Expression, Subcellular Localization, and Regulation of Sigma Receptor in Retinal Müller Cells

Guoliang Jiang,[1] *Barbara Mysona,*[1] *Ying Dun,*[1] *Jaya P. Gnana-Prakasam,*[2] *Navjotsin Pabla,*[1] *Weiguo Li,*[1] *Zheng Dong,*[1] *Vadivel Ganapathy,*[2] *and Sylvia B. Smith*[1,3]

Purpose. Sigma receptors (σRs) are nonopioid, nonphencyclidine binding sites with robust neuroprotective properties. Type 1 σR1 (σR1) is expressed in brain oligodendrocytes, but its expression and binding capacity have not been analyzed in retinal glial cells. This study examined the expression, subcellular localization, binding activity, and regulation of σR1 in retinal Müller cells.

Methods. Primary mouse Müller cells (MCs) were analyzed by RT-PCR, immunoblotting, and immunocytochemistry for the expression of σR1, and data were compared with those of the rat Müller cell line (rMC-1) and the rat ganglion cell line (RGC-5). Confocal microscopy was used to determine the subcellular σR1 location in primary mouse MCs. Membranes prepared from these cells were used for binding assays with [^{3}H]-pentazocine (PTZ). The kinetics of binding, the ability of various σR1 ligands to compete with σR1 binding, and the effects of donated nitric oxide (NO) and reactive oxygen species (ROS) on binding were examined.

Results. σR1 is expressed in primary mouse MCs and is localized to the nuclear and endoplasmic reticulum membranes. Binding assays showed that in primary mouse MCs, rMC-1, and RGC-5, the binding of PTZ was saturable. [^{3}H]-PTZ bound with high affinity in RGC-5 and rMC-1 cells, and the binding was similarly robust in primary mouse MCs. Competition studies showed marked inhibition of [^{3}H]-PTZ binding in the presence of σR1-specific ligands. Incubation of cells with NO and ROS donors markedly increased σR1 binding activity.

Conclusions. MCs express σR1 and demonstrate robust σR1 binding activity, which is inhibited by σR1 ligands and is stimulated during oxidative stress. The potential of Müller cells to bind σR1 ligands may prove beneficial in retinal degenerative diseases such as diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2006;47:5576–5582) DOI:10.1167/iovs.06-0608

Sigma receptors (σRs) are nonopiate, nonphencyclidine binding sites[1] whose ligands have robust neuroprotective properties. The endogenous ligand and the physiological function of σR1 have not been elucidated. σRs consist of several subtypes distinguishable by biochemical and pharmacologic means.[2] Among these, type 1 σR (σR1) is best characterized. The cDNA encoding σR1 was cloned originally from guinea pig liver[3] and subsequently from human, mouse, and rat.[4–7] The σR1 cDNA predicts a protein of 223 amino acids (M_r, 25–28 kDa).[3] Initial hydropathy analysis of the deduced σR1 amino acid sequence suggested a single transmembrane segment.[3,4,6] Recently, Aydar et al.[8] showed that, when expressed in *Xenopus laevis* oocytes, σR1 has two transmembrane segments with the NH_2 and COOH termini on the cytoplasmic side of the membrane.

σR1 distribution has been analyzed in brain, and its association with neurons is well established.[9] The receptors modulate ion channel activities at the plasma membrane, neuronal firing, and release of certain neurotransmitters. These receptors are of interest because of their profound capacity to prevent neuronal cell death. They inhibit ischemia-induced glutamate release,[10,11] attenuate postsynaptic glutamate-evoked Ca^{2+} influx,[12,13] depress neuronal responsivity to NMDA receptor stimulation ,[14–16] and reduce NO production.[17]

In retina, the role of σR1 recognition sites in ischemia–reperfusion injury in controlling intraocular pressure and in protection against glutamate-induced neurotoxicity has been reported.[18–20] Binding assays making use of bovine retinal membranes suggested the presence of σRs,[21,22] but the studies did not disclose in which retinal cell types σRs were present, nor did they establish unequivocally the molecular identity of the receptor. Recently, we used molecular and biochemical methods to study σR in mouse retina and reported widespread expression of σR1.[23] RT-PCR analysis amplified σR1 in neural retina, RPE-choroid complex, and lens. In situ hybridization studies revealed abundant expression of σR1 in the ganglion cell layer, inner nuclear layer, inner segments of photoreceptor cells, and RPE cells. Immunohistochemical analysis confirmed these observations. Subsequent studies focused on ganglion cells because of their vulnerability in diabetic retinopathy and revealed that σR1 continues to be expressed under hyperglycemic conditions and during diabetic retinopathy,[24] making it a promising target for neuroprotection against cell death. More recent work using the rat ganglion cell line RGC-5 showed that (+)-pentazocine, a σR1-specific compound, can block RGC-5 cell death induced by homocysteine and glutamate.[25]

Despite evidence that σR1 is present in RGCs and other retinal cell types, no studies have been published of σR1 binding activity in isolated retinal cells. In this study, we examined σR1 binding in retinal Müller cells (MCs) using the rat cell line (rMC-1), primary MCs, and RGC-5 cells. The rationale for studying σR1 binding activity in Müller cells is that recent studies have demonstrated a possible role for σR in glial cell maintenance. Pharmacology studies showed that C6 glioma cells have σR binding sites.[26] Immunohistochemistry studies demonstrated that, in addition to neurons, σR1 is present in oligodendrocytes[27] and Schwann cells.[28] Hayashi and Su[29] confirmed σR1 presence in oligodendrocytes and localized it to the endoplasmic reticulum (ER) forming galactosylceramide-enriched lipid rafts in the myelin sheet of mature oligodendrocytes. They speculated that σRs are important for oligodendro-

From the Departments of [1]Cellular Biology and Anatomy, [2]Biochemistry and Molecular Biology, and [3]Ophthalmology, Medical College of Georgia, Augusta, Georgia.

Supported by National Institutes of Health Grant R01 EY014560.

Submitted for publication June 5, 2006; revised July 26, 2006; accepted September 26, 2006.

Disclosure: **G. Jiang**, None; **B. Mysona**, None; **Y. Dun**, None; **J.P. Gnana-Prakasam**, None; **N. Pabla**, None; **W. Li**, None; **Z. Dong**, None; **V. Ganapathy**, None; **S.B. Smith**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Sylvia B. Smith, Department of Cellular Biology and Anatomy, Medical College of Georgia, 1459 Laney-Walker Boulevard, CB 2820, Augusta, GA 30912-2000; sbsmith@mail.mcg.edu.

cyte differentiation and may play a role in the pathogenesis of certain demyelinating diseases.

