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C-TYPE NATRIURETIC PEPTIDE MODULATES GLUTAMATE RECEPTORS ON CULTURED RAT RETINAL AMACRINE CELLS

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Abstract—C-type natriuretic peptide, widely distributed in the CNS, may work as a neuromodulator. In this work, we investigated modulation by C-type natriuretic peptide of functional properties of glutamate receptors in rat retinal GABAergic amacrine cells in culture. Immunocytochemical data revealed that natriuretic peptide receptor-B was strongly expressed on the membrane of cultured GABAergic amacrine cells. By whole cell recording techniques we further identified the glutamate receptor expressed on the GABAergic amacrine cells as an AMPA-preferring subtype. Incubation with C-type natriuretic peptide suppressed the AMPA receptor-mediated current of these cells in a dose-dependent manner by decreasing the efficacy and apparent affinity for glutamate. The effect of C-type natriuretic peptide was reversed by HS-142-1, a guanylyl cyclase-coupled natriuretic peptide receptor-A/B antagonist. Meanwhile, the selective natriuretic peptide receptor-C agonist cANF did not change the glutamate current. In conjunction with the immunocytochemical data, these results suggest that the C-type natriuretic peptide effect may be mediated by natriuretic peptide receptor-B. Furthermore, incubation of retinal cultures in the C-type natriuretic peptide-containing medium elevated cGMP immunoreactivity in the GABAergic amacrine cells, and the C-type natriuretic peptide effect on the glutamate current was mimicked by application of 8-Br-cGMP. It is therefore concluded that C-type natriuretic peptide may modulate the glutamate current by increasing the intracellular concentration of cGMP in these cells via activation of natriuretic peptide receptor-B.

Key words: C-type natriuretic peptide, glutamate receptor, cGMP, amacrine cells, retina.

C-type natriuretic peptide (CNP) belongs to the natriuretic peptide (NP) family, other two members of which are atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). CNP has considerable sequence homology with ANP and BNP (Sudoh et al., 1990). It is now known that a 17-membraned disulfide ring structure is shared by all of the NPs (Kourie and Rive, 1999; Fowkes and McArdle, 2000). There are three classes of receptors that have been characterized (Nakao et al., 1992): natriuretic peptide receptor (NPR)-A, which is sensitive to ANP and BNP (Garbers, 1992), NPR-B, which is highly specific for CNP (Koller et al., 1991), and NPR-C, which binds all the three NPs with similar affinities (Garbers, 1992). NPR-A and NPR-B, having an intrinsic guanylyl cyclase (GC) domain, belong to the GC receptor family and transduce their biological effects via cGMP (Koller et al., 1991; Garbers, 1992; Kourie and Rive, 1999), whereas NPR-C lacks the GC domain and signals through inhibition of cAMP (Anand-Srivastava et al., 1990; Levin et al., 1998).

NPs are involved in the regulation of various physiological functions, such as diuresis, natriuresis, blood flow (Levin et al., 1998). NPs, along with NPRs, have been also found in the CNS, including hypothalamus, cerebellum, spinal cord etc. and they may work as neuromodulators (Komatsu et al., 1991; Herman et al., 1993; Minamino et al., 1993; Fowkes and McArdle, 2000; DiCicco-Bloom et al., 2004). Accumulating evidence suggests the presence of NPs in the retinas of several species (Shyjan et al., 1992; Wolfensberger et al., 1994; Moriwaki et al., 1998; Blute et al., 2000; Cao et al., 2004; Rollin et al., 2004), and they are distributed with different profiles in rat retina (Cao et al., 2004). There is also evidence that NPRs may exist in retinal neurons (Blute et al., 2000; Rollin et al., 2004). It is of particular interest that ANP and CNP were found to induce cGMP synthesis dramatically in rat retina, suggesting the existence of the NP/cGMP pathway (Moriwaki et al., 1998; Blute et al., 2000). We recently report that BNP modulates $GABA_A$ receptors on rat retinal ON-type bipolar cells through activating NPR-A (Yu et al., 2006).

Retinal amacrine cells, located in the inner nuclear layer (INL), extend their dendrites to the inner plexiform layer (IPL). These cells are involved in shaping response properties of ganglion cells, output neurons of the retina. The majority of the amacrine cell population is GABAergic cells in various vertebrate species (Yazulla, 1986; Strettoi and Masland, 1996; Kolb, 1997). GABAergic amacrine cells have rather wide dendritic fields in the mammalian retina and mediate lateral inhibition (Wässle and Boycott, 1991), and they could send signals back to bipolar cells (feedback inhibition) and to other amacrine cells (mutual inhibition) and ganglion cells (feedforward inhibition) (Tachibana and Kaneko, 1988; Dong and Werblin, 1998; Hartveit, 1999; Watanabe et al., 2000; Shen and Slaughter, 2001; Zhang et al., 2004). The lateral inhibition mediated by GABAergic amacrine cells may contribute to forming the center-surround organization of the ganglion cell

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GC, guanylyl cyclase; IBMX, 3-isobutyl-1-methylxanthine; INL, inner nuclear layer; IPL, inner plexiform layer; NP, natriuretic peptide; NPR, natriuretic peptide receptor; PBS, phosphate-buffered saline; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase.

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receptive field (Cook and McReynolds, 1998; Flores-Herr et al., 2001; Shields and Lukasiewicz, 2003).

In the present work, we found strong expression of NPR-B on the membrane of rat GABAergic amacrine cells in culture by immunocytochemistry. We further demonstrated, using the patch clamp technique, that CNP suppressed glutamate receptor-mediated currents of these cells via the activation of NPR-B.

EXPERIMENTAL PROCEDURES

Cell culture

Cell culture was performed following the procedure described by Koizumi et al. (2001), with minor modifications. All procedures conformed to the National Institutes of Health guidelines for animal experimentation. In brief, retinas were removed from newborn S.D. rats after cryoanesthesia and were incubated for 25 min in Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution with HEPES (10 mM) supplemented with 1 mg/ml trypsin at 37 °C. The preparations were then rinsed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum. Retinal cells, mechanically dissociated, were seeded onto glass coverslips pretreated with 0.5 mg/ml poly-L-lysine and cultured in DMEM added with 44 mM $NaHCO_3$, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% heat-inactivated fetal bovine serum in a 5% CO_2 environment at 37 °C. Since amacrine cells are fully matured by day 10 in culture (Koizumi et al., 2001), all experiments reported in this work were conducted on cells cultured for 10–14 days.

