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Expression, subcellular localization and regulation of sigma receptor in retinal Müller cells

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Abstract

Purpose—Sigma receptors (σ R) are non-opioid, non-phencyclidine binding sites with robust neuroprotective properties. σ R1 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 σ R1 in retinal Müller cells.

Methods—Primary mouse Müller cells (1^oMC) were analyzed by RT-PCR, immunoblotting and immunocytochemistry for the expression of σ R1 and data were compared to the rat Müller cell line, rMC-1 and rat ganglion cell line, RGC-5. Confocal microscopy was used to determine the subcellular σ R1 location in 1^oMC. Membranes prepared from these cells were used for binding assays using [³H]-pentazocine (PTZ). The kinetics of binding, the ability of various σ R1 ligands to compete with σ R1 binding and the effects of nitric oxide (NO) and reactive oxygen species (ROS) donors on binding were examined.

Results— σ R1 is expressed in 1^oMC and is localized to the nuclear and endoplasmic reticulum membranes. Binding assays showed that in 1^oMCs, rMC-1 and RGC-5 cells, the binding of PTZ was saturable. [³H]-PTZ bound with high affinity in RGC-5 and rMC-1 cells and the binding was similarly robust in 1^oMC. Competition studies showed marked inhibition of [³H]-PTZ binding in the presence of σ R1-specific ligands. Incubation of cells with NO and ROS donors markedly increased σ R1 binding activity.

Conclusions—Müller cells express σ R1 and demonstrate robust σ R1 binding activity, which is inhibited by σ R1 ligands and is stimulated during oxidative stress. The potential of Müller cells to bind σ R1 ligands may prove beneficial in retinal degenerative diseases such as diabetic retinopathy.

Keywords

sigma receptor; Müller cells; neuroprotection; ganglion cells; nitric oxide; oxidative stress

Introduction

Sigma receptors (σ Rs) are nonopiate, nonphencyclidine binding sites [1], whose ligands have robust neuroprotective properties. The endogenous ligand and physiological function of σ R1 have not been elucidated. σ Rs consist of several subtypes distinguishable by

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biochemical and pharmacological means [2]. Among these, type 1 σ R (σ R1) is best characterized. The cDNA encoding σ R1 was cloned originally from guinea pig liver [3] and subsequently from human, mouse and rat [4-7]. The σ R1 cDNA predicts a protein of 223 amino acids (M_r 25-28 kD) [3]. Initial hydropathy analysis of the deduced σ R1 amino acid sequence suggested a single transmembrane segment [3,4,6]; recently Aydar and colleagues [8] showed that, when expressed in *Xenopus laevis* oocytes, σ R1 has two transmembrane segments with the NH₂ and COOH termini on the cytoplasmic side of the membrane.

σ R1 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 Ca²⁺ influx [12,13], depress neuronal responsivity to NMDA receptor stimulation [14-16], and reduce NO production [17].

In retina, the role of σ R1 recognition sites in ischemia-reperfusion injury, in controlling intraocular pressure and in protection against glutamate-induced neurotoxicity has been reported [18 – 20]. Binding assays, using bovine retinal membranes, suggested the presence of σ R_s [21, 22], but the studies did not disclose in which retinal cell types σ R_s were present, nor did they establish unequivocally the molecular identity of the receptor. Recently, we used molecular and biochemical methods to study σ R in mouse retina and reported widespread expression of σ R1 [23]. RT-PCR analysis amplified σ R1 in neural retina, RPE-choroid complex and lens. *In situ* hybridization studies revealed abundant expression of σ R1 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, owing to their vulnerability in diabetic retinopathy, and revealed that σ R1 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 σ R1 specific compound, can block RGC-5 cell death induced by homocysteine and glutamate [25].