Müller cells, the key retinal glial cell, span the retinal thickness, contacting and ensheathing neuronal cell bodies and processes. They are crucial role for neuronal survival and provide trophic substances and precursors of neurotransmitters to neurons.[30] Most retinal diseases are associated with reactive MC gliosis, which may contribute to neuronal cell death; hence, we sought to characterize σRs in these cells. Our earlier work with the rat MC line rMC-1 suggested that σR1 mRNA was present in these cells.[24] In this study, we confirmed that finding and extended the analysis to MCs isolated from mouse retina (primary cell culture). We analyzed the subcellular localization of σR in primary mouse MCs. Additionally, we used rMC-1, RGC-5, and primary mouse MCs to analyze the binding characteristics of σR1. Our study represents the first comprehensive analysis of σR1 in MCs and the first information about the binding characteristics of this receptor in any isolated retinal cell type.

Materials and Methods

Reagents

Materials were obtained as follows: DMEM/F12, reagent (TRIzol) and penicillin–streptomycin (Gibco-Invitrogen Corp., Grand Island, NY); fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA); [1,3-^{3}H](+)-pentazocine (specific radioactivity, 37 Ci/mmol), [2,3-^{3}H]-(+)-3-(3-hydroxyphenyl)N-(1-propyl)piperidine [^{3}H]-(+)-3-PPP] (specific radioactivity, 92.4 Ci/mmol) (DuPont-NEN, Boston, MA); IgG–horseradish peroxidase (HRP; Santa Cruz Biotechnology, Santa Cruz, CA); Cy-3 IgG, 488-IgG, and 568-IgG (Alexa Fluor; Invitrogen, Carlsbad, CA); monoclonal anti–lamin A and monoclonal anti-PDI (Abcam Inc., Cambridge, MA); polyclonal antivimentin (Chemicon International, Temecula, CA); enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Inc., Rockford, IL); Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA); commercial blocker (PowerBlock; BioGenex, San Ramon, CA); protease inhibitors (Complete Mini Protease Inhibitor Cocktail tablets; GeneAmp RNA PCR Kit; Applied Biosystems/Roche Molecular System, Branchburg, NJ); DNA polymerase (TaKaRa *Taq*; Takara Bio Inc., Otsu, Shiga, Japan); sense and antisense primers for rat and mouse σR1 (Integrated DNA Technologies, Inc., Coralville, IA); (+)-3-PPP, carbetapentane, 1,3-di-(2-tolyl)guanidine (DTG), haloperidol, and phenytoin (Research Biochemicals, Natick, MA); (+)-pentazocine, anti–β-actin, 3-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (SNOG), 3-morpholinosydnonimine (SIN-1), hydrogen peroxide 30% (wt/wt) solution, xanthine, xanthine oxidase (X/XO), and all other chemicals (Sigma-Aldrich Chemical Co., St. Louis, MO). rMC-1 cells and RGC-5 cells were kind gifts, respectively, of Vijay P. Sarthy and Neeraj Agarwal. Anti-CRALBP was a generous gift of John Saari.

Animals and Isolation of Müller Cells

C57Bl/6 mouse breeding pairs (Harlan Sprague–Dawley, Indianapolis, IN) were maintained in our colony in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Müller cells were isolated from 6- to 7-day-old mice following our published methods,[31] which were adapted from those of Hicks and Courtois.[32] Briefly, eyeballs were removed, placed in Dulbecco modified Eagle medium (DMEM) with gentamicin, and soaked for 3 hours at 25°C in the dark. Then they were rinsed in PBS and were incubated in buffer containing trypsin, EDTA, and collagenase. Retinas were removed from eyeballs (taking care to avoid contamination by pigmented RPE), placed in DMEM supplemented with glucose, FBS, and penicillin/streptomycin, and gently pipetted into small aggregates at a density of 10 to 16 retinas per dish. Isolated cells were detected within 1 to 3 days. By 3 to 5 days, substantial cell growth ensued. Cultures were washed vigorously with medium until only a strongly adherent flat cell population remained. Cells were passaged 1 to 3 days after washing and were seeded into culture flasks (50,000 cells/cm^2); culture media were changed three times per week. Purity of the cultures was verified using antibodies that are known markers of Müller cells (CRALBP, vimentin, glutamine synthetase, GLAST). Glial fibrillary acidic protein (GFAP), typically considered a marker of Müller cells under stress, was detected only at a low level.[31] Immunocytochemical studies using markers for neurons (neurofilament-L, a major component of neuronal cytoskeleton) and RPE (RPE-65) showed minimal detection.

Cell Culture

Primary mouse MCs, rMC-1, and RGC-5[31,33,34] were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and were maintained at 37°C in a humidified chamber of 5% CO_2. Medium was replaced every other day. On confluence, cultures were passaged by dissociation in 0.05% (wt/vol) trypsin in PBS.

Semiquantitative RT-PCR Analysis of σR1 mRNA

Total RNA was prepared from confluent primary mouse MCs, rMC-1, and RGC-5 cells using reagent (TRIzol; Gibco-Invitrogen Corp.). For rMC-1 and RGC-5, two cell lines derived from rat,[33,34] RT-PCR was carried out with primer pairs specific for rat σR1[7]: sense, 5′-GTTTCT-GACTATTGTGGCGGTGCTG-3′; antisense, 5′-CAAATGCCAGGGTA-GACGGAATAAC-3′ (nucleotide positions 80–104 and 567–591; expected PCR product size, 512 bp). For the primary mouse MCs isolated from mouse, RT-PCR was carried out using primer pairs specific for mouse[6]: sense, 5′-CTCGCTGTCTGAGTACGTG-3′; antisense, 5′AA-GAAAGTGTCGGCTGCTAGTGCAA-3′ (nucleotide positions 315–333 and 572–593; expected PCR product size, 279 bp). 18S RNA was the internal standard. RT-PCR was performed at 35 cycles, with a denaturing phase of 1 minute at 94°C, an annealing phase of 1 minute at 59°C, and an extension of 2 minutes at 72°C. Twenty microliters of the PCR products were gel electrophoresed and stained with ethidium bromide.

Immunoblot Analysis of σR1

Immunodetection of σR1 in RGC-5, rMC-1, and primary mouse MCs followed our published methods.[24,35] Protein samples were subjected to SDS-PAGE and were transferred to nitrocellulose membranes, which were blocked for 1.5 hours with Tris-buffered saline–0.05% Tween-20 containing 5% nonfat milk. Membranes were incubated with anti–σR1 antibody (1:1000)[24] overnight at 4°C, followed by incubation with HRP-conjugated goat anti–rabbit IgG antibody (1:5000). After washing, proteins were visualized with the ECL Western blot detection system. Membranes were washed three times, blocked with 5% nonfat milk for 2 hours, and reprobed with mouse monoclonal anti–β-actin antibody (1:5000) as a loading control.