Immunocytochemistry and Western blot analysis

Immunocytochemical experiments on retinal cells in culture were carried out following a procedure that was previously described in detail (Tian et al., 2003), with modifications. The primary antibodies used in this work were divided into three groups: 1. mouse anti-HPC-1 antibody (1:4000 dilution, Sigma, St. Louis, MO, USA) for labeling amacrine cells and rabbit anti-GABA antibody (1:2000 dilution, Sigma) for labeling GABAergic neurons; 2. mouse anti-GAD67 antibody (1:800 dilution, Chemicon, Temecula, CA, USA) for labeling GABAergic neurons and rabbit anti-NPR-A, NPR-B or NPR-C antibodies (1:200, 1:400 and 1:800 dilution respectively, Abcam, Cambridge, UK) for labeling three types of NPRs; 3. mouse anti-GAD67 antibody and rabbit anti-cGMP antibody (1:500 dilution, Chemicon) for labeling cGMP. These antibodies were diluted with 0.01 M PBS containing 3% normal donkey serum and 1% bovine serum albumin. The secondary antibodies used were donkey anti-mouse IgG tagged with fluorescein isothiocyanate (FITC, 1:200 dilution, Jackson Immunoresearch Laboratories, West Grove, PA, USA) and donkey anti-rabbit IgG tagged with Rhodamine Red-X (1:200 dilution, Jackson Immunoresearch Laboratories).

Primary cultured neurons placed on the coverslips were first washed three times with 0.01 M phosphate-buffered saline (PBS) and were then fixed with 4% paraformaldehyde for 30 min and preincubated for 1 h in 6% normal donkey serum (v/v) in PBS plus 0.1% Triton X-100 at room temperature. The cultures were incubated overnight at 4 °C with one of the primary antibodies in a humidified air chamber. After incubation for 12–24 h, they were rinsed with PBS for 10 min×3 and then incubated with the secondary antibody to reveal binding sites. After incubation for 1 h at 4 °C with the secondary antibody, the coverslips were rinsed twice in PBS and were mounted onto glass slides. For double immunofluorescence labeling, the cultures were incubated sequentially in a mixture of the two primary antibodies and two secondary antibodies. To detect changes in cGMP immunoreactivity of cultured amacrine cells following CNP treatment, retinal cultures were washed twice with serum-free DMEM, and then incubated for 5 min at 37 °C in serum-free DMEM, containing one of the following: (1) 1 mM 3-isobutyl-1-methylxanthine (IBMX); (2) a mixture of 1 mM IBMX and 1 μM CNP. Immunocytochemical assay was performed for the cultures using the procedure described above. Different controls were performed by omitting one or both of the two primary antibodies during the incubation. Omission of one primary antibody yielded only the immunoreactivity for the remaining antibody and omission of both abolished any immunolabeling. Staining by a mixture of the two secondary antibodies after incubating with one of the two primary antibodies showed no cross-reactivity of species-specific secondary antibodies. Fluorescently labeled cultures were visualized with a Leica SP2 confocal laser scanning microscope (Leica, Mannheim, Germany) using a 63× oil-immersion objective lens. Double labeling was precisely evaluated by sequential scanning on single-layer optical sections.

Western blot analysis was conducted as described previously in detail (Chen et al., 2004). The rat retina extract samples (2.0 mg/ml, 20 μl) were loaded, subjected to 12% SDS-PAGE and electroblotted onto PVDF membranes using Mini-PROTEAN 3 electrophoresis system and Mini Trans-Blot Electrophoretic Transfer System (Bio-Rad; Hercules, CA, USA). The membranes were blocked in blocking buffer consisting of 20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20, and 20% nonfat milk at room temperature for 2 h, and then incubated with the antibody against NPR-A, NPR-B or NPR-C, at working dilutions of 1:1000, 1:1500 and 1:2000, respectively, overnight at 4 °C. The blots were washed, incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 4 °C, and finally visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Whole cell patch-clamp recording

A coverslip with cultured cells was placed in a recording chamber, mounted on the stage of an inverted microscope (CK 2, Olympus, Japan). Whole-cell membrane currents were conventionally recorded under voltage-clamp conditions at −60 mV, using an Axopatch 200B amplifier, Digidata 1322A data acquisition board, and Clampex 8.0 software (Axon Instruments, Foster City, CA, USA). Patch electrodes were pulled from borosilicate glass (Sutter, Novato, CA, USA) with a horizontal puller (P97, Sutter), having a resistance of about 10 MΩ when filled with the pipette solution. An Ag–AgCl wire connected to the bath via an agar–NaCl bridge served as a reference electrode. Fast capacitance and cell capacitance were cancelled by the circuit of the amplifier as much as possible. Seventy percent 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 analysis. All recordings were made at room temperature (23～25 °C).

Solutions and drug application

The standard extracellular solution was Ringer's containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose; pH was adjusted to 7.4 with NaOH. To block synaptic transmission, $MgCl_2$ was replaced by $CoCl_2$. The standard pipette solution contained (in mM) 5 NaCl, 40 KCl, 100 K gluconate, 1 $CaCl_2$, 2 $MgCl_2$, 1.1 EGTA, 10 HEPES, 2 ATP-Na_2 and 0.25 GTP-Na, pH 7.3 adjusted with KOH. All chemicals were obtained from Sigma Chemical Company except cANF (Peninsula Laboratories, San Carlos, CA, USA) and HS-142-1 (a kind gift of Prof. Chiming Wei at Johns Hopkins University). Most of drugs were freshly dissolved in the extracellular solution. IBMX and GYKI-52466 were first dissolved in dimethyl sulfoxide (DMSO) and then added to the extracellular solution on the day of experiment. The final concentration of DMSO was less than 0.1% that had no

effects on glutamate-induced currents from cultured amacrine cells.

Drugs were delivered through a pair of parallel polyethylene flow pipes (500 μm inner diameter, Biotrol Diagnostic, France). Each flow pipe was supplied by eight pressurized 5 ml reservoirs, each with its own control valve (DAD-8VCSP, ALA Scientific Instruments, Westbury, NY, USA) to feed fluid through an eight-to-one tubing manifold (500 μm inner diameter, ALA Scientific Instruments). The open/close switch of each valve was under computer control. Once the valve was open, the solution in the corresponding reservoir was pressure ejected by nitrogen gas along the pipes. With the large flow pipes, 10–90% whole-cell solution exchanges were achieved in less than 2.5 ms.