Despite evidence that σ R1 is present in RGCs and other retinal cell types; there have been no studies of σ R1 binding activity in isolated retinal cells. In this study, we examined σ R1 binding in retinal Müller cells using the rat cell line (rMC-1) and primary Müller cells (1^oMCs) and in RGC-5 cells. The rationale for studying σ R1 binding activity in Müller cells is that recent studies have demonstrated a possible role for σ R in glial cell maintenance. Pharmacological studies showed that C6 glioma cells have σ R binding sites [26]. Immunohistochemical studies demonstrated that, in addition to neurons, σ R1 is present also in oligodendrocytes [27] and Schwann cells [28]. Hayashi and Su confirmed σ R1 presence in oligodendrocytes and localized it to the endoplasmic reticulum (ER) forming galactosylceramide-enriched lipid rafts in the myelin sheet of mature oligodendrocytes [29]. They speculated that σ R_s are important for oligodendrocyte 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 providing trophic substances and precursors of neurotransmitters to neurons [30]. Most retinal diseases are associated with reactive Müller cell gliosis, which may contribute to neuronal cell death; hence, we sought to characterize σ R_s in these cells. Our earlier work with the rat Müller cell line, rMC-1, suggested that σ R1 mRNA was present in these cells [24]. In this study we confirmed that finding and extended the analysis to Müller cells isolated from mouse retina

(primary cell culture). We analyzed the subcellular localization of σR in $1^\circ MCs$. Additionally, we used rMC-1, RGC-5 and $1^\circ MCs$ to analyze the binding characteristics of $\sigma R1$. Our study represents the first comprehensive analysis of $\sigma R1$ in Müller cells and the first information about the binding characteristics of this receptor in any isolated retinal cell type.

Methods and Materials

Reagents

Reagents were obtained from the following sources: DMEM:F12, TRIzol, penicillin-streptomycin (Gibco-Invitrogen Corp., Grand Island, NY); fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA); [1,3- 3H] (+)-pentazocine (specific radioactivity 37 Ci/mmol), [2,3- 3H] -(+)-3-(3-hydroxyphenyl)N-(1-propyl)piperidine [3H]-(+)-3-PPP (specific radioactivity 92.4 Ci/mmol) (DuPont-NEN, Boston, MA); IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA); Cy-3 IgG, Alexa Fluor 488-IgG and 568-IgG (In Vitrogen, Carlsbad, CA); monoclonal anti-Lamin A and monoclonal anti-PDI (Abcam Inc., Cambridge, MA), polyclonal anti-vimentin (Chemicon Intl., Temecula, CA); ECL detection kit (Pierce Biotechnology, Inc., Rockford, IL); Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA); Power Block (BioGenex, San Ramon, CA); complete Mini Protease Inhibitor Cocktail tablets, GeneAmp RNA PCR Kit (Applied Biosystems/Roche Molecular System, Branchburg, NJ); TaKaRa TaqTM (Takara Bio Inc, Otsu, Shiga, Japan); sense and antisense primers for rat and mouse $\sigma R1$ (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-morpholiniosydnominine (SIN-1), hydrogen peroxide 30% (W/W) 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 Dr. V. P. Sarthy and Dr. N. Agarwal. Anti-CRALBP was a generous gift from Dr. J. Saari.

Animals and isolation of Müller cells

C57Bl/6 mouse breeding pairs, purchased from Harlan Sprague-Dawley, (Indianapolis, IN), were maintained in our colony following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Müller cells were isolated from 6-7 day mice following our published methods [31], which were adapted from those of Hicks and Courtois [32]. Briefly, eyeballs are removed, placed in DMEM with gentamicin and soaked 3 h at 25°C in the dark. They are rinsed in PBS, incubated in buffer containing trypsin, EDTA and collagenase. Retinas are removed from eyeballs (taking care to avoid contamination by pigmented RPE), and placed in DMEM supplemented with glucose, FBS, penicillin/streptomycin and gently pipetted into small aggregates at a density of 10-16 retinas/dish. Isolated cells are detectable within 1-3 days. By 3-5 days substantial cell growth ensues. Cultures are washed vigorously with medium until only a strongly adherent flat cell population remains. Cells are passaged 1-3 days after washing, seeded into culture flasks (50,000 cells/cm²); culture media is changed three times per week. The purity of the cultures is verified using antibodies that are known markers of Müller cells (CRALBP, vimentin, glutamine synthetase, GLAST). It is noteworthy that GFAP, typically considered a marker of Müller cells under stress, is 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) show minimal detection.

Cell Culture

Primary mouse Müller cells (1°MC), rMC-1 and RGC-5 cells [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% CO₂. Medium was replaced every other day. Upon confluence, cultures were passaged by dissociation in 0.05% (w/v) trypsin in PBS.