Immunocytochemical Analysis of σR1

σR1 detection in RGC-5, rMC-1, and primary mouse MCs followed our published protocol.[23–25,35] Cells were incubated with polyclonal anti–σR1 antibody (1:100)[24] and subsequently with Cy-3–conjugated anti–rabbit IgG (1:200). Negative control experiments were performed by incubating the slides without the primary antibody. σR1 was detected by epifluorescence (Axioplan-2 microscope and AxioVision program; Carl Zeiss, Göttingen, Germany). For σR1 subcellular localization studies, primary mouse MCs were seeded on coverslips, grown for 24 hours, fixed in ice-cold methanol for 10 minutes, air dried, washed in PBS, blocked (PowerBlock; BioGenex), and incubated overnight at 4°C with the σR1 antibody (1:100)[24] and either monoclonal anti-lamin A (nuclear membrane marker, 1:25) or monoclonal anti-PDI (ER marker, 1:25). Cells were then incubated for 30 minutes with goat anti–rabbit IgG coupled to dye conjugates and goat anti—mouse IgG (Alexa Fluor 488 and 568; 1:1500; Invitrogen). Negative control sections were treated identically except that PBS replaced the primary antibodies. Coverslips containing the cells were mounted with mounting medium (Vectashield Hardset; Vector Laboratories, Burlingame, CA) on micro-

scope slides and examined under a confocal microscope with imaging software (LSM 510 Meta; Carl Zeiss).

Preparation of Cell Membranes for Binding Assays

RGC-5, rMC-1, and primary mouse MCs were cultured as described. Cell monolayers were chilled on ice, washed with ice-cold PBS (pH 7.5), and lysed with 5 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.5). The suspension was centrifuged for 30 minutes at 56,800*g*; the final membrane pellets were rinsed and suspended in 5 mM K_2HPO_4–KH_2PO_4 buffer and homogenized 20 times using a 25-gauge needle. Protein concentration in the final membrane preparation was measured with the protein assay (Bio-Rad Protein Assay Reagent; Bio-Rad).

Ligand-Binding Assay

[^{3}H]-(+)-pentazocine binding to membrane preparations was assayed as described with minor modifications.[36] Samples were incubated with [^{3}H]-(+)-pentazocine (10 nM) in 250 μL of 5 mM K_2HPO_4–K_2PO_4 buffer, pH 7.5, at 25°C for 90 minutes Binding was terminated by the addition of ice-cold binding buffer, and the mixture was filtered on a Whatman GF/F glass fiber filter, presoaked in 0.3% polyethylenimine. The filter was washed three times with ice-cold binding buffer, and radioactivity associated with the filter was determined by liquid scintillation spectrometry. To study the inhibition of [^{3}H]-pentazocine binding to the σR1, several competitive inhibitors—(+)-3-PPP, carbetapentane, DTG, haloperidol, and (+)-pentazocine—were used. Concentrations for the [^{3}H]-pentazocine and the inhibitors were 50 nM and 1 μM, respectively. To investigate the allosteric effects of phenytoin on σR1, 150 μL rMC-1 membrane preparation containing 300 μg protein was incubated with 50 μL of 125 nM of [^{3}H](+)-3-PPP (final concentration, 25 nM) and 50 μL of 250 μM phenytoin (final concentration, 50 μM) or its solvent (5 mM K_2HPO_4 buffer containing 2.5% DMSO, pH 7.5) for 90 minutes at 25°C. The final volume of the reaction system was 250 μL of 50 μM unlabeled (+)-pentazocine and was used to define nonspecific binding.

Effects of donor NO and ROS on σR1 binding activity were examined by treating rMC-1 cells on the second day after seeding for 6 hours with NO donor SNAP (250 μM), SNOG (250 μM), and SIN-1 (100 μM) or ROS donor H_2O_2 (0.00025%) and xanthine (25 μM)/xanthine oxidase (10 mU/mL), after which the binding activity was assayed as described.

Data Analysis

Experiments were performed in duplicate or triplicate; each experiment was repeated at least twice. Results are expressed as mean ± SE. Equilibrium saturation-binding parameters, dissociation constant (K_d), and maximum number of binding sites (B_{max}) were calculated by nonlinear regression analysis of the equation for a rectangular hyperbola (SigmaPlot 2001 for Windows, version 7.0; Systat Software Inc., Richmond, CA), and statistical significance ($P < 0.05$) was determined (SigmaStat, version 2; Systat).

Results

Analysis of σR1 Expression in Müller and Ganglion Cells

Purity of the mouse Müller cells[31] was confirmed using known antibody markers for Müller cells, including CRALBP and vimentin (Fig. 1A) and glutamine synthetase (data not shown). The cells were not positive for neuronal markers such as NF-L[31] or for cytokeratin-8, which labels RPE cells (Fig. 1A). For expression and ligand-binding studies, primary mouse MCs were used at passage number 5. RT-PCR of the mouse primary mouse MCs amplified the expected PCR product (279 bp; Fig. 1B), indicating that σR1 is expressed in these cells. RT-PCR analysis of the rat cell lines amplified the expected product (512 bp), consistent with our earlier data using these cell lines.[23] Immunoblotting with an antibody specific for σR1[24] detected a protein band of the expected size (M_r ~27 kDa) in primary mouse MCs, rMC-1, and RGC-5 cells. Immunocyto-