Data processing

Desensitization of the responses to glutamate was fitted to a mono-exponential equation: $I(t)=A\times\exp(-t/\tau)+C$, where A is the peak current; t is time; τ is the time constant and C is an offset. Dose-response relationships of glutamate-induced currents were fitted to the equation: $I/Imax=1/[1+(EC_{50}/[glu])n]$, where I is the current response elicited by a given glutamate concentration [glu]; Imax is the response at a saturating concentration of glutamate; EC_{50} is the concentration of glutamate eliciting a half-maximal response; and n is the Hill coefficient. The data were all presented as mean±S.E. Paired Student's test was performed for statistical analysis.

RESULTS

Identification of GABAergic amacrine cells

Amacrine cells in culture were identified by the amacrine cell marker HPC-1 (Wexler et al., 1998; and Koizumi et al., 2001). These cells with somata larger than 10 μm in diameter had multiple long processes, some of which may extend over hundreds of micrometers. Almost all of the large multipolar cells (>10 μm in diameter) in culture were HPC-1 positive, and most of them (>93%) were also immunoreactive to GABA. Fig. 1A shows the bright field image of several amacrine cells cultured for 10 days. It was noteworthy that the larger multipolar cells (asterisks), including the somata and all processes, were strongly HPC-1 labeled, whereas the smaller cells (arrows) were HPC-1 negative (Fig. 1B). Furthermore, these HPC-1 immunoreactive cells were also GABA positive (Fig. 1C, D). According to these results and those obtained in previous studies (Wexler et al., 1998; Koizumi et al., 2001), cell size and multipolar morphology could be used as useful criteria for the identification of GABAergic amacrine cells.

AMPA receptor-mediated currents of cultured GABAergic amacrine cells

Glutamate induced inward currents in all GABAergic amacrine cells tested (n=135). Fig. 2A shows current responses of an amacrine cell induced by glutamate of increasing concentrations. The responses induced by concentrations larger than 30 μM showed significant desensitization. That is, the currents reached a peak quickly and then decayed to a steady state level during glutamate application. The saturating concentration that induced a maximum current was around 10 mM, and the peak amplitude of the maximum current was 5.08±0.59 nA (n=8) with a 10–90% rise time of less than 2.5 ms. The time constant of the current desensitization was 6.09±0.52 ms (n=8). Fig. 2B shows the average dose-response relationship of the peak currents obtained in eight cells. The data were well fitted by the curve, yielding an EC_{50} of 169.7±10.4 μM and a Hill coefficient of 1.06±0.06. To elucidate the glutamate receptor subtype(s) present on cultured GABAergic amacrine cells, we examined the effect of the specific AMPA receptor antagonist GYKI-52466 (Donevan and Rogawski, 1993) on the glutamate currents. As illustrated in Fig. 2C, 30 μM GYKI-52466 almost completely abolished the response of the cell to 1 mM glutamate. Similar results were obtained in five other cells. On the other hand, NMDA of concentrations ranging from 100 μM to 10 mM failed to induce any current from this cell type, even with coapplication of 10 μM glycine and removal of extracellular Mg^{2+} (Fig. 2D).

Expression of NPR-B on the membrane of cultured GABAergic amacrine cells

The specificity of the antibodies against NPR-A, NPR-B and NPR-C was first tested using Western blot analysis and the results are shown in the right column of Fig. 3. Each of the three antibodies revealed a single immunoreactive band at approximately 120 kDa (for NPR-A, top), 110 kDa (NPR-B, middle) and 65 kDa (NPR-C, bottom), precisely corresponding to the predicted molecular weight of the receptor (Lowe and Fendly, 1992; Bonhomme et al., 1998; Sakaguchi and Takei, 1998), indicating that the proteins recognized by these antibodies were indeed NPR-A, NPR-B and NPR-C respectively. Fig. 3A–C are confocal laser scanning micrographs of retinal cultures, showing

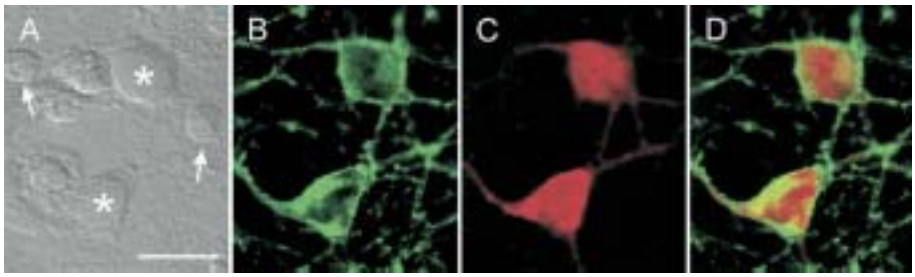

Fig. 1. Confocal laser scanning micrographs of rat amacrine cells cultured for 10 days. (A) Bright field image of cultured amacrine cells. Large multipolar cells and some of small cells are indicated by asterisks and arrows, respectively. (B) Large multipolar cells are positive to the HPC-1 antibody (green), whereas the small cells are negative. (C) HPC-1 positive cells, but not the small ones, are immunoreactive to GABA (red). (D) Overlay of (B) and (C) shows that the large multipolar cells are double-labeled, which appear yellowish. Scale bar=20 μm.

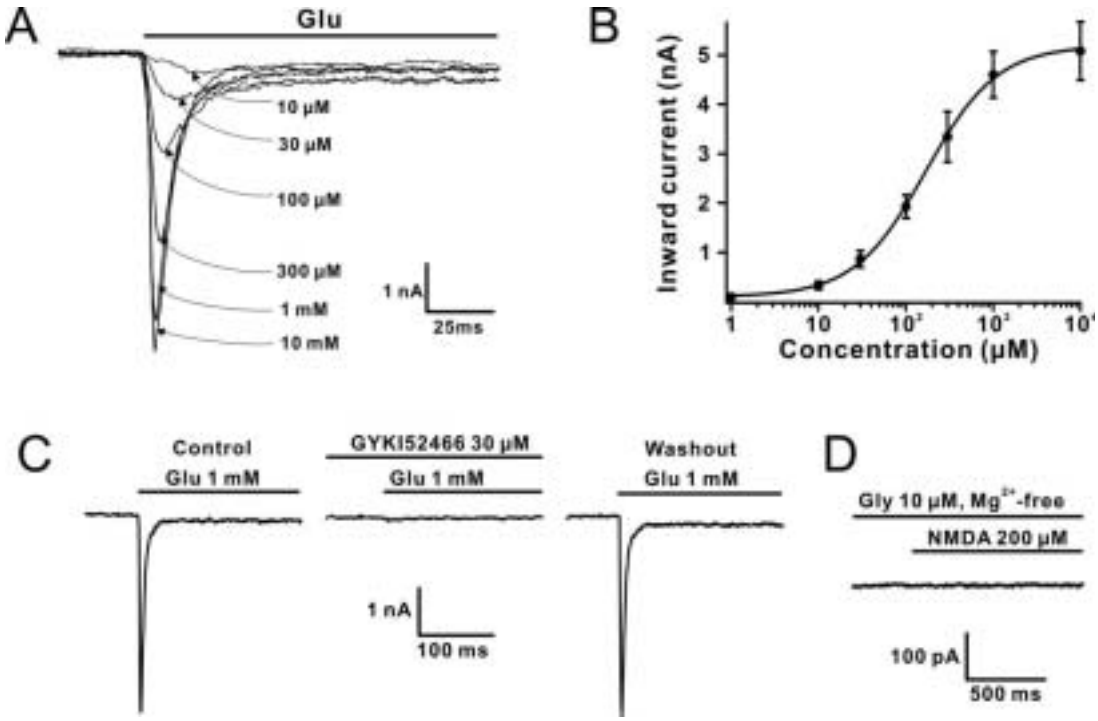

Fig. 2. Glutamate-induced currents from rat GABAergic amacrine cells in culture. (A) Responses of an amacrine cell to glutamate of increasing concentrations. Peak currents steadily increased with the increase of glutamate concentration (indicated by arrows). Note that the currents induced by glutamate of concentrations larger than 30 μM showed significant desensitization. (B) Average dose-response relationship for the peak glutamate currents elicited by 300 ms applications from eight amacrine cells. Curve fitting yielded an EC_{50} of 169.7±10.4 μM and a Hill coefficient of 1.06±0.06. (C) The current induced by 1 mM glutamate (left) was almost completely blocked by addition of 30 μM GYKI-52466 (middle). The current fully recovered on washout (right). (D) NMDA of 200 μM did not induce any current from another amacrine cell, even with coapplication of 10 μM glycine and removal of extracellular Mg^{2+}. Pipette potential was held at −60 mV.

that these cells were all strongly labeled by the antibody against GAD67, a marker of GABAergic neurons. Fig. 3A′–C′ are micrographs of the cultures double-labeled by the antibodies against NPRs (A′ for NPR-A, B′ for NPR-B and C′ for NPR-C), and Fig. 3A″–C″ are the merged images of A and A′, B and B′, C and C′, respectively. Double-labeled elements appeared yellowish. It was evident that these three NPRs had rather different expression patterns. The staining for NPR-A was predominantly localized in the cytoplasm of the GAD67 positive cells (Fig. 3A′, A″), whereas the GAD67-positive cells were not labeled by NPR-C at all (Fig. 3C′, C″). On the other hand, the labeling for NPR-B clearly delineated the GAD67 positive cells, suggesting the expression of this receptor on the plasma membrane of the somata (Fig. 3B′, B″). Moreover, the dendrites also showed strong NPR-B immunoreactivity. The staining for NPR-B was strongly present on the majority (>70%) of the GABAergic amacrine cells tested, but weakly in less than 30% of the cell population. These different expression patterns of three NPRs were consistently observed in cell cultures made at different times.

Suppression of glutamate currents of GABAergic amacrine cells by CNP

We then tested effects of CNP on glutamate currents of GABAergic amacrine cells. In 49 of 75 cells tested, application of 100 nM CNP significantly suppressed the glutamate currents, and an example is shown in Fig. 4A–F. With incubation of 10 nM CNP, the peak current induced by 1 mM glutamate in Ringer's (Fig. 4A) was not much changed in size (Fig. 4B) (from 5.93 nA to 5.77 nA). When CNP concentration was increased to 100 nM, however, both peak and steady-state currents of the response were gradually decreased in size with time. The peak current declined from the control value (5.93 nA) to a lower stable level (4.21 nA), whereas the steady-state one from 325 pA (control) to 235 pA (Fig. 4C). With the further increase of CNP concentration to 1 μM, both peak and steady currents were further reduced to 3.67 nA and 196 pA, respectively (Fig. 4D). Full recovery of the current response to the control level could be obtained after washout with Ringer's for 3 min (Fig. 4E). When the response traces shown in Fig. 4B, C and D were normalized and then superimposed on the control response (Fig. 4A) at a much faster time scale (Fig. 4F), it immediately became evident that these normalized traces coincided quite well, indicating that CNP did not change the 10–90% rise time (2.25±0.19 ms for 100 nM CNP and 2.32±0.18 ms for 1 μM CNP vs. 2.23±0.20 ms (control), n=6, P>0.05) and the time constant of desensitization was also not changed (6.19±0.73 ms for 100 nM CNP and 6.27±0.92 ms for 1 μM CNP vs. 6.13±0.76 ms (control), n=6, P>0.05). Based on the data obtained in six amacrine cells, 10 nM CNP did not much change the peak currents (a reduction of 2.28±1.36%,

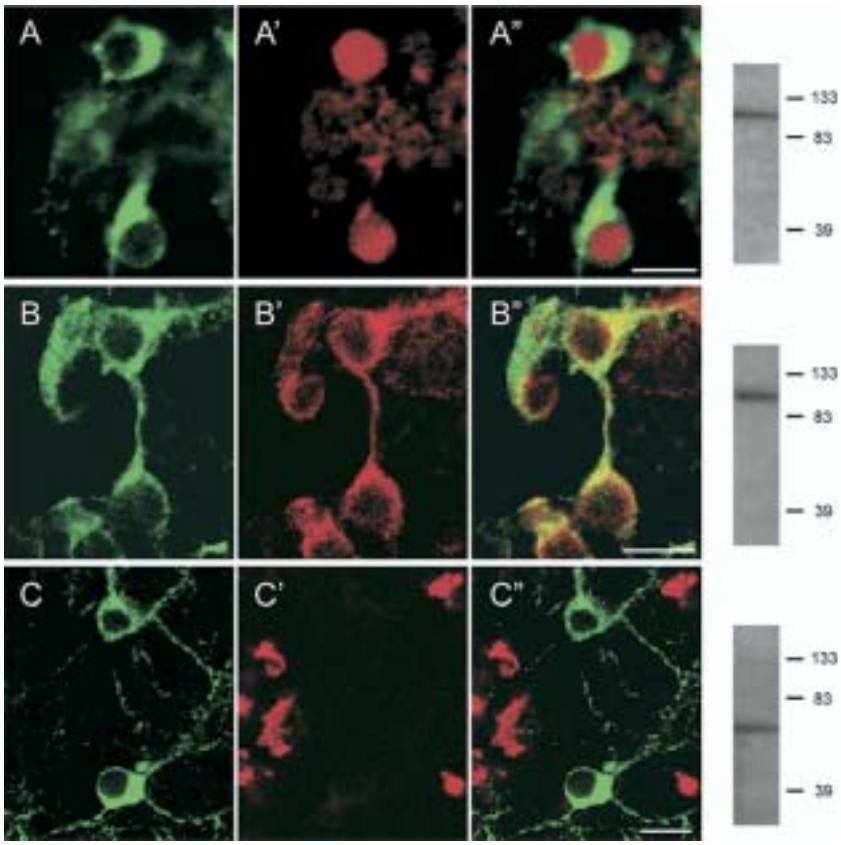

Fig. 3. Confocal fluorescence micrographs of cultured amacrine cells double labeled by GAD67, along with NPR-A, -B or -C. (A–A″) Showing labeling for GAD67 (A) and NPR-A (A′). NPR-A is mainly localized in the cytoplasm. (B–B″) Labeling for NPR-B (B′) is strongly present on the membrane of GAD67-positive cells (B). The staining could also be sparsely seen in the cytoplasm. (C–C″) No immunolabeling for NPR-C (C′) is observed in GAD67 positive cells (C). (A″–C″) The merged images of A and A′, B and B′, C and C′, respectively. Double-labeled elements appear yellowish. Western blots of whole rat retina extracts, using the anti-NPR-A (top), NPR-B (middle) and NPR-C (low) antibodies, are shown in the right column, revealing a single immunoreactive band at approximately 120, 110 and 65 kD, respectively. Scale bar=20 μm.

$P>0.05$), but CNP of both 100 nM and 1 μM significantly reduced those by 27.7±2.86% ($P<0.01$) and 38.7±3.26% ($P<0.01$), respectively (Fig. 4G). It should be emphasized that CNP of 100 nM or 1 μM applied alone did not evoke any currents in these cells.

We also investigated effects of CNP on kainate-induced currents (Fig. 4H). The current induced by 50 nM kainate (gray trace) from the cell was sustained and did not show desensitization. CNP of 100 nM considerably reduced the current from 2.99 nA to 1.91 nA. Similar results were obtained in four other cells, and the relative decrease of the peak response amplitude caused by 100 nM CNP was 28.1±3.06% ($n=5$, $P<0.05$).

The dose-response relationships of the glutamate currents determined in the absence and presence of CNP are shown in Fig. 5. All the responses of each cell to glutamate of various concentrations recorded both in Ringer's and in the presence of 100 nM CNP were normalized by the response of that cell to glutamate of 10 mM, a saturating concentration, in Ringer's. Relative reduction caused by CNP was calculated for each of the doses, and the data obtained from five to eight cells were averaged. CNP of 100 nM suppressed the maximal peak current (I_{max}) to 10 mM glutamate by 22.5% ($P<0.01$), suggesting a reduction of the efficacy for glutamate. When the data obtained in the presence of CNP (squares) were further normalized by the maximal response to 10 mM glutamate obtained in normal Ringer's, it was found that the EC_{50} was shifted rightward from 167.9±11.2 μM (control) to 263.2±11.5 μM, indicating a decrease of the apparent affinity for glutamate. It was noteworthy that the Hill coefficient was not significantly changed (1.05±0.06 vs. 1.10±0.04).

NPR-B mediates CNP-induced suppression of glutamate currents

To explore NPR subtypes that mediate the CNP-induced suppression of glutamate currents, we examined if HS-142-1, a GC-coupled NPR-A/B antagonist (Matsuda and Morishida, 1993), may alter the CNP effect. Fig. 6A shows the result of such an experiment. Similar to that shown in Fig. 4C, the current (gray dotted trace, control) of this cell in response to 1 mM glutamate was clearly reduced in size in the presence of 100 nM CNP (from 3.92 nA to 2.87 nA). When 100 nM CNP was substituted by a mixture of 100 μg/ml HS-142-1 and 100 nM CNP, however, the reduced current gradually recovered to the control level in about 2 min. The blockade of the CNP effect by HS-142-1

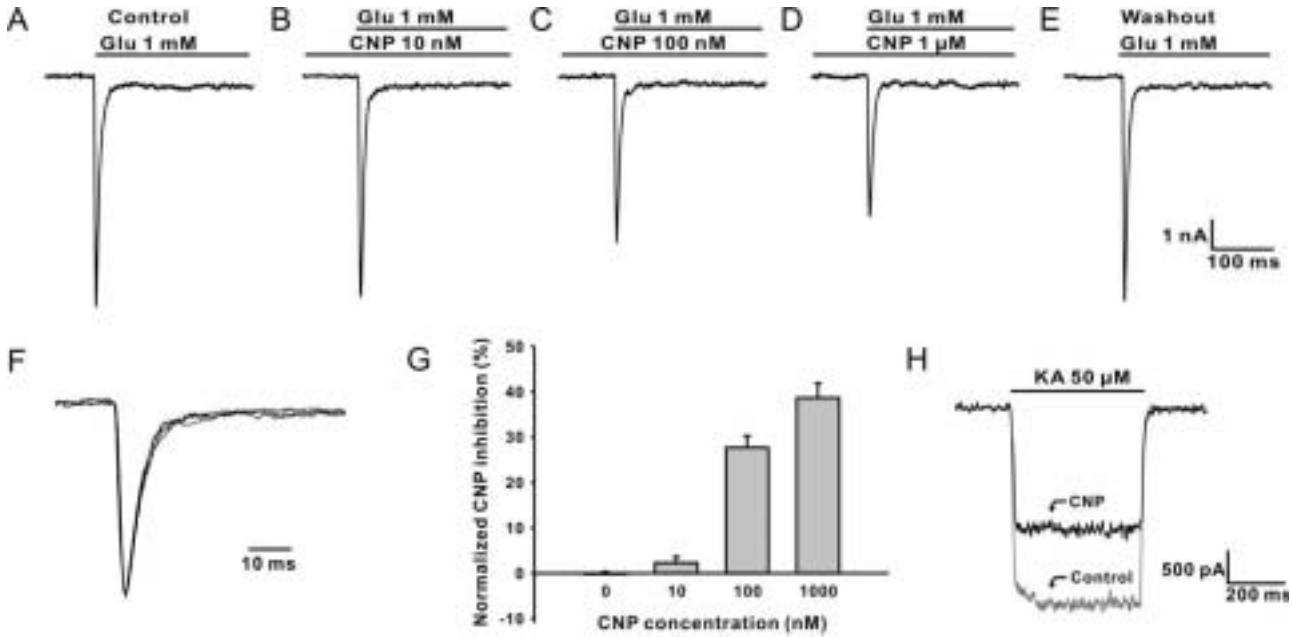

Fig. 4. Suppression by CNP of glutamate-induced currents from cultured amacrine cells. (A–D) Current responses of an amacrine cell to 1 mM glutamate recorded in normal Ringer's (control, A) and in the presence of CNP of 10 nM (B), 100 nM (C) and 1 μM (D). The current was suppressed by CNP in a dose-dependent manner. (E) The response of the cell was obtained after washout with normal Ringer's for 4 min. (F) The responses shown in B, C and D, when normalized, are superimposed on the control response at a much faster time scale. The three normalized traces coincide well with the control one. (G) Relative suppression of currents induced by 1 mM glutamate by CNP of increasing concentrations. Data obtained from each cell at different concentrations of CNP were normalized to the current of that cell recorded in normal Ringer's and then averaged (n=5). (H) Effect of CNP on kainate-induced current from an amacrine cell. The sustained current induced by 50 μM kainate (gray trace, control) was significantly suppressed by 100 nM CNP (dark trace).

was consistently observed in four other cells. A statistical analysis indicated that the data obtained in presence of a mixture of 100 μg/ml HS-142-1 and 100 nM CNP were not different from that obtained in Ringer's (n=5, P>0.05). Since no specific antagonist of NPR-C is now available, we instead used the selective NPR-C agonist cANF (Anand-Srivastava et al., 1990) to investigate whether NPR-C might be involved in the CNP effect. As shown in Fig. 6B, when the cell was incubated in 50 nM cANF for 2 min or even longer, the current response to 1 mM glutamate (control) was hardly changed (4.71 vs. 4.76 nA, n=5, P>0.05).

Involvement of cGMP in CNP-induced suppression of glutamate currents

NPR-B is known to be a GC-coupled receptor and to transduce the biological effects via cGMP (Koller et al., 1991; Garbers, 1992; Moriwaki et al., 1998). Effects of CNP on intracellular levels of cGMP were studied by determining changes of cGMP immunoreactivity in GABAergic amacrine cells when the cultures were exposed to CNP. For these experiments, we used the non-specific phosphodiesterase (PDE) inhibitor IBMX of 1 mM to block endogenous PDE activity. As shown in Fig. 7A, A″, no cGMP labeling was detected in GAD67 positive GABAergic amacrine cells without addition of CNP. When the cultures were incubated with 1 μM CNP for 5 min, the staining for cGMP was clearly seen in these cells (Fig. 7B, B″), indicative of increased intracellular levels of cGMP, and the elevation could be observed in both somata and dendrites. This elevation was observed in 105 of 161 cells tested, while no significant changes in cGMP were detected in the

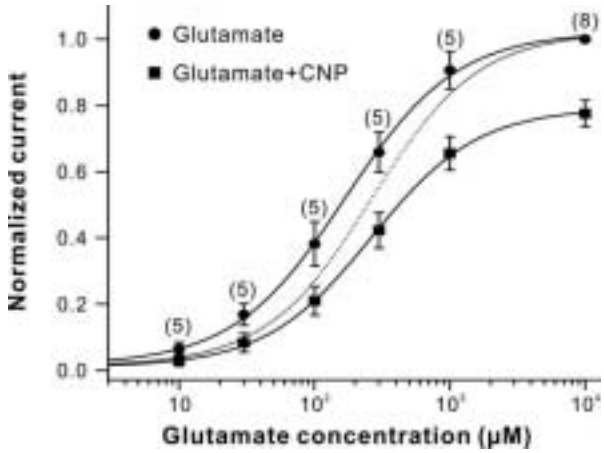

Fig. 5. CNP decreases receptor efficacy and affinity for glutamate in cultured amacrine cells. Dose-response relationships for glutamate currents of amacrine cells obtained in Ringer's and in the presence of CNP are represented by circles and squares, respectively. All the responses of each cell to glutamate both in Ringer's and in the presence of 100 nM CNP were normalized by the response of that cell to 10 mM glutamate (a saturating concentration) in normal Ringer's. Relative reduction was calculated for each of the doses determined in five to eight cells. Curve fitting was performed for the averaged data, yielded an EC_{50} of 167.9±11.2 μM and 263.2±11.5 μM in the absence and presence of 100 nM CNP, respectively. The dotted curve is the dose-response relationship obtained in the presence of 100 nM CNP, normalized by that obtained in normal Ringer's. Values in the parentheses indicate the number of cells tested for each dose.

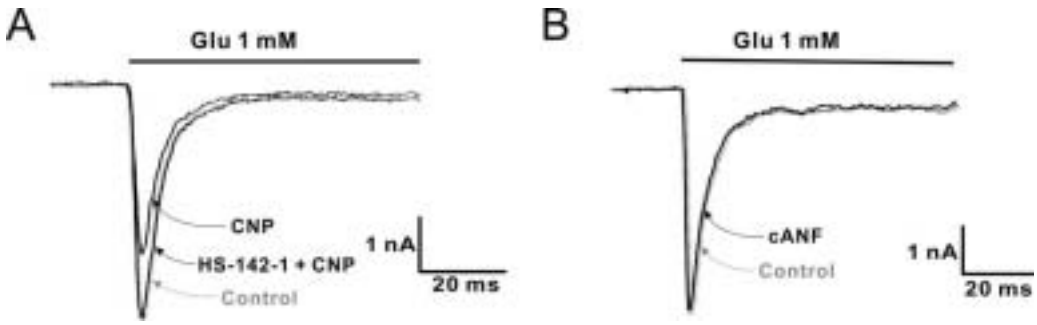

Fig. 6. Effects of HS-142-1 and cANF on CNP-caused suppression of glutamate currents. (A) The response to 1 mM glutamate (gray trace, control) was suppressed by 100 nM CNP. When 100 nM CNP was substituted by a mixture of 100 μg/ml HS-142-1 and 100 nM CNP, the suppressed current recovered to the control level. (B) cANF of 50 nM did not change the current induced by 1 mM glutamate (gray trace, control).

remaining ones (56). We further tested effects of 8-Br-cGMP, a membrane permeable cGMP analog, on glutamate currents of the amacrine cells. When the cell was incubated with 1 mM 8-Br-cGMP for 2 min, the amplitude of the response to 1 mM glutamate (gray trace) was substantially reduced (Fig. 8A) from 3.95 nA to 2.01 nA. Again, the reduced current trace, when normalized, coincided well with the control trace (see the inset), thus mimicking the CNP effect. On average, 1 mM 8-Br-cGMP reduced the glutamate current by 46.7±4.98% of control (n=5, P<0.01). When the cultures were further incubated with a mixture of 1 mM 8-Br-cGMP and 100 nM CNP for another 2 min, no reduction of the glutamate current was observed (96.5±3.05% of the value obtained before the addition of CNP, n=5, P>0.05). After washing out with normal Ringer's for 3 min, the current recovered to the control level (Fig. 8B). Determination of dose-response relationships of the glutamate currents in the absence and presence of 1 mM 8-Br-cGMP indicated an averaged reduction of 36.5% of the maximum peak amplitude and a rightward shift of the EC_{50} (from 159.1±8.7 μM to 305.8±5.2 μM) (data not shown). These results suggest that cGMP, like CNP, caused a decrease in both efficacy and apparent affinity of the AMPA receptor for glutamate.

DISCUSSION

AMPA receptor subtype on GABAergic amacrine cells

Glutamate-induced currents from the GABAergic amacrine cells showed significant desensitization and were almost completely blocked by GYKI 52466, suggesting that these cells predominantly express the AMPA-preferring subtype. This result is similar to those obtained in amacrine cells of other species, using freshly dissociated cells (Shen et al., 1999 for carp; Tran et al., 1999 for salamander). Furthermore, NMDA failed to induce any current from these cells, even with coapplication of glycine and removal of extracellular Mg^{2+} (Fig. 2D), indicating no expression of NMDA receptors. This is different from the observation made in

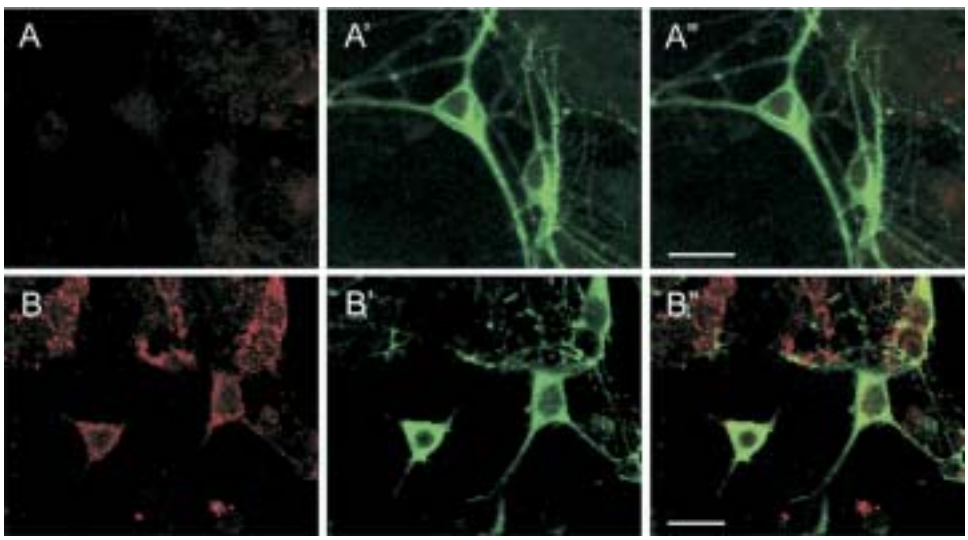

Fig. 7. Immunolabeling of cGMP and GAD67 in cultured amacrine cells. (A) cGMP immunoreactivity was hardly detected when the cultures were incubated with IBMX alone. (A′) Micrograph of the same field labeled with the antibody against GAD67, a marker of GABAergic neurons. (A″) Overlapped images of A and A′. No double labeling was observed in the GAD-positive cells. (B) cGMP immunoreactivity was clearly seen in the cultures which were incubated with a mixture of IBMX and CNP. (B′) GAD-positive cells in the same field. (B″) Overlapped images of B and B′, showing that the cells were double-labeled (yellowish). Scale bar=20 μm.

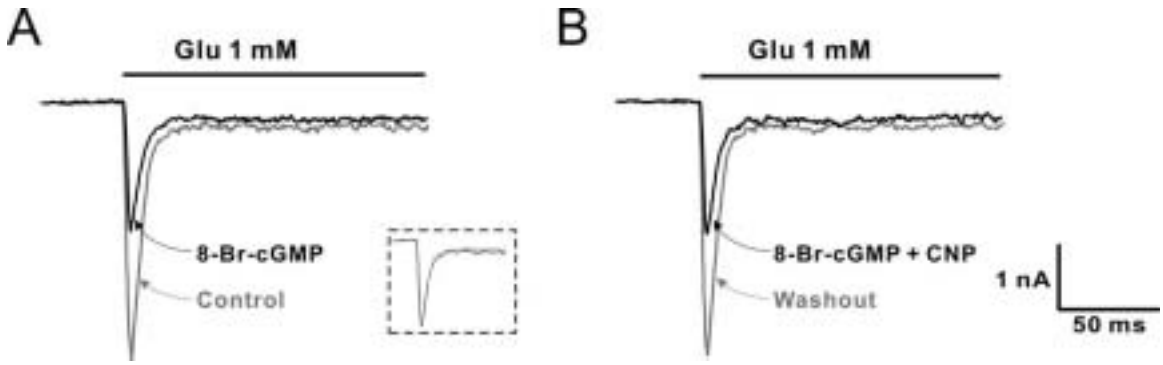

Fig. 8. CNP-caused suppression of glutamate currents may be mediated by an increase of intracellular levels of cGMP. (A) The response to 1 mM glutamate (gray trace, control) was considerably suppressed by 1 mM 8-Br-cGMP. In the inset the suppressed response, when normalized, is superimposed on the control response. The two traces coincide well. (B) In the presence of 1 mM 8-Br-cGMP, application of 100 nM CNP failed to further change the current, and the current fully recovered after washing out with normal Ringer's for 3 min.

cultured chick GABAergic amacrine cells, in which NMDA could induce currents that were blocked by the selective NMDA receptor antagonist MK 801 and potentiated by glycine (Huba and Hofmann, 1991). In rat retina it is shown that NMDA subunits are expressed in a subpopulation of amacrine cells (Fletcher et al., 2000; Kalloniatis et al., 2004). Whether these cells are GABAergic ones are uncertain.

CNP modulates glutamate currents through NPR-B

Our results showed that CNP suppressed glutamate currents of the GABAergic amacrine cells in a dose-dependent manner by reducing both efficacy and apparent affinity of the AMPA receptor for glutamate. CNP did not change desensitization kinetics of the glutamate current, suggesting that CNP might not interfere with physiological and biochemical processes which are responsible for receptor desensitization (Jones and Westbrook, 1996; Sun et al., 2002). The CNP effect was not mimicked by the selective agonist of NPR-C cANF, which is consistent with the fact that NPR-C is not present in these cells (Fig. 3C–C″). The CNP-caused suppression of glutamate currents was not observable in the presence of the NPR-A/B antagonist HS-142-1, indicative of the involvement of NPR-B. In combination with our immunocytochemical data showing that the labeling for NPR-B was clearly seen on the membrane of the GABAergic amacrine cells, including the somata and dendrites, it seemed most likely that the CNP effect was mediated by NPR-B.

NPR-B has an intrinsic GC domain and may thus play its physiological role by elevating intracellular levels of cGMP (Koller et al., 1991; Garbers, 1992; Moriwaki et al., 1998). Immunocytochemical detection of cGMP following exposure to NO donors or NPs has proven an effective method of identifying cells that express GC (Blute et al., 2000; Baldridge and Fischer, 2001). In turtle, incubation with CNP increases cGMP immunoreactivity in multistratified amacrine cells in the central retina and in select amacrine and bipolar cells in the peripheral retina (Blute et al., 2000). Consistent with this result, our result showed a significant elevation of cGMP immunoreactivity in subpopulations of GABAergic amacrine cells after stimulation of retinal cultures by CNP (Fig. 7), indicating an increased intracellular cGMP concentration in these cells. Indeed, we found that 8-Br-cGMP, like CNP, considerably suppressed glutamate currents of the GABAergic amacrine cells, by reducing both efficacy and apparent affinity of the receptor. Moreover, the CNP effect was no longer observed in the 8-Br-cGMP incubated amacrine cells. It is well-documented that cGMP suppresses glutamate receptor-mediated responses in a variety of neurons, including cerebellar Purkinje neurons (Linden et al., 1995; Boxall and Garthwaite, 1996), retinal horizontal cells (McMahon and Ponomareva, 1996), hippocampal neurons (Lei et al., 2000), and striatal spiny neurons (Calabresi et al., 1999).

How the elevation of the intracellular cGMP concentration suppresses glutamate currents in the GABAergic amacrine cells remains to be explored. It could be due to the activation of cGMP-dependent protein kinase (PKG), which is responsible for regulation of glutamatergic transmission by cGMP in neurons of the cerebral cortex, cerebellum and hippocampus (Levenes et al., 1998; Wu et al., 1998; Calabresi et al., 1999), and/or a direct inhibition of the activity of AMPA channels by cGMP, as reported in CA1 hippocampal neurons (Lei et al., 2000). PKG is also found to be involved in modulation of GABA receptor by cGMP in retinal bipolar cells (Yu et al., 2006). Moreover, cGMP-caused changes of AMPA responses during long-term depression in cerebellar Purkinje neurons are mediated by indirectly activating the PKC pathway, but not modulating a cGMP kinase (Linden et al., 1995).

Possible physiological roles of CNP in the inner retina

Immunocytochemical data show that CNP immunoreactivity is strongly present in the IPL and numerous neurons in the INL, including bipolar cells in rat (Cao et al., 2004). This result is basically similar to that reported in turtle retina, in which CNP is present in the IPL and neurons in the INL (Blute et al., 2000). Moreover, major processes of Müller cells in rat are also CNP-positive. It seems reasonable to speculate that bipolar cells and/or Müller cells may release CNP, which modulates glutamate currents of GABAergic amacrine cells. Given the fact that glutamate released from bipolar cells mediates transmission of excitatory signal to

amacrine cells, CNP may regulate the strength of this synaptic transfer. GABAergic amacrine cells, making up the majority of amacrine cells, are a major source of GABA in rat retina (Vaughn et al., 1981; Yazulla, 1986; Wässle and Boycott, 1991; Strettoi and Masland, 1996; Kolb, 1997). GABA plays lots of important roles in the inner retina. Feedback through reciprocal synapses between bipolar cells and amacrine cells is primarily mediated by GABA receptors, which is responsible for making responses of amacrine and ganglion cells transient (Dong and Werblin, 1998; Lagnado, 1998; Shen and Slaughter, 2001). The inhibitory feedforward pathway from GABAergic amacrine cells to ganglion cells, as well as the inhibition through serial synapses between amacrine cells, is also mediated by GABA (Watanabe et al., 2000; Flores-Herr et al., 2001; Zhang et al., 2004). GABAergic amacrine cells have been shown to contribute to the center-surround receptive field organization of ganglion cells (Cook and McReynolds, 1998; Flores-Herr et al., 2001; Shields and Lukasiewicz, 2003). Modulation by CNP of glutamate currents, therefore, is supposed to make responses of amacrine cells and ganglion cells less transient and the receptive field surround of ganglion cells weaker in strength.

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