Functional N-Methyl-D-Aspartate Receptors Are Expressed in Cone-Driven Horizontal Cells in Carp Retina

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Introduction

Ionotropic glutamate receptors have been described as either N-methyl-D-aspartate (NMDA), or non-NMDA subtypes, depending on whether they are activated by NMDA. NMDA receptors are heteromeric in that they are composed of the NR1 subunit (which binds glycine) and the NR2 subunit (which binds glutamate) [1]. NMDA receptors are up to 70 times more permeable to Ca\(^{2+}\) than non-NMDA receptors, and once activated, produce long lasting effects on the postsynaptic neurons through changing the Ca\(^{2+}\) permeability [2, 3]. NMDA receptors are known to be involved in a variety of physiological and pathological processes in the CNS. While these receptors have been shown to play functional roles in the inner retina [4–6], the data concerning functions of NMDA receptors in the outer retina are rather fragmentary. As far as horizontal cells (HCs) that are responsible for lateral interaction in the distal retina are concerned, it is generally thought that NMDA receptors are absent from these cells [6, 7] and it is the AMPA receptor that mediates signal transfer from photoreceptors to these cells in both mammalian and non-mammalian retinas [8–10]. The only exception from this general scheme is the catfish HC, which expresses NMDA receptors [11, 12]. It should be noted, however, that there is anatomical evidence, suggesting the existence of these receptors in HCs of rat and human [13–15].

Key Words
NMDA · NMDA receptor · Retina · Horizontal cell · Patch clamp · Ca\(^{2+}\)

Abstract

Glutamate works as a major excitatory neurotransmitter in the vertebrate retina. Whole-cell recordings made from isolated carp cone horizontal cells (H1 cells) showed that N-methyl-D-aspartate (NMDA), co-applied with glycine, induced inward currents that were blocked by the NMDA receptor competitive antagonist D-2-amino-5-phosphono-pentanoate (D-AP5) and 5,7-dichlorokynurenic acid (DCKA), a selective NMDA receptor antagonist acting at the glycine site on the NMDA receptor complex. Moreover, calcium imaging showed that NMDA caused a significant elevation of intracellular calcium levels ([Ca\(^{2+}\)]\(_i\)) of H1 cells, which was also blocked by D-AP5. In contrast, neither inward currents nor changes in [Ca\(^{2+}\)]\(_i\), could be induced by NMDA in rod horizontal cells (H4 cells). Intracellular recordings made from H1 cells in the isolated retina, superfused with Ringer’s containing 1 mM Mg\(^{2+}\), in the dark demonstrated that NMDA reduced the light-off overshoot of H1 cells. We therefore conclude that the functional NMDA receptor is expressed in carp H1 cells, from which this receptor has been thought to be absent, and this receptor may play a role in modulating cone-driven signal of horizontal cells in the dark.

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In the present work we show by patch clamp techniques and calcium imaging that NMDA application induced a whole-cell current and an increase in intracellular calcium levels \([\text{Ca}^{2+}]\), in carp cone-driven HCs (H1 cells), but not in rod-driven ones (H4 cells), which was suppressed by D-2-amino-5-phosphonopentanoate (D-AP5), suggesting the presence of functional NMDA receptors in the H1 cells. Possible functional roles of these receptors were analyzed by intracellular recording in the isolated superfused retina.

**Materials and Methods**

**Animals**

Experiments were performed on the adult Crucian carp (*Carassius auratus*) retina, as described previously [16]. The animal, maintained under a 12:12 h light:dark cycle for at least 1 week, was dark-adapted for 20 min prior to an experiment and then decapitated. The eyeball was enucleated and hemisected. Adequate care was taken to minimize pain and discomfort to animals in accordance with the National Institutes of Health guidelines for animal experimentation.

**Whole-Cell Patch Clamp Recording**

Dissociation of retinal neurons and whole-cell recording were conducted following the procedures described in detail previously [8]. In brief, freshly dissociated HCs were commonly bathed in Mg\(^{2+}\)-free Ringer’s containing (in mM) NaCl 145, KCl 5, CaCl\(_2\) 2, HEPES 10 and glucose 16, pH adjusted to 7.4 with NaOH. H1 and H4 cells were identified by their characteristic morphology [17]. Whole-cell membrane currents of HCs, voltage-clamped at –60 mV, were recorded with pipettes of 5–7 MΩ. That is, the liquid junction potential was compensated online. That is, the liquid potential for the solution used was calculated and then added to the nominal holding potential for the amplifier. Fast capacitance and cell capacitance transients were cancelled by the circuit of the amplifier. 70% of the series resistance of the recording electrode was compensated. Analog signals were filtered at 2 kHz, sampled at 10 kHz, and stored on PC hard disk for further off-line analysis. The data were all presented as mean ± SEM. Paired Student’s t test was performed for statistical analysis.

All solutions were delivered using a stepper motor-based rapid solution changer RSC-100 (Bio-Logic Science Instruments, France), with which solution exchange could be completed in a few milliseconds [8]. The recorded cells were lifted from the dish bottom and completely bathed in the solution. In all experiments, NMDA was always co-applied with 40 μM glycine, which alone could not induce detectable current, unless otherwise specified.

**Calcium Imaging**

[Ca\(^{2+}\)], in HCs were monitored with fura-2 AM (Dojindo, Kumamoto, Japan), a membrane permeable indicator, following a procedure described previously [18]. Fura-2 AM (1 mM) was dissolved in 20% Pluronic F-127 (w/v, DMSO) and then added to Ringer’s at a dilution of 1:500. Fura-2 AM-containing Ringer’s was added to a chamber to give a final concentration of 2 μM fura-2 AM. Isolated HCs were incubated in the dye solution for 30 min at room temperature and then perfused with dye-free Ringer’s for 5 min before an experiment. Fluorescence images were acquired with an inverted microscope (IX-70; Olympus Optical, Tokyo, Japan) equipped with a digital CCD camera (C4742–95-12NBB; Hamamatsu Photonics, Hamamatsu, Japan). A high-speed scanning polychromatic light source (C7773; Hamamatsu Photonics) was used for alternate excitations at wavelengths of 340 and 380 nm. The fluorescence intensities at both wavelengths (F340 and F380) were measured every 0.5 s, and images were obtained using PC-based software (Aquacosmos version 1.2; Hamamatsu Photonics, Hamamatsu, Japan). The ratio between the two was proportional to [Ca\(^{2+}\)], of the cell under study. Before an experiment, a bath ground level of fluorescence (attributable to autofluorescence and camera noise) was determined and subtracted from all the data obtained. Solutions were delivered using a DAD-VC voltage command valve control system PR-10 (Scientific Instrument, New York, N.Y., USA).

**Intracellular Recording and Photostimulation**

Intracellular recordings were conventionally made from HCs in the isolated, superfused retina [16], superfused with oxygenated (95% O\(_2\), 5% CO\(_2\)) Ringer’s containing 1 mM Mg\(^{2+}\) buffered to pH 7.7. The retina was illuminated diffusely from the photoreceptor side by a dual-beam photostimulator, which provided two coincident 8 mm-diameter spots around the electrode tip. Light intensities and wavelengths of the two beams were changed by calibrated neutral density and interference filters. All light intensities referred to in the text are in log units relative to the maximum intensity (log I = 0), which was 5.5 × 10\(^{13}\) quanta cm\(^{-2}\) s\(^{-1}\).

Glass microelectrodes filled with 4 M potassium acetate and having a tip resistance of 70–150 MΩ, in combination with an amplifier (MEZ 8300; Nihon Koden Corporation, Tokyo, Japan), were employed to record intracellular potentials. Light responses of H1 cells were identified by reference to previously well-established criteria [19].

**Chemicals**

All chemicals were obtained from Sigma Chemical Company (St. Louis, Mo., USA), unless otherwise specified.

**Results**

**NMDA Induces Inward Currents from Isolated H1 Cells**

In 96 out of 98 H1 cells tested, NMDA induced inward currents. Figure 1a shows desensitizing current responses induced by NMDA of increasing concentrations from an isolated H1 cell, voltage-clamped at –60 mV. The threshold concentration of NMDA was around 10 μM. With the increase of NMDA concentration, the peak current steadily increased in size and reached a maximum value at 1 mM. The desensitization course of the current...
induced by 1 mM NMDA was well fitted by equation
\[ I(t) = A \times e^{-t/\tau} + C, \]
yielding a time constant (\(\tau\)) of 157 ms (fig. 1b). The average value of \(\tau\) was 163.3 ± 13.4 ms (n = 7).

Dose-response relationships for peak currents were determined in six H1 cells (fig. 1C). The averaged data were well fitted by the curve described by
\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + (\text{EC}_{50}/\text{NMDA})^n} \]
with a EC\(_{50}\) of 52.3 ± 4.3 μM, which is similar to that of catfish HCs (37 μM) [12].

Current responses of H1 cells to 100 μM NMDA were recorded at different holding potentials (40, 20, 0, −20, −40 and −60 mV), and currents commonly reversed polarity between 0 mV and −20 mV. The averaged I-V curve based on the data obtained in five H1 cells was linear in the whole voltage range from −60 to 40 mV, and the reversal potential derived from the curve was −8.1 mV (fig. 1d).

NMDA-induced currents from H1 cells (n = 36) were reversibly blocked by D-AP5 of 100 μM, an NMDA receptor competitive antagonist (fig. 2a). The NMDA currents could be also blocked by the NMDA receptor glycine site blocker 5,7-dichlorokynurenic acid (DCKA) of 20 μM (n = 9), as illustrated in figure 2b. On the other hand, the glycine receptor antagonist strychnine (20 μM) failed to affect the NMDA currents (fig. 2c, n = 8). Furthermore, the NMDA currents exhibited marked voltage-dependence in the presence of extracellular Mg\(^{2+}\), as shown in mammalian central neurons [2]. Figure 2d shows that 100 μM NMDA induced a small inward current of less than 10 pA in Ringer’s containing 1 mM Mg\(^{2+}\), when clamped at −60 mV, and the inward current was maximal around −20 mV. An outward current was induced when the holding potential was shifted to a voltage more positive than 0 mV.

In contrast, no currents could be induced from H4 cells (n = 7) by NMDA application, even at a concentration as high as 1 mM (data not shown).
Increase of Intracellular Calcium Levels in H1 Cells by Activation of NMDA Receptors

There is abundant evidence that activation of NMDA receptors modulated \([Ca^{2+}]_i\) by changing the permeability to \(Ca^{2+}\) \([3, 11, 20]\). This was also the case in the H1 cells. Figure 4 shows the result obtained in an H1 cell, by monitoring NMDA-induced changes in \([Ca^{2+}]_i\). After puffing 100 \(\mu M\) NMDA to the cell for 3 s in the presence of 40 \(\mu M\) glycine, \([Ca^{2+}]_i\), represented as the ratio (F340/F380), increased from 0.577 to 1.104, and the change was blocked in the presence of 100 \(\mu M\) D-AP5. \(a, b\) CCD pictures of the cell taken before NMDA application and when \([Ca^{2+}]_i\) reached a maximum, respectively. Average changes in \([Ca^{2+}]_i\), caused by 100 \(\mu M\) NMDA obtained in seven H1 cells are summarized in the bar chart shown in the inset. Data obtained in individual cells are represented by solid lines. No change in \([Ca^{2+}]_i\) was produced by 100 \(\mu M\) NMDA in the H4 cell (black line). \(c, d\) CCD pictures of the H4 cell taken before and after NMDA application, respectively.

Effects of NMDA on the Membrane Potential and Light Responses of H1 Cells

Figure 4a shows the effects of NMDA on the membrane potential and light responses of an H1 cell recorded in the isolated retina, superfused with Ringer’s containing 1 mM Mg\(^{2+}\). Test flashes of 500 nm (log I = –0.6) and 680 nm (log I = –0.6) were alternately presented in the dark, which activate short wavelength-sensitive cones, most likely green-sensitive cones, and red-sensitive cones \([21]\). NMDA of 100 \(\mu M\) depolarized the membrane potential from –25 to –15 mV, which was associated with a decrease of the hyperpolarizing light responses (see also fig. 4b). The relative reduction of the hyperpolarizing response were 23.01 ± 9.96% and 25.93 ± 10.89% (n = 6) for red and green test lights, respectively. It is of interest that the off-overshoot of the green light-induced response
was reduced in size and the steady-state membrane potential following the overshoot tended to be increased, as clearly seen in figure 4c. The average reduction of the off-overshoot caused by 100 μM NMDA in seven H1 cells was 47.82 ± 22.14%.

**Discussion**

**Expression of Functional NMDA Receptors in H1 Cells, but Not H4 Cells**

While functional NMDA receptors are extensively expressed in the inner retina, which are involved in signaling from bipolar cells to amacrine and ganglion cells [5, 22, 23], it is generally thought that HCs express the AMPA receptor subtype, and the NMDA receptor is absent from HCs. In the present work, however, we showed that NMDA induced inward currents from H1 cells, but not from H4 cells, in a dose-dependent manner. These currents showed desensitization, with a time constant of ~160 ms, similar to that obtained in other central neurons [24], but much longer than that for the AMPA receptor-mediated current of this cell type (~1.5 ms) [8]. The NMDA-induced currents were completely blocked by D-AP5 and DCKA, but not by strychnine, and they showed voltage-dependence in the presence of Mg²⁺. Moreover, NMDA induced a significant increase in [Ca²⁺]ᵢ in H1 cells, but not in H4 cells, and the increase was blocked by D-AP5. All these results suggest that the carp H1 cell expresses the functional NMDA receptor, in addition to the AMPA receptor [8], and this receptor type is involved in modulation of signal transfer between cones, but not rods, and HCs. Expression of NMDA receptors in HCs might have been overlooked previously in this species and other lower vertebrates. It is noteworthy that 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective antagonist of non-NMDA receptors, blocks the actions of kaniate and AMPA in salamander HCs, but not that of glutamate, indicative of the existence of receptors other than AMPA receptors [25, 26]. It will be of interest to determine whether functional NMDA receptors may also be expressed in HCs of other species.

**Possible Physiological Roles of NMDA Receptors of Carp H1 Cells**

Due to the voltage-dependent block of NMDA receptors by Mg²⁺, these receptors are not quite activated in external medium containing physiological concentra-
tions of Mg$^{2+}$ (~1 mM) at resting membrane potentials of most central neurons [2]. The situation in HCs, however, is quite different in that these cells are depolarized to around ~20 mV in the dark due to the activation of the AMPA receptor by glutamate tonically released from photoreceptors. In carp H1 cells the NMDA receptor-mediated inward current was maximal at around ~20 mV, which is in agreement with that reported in other central neurons [2]. These suggest that this receptor may play a physiological role in signal transfer from cones to HCs in the dark. As shown in figure 4c, NMDA reduced the size of the off-overshoot and tended to increase the depolarized steady-state membrane potential. This effect of NMDA is reminiscent of a recent study of Davis and Linn [11]. They reported that activation of NMDA receptors in isolated catfish HCs produced a down regulation of voltage-gated Na$^+$ and Ca$^{2+}$ currents, which plays a role in shaping the off-overshoot under current clamp [27], and thus reduced the simulated depolarized off-overshoot and steady-state membrane potential elicited by injection of depolarizing currents. These changes of the off response of the H1 cell suggest that NMDA receptors in the outer retina may be involved in modulating visual information concerning dark/light transition.

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