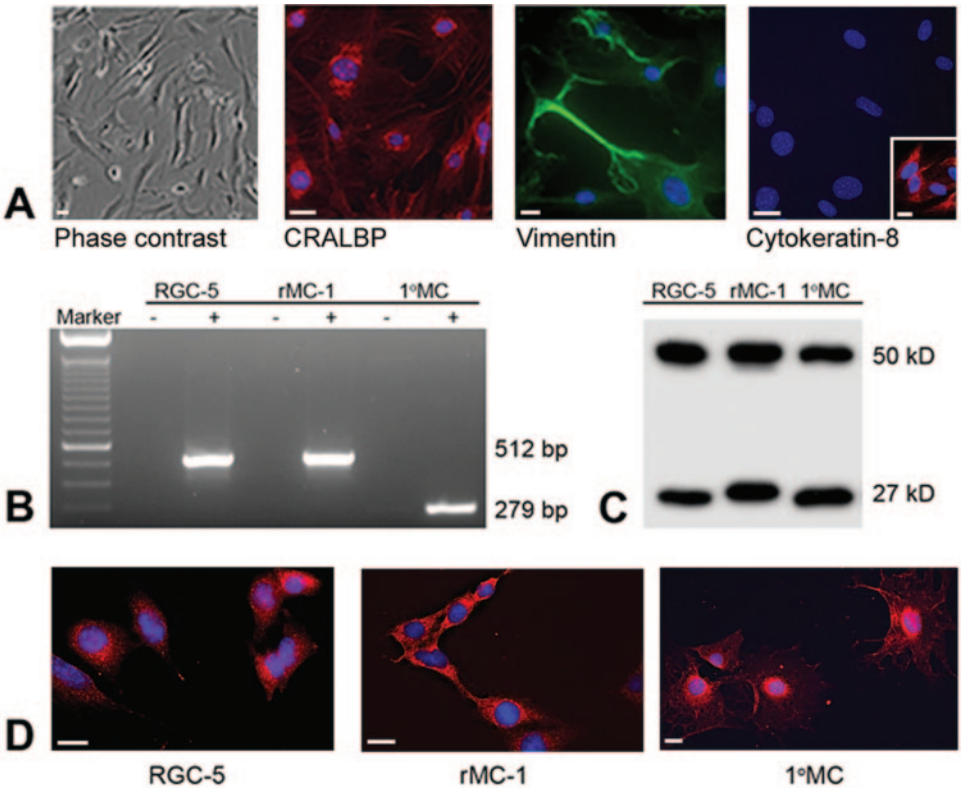

Figure 1. Analysis of σR1expression in retinal cell lines and in primary mouse MCs. (**A**) Verification of the purity of the primary mouse MCs used in these studies. *Left*: phase-contrast image of cells grown for 3 days in culture. *Middle*: positive immunostaining for CRALBP (*red fluorescence*) and vimentin (*green fluorescence*). *Right*: immunolabeling with cytokeratin-8, which is negative in the Müller cells but is positive in ARPE-19 cells (*red fluorescence*, *inset*). (**B**) RT-PCR showing expression of σR1 mRNA in rat ganglion (RGC-5) and Müller (rMC-1) cell lines (512 bp) and primary mouse MCs (279 bp). For each cell type, PCR was run in the absence (–) or presence (+) of reverse transcriptase. (**C**) Immunoblotting of RGC-5, rMC-1, and primary mouse MCs showing the detection of σR1 (M_r ~27 kDa) and β-actin (M_r ~50 kDa). (**D**) Cell lines or primary mouse MCs were cultured and subjected to immunocytochemistry to detect σR1, followed by a Cy-3–conjugated secondary antibody (*red*). DAPI was used to label nuclei (*blue*). σR1 was detected in the cell lines (RGC-5, rMC-1) and in primary mouse MCs. Bar, 15 μm.

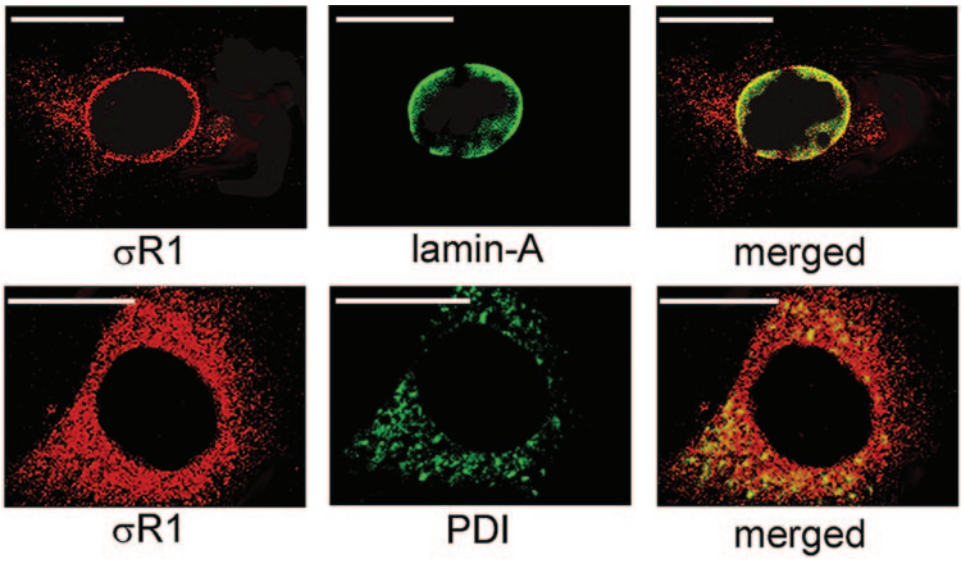

FIGURE 2. Subcellular localization of σR1 in primary mouse MCs. MCs were isolated from mouse retina and cultured as described. They were subjected to double-labeling immunocytochemical analysis using a polyclonal antibody specific for σR1 (*red*) and monoclonal antibodies (*green*) that label the nuclear membrane (lamin-A) or the endoplasmic reticulum (PDI). Optical sectioning by confocal microscopy detected colocalization of σR1 with lamin-A (merged image) and with PDI (merged image). In the merged images, the *orange* signal was detected when the *red* and *green* fluorescence overlapped, indicating colocalization. Bar, 15 μm.

chemical analysis of the cells detected σR1 in RGC-5, rMC-1, and primary mouse MCs (Fig. 1D).

To analyze the subcellular location of σR1 in primary mouse MCs, markers for the nuclear and ER membranes were used in double-labeling experiments with the σR1 antibody. As shown in Figure 2A, σR1 was present in the area, consistent with the nuclear membrane, and the merged image of the σR1 plus the nuclear membrane marker, lamin-A, showed marked colocalization, suggesting that σR1 is present on the nuclear membrane. Similarly, optical sectioning at a slightly different cell plane showed intense σR1 levels in the perinuclear area, consistent with ER localization. The merged image of the σR1 and the ER membrane protein, PDI (Fig. 2), provided strong evidence that σR1 was also present in the ER.

Characterization of [^{3}H](+)-Pentazocine Binding in Retinal Cell Membranes

σR1 binding in RGC-5, rMC-1, and primary mouse MCs was characterized using (+)-pentazocine, a high-affinity σR1 ligand.[37] Binding was saturable over a (+)-pentazocine concentration range of 1.25 to 75 nM (Fig. 3). Apparent K_d was 25.0 ± 5.9 nM, 21.5 ± 2.6, and 18.9 ± 5.6 nM for RGC-5, rMC-1, and primary mouse MCs, respectively. Thus, in the cell lines derived from rat and in primary mouse MCs, the affinity constant for the protein was comparable to the cloned σR1 from rat and mouse. Scatchard analysis of the binding data revealed the presence of a single binding site in each cell type. The binding constants (B_{max}) calculated for the RGC-5, rMC-1, and primary mouse MCs were 1.53 ± 0.14, 1.84 ± 0.08, and 1.32 ± 0.13 pmol/mg protein, respectively. These data suggest that the receptor density was comparable between the retinal cell lines and the primary mouse MCs.

The binding of [^{3}H](+)-pentazocine (final concentration, 25 nM) to RGC-5, rMC-1 and primary mouse MC membranes was inhibited by several σR1 ligands (Fig. 4). The order of potency differed among the cell types studied, such that in RGC-5 cells it was (+)-pentazocine > haloperidol > carbetapentane > 3-PPP > DTG and in rMC-1 cells it was haloperidol > (+)-pentazocine > carbetapentane = DTG > 3-PPP. In the primary mouse MCs, the order of potency was carbetapentane = haloperidol > (+)-pentazocine > 3-PPP > DTG.

A key pharmacologic characteristic of σR1, which distinguishes these receptors from type 2 receptors, is the allosteric enhancement of ligand binding to the σR1 in the presence of phenytoin.[9] Stimulation of binding of the σR1 agonist (+)-3-PPP in the presence of phenytoin was reported recently in guinea pig brain.[38] We used primary mouse MCs to confirm that the σR binding we were studying was specific to type 1 (σR1). In these studies, the binding of [^{3}H](+)-3-PPP was examined in the absence and presence of 50 μM phenytoin. There was a 35% stimulation of binding activity in the presence of phenytoin (Fig. 5). The data suggest that the binding activity measured in the isolated Müller cells was specific to σR1.

The ability to analyze σR1 binding activity in individual retinal cell types affords the opportunity to investigate the regulation of this activity in the presence of factors implicated in retinal disease. Although it was not the focus of this study to investigate exhaustively the regulation of σR1, it was important to ascertain whether binding activity was likely to be altered when retinal cells were subjected to stress. To this end, we performed preliminary studies in rMC-1 cells and analyzed σR1 binding activity when cells were exposed to NO or ROS donors. These stressors were selected because NO [39–42] and oxidative stress[43] have been implicated in the pathogenesis of diabetic retinopathy. The cells were exposed to the NO donors SNAP, SNOG, and SIN-1 or to the ROS donors H_2O_2 and X/XO for 6 hours, after which the binding activity of σR1 was assayed (Fig. 6). Treatment of rMC-1 cells with all three NO donors resulted in a marked increase in binding activity, with the greatest effect observed when cells were incubated with SIN-1. There was an approximate threefold increase in binding activity after 6-hour exposure to this NO donor. In experiments using ROS donors, the effects were similarly profound, with a threefold increase in binding activity after 6-hour exposure to H_2O_2 and a nearly fourfold increase in the presence of X/XO. The findings were observed in three independent experiments.

DISCUSSION

In this study, we examined σR1 expression in isolated retinal cell types and comprehensively characterized σR1 binding activity in these cells. We used molecular techniques and demonstrated that σR1 is present in primary mouse MCs isolated from the mouse retina. The location of the σR1 appeared to be perinuclear. We explored this through laser scanning confocal microscopy (LSCM) with antibodies that recognize the nuclear and ER membranes. Our studies demonstrated that in primary mouse MCs, σR1 is localized to both sites. σR1 to the ER was consistent with findings by Hyashi and Su,[29] who localized σR1 to the ER of oligodendrocytes. They did not

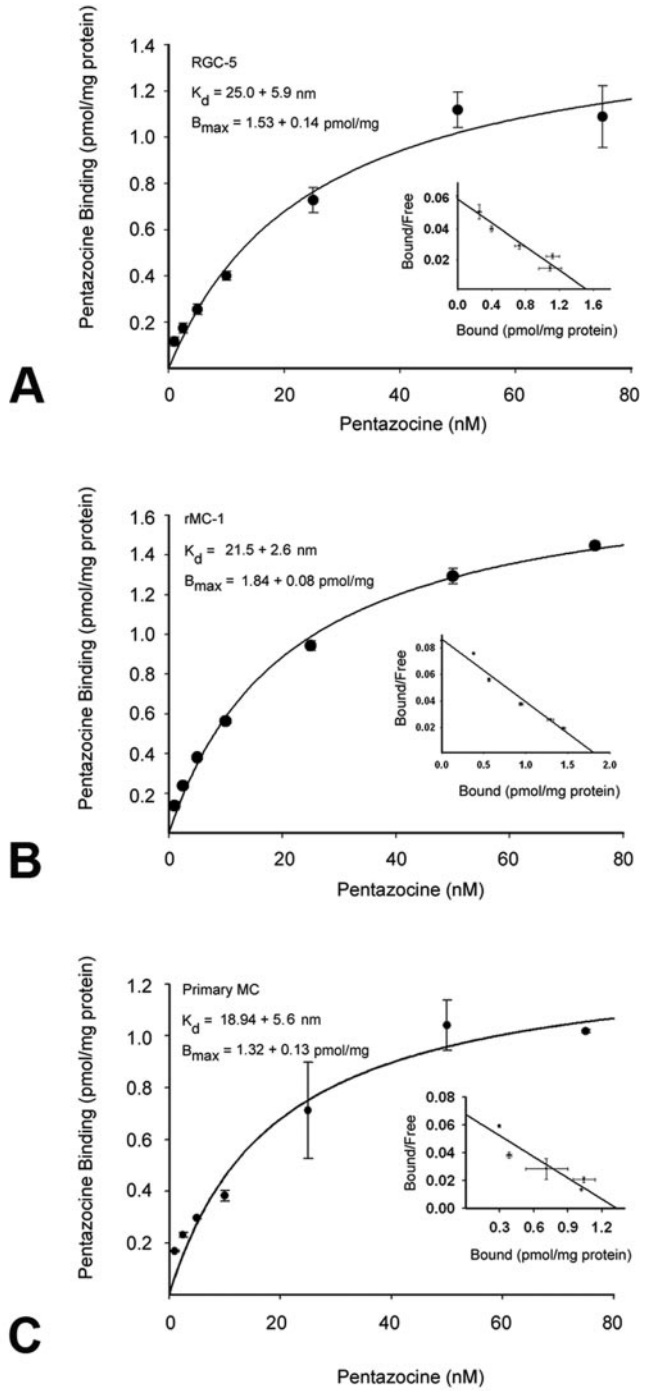

FIGURE 3. Saturation kinetics of σR1 binding in membrane preparations. The two retinal cell lines (RGC-5 and rMC-1) and primary mouse MCs were cultured as described, membranes were prepared, and the binding of [^{3}H](+)-pentazocine over a broad concentration range was measured in (**A**) RGC-5 cells, (**B**) rMC-1 cells, and (**C**) primary mouse MCs. Values are the mean ± SE for two independent experiments performed in triplicate. *Inset*: Scatchard plot.

report localization to the nuclear membrane; however, they did not use antibody markers, so it is unknown whether oligodendrocytes might also place σR1 on the nuclear membrane.

Previous studies of σR in retina have demonstrated σR1 binding activity; however, these studies used whole retina from large models (bovine)[21,22] and did not attempt to study the σR1 binding activities of individual retinal cell types. If we are to postulate a role for σRs in mediating neuroprotection in retinal cells, it is essential to be able to quantify the σR1 binding activity in the isolated cells. We were interested in σR1 binding activity in ganglion cells and in Müller cells. Ganglion cells die in several retinal diseases,[44–46] and we predict that activation of the σR1 may be beneficial to their survival; Müller cells play a key role in neuronal survival[30] and may activate σR1 during stressful episodes, providing protection for adjacent neurons. In the present studies, we exploited the retinal ganglion cell line RGC-5 to study σR1 binding activity in neurons. Although isolation of ganglion cells from mouse retina is

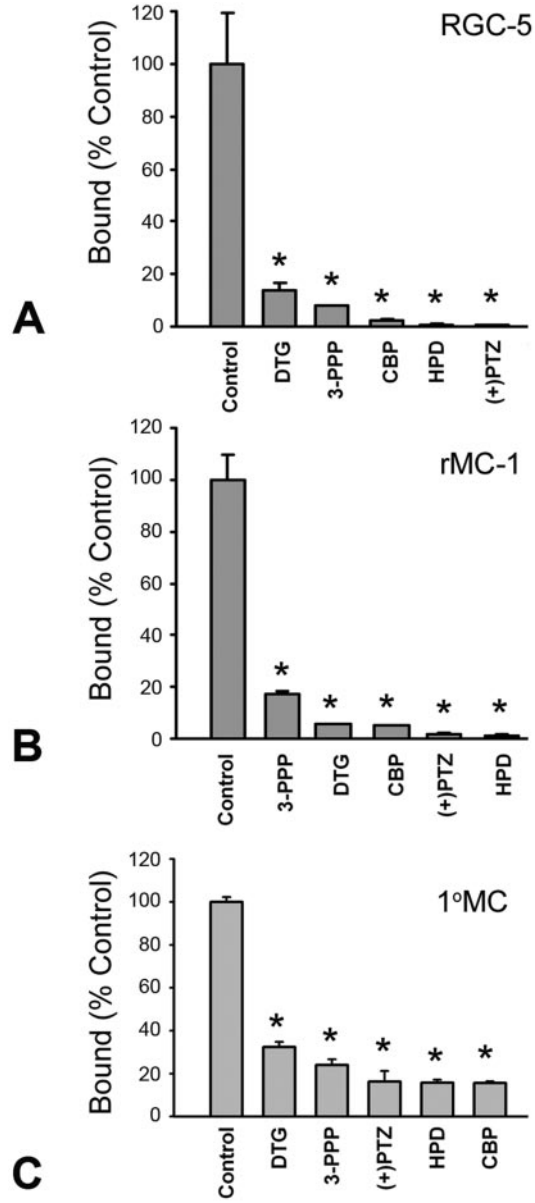

FIGURE 4. Inhibition of [^{3}H](+)-pentazocine binding by σR1 ligands. RGC-5, rMC-1, and primary mouse MCs were cultured as described, membranes were prepared, and the inhibition of the binding of [^{3}H](+)-pentazocine was measured using several known ligands for σR1. (**A**) RGC-5 cells, (**B**) rMC-1 cells, and (**C**) primary mouse MCs. Data are shown as relative percentage of control binding (100%) measured in the absence of ligands. Values are the mean ± SE for two independent experiments performed in triplicate. 3-PPP, (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine; CBP, carbetapentane; DTG, 1,3-di-(2-tolyl)guanidine; HPD, haloperidol; (+)-PTZ, (+)-pentazocine. *Significantly different from control; $P < 0.01$.

possible,[35] these cells are terminally differentiated, making it difficult to obtain sufficient numbers of these neurons to carry out σR1 binding assays. For studies of σR1 in Müller cells, we used the rMC-1 cell line and complemented the data using primary MCs isolated from mouse. Given the plethora of genetic defects that cause retinal abnormalities in commercially available strains of mice, the ability to study σR1 expression and activity in primary mouse MC cultures afforded an excellent opportunity to dissect σR1 activity and expression in Müller cells of various mouse models of retinal disease.

Our studies of σR1 binding characteristics in Müller cells showed that the affinity of σR1 in primary mouse MCs was comparable to that of two retinal cell lines, RGC-5 and rMC-1. In all three cases, σR1 bound its ligand, (+)-pentazocine, with great affinity. The density of receptors, as indicated by the B_{max} value, was similar between the primary mouse MCs and the two retinal cell lines. The ability of various ligands to inhibit the binding of (+)-pentazocine differed slightly among the cell types. The two cell lines seemed more sensitive to the inhibitory effects of CBP, HPD, and (+)-pentazocine, whereas the primary mouse MCs seemed slightly less sensitive to these inhibitors. Nonetheless, the binding in these primary mouse MCs was inhibited by 75% to 80% over a wide range of ligands tested. Finally, the known allosteric effects of phenytoin to stimulate binding of 3-PPP were borne out in these studies and provide strong evidence that the binding studied in these retinal cells was indeed mediated by σR1.

The value of the present work is that it forms a scaffold on which to study the regulation of σR1 activity in isolated retinal cells. We now have substantial baseline data for two retinal cell lines and primary mouse MCs about the dissociation constant, receptor density, and ligand-specificity characteristics of these receptors. Thus, we are poised to compare these benchmarks under conditions that are known to represent disease states in the retina. For example, in diabetic retinopathy, a variety of factors such as hyperglycemia, oxidative stress, increased levels of NO, and inflammation are thought to figure prominently in the eventual compromised functions of several retina cell types.[44] It will now be possible to evaluate the effects of these factors on the σR1 binding characteristics in isolated retinal cells. Although we did not explore this comprehensively in the present study, we performed preliminary experiments using NO donors and ROS donors to determine the effects on σR1 binding. Our studies showed that oxidative stress led to increased σR1 binding activity. Future studies will investigate this phenomenon comprehensively, determining whether gene and protein expression are altered under conditions of oxidative stress and characterizing the kinetic parameters associated with this increased activity. In addition to characterizing the binding activity under conditions of oxidative stress, we will use the subcellular localization information obtained in this study for future experiments designed to determine whether oxidative stress induces σR1 to translocate from its typical location at the nuclear and ER membranes to other cellular sites, such as the plasma membrane.

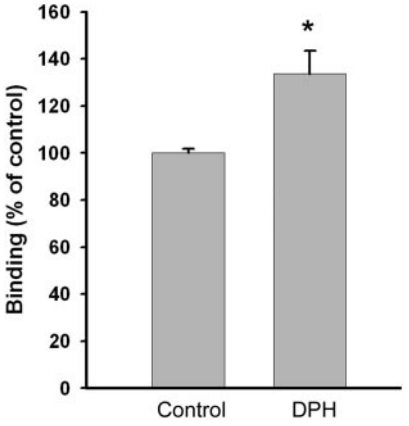

FIGURE 5. Allosteric effects of phenytoin on the binding of [^{3}H]-3-PPP. The rMC-1 cell line was cultured and membranes were prepared as described. The allosteric effect on [^{3}H]-3-PPP binding activity in the absence (Control) or presence of phenytoin (DPH) was measured after 90-minute incubation, as described. Values are mean ± SE for two experiments performed in triplicate. *Significantly different from control; $P < 0.01$.

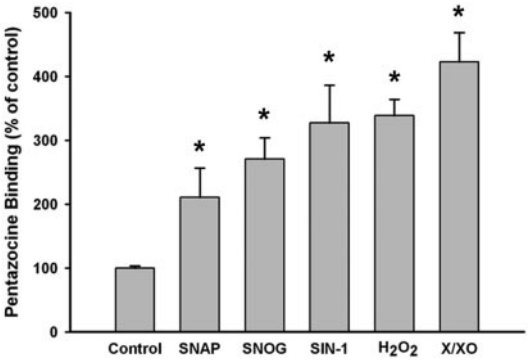

FIGURE 6. Regulation of σR1 binding activity by NO and ROS donors. rMC-1 cells were cultured as described and were treated for 6 hours with NO (3-nitroso-N-acetylpenicillamine [SNAP, 250 μM], S-nitrosoglutathione [SNOG, 250 μM], or 3-morpholinosydnonimine [SIN-1, 100 μM]), ROS (hydrogen peroxide [H_2O_2, 0.00025%, wt/wt] solution), or xanthine/xanthine oxidase (X/XO, 25 μM/10 mU/mL), after which the binding activity of σR1 was assayed. Values are mean ± SE for two experiments performed in triplicate. *Significantly different from control; $P < 0.01$.

Acknowledgments

The authors thank Vijay Sarthy (Northwestern University) and Neeraj Agarwal (University of North Texas Health Science Center) for rMC-1 and RGC-5 cell lines, respectively. They thank John Saari for the antibody against CRALBP. They also thank Tracy Van Ells, Leena Amarnath, and Angeline Martin-Studdard for their assistance in the use of the Carl Zeiss Axioplan 2 microscope.

References

1. Monnet FP, Maurice T. The sigma-1 protein as a target for the non-genomic effects of neuro(active)steroids: molecular, physiological and behavioral aspects. *J Pharmacol Sci*. 2006;100:93–118.
2. Quirion R, Bowen WD, Itzhak Y, et al. A proposal for the classification of sigma binding sites. *Trends Pharmacol Sc*i. 1992;13:85–86.
3. Hanner M, Moebius FF, Flandorfer A, et al. Purification, molecular cloning, and expression of the mammalian sigma 1-binding site. *Proc Natl Acad Sci USA*. 1996;93:8072–8077.
4. Kekuda R, Prasad PD, Fei YJ, Leibach FH, Ganapathy V. Cloning and functional expression of the human type 1 sigma receptor (hσR1). *Biochem Biophys Res Commun*. 1996;229:553–558.
5. Prasad PD, Li HW, Fei YJ, et al. Exon-intron structure, analysis of promoter region, and chromosomal localization of the human type 1 sigma receptor gene. *J Neurochem*. 1998;70:443–451.
6. Seth P, Leibach FH, Ganapathy V. Cloning and structural analysis of the cDNA and the gene encoding the murine type 1 sigma receptor. *Biochem Biophys Res Comm*. 1997;241:535–540.
7. Seth P, Fei Y-J, Li HW, Huang W, Leibach FH, Ganapathy V. Cloning and functional characterization σ from rat brain. *J Neurochem*. 1998;70:922–931.
8. Aydar E, Palmer C, Klachko V, Jackson M. Sigma receptor as a ligand-modulated auxiliary potassium channel subunit. *Neuron*. 2002;34:399–410.

9. Maurice T, Urani A, Phan VL, Romieu P. The interaction between neuroactive steroids and the sigma1 receptor function: behavioral consequences and therapeutic opportunities. *Brain Res Brain Res Rev*. 2001;37:116–132.
10. Lobner D, Lipton P. σ-Ligands and non-competitive NMDA antagonists inhibit glutamate release during cerebral ischemia. *Neurosci Lett*. 1990;117:169–174.
11. Lockhart BP, Soulard P, Benicourt C, Privat A, Junien J-L. Distinct neuroprotective profiles for σ-ligands against N-methyl-D-aspartate (NMDA), and hypoxia-mediated neurotoxicity in neuronal culture toxicity studies. *Brain Res*. 1995;675:110–120.
12. DeCoster MA, Klette KL, Knight KS, Tortella SC. σ Receptor-mediated neuroprotection against glutamate toxicity in primary rat neuronal cultures. *Brain Res*. 1995;671:45–53.
13. Klette KL, DeCoster MA, Moreton JE, Tortella FC. Role of calcium in sigma-mediated neuroprotection in rat primary cortical cultures. *Brain Res*. 1995;704:31–41.
14. Bhardwaj A, Sawada M, London ED, Koehler RC, Traystman RJ, Kirsch JR. Potent σ1-receptor ligand 4-phenyl-1-(4-phenylbutyl)-piperidine modulates basal and N-methyl-D-aspartate–evoked nitric oxide production in vivo. *Stroke*. 1998;29:2404–2411.
15. Carter C, Benavides J, Legendre P, et al. Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents, II: evidence for N-methyl-D-aspartate σ-receptor antagonist properties. *J Pharmacol Exp Ther*. 1988;247:1222–1232.
16. Yamamoto H, Yamamoto T, Sagi N, et al. Sigma ligands indirectly modulate the NMDA receptor-ion channel complex on intact neuronal cells via sigma 1 site. *J Neurosci*. 1995;15:731–736.
17. Goyagi T, Goto S, Bhardwaj A, Dawson VL, Hurn PD, Kirsch JR. Neuroprotective effect of sigma(1)-receptor ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) is linked to reduced neuronal nitric oxide production. *Stroke*. 2001;32:1613–1620.
18. Bucolo C, Drago F. Effects of neurosteroids on ischemia-reperfusion injury in the rat retina: role of sigma1 recognition sites. *Eur J Pharmacol*. 2004;498:111–114.
19. Campana G, Bucolo C, Murari G, Spampinato S. Ocular hypotensive action of topical flunarizine in the rabbit: role of sigma 1 recognition sites. *J Pharmacol Exp Ther*. 2002;303:1086–1094.
20. Bucolo C, Campana G, Di Toro R, Cacciaguerra S, Spampinato S. Sigma1 recognition sites in rabbit iris-ciliary body: topical sigma1-site agonists lower intraocular pressure. *J Pharmacol Exp Ther*. 1999;289:1362–1369.
21. Senda T, Mita S, Kaneda K, Kikuchi M, Akaike A. Effect of SA4503, a novel sigma1 receptor agonist, against glutamate neurotoxicity in cultured rat retinal neurons. *Eur J Pharmacol*. 1998;342:105–111.
22. Senda T, Matsuno K, Mita S. The presence of sigma receptor subtypes in bovine retinal membranes. *Exp Eye Res*. 1997;64:857–860.
23. Ola MS, Moore PM, El-Sherbeny A, et al. Expression pattern of sigma receptor 1 mRNA and protein in mammalian retina. *Brain Res Mol Brain Res*. 2001;95:86–95.
24. Ola MS, Martin PM, Maddox D, et al. Analysis of sigma receptor (σ R1) expression in retinal ganglion cells cultured under hyperglycemic conditions and in diabetic mice. *Brain Res Mol Brain Res*. 2002;107:97–107.
25. Martin PM, Ola MS, Agarwal N, Ganapathy V, Smith SB. The sigma receptor (σR) ligand (+)-pentazocine prevents retinal ganglion cell death induced in vitro by homocysteine and glutamate. *Brain Res Mol Brain Res*. 2004;123:66–75.
26. Georg A, Friedl A. Characterization of specific binding sites for [^{3}H]-1,3-di-o-tolyl-guanidine (DTG) in the rat glioma cell line C6-BU-1. *Glia*. 1992;6:258–263.
27. Palacios G, Muro A, Vela JM, et al. Immunohistochemical localization of the sigma1-receptor in oligodendrocytes in the rat central nervous system. *Brain Res*. 2003;961:92–99.
28. Palacios G, Muro A, Verdu E, Pumarola M, Vela JM. Immunohistochemical localization of the sigma1 receptor in Schwann cells of rat sciatic nerve. *Brain Res*. 2004;1007:65–70.
29. Hayashi T, Su TP. Sigma-1 receptors at galactosylceramide-enriched lipid microdomains regulate oligodendrocyte differentiation. *Proc Natl Acad Sci USA*. 2004;101:14949–14954.
30. Bringmann A, Reichenbach A. Role of Müller cells in retinal degenerations. *Front Biosci*. 2001;6:77–92.
31. Umapathy NS, Li W, Mysona BA, Smith SB, Ganapathy V. Expression and function of glutamine transporters SN1 (SNAT3) and SN2 (SNAT5) in retinal Müller cells. *Invest Ophthalmol Vis Sci*. 2005;46:3980–3987.
32. Hicks D, Courtois Y. The growth and behavior of rat retinal Müller cells in vitro, I: an improved method for isolation and culture. *Exp Eye Res*. 1990;51:119–129.
33. Sarthy VP, Brodjian SJ, Dutt K, Kennedy BN, French RP, Crabb JW. Establishment and characterization of a retinal Müller cell line. *Invest Ophthalmol Vis Sci*. 1998;39:212–216.
34. Krishnamoorthy RR, Agarwal P, Prasanna G, et al. Characterization of a transformed rat retinal ganglion cell line. *Brain Res Mol Brain Res*. 2001;86:1–12.
35. Dun Y, Mysona B, Van Ells TK, et al. Expression of the glutamate-cysteine (x_c^-) exchanger in cultured retinal ganglion cells and regulation by nitric oxide and oxidative stress. *Cell Tiss Res*. 2006;324:189–202.
36. Ramamoorthy JD, Ramamoorthy S, Mahesh VB, Leibach FH, Ganapathy V. Cocaine-sensitive sigma-receptor and its interaction with steroid hormones in the human placental syncytiotrophoblast and in choriocarcinoma cells. *Endocrinology*. 1995;136:924–932.
37. Su TP. Evidence for sigma opioid receptor: binding of [^{3}H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. *J Pharmacol Exp Ther*. 1982;223:284–290.
38. Cobos EJ, Baeyens JM, Del Pozo E. Phenytoin differentially modulates the affinity of agonist and antagonist ligands for sigma 1 receptors of guinea pig brain. *Synapse*. 2005;55:192–195.
39. Goldstein IM, Ostwald P, Roth S. Nitric oxide: a review of its role in retinal function and disease. *Vision Res*. 1996;36:2979–2994.
40. Schmetterer L, Findl O, Fasching P, et al. Nitric oxide and ocular blood flow in patients with IDDM. *Diabetes*. 1997;46:653–658.
41. Du Y, Sarthy VP, Kern TS. Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats. *Am J Physiol Regul Integr Comp Physiol*. 2004;287:R735–R741.
42. Yilmaz G, Esser P, Kociek N, Aydin P, Heimann K. Elevated vitreous nitric oxide levels in patients with proliferative diabetic retinopathy. *Am J Ophthalmol*. 2000;130:87–90.
43. Frank RN. Diabetic retinopathy. *N Engl J Med*. 2004;350:48–58.
44. Levin LA, Gordon LK. Retinal ganglion cell disorders: types and treatments. *Prog Retina Eye Res*. 2002;21:465–484.
45. Martin PM, Roon P, Van Ells TK, Ganapathy V, Smith SB. Death of retinal neurons in streptozotocin-induced diabetic mice. *Invest Ophthalmol Vis Sci*. 2004;45:3330–3336.
46. Barber AJ, Antonetti DA, Kern TS, et al. The Ins2^{Akita} mouse as a model of early retinal complications in diabetes. *Invest Ophthalmol Vis Sci*. 2005;46:2210–2218.