Semiquantitative RT-PCR analysis of σ R1 mRNA

Total RNA was prepared from confluent 1°MC, rMC-1 and RGC-5 cells using TRIzol. For the two cells lines (rMC-1 and RGC-5), which were derived from rat [33,34], RT-PCR was carried out using primer pairs specific for rat σ R1 [7]: 5'-GTT TCT GAC TAT TGT GGC GGT GCT G -3' (sense) and 5'-CAA ATG CCA GGG TAG ACG GAA TAA C -3' (antisense) (nucleotide position 80-104 and 567-591; expected PCR product size: 512 bp). For the 1°MCs, isolated from mouse, RT-PCR was carried out using primer pairs specific for mouse [6]: 5'- CTC GCT GTC TGA GTA CGT G -3' (sense) and 5'-AAG AAA GTG TCG GCT GCT AGT GCA A -3' (antisense) (nucleotide position 315-333 and 572-593; expected PCR product size: 279 bp). 18S RNA was the internal standard. RT-PCR was done at 35 cycles, with a denaturing phase of 1 min at 94°C, annealing phase of 1 min at 59°C, and an extension of 2 min at 72°C. 20 µl of the PCR products were gel electrophoresed and stained with ethidium bromide.

Immunoblot analysis of σ R1

Immunodetection of σ R1 in RGC-5, rMC-1, and 1°MCs followed our published methods [24, 35]. Protein samples were subjected to SDS-PAGE and were transferred to nitrocellulose membranes, which were blocked 1.5 h with Tris-buffered-saline-0.05% Tween-20 containing 5% non-fat milk. Membranes were incubated with anti- σ R1 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 using the ECL western blot detection system. Membranes were washed thrice, blocked with 5% non-fat milk for 2 h and were reprobed with mouse monoclonal anti- β -actin antibody (1:5000) as a loading control.

Immunocytochemical analysis of σ R1

σ R1 detection in RGC-5, rMC-1 and 1°MCs cells followed our published protocol [23-25, 35]. Cells were incubated with polyclonal anti- σ R1 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. σ R1 was detected by epifluorescence using a Zeiss Axioplan-2 microscope (Carl Zeiss, Göttingen, Germany) and the axiovision program. For σ R1 subcellular localization studies, 1°MCs, cells were seeded on coverslips, grown for 24 h, fixed in ice-cold methanol 10 min, air dried, washed in PBS, blocked with Power Block and were incubated overnight at 4°C with the σ R1 antibody (1:100) [24] and either the monoclonal anti-Lamin A (nuclear membrane marker, 1:25) or the monoclonal anti-PDI (ER marker, 1:25). Cells were then incubated 30 min with goat anti-rabbit IgG coupled to Alexa Fluor 568 and goat anti-mouse IgG coupled to Alexa Fluor 488 (1:1500). Negative control sections were treated identically except that PBS replaced the primary antibodies. Coverslips containing the cells were mounted with Vectashield Hardset mounting media on microscope slides and examined with a Zeiss confocal microscope using LSM 510 Meta imaging software.

Preparation of cell membranes for binding assays

RGC-5, rMC-1 and 1°MCs were cultured as described above. Cell monolayers were chilled on ice, washed with ice-cold PBS (pH 7.5), lysed using 5 mM K₂HPO₄-KH₂PO₄ buffer (pH

7.5). The suspension was centrifuged 30 min at 25,000 rpm; the final membrane pellets were rinsed and suspended in 5 mM K_2HPO_4 - KH_2PO_4 buffer and rehomogenized 20 times using a 25g needle. The protein concentration in the final membrane preparation was measured using the Bio-Rad protein assay.

Ligand binding assay

[3H]-(+)-pentazocine binding to membrane preparations was assayed as described with minor modifications [36]. Samples were incubated with [3H]-(+)-pentazocine (10 nM) in 250 μ l 5 mM K_2HPO_4 - K_2PO_4 buffer, pH 7.5, 25°C, 90 min. Binding was terminated by adding ice-cold binding buffer and the mixture was filtered on a Whatman GF/F glass fiber filter, presoaked in 0.3% polyethyleneimine. The filter was washed thrice with ice-cold binding buffer and radioactivity associated with the filter was determined by liquid scintillation spectrometry. To study the inhibition of [3H]-pentazocine binding to the $\sigma R1$, several competitive inhibitors (+)-3-PPP, carbetapentane, DTG, haloperidol, and (+)-pentazocine were used. The concentrations for the [3H]-pentazocine and the inhibitors were 50 nM and 1 μ M, respectively. To investigate the allosteric effects of phenytoin on $\sigma R1$, 150 μ l of rMC-1 membrane preparation containing 300 μ g of protein was incubated with 50 μ l of 125 nM of [3H](+)-3-PPP (final concentration 25 nM) and 50 μ l of 250 μ M phenytoin (final concentration 50 μ M) or its solvent (5 mM K_2HPO_4 buffer containing 2.5% DMSO, pH 7.5) for 90 min at 25°C. The final volume of the reaction system was 250 μ l. 50 μ M unlabeled (+)-pentazocine was used to define non-specific binding.

