responses are mediated by receptors
616 expressed by the cells recorded from, as opposed to being mediated by specific
617 receptors expressed by other types of neurons and conveyed to the cells recorded
618 from through synaptic network mechanisms.
619 With respect to pharmacological specificity, two sets of experiments argue for
620 the involvement of specific NMDA receptors. First, the responses evoked by
621 application of NMDA could be completely blocked by CPP, a competitive antagonist
622 that is specific for NMDA receptors (Davies et al. 1986). Second, the responses were
623 also abolished (for AII amacrines) and strongly suppressed (for A17 amacrines)
624 when the noncompetitive NMDA receptor antagonist and open channel blocker
625 MK-801 (Wong et al. 1986; Huettner and Bean 1988) was applied intracellularly. The
626 difference between AII and A17 amacrine cells is most likely due to lower
627 concentrations of MK-801 that reach the location of the receptors in A17 amacrines
628 because these cells have very long and thin processes that restrict and delay spread
629 by diffusion intracellularly.
630 With respect to functional properties, we demonstrated that the responses
631 evoked by NMDA in whole-cell recordings from both AII and A17 amacrine cells
632 displayed *I-V* properties that are unique for NMDA receptors. First, the *I-V* curves
633 displayed the classical J-shape, with negative slope conductance between -80 and -30

mV (Nowak et al. 1984). From detailed biophysical investigations, we know that this reflects a Mg^{2+}-dependent voltage block of the ion channel associated with the NMDA receptor complex (Nowak et al. 1984). Second, when Mg^{2+} was removed from the extracellular solution, the voltage block disappeared and the shape of the *I-V* curves became more linear for both AII and A17 amacrine cells. For both AII and A17 amacrine cells, the E_{rev} of the NMDA-evoked current was close to 0 mV, consistent with activation of non-selective cation channels.

The *I-V* relationship of the NMDA response and the results with MK-801 also provide strong evidence that the NMDA receptors must be localized on the AII and A17 amacrine cells themselves. First, if the NMDA receptors were not localized on the cell from which the whole-cell recording was performed, it is unlikely that changing the holding potential, as was done in the experiments investigating *I-V* properties, would have changed the driving force to generate the observed *I-V* curves. For example, AII amacrine cells are coupled to ON-cone bipolar cells via electrical synapses mediated by gap junctions (Strettoi et al. 1992, 1994; Veruki and Hartveit 2002b) and when recording from ON-cone bipolar cells that do not express non-NMDA receptors, it is possible to measure responses evoked by non-NMDA receptor agonists, presumably mediated by activating receptors on AII amacrines (Hartveit 1997). Importantly, however, it is not possible to reverse the direction of the evoked currents, presumably because the resistance of the electrical synapses is too high to adequately control the voltage at the location of the ion channels being activated.

Second, because the NMDA receptor antagonist MK-801 blocked responses mediated by NMDA in both AII and A17 amacrine cells after being applied intracellularly, the most likely interpretation is that the responses were mediated by receptors located in the AII and A17 amacrine cells themselves, where MK-801 could diffuse to the receptors within the processes of these cells. It has to be taken into account, however, that with puffer pipette application of drugs to cells in *in vitro* slice preparations, it is difficult to control the spatial extent of application and we do

not know the extent to which pressure-applied NMDA, directed at a specific cell, could also reach neighboring cells. Whereas MK-801 strongly suppressed NMDA-evoked responses in both AII and A17 amacrine cells, we cannot exclude the possibility that MK-801 could diffuse across gap junctions that couple these cells to other neurons. On the other hand, current knowledge of the possibilities available for such coupling suggests that if such difusion of MK-801 should occur, it would not change the conclusion. For homologous coupling, i.e. gap junctions that couple AII amacrines to other AII amacrines (Strettoi et al. 1992; Veruki and Hartveit 2002a) and A17 amacrines to other A17 amacrines (Li et al. 2002; Grimes et al. 2014), any indirectly mediated NMDA receptor-mediated responses blocked by diffusion of MK-801 would originate from NMDA receptors in cells of the same type as recorded from. There is also the possibility that heterologous coupling between AII amacrine cells and ON-cone bipolar cells (Strettoi et al. 1992, 1994; Veruki and Hartveit 2002b) could mediate indirect responses, but existing evidence suggests that ON-cone bipolar cells do not express NMDA receptors (Hartveit 1997). Irrespective of the possibilities for indirect effects mediated via gap junction coupling, NMDA-evoked responses in both AII and A17 amacrine cells were resistant to the gap junction blocker MFA, strongly suggesting that the NMDA-evoked responses of these neurons were mediated by receptors expressed on the cells themselves.

Because neurons are branched structures with dendritic processes that can extend over hundreds of μm, it can be challenging and even impossible to obtain the degree of experimental control that is required for rigorous biophysical investigations of the functional properties of ion channels and receptors expressed along these processes. Whereas investigating the functional properties in isolated membrane patches, either conventional outside-out patches or nucleated patches, can to a large extent compensate for this (e.g. Hartveit and Veruki 2007), we were unfortunately unsuccessful in using this approach for more detailed investigations of the NMDA receptors expressed by AII and A17 amacrine cells. Apart from the apical dendrites of AII amacrines, the processes of both types of cells are too thin for

692 excising outside-out patches. Nucleated patches can be isolated for both types of
693 cells, but the NMDA-evoked responses were unfortunately too small and infrequent
694 to allow adequate analysis. As a control that the lack of responses was not due to
695 technical or methodological problems, we performed experiments where we applied
696 the receptor ligands GABA and glycine to the same patches. These ligands evoked
697 robust responses, confirming previous results for non-NMDA-type glutamate
698 receptors (Mørkve et al. 2002; Veruki et al. 2003) and glycine receptors (Gill et al.
699 2006) at the cell bodies of AII amacrine cells and indicating that the lack of responses
700 obtained with NMDA was due to lack of the receptors themselves and not technical
701 problems. The results for A17 amacrine cells suggest that functional properties of
702 GABA and glycine receptors in these cells can be fruitfully investigated by this
703 technique in future studies.
704
705 **Activity-dependent suppression of NMDA receptors in AII and A17 amacrine cells**
706 The reduction of NMDA-evoked responses in AII amacrine cells previously observed
707 in our laboratory was interpreted as reflecting rundown (Hartveit and Veruki 1997).
708 Rundown is a phenomenon observed for several types of receptors and ion channels
709 and is considered to be a consequence of washout of the intracellular environment
710 after establishing the whole-cell recording configuration (Horn and Korn 1992).
711 Accordingly, the response reduction will occur simply as a function of time. This
712 kind of mechanism is not consistent with the observations made in the current study.
713 First, by restricting the frequency of application of NMDA to once every 60 s, the
714 response was very stable for recording periods up to 25 - 50 min. The ability to
715 maintain stable NMDA responses for such extended time periods did not depend on
716 recording with high series resistance or other measures (e.g. perforated patch) to
717 slow or prevent rundown. This suggests that the NMDA receptors in AII and A17
718 amacrine cells are not particularly susceptible to rundown as such.
719 Second, by increasing the frequency of stimulation, corresponding to
720 intervals of 10 - 15 s, we observed an immediate reduction in the response to NMDA

721 that reversed quickly after reducing the frequency of stimulation. Thus, our results
722 suggest that the suppression of NMDA-evoked responses is an expression of an
723 activity-dependent feedback mechanism. Although our results do not provide a
724 mechanistic explanation, a possible explanation is Ca^{2+}-dependent inactivation which
725 constitutes one form of desensitization of NMDA receptors (for review, see Gibb
726 2010) and has been observed for both native receptors and receptors in heterologous
727 expression systems (Rosenmund and Westbrook 1993; Ehlers et al. 1996). This is
728 supported by our observation of an increased response to NMDA in nominally Ca^{2+}-
729 free extracellular solution for both AII and A17 amacrine cells. Although we have not
730 directly verified Ca^{2+} influx through NMDA receptor channels in either AII or A17
731 amacrine cells, NMDA receptors have been found to be the predominant source of
732 Ca^{2+} signals in several types of neurons, both because of their high Ca^{2+} permeability
733 and their slow kinetics (reviewed by Higley and Sabatini 2012). Further work is
734 required to decide if this mechanism is operative for the NMDA receptors in AII and
735 A17 amacrine cells and whether it plays a specific functional role for signal
736 processing in these cells.
737
738 **Functional role of NMDA receptors in AII and A17 amacrine cells**
739 Despite what we believe is strong evidence for the expression of NMDA receptors by
740 both AII and A17 amacrine cells, very little is known with respect to any functional
741 role of these receptors for the signal processing that takes place in these cells. For AII
742 amacrine cells, there is no evidence for an NMDA receptor-mediated component in
743 the response evoked by depolarization of a presynaptic rod bipolar cell (Singer and
744 Diamond 2003). To our knowledge, nothing is known with respect to the receptors
745 mediating input from (some) OFF-cone bipolar cells to the lobular appendages of AII
746 amacrine cells (Strettoi et al. 1992, 1994; Veruki et al. 2003). Immunocytochemical
747 investigations at the ultrastructural level have not found evidence for NMDA
748 receptors postsynaptic to rod bipolar cells (Fletcher et al. 2000), but there is evidence
749 for a presumed extrasynaptic location of the GluN1 subunit of the NMDA receptor at

arboreal dendrites of AII amacrine cells (Kothmann et al. 2012). Interestingly, GluN1 was colocalized with Cx36 that mediates gap junction coupling between AII amacrine cells and Kothmann et al. (2012) found evidence that NMDA receptors could be involved in regulating the strength of coupling between these cells.

For A17 amacrine cells, the reciprocal feedback inhibition that is triggered by depolarization and glutamate release from rod bipolar cells is not blocked in the presence of the NMDA receptor antagonist CPP (Hartveit 1999), although similar experiments with Mb1 bipolar cells in the goldfish retina suggest that NMDA receptors can contribute to the feedforward excitation of inhibitory amacrine cells, at least under some conditions (Vigh and von Gersdorff 2005). Rod bipolar cells seem to constitute the only source of glutamatergic, bipolar cell input to A17 amacrine cells (Nelson and Kolb 1985). If NMDA receptors in the A17 are not localized postsynaptic to rod bipolars, the only alternative left open would seem to be an extrasynaptic location.

As expression of NMDA receptors now seems to be a property that is shared by both types of amacrine cells postsynaptic to rod bipolar cells, it is possible that information about the subunit composition of the NMDA receptors expressed by the two cell types can provide valuable information. For many regions and cell types in the CNS, it is known that the NMDA receptor subunit expression and composition displays striking variation, not only between regions, but also between cell types and even between different subcellular regions within single neurons (Nusser 2008; Paoletti et al. 2013). From *in situ* hybridization studies, we know that the GluN2A, GluN2B, and GluN2C subunits of the NMDA receptor are expressed in the rat retina, with considerable heterogeneity among amacrine cells in the INL, but the resolution is not sufficient to decide the specific identity of the various cells (Brandstätter et al. 1994). This, together with the mechanisms and conditions for activation of NMDA receptors on AII and A17 amacrine cells, remain important questions for future investigations. NMDA receptors might contribute directly to signaling in these amacrine cells or their function could be important for modulation and plasticity of

779 other ion channels that are more directly responsible for signal processing and
780 integration.
781
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788
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791
792 **AUTHOR CONTRIBUTIONS**
793 Author contributions: Y.Z. and B.T. performed experiments; Y.Z., B.T., and M.L.V.
794 analyzed data; M.L.V. prepared figures; E.H. and M.L.V. edited and revised
795 manuscript; E.H. and M.L.V. conception and design of research; E.H. and M.L.V.
796 interpreted results of experiments; E.H. and M.L.V. drafted manuscript; Y.Z., B.T.,
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REFERENCES

Bloomfield SA, Dacheux RF. Rod vision: pathways and processing in the mammalian retina. *Prog Ret Eye Res* 20: 351–384, 2001.

Bloomfield SA, Xin D. Surround inhibition of mammalian AII amacrine cells is generated in the proximal retina. *J Physiol* 523: 771-783, 2000.

Boos R, Schneider H, Wässle H. Voltage- and transmitter-gated currents of AII-amacrine cells in a slice preparation of the rat retina. *J Neurosci* 13: 2874-2888, 1993.

Brandstätter JH, Hartveit E, Sassoè-Pognetto M, Wässle H. Expression of NMDA and high-affinity kainate receptor subunit mRNAs in the adult rat retina. *Eur J Neurosci* 6: 1100-1112, 1994.

Castilho Á, Ambrósio AF, Hartveit E, Veruki ML. Disruption of a neural microcircuit in the rod pathway of the mammalian retina by diabetes mellitus. *J Neurosci* 35: 5422-5433, 2015a.

Castilho Á, Madsen E, Ambrósio AF, Veruki ML, Hartveit E. Diabetic hyperglycemia reduces Ca^{2+} permeability of extrasynaptic AMPA receptors in AII amacrine cells. *J Neurophysiol* 114: 1545-1553, 2015b.

Chávez AE, Singer JH, Diamond JS. Fast neurotransmitter release triggered by Ca influx through AMPA-type glutamate receptors. *Nature* 443: 705-708, 2006.

Copenhagen DR, Mittman S, Taylor WR, Dixon DB. Glutamatergic synaptic excitation of retinal amacrine and ganglion cells. In: *Contrast Sensitivity*, edited by Shapley R and Lam D M-K. Cambridge, MA: MIT Press, 1993, p. 41-57.

Davies J, Evans RH, Herrling PL, Jones AW, Olverman HJ, Pook P, Watkins JC. CPP, a new potent and selective NMDA antagonist. Depression of central neuron responses, affinity for [^{3}H]D-AP5 binding sites on brain membranes and anticonvulsant activity. *Brain Res* 382: 169-173, 1986.

Eggers ED, Lukasiewicz PD. $GABA_A$, $GABA_C$ and glycine receptor-mediated inhibition differentially affects light-evoked signalling from mouse retinal rod

bipolar cells. *J Physiol* 572: 215-225, 2006.

Ehlers MD, Zhang S, Bernhardt JP, Huganir RL. Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* 84: 745-755, 1996.

Engelman HS, MacDermott AB. Presynaptic ionotropic receptors and control of transmitter release. *Nat Rev Neurosci* 5: 135-145, 2004.

Fletcher EL, Hack I, Brandstätter JH, Wässle H. Synaptic localization of NMDA receptor subunits in the rat retina. *J Comp Neurol* 420: 98-112, 2000.

Gibb A. NMDA receptors. In: *Ion Channels. From Structure to Function*, edited by Kew JNC and Davies CH. New York: Oxford University Press, 2010, p. 309-325.

Gill SB, Veruki ML, Hartveit E. Functional properties of spontaneous IPSCs and glycine receptors in rod amacrine (AII) cells in the rat retina. *J Physiol* 575: 739-759, 2006.

Grimes WN, Zhang J, Graydon CW, Kachar B, Diamond JS. Retinal parallel processors: more than 100 independent microcircuits operate within a single interneuron. *Neuron* 65: 873-885, 2010.

Grimes WN, Hoon M, Briggman KL, Wong RO, Rieke F. Cross-synaptic synchrony and transmission of signal and noise across the mouse retina. *eLife* 3: e03892, 2014.

Hartveit E. Membrane currents evoked by ionotropic glutamate receptor agonists in rod bipolar cells in the rat retinal slice preparation. *J Neurophysiol* 76: 401-422, 1996.

Hartveit E. Functional organization of cone bipolar cells in the rat retina. *J Neurophysiol* 77: 1716-1730, 1997.

Hartveit E. Reciprocal synaptic interactions between rod bipolar cells and amacrine cells in the rat retina. *J Neurophysiol* 81: 2923-2936, 1999.

Hartveit E, Veruki ML. AII amacrine cells express functional NMDA receptors. *Neuroreport* 8: 1219-1223, 1997.

Hartveit E, Veruki ML. Studying properties of neurotransmitter receptors by non-stationary noise analysis of spontaneous postsynaptic currents and agonist-evoked responses in outside-out patches. *Nat Prot* 2: 434-448, 2007.

Hartveit E, Veruki ML. Electrical synapses between AII amacrine cells in the retina: Function and modulation. *Brain Res* 1487: 160-172, 2012.

Hassel B, Dingledine R. Glutamate and glutamate receptors. In: *Basic Neurochemistry*, edited by Brady ST et al. Amsterdam: Elsevier, 8th ed., 2012, p. 342-366.

Higley MJ, Sabatini BL. Calcium signaling in dendritic spines. *Cold Spring Harb Perspect Biol* 4: a005686, 2012.

Horn R, Korn SJ. Prevention of rundown in electrophysiological recording. In: *Methods in Enzymology: Ion Channels*, edited by Rudy B, Iverson LE. San Diego, CA: Academic Press, vol. 207, 1992, p. 149-155.

Huettner JE, Bean BP. Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc Natl Acad Sci USA* 85: 1307-1311, 1988.

Karschin A, Wässle H. Voltage- and transmitter-gated currents in isolated rod bipolar cells of rat retina. *J Neurophysiol* 63: 860-876, 1990.

Kleckner NW, Dingledine R. Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science* 241: 835-837, 1988.

Kolb H, Famiglietti EV. Rod and cone pathways in the inner plexiform layer of cat retina. *Science* 186: 47-49, 1974.

Kothmann W, Massey SC, O'Brien J. Nonsynaptic NMDA receptors mediate activity-dependent plasticity of gap junctional coupling in the AII amacrine cell network. *J Neurosci* 32: 6747-6759, 2012.

Li W, Zhang J, Massey SC. Coupling pattern of S1 and S2 amacrine cells in the rabbit retina. *Visual Neurosci* 19: 119-131, 2002.

Lo W, Molloy R, Hughes TE. Ionotropic glutamate receptors in the retina: moving from molecules to circuits. *Vision Res* 38: 1399-1410, 1998.

884 **Malinow R, Mainen ZF, Hayashi Y.** LTP mechanisms: from silence to four-lane
885 traffic. *Curr Op Neurobiol* 10: 352-357, 2000.
886 **Manookin MB, Beaudoin DL, Ernst ZR, Flagel LJ, Demb JB.** Disinhibition combines
887 with excitation to extend the operating range of the OFF visual pathway in
888 daylight. *J Neurosci* 28: 4136-4150, 2008.
889 **Manookin MB, Weick M, Stafford BK, Demb JB.** NMDA receptor contributions to
890 visual contrast coding. *Neuron* 67: 280-293, 2010.
891 **Masland RH.** The fundamental plan of the retina. *Nat Neurosci* 4: 877-886, 2001.
892 **Massey SC, Maguire G.** The role of glutamate in retinal circuitry. In: *Excitatory*
893 *Amino Acids and Synaptic Transmission*, edited by Wheal H, Thomson A.
894 London: Academic Press, 2nd ed., 1995, p. 201-221.
895 **Menger N, Wässle H.** Morphological and physiological properties of the A17
896 amacrine cell in the rat retina. *Visual Neurosci* 17: 769-780, 2000.
897 **Mørkve SH, Veruki ML, Hartveit E.** Functional characteristics of non-NMDA-type
898 ionotropic glutamate receptor channels in AII amacrine cells in rat retina. *J*
899 *Physiol* 542: 147-165, 2002.
900 **Münch TA, da Silveira RA, Siegert S, Viney TJ, Awatramani GB, Roska B.**
901 Approach sensitivity in the retina processed by a multifunctional neural
902 circuit. *Nat Neurosci* 12: 1308–1316, 2009.
903 **Nelson R, Kolb H.** A17: A broad-field amacrine cell in the rod system of the cat
904 retina. *J Neurophysiol* 54: 592-614, 1985.
905 **Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A.** Magnesium gates
906 glutamate-activated channels in mouse central neurones. *Nature* 307: 462-465,
907 1984.
908 **Nusser Z.** Subcellular distribution of neurotransmitter receptors and voltage-gated
909 ion channels. In: *Dendrites*, edited by Stuart G, Spruston N, and Häusser M.
910 Oxford: Oxford University Press, 2nd ed., 2008, p. 155-187.

911 **Paoletti P, Bellone C, Zhou Q.** NMDA receptor subunit diversity: impact on
912 receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 14: 383-
913 400, 2013.
914 **Perkel DJ, Hestrin S, Sah P, Nicoll RA.** Excitatory synaptic currents in Purkinje
915 cells. Proc R Soc Lond B 241: 116-121, 1990.
916 **Raviola E, Dacheux RF.** Excitatory dyad synapse in rabbit retina. *Proc Natl Acad Sci*
917 *USA* 84: 7324-7328, 1987.
918 **Rosenmund C, Westbrook GL.** Rundown of N-methyl-D-aspartate channels during
919 whole-cell recording in rat hippocampal neurons: role of Ca^{2+} and ATP. *J*
920 *Physiol* 470: 705-729, 1993.
921 **Salter MW, Dong Y, Kalia LV, Liu XJ, Pitcher G.** Regulation of NMDA receptors by
922 kinases and phosphatases. In: *Biology of the NMDA Receptor*, edited by
923 VanDongen AM. Boca Raton, FL: CRC Press, 2009, p. 123-148.
924 **Sheng M, Kim E.** The postsynaptic organization of synapses. *Cold Spring Harb*
925 *Perspect Biol* 4: a005678, 2012.
926 **Singer JH, Diamond JS.** Sustained Ca^{2+} entry elicits transient postsynaptic currents
927 at a retinal ribbon synapse. *J Neurosci* 23: 10923-10933, 2003.
928 **Smart TG, Paoletti P.** Synaptic neurotransmitter-gated receptors. *Cold Spring Harb*
929 *Perspect Biol* 4: a009662, 2012.
930 **Stafford BK, Manookin MB, Singer JH, Demb JB.** NMDA and AMPA receptors
931 contribute similarly to temporal processing in mammalian retinal ganglion
932 cells. *J Physiol* 592: 4877-4889.
933 **Strettoi E, Dacheux RF, Raviola E.** Synaptic connections of rod bipolar cells in the
934 inner plexiform layer of the rabbit retina. *J Comp Neurol* 295: 449-466, 1990.
935 **Strettoi E, Raviola E, Dacheux RF.** Synaptic connections of the narrow-field,
936 bistratified rod amacrine cell (AII) in the rabbit retina. *J Comp Neurol* 325: 152-
937 168, 1992.
938 **Strettoi E, Dacheux RF, Raviola E.** Cone bipolar cells as interneurons in the rod
939 pathway of the rabbit retina. *J Comp Neurol* 347: 139-149, 1994.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* 62: 405-496, 2010.

Veruki ML, Hartveit E. AII (rod) amacrine cells form a network of electrically coupled interneurons in the mammalian retina. *Neuron* 33: 935-946, 2002a.

Veruki ML, Hartveit E. Electrical synapses mediate signal transmission in the rod pathway of the mammalian retina. *J Neurosci* 22: 10558-10566, 2002b.

Veruki ML, Hartveit E. Meclofenamic acid blocks electrical synapses of retinal AII amacrine and ON-cone bipolar cells. *J Neurophysiol* 101: 2339-2347, 2009.

Veruki ML, Mørkve SH, Hartveit E. Functional properties of spontaneous EPSCs and non-NMDA receptors in rod amacrine (AII) cells in the rat retina. *J Physiol* 549: 759-774, 2003.

Vigh J, von Gersdorff H. Prolonged reciprocal signaling via NMDA and GABA receptors at a retinal synapse. *J Neurosci* 25: 11412-11423, 2005.

Wenzel A, Benke D, Mohler H, Fritschy J-M. N-methyl-D-aspartate receptors containing the NR2D subunit in the retina are selectively expressed in rod bipolar cells. *Neuroscience* 78: 1105-1112, 1997.

Wong EHF, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iversen LL. The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc Natl Acad Sci USA* 83: 7104-7108, 1986.

Zhang S, Ehlers MD, Bernhardt JP, Su CT, Huganir RL. Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* 21: 443-453, 1998.

Zhou C, Dacheux RF. All amacrine cells in the rabbit retina possess AMPA-, NMDA-, GABA-, and glycine-activated currents. *Visual Neurosci* 21: 181-188, 2004.

967 **FIGURE LEGENDS**

968 Fig. 1. Identification of rod amacrine cells (AII and A17) and rod bipolar cells in the
969 rat retinal slice preparation. *A*: *left,* infrared differential interference contrast (IR-DIC)
970 videomicrograph of an A17 amacrine cell in a retinal slice. Whole-cell recording with
971 upper pipette (located at cell body). Lower pipette for drug application. *Middle*, IR-
972 DIC videomicrograph of an AII amacrine cell in a retinal slice. Whole-cell recording
973 with upper pipette (located at cell body). Lower pipette for drug application. *Right*,
974 IR-DIC videomicrograph of a rod bipolar cell in a retinal slice. Whole-cell recording
975 with pipette located at cell body. *B*: maximum intensity projections (along Z-axis)
976 generated from wide-field fluorescence image stacks (after deconvolution) for cells
977 recorded in retinal slices and filled with Alexa Fluor 594 via patch pipettes: A17
978 amacrine cell (*left*), AII amacrine cell (*middle*), and rod bipolar cell (*right*). *C*: *left*,
979 IR-DIC videomicrograph of a nucleated patch pulled from an AII amacrine cell in the
980 retinal slice and a multibarrel pipette for drug application. *Right:* IR-DIC
981 videomicrograph of a nucleated patch pulled from an AII amacrine cell in the retinal
982 slice and a single pipette for drug application. Scale bars: ***A***-***C***, 10 μm.

983

984 Fig. 2. AII and A17 amacrine cells, but not rod bipolar cells, express functional
985 (conductance-increasing) NMDA receptors. *A*: schematic diagram of retinal
986 microcircuit consisting of a rod bipolar cell (RBC) and AII and A17 amacrine cells.
987 Arrows indicate direction and sign of synaptic transmission between the cells:
988 feedforward glutamatergic transmission from RBCs to AII and A17 amacrines (solid
989 arrows) and feedback GABAergic transmission from A17 amacrine to RBC (open
990 arrow). *B*: current activated in an AII amacrine cell (V_{hold} = -60 mV) by application of
991 NMDA (1 mM, 1 s) from a puffer pipette. Except where indicated, NMDA was
992 always applied together with the co-agonist D-serine (200 μM). Here and later,
993 recording configuration indicated by the schematic figure (*right*). In this and
994 subsequent figures, the duration of drug application is indicated by the horizontal
995 bar above the current trace and, unless otherwise noted, recordings were performed

996 in nominally Mg^{2+}-free extracellular solution (both in the bath and in the drug
997 pipette). *C*: current activated in an A17 amacrine cell (V_{hold} = -60 mV) by application
998 (1 s) of NMDA (1 mM) from a puffer pipette. ***D***, No current activated in a rod bipolar
999 cell (V_{hold} = -60 mV) by application of NMDA (1 mM, 1 s) from a multibarrel puffer
1000 pipette. The two traces correspond to application directed towards the soma-
1001 dendritic region (1) and the axon terminal region (2), obtained by moving the
1002 multibarrel pipette as indicated in the schematic figure (*right*). To avoid confounding
1003 effects caused by application of the vehicle solution itself, application of NMDA was
1004 preceded and followed by application of vehicle solution without agonist from a
1005 different barrel of the multibarrel pipette (broken horizontal lines). The extracellular
1006 solution contained strychnine, bicuculline, TPMPA, CNQX, and TTX (see Methods).
1007
1008 Fig. 3. AII and A17 amacrine cells express NMDA receptors blocked by the specific
1009 antagonist CPP. *A*: currents activated in an AII amacrine cell (V_{hold} = -60 mV) by
1010 application (1.5 s) of NMDA (1 mM) from a multibarrel puffer pipette. *Top* trace:
1011 response evoked by NMDA in the control condition. *Middle* trace: no response to
1012 NMDA when co-applied with CPP (400 μM) in the same pipette barrel. *Bottom* trace:
1013 recovery of response to NMDA after washing out CPP. Here and in *B*, the
1014 extracellular solution contained strychnine, bicuculline, CNQX, and TTX (see
1015 Methods). *B*: currents activated in an A17 amacrine cell (V_{hold} = -70 mV) by
1016 application (1.5 s) of NMDA (1 mM) from a multibarrel puffer pipette. *Top* trace:
1017 response evoked by NMDA in the control condition. *Middle* trace: no response to
1018 NMDA when co-applied with CPP (400 μM) in the same pipette barrel. *Bottom* trace:
1019 recovery of response to NMDA after washing out CPP.
1020
1021 Fig. 4. AII and A17 amacrine cells express NMDA receptors blocked by intracellular
1022 application of the specific noncompetitive antagonist (open-channel blocker) MK-
1023 801. *A*: currents recorded in an AII amacrine cell (V_{hold} = -60 mV) with application (1
1024 s) of NMDA (1 mM) from a puffer pipette. MK-801 (2 mM) added to the intracellular

solution in the recording pipette. Here and in *B*, time points at the right of each trace indicate the approximate time after breaking into the cell and establishing the whole-cell recording configuration. Notice lack of response to NMDA even during the first application. Here and in *B*, the extracellular solution contained strychnine, bicuculline, CNQX, and TTX (see Methods). *B*: currents recorded in an A17 amacrine cell (V_{hold} = -70 mV) with application (1 s) of NMDA (1 mM) from a puffer pipette. MK-801 (2 mM) added to the intracellular solution in the recording pipette. Notice response to NMDA during the first application and gradual reduction of response amplitude during subsequent applications reflecting increasing block of NMDA receptors by MK-801.

Fig. 5. Current-voltage (*I-V*) relationships of NMDA-evoked whole-cell currents responses in AII and A17 amacrine cells. *A*: NMDA-evoked (1 mM, 1 s) responses in an AII amacrine cell, normal (1 mM) Mg^{2+} extracellularly. Here and in subsequent figures, voltages next to the current traces indicate holding potential of cell (-80 to +60 mV) and circle below traces indicates position of time window for averaging the response amplitude. Here and in *B*, the extracellular solution contained CNQX and TTX (see Methods). *B*: NMDA-evoked (1 mM, 1 s) responses in an AII amacrine cell, nominally Mg^{2+}-free extracellular solution. Notice larger NMDA-evoked responses at negative holding potentials compared to *A*. *C*: *left*, *I-V* relationship for NMDA-evoked peak responses in six AII amacrines in normal Mg^{2+} extracellularly (as in *A*, filled circles) and five AII amacrines in nominally Mg^{2+}-free extracellular solution (as in *B*, open circles). Here and in subsequent figures, the NMDA-evoked current at each holding potential is plotted as mean ± SE; n = 4 - 6 cells for each data point). Data points connected by straight lines. *Right*, *I-V* relationship for NMDA-evoked peak responses, same data as in left panel, but data points normalized to the current at +40 mV. Data points connected by straight lines. *D*: NMDA-evoked (1 mM, 1 s) responses in an A17 amacrine cell, normal (1 mM) Mg^{2+} extracellularly. Here and in *E*, the extracellular solution contained strychnine, bicuculline, CNQX, and TTX (see

Methods). *E*: NMDA-evoked (1 mM, 1.5 s) responses in an A17 amacrine cell, nominally Mg^{2+}-free extracellular solution. Notice larger NMDA-evoked responses at negative holding potentials compared to *D*. *F*: *left*, *I-V* relationship for NMDA-evoked peak responses in seven A17 amacrines in normal Mg^{2+} extracellularly (as in *D*, filled circles) and six A17 amacrines in nominally Mg^{2+}-free extracellular solution (as in *E*, open circles; n = 4 -7 cells for each data point). Data points connected by straight lines. *Right*, *I-V* relationship for NMDA-evoked peak responses, same data as in left panel, but with data points normalized to the current at +40 mV. Data points connected by straight lines.

Fig. 6. Blocking gap junction coupling pharmacologically with meclofenamic acid (MFA) does not block NMDA-evoked responses in AII or A17 amacrine cells. *A*: currents activated in an AII amacrine cell (V_{hold} = -60 mV) by application of NMDA (1 mM, 1 s) from a puffer pipette at 7 (*top*), 27 (*middle*), and 45 (bottom) min of whole-cell recording with MFA (100 μM) in the extracellular solution. *B*: currents activated in an A17 amacrine cell (V_{hold} = -60 mV) by application of NMDA (1 mM, 1 s) from a puffer pipette at 7 (*top*), 23 (*middle*), and 38 (*bottom*) min of whole-cell recording with MFA (100 μM) in the extracellular solution. NMDA was applied every 60 s (*A*, *B*). Notice how NMDA-evoked responses are maintained in the presence of MFA (*A*, *B*).

Fig. 7. Nucleated patches from AII amacrine cells do not respond to NMDA. *A*: no response of nucleated patch to application of NMDA (1 mM, 3 s; upper right traces) at -60 or +20 mV (as indicated), normal (1 mM) Mg^{2+} extracellularly. Here and in *B*, NMDA was applied together with the co-agonist glycine (10 μM) and strychnine (10 μM) and picrotoxin (100 μM) to block conventional glycine receptors. Robust responses of same nucleated patch to application of glycine (1 mM, 100 ms; lower left traces) and GABA (1 mM, 500 ms; lower right traces) at -60 mV (inward currents) and +20 mV (outward currents). *B*: clear response to NMDA of AII amacrine cell (voltage clamped at +40 mV, normal Mg^{2+} extracellularly) during whole-cell

recording in retinal slice (upper right trace; recording configuration indicated in upper left panel), but no response when tested with NMDA after pulling nucleated patch (lower right trace; recording configuration indicated in lower left panel).

Fig. 8. Nucleated patches from A17 amacrine cells show no or only minimal response to NMDA. *A*: no response of nucleated patch to application of NMDA (1 mM, 1 s; upper right traces) at -60 or +20 mV (as indicated). Robust responses of same nucleated patch to application of glycine (1 mM, 500 ms; lower left traces) and GABA (1 mM, 500 ms; lower right traces) at -60 mV (inward currents) and +20 mV (outward currents). *B*: clear response to NMDA of A17 amacrine cell (voltage clamped at -60 mV) during whole-cell recording in retinal slice (upper right trace; recording configuration indicated in upper left panel), but only minimal response with increase in membrane noise when tested with NMDA after pulling nucleated patch (lower right trace; recording configuration indicated in lower left panel).

Fig. 9. NMDA-evoked responses in AII and A17 amacrine cells display temporal stability without rundown. *A*: *top*, time series of peak response amplitude of an AII amacrine cell (V_{hold} = -60 mV) to repeated application of NMDA (1 mM, 1 s duration, 60 s interval) during 50 min. Notice stability of response amplitude. *Bottom*, time series of peak response amplitude of AII amacrine cells (as in top panel; n = 4 cells) to repeated application of NMDA (1 mM, 1 s). Here and in *B*, time "0" indicates the time of breaking into the cell and establishing the whole-cell recording configuration, data points plotted as means ± SE and normalized to the current evoked by the first application for each cell (n = 1 - 4 cells for each data point, depending on duration of recording). *B*: *top*, time series of peak response amplitude of an A17 amacrine cell (V_{hold} = -70 mV) to repeated application of NMDA (1 mM, 1 s duration, 60 s interval) during 26 min. Notice stability of response amplitude. *Bottom*, time series of peak response amplitude of A17 amacrine cells (as in top panel; n = 6 cells) to repeated

application of NMDA (1 mM, 1 s; n = 3 - 6 cells for each data point, depending on duration of recording).

Fig. 10. Frequency-dependent suppression of NMDA-evoked responses in AII and A17 amacrine cells. *A*: *top*, time series of peak response amplitude of an AII amacrine cell (V_{hold} = -60 mV) to repeated application of NMDA (1 mM, 1 s duration). For the cell illustrated, testing started after about 20 min of recording. Interval between pulses: 60 s (22 - 29 min), 10 s (29 - 33 min), 60 s (34 - 43 min), 10 s (43 - 46 min), and 60 s (47 - 56 min). Notice response reduction during higher-frequency application of NMDA. Numbers (1, 2, and 3) correspond to raw data traces (*bottom*) displaying responses evoked by NMDA application during lower- (1, 3) and higher-frequency (2) stimulation periods. Here and in *C*, time "0" indicates the time of breaking into the cell and establishing the whole-cell recording configuration. *Middle*, time series of corresponding estimates of series resistance (R_s) for whole-cell recording in top panel. R_s estimate updated between each application of NMDA. Notice relative stability of R_s. *B*: peak amplitude of NMDA-evoked responses (here and below, bars represent means ± SE) in AII amacrine cells (n = 7 cells) in control condition with 60 s intervals between NMDA-applications (Control; *left* bar), during higher-frequency stimulation with 10 - 15 s intervals between NMDA-applications (High-freq.; *middle* bar), and after return to the control condition (Recovery; *right* bar). Here and later, data points for the same cell are connected by lines and the results from statistical comparisons between averages are indicated by a single asterisk (statistically significant difference; $P < 0.05$). *C*: *top*, time series of peak response amplitude of an A17 amacrine cell (V_{hold} = -70 mV) to repeated application of NMDA (1 mM, 1 s duration). Interval between pulses: 60 s (3 - 9 min), 12 s (9 - 15 min), 60 s (17 - 25 min), and 12 s (26 - 32 min). Notice response reduction during higher-frequency application of NMDA. Numbers (1, 2, and 3) correspond to raw data traces (*bottom*) displaying responses evoked by NMDA application during lower- (1, 3) and higher-frequency (2) stimulation periods. *Middle*, time series of corresponding estimates of

R_s for whole-cell recording in top panel. R_s estimate updated between each application of NMDA. Notice relative stability of R_s. *D*: peak amplitude of NMDA-evoked responses in A17 amascrine cells (n = 8 cells) in control condition with 60 s intervals between NMDA-applications (Control; *left* bar), during higher-frequency stimulation with 10 - 15 s intervals between NMDA-applications (High-freq.; *middle* bar), and after return to the control condition (Recovery; *right* bar).

Fig. 11. Ca^{2+}-dependent suppression of NMDA-evoked responses in AII and A17 amacrine cells. *A*: *top*, time series of peak response amplitude of an AII amacrine cell (V_{hold} = -60 mV) to repeated application of NMDA (1 mM, 1 s duration) at intervals of 60 s, first in normal extracellular solution ($[Ca^{2+}]_o$ = 2.5 mM), followed by nominally Ca^{2+}-free extracellular solution (continuous line), and return to normal extracellular solution. Notice response increase in Ca^{2+}-free solution. Numbers (1, 2, and 3) correspond to raw data traces (*bottom*) displaying responses evoked by NMDA application in normal (1, 3) and Ca^{2+}-free (2) bath solution. *B*: peak amplitude of NMDA-evoked responses in AII amacrine cells (n = 5 cells) in control solution with $[Ca^{2+}]_o$ = 2.5 mM (Control; *left* bar), in nominally Ca^{2+}-free extracellular solution ($[Ca^{2+}]_o$ = 0; *middle* bar), and after return to control solution with $[Ca^{2+}]_o$ = 2.5 mM (Recovery; *right* bar). *C*: *top*, time series of peak response amplitude of an A17 amacrine cell (V_{hold} = -60 mV) to repeated application of NMDA (1 mM, 1 s duration) at intervals of 60 s, first in normal extracellular solution ($[Ca^{2+}]_o$ = 2.5 mM), followed by nominally Ca^{2+}-free extracellular solution (continuous line), and return to normal extracellular solution. Notice response increase in Ca^{2+}-free solution. Numbers (1, 2, and 3) correspond to raw data traces (*bottom*) displaying responses evoked by NMDA application in normal (1, 3) and Ca^{2+}-free (2) bath solution. *D*: peak amplitude of NMDA-evoked responses in A17 amacrine cells (n = 5 cells) in control solution with $[Ca^{2+}]_o$ = 2.5 mM (Control; *left* bar), in nominally Ca^{2+}-free extracellular solution ($[Ca^{2+}]_o$ = 0; *middle* bar), and after return to control solution with $[Ca^{2+}]_o$ = 2.5 mM (Recovery; *right* bar).