The effects of donors of nitric oxide (NO) and reactive oxygen species (ROS) on $\sigma R1$ binding activity were examined by treating rMC-1 cells on the 2nd day following seeding for 6 h with the NO donors SNAP (250 μ M), SNOG (250 μ M) and SIN-1 (100 μ M) or the ROS donors H_2O_2 (0.00025%) and xanthine (25 μ M)/xanthine oxidase (10 mU/ml), after which the binding activity was assayed as described above.

Data analysis

Experiments were performed in duplicate or triplicate; each experiment was repeated at least two times. Results are expressed as mean $\pm$ SE. The equilibrium saturation binding parameters, dissociation constant (K_d), and the maximum number of binding sites (B_{max}) were calculated by nonlinear regression analysis of the equation for a rectangular hyperbola using SigmaPlot 2001 for Windows version 7.0. Statistical significance ($p < 0.05$) was determined using SigmaStat, Version 2.

Results

Analysis of $\sigma R1$ expression in Müller and ganglion cells

The purity of the mouse Müller cells [31] was confirmed using known antibody markers for Müller cells, including CRALBP and vimentin (Fig. 1 A) and glutamine synthetase (data not shown). The cells were not positive for neuronal markers such as NF-L [31] nor were they positive for cytokeratin-8, which labels RPE cells (Fig. 1A). For expression and ligand-binding studies, 1^oMCs were used at passage number 5. RT-PCR of the mouse 1^oMCs amplified the expected PCR product (279 bp) (Fig. 1B), indicating that $\sigma R1$ is expressed in these cells. The 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 $\sigma R1$ [24] detected a protein band of the expected size ($M_r \sim 27$ kD) in 1^oMCs, rMC-1 and RGC-5 cells. Immunocytochemical analysis of the cells detected $\sigma R1$ in RGC-5, rMC-1, and 1^oMCs (Fig. 1D).

To analyze the subcellular location of σ R1 in 1° MCs markers for the nuclear and ER membranes were used in double-labeling experiments with the σ R1 antibody. As shown in Fig. 2A, σ R1 was present in the area consistent with the nuclear membrane and the merged image of the σ R1 plus the nuclear membrane marker, lamin-A, showed marked co-localization suggesting that σ R1 is present on the nuclear membrane. Similarly, optical sectioning at a slightly different plane of the cells showed intense σ R1 levels in the perinuclear area, consistent with ER localization. The merged image of the σ R1 and the ER membrane protein, PDI (Fig. 2) provides strong evidence that σ R1 is present also in the ER.

Characterization of [3 H](+)-pentazocine binding in retinal cell membranes

σ R1 binding in RGC-5, rMC-1 and 1° MC cells was characterized using (+)-pentazocine, a high-affinity σ R1 ligand [37]. The binding was saturable over a (+)-pentazocine concentration range of 1.25-75 nM (Fig. 3). The apparent dissociation constants (K_d) were 25.0 ± 5.9 nM, 21.5 ± 2.6 and 18.9 ± 5.6 nM for RGC-5, rMC-1 and 1° MCs, respectively. Thus, in the cell lines derived from rat and in 1° MCs obtained from mouse, the affinity constant for the protein is comparable to the cloned σ R1 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 1° 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 is comparable between the retinal cell lines and the 1° MCs.

The binding of [3 H](+)-pentazocine (final concentration 25 nM) to RGC-5, rMC-1 and 1° MC membranes was inhibited by several σ R1 ligands (Fig. 4). The order of potency differed somewhat between the cell type being 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 1° MCs the order of potency was carbetapentane = haloperidol > (+)-pentazocine > 3-PPP > DTG.

A key pharmacological characteristic of type 1 sigma receptors, which distinguishes them from the type 2 receptors, is the allosteric enhancement of binding of ligands to the σ R1 in the presence of phenytoin [9]. Stimulation of binding of the σ R1 agonist (+)-3-PPP in the presence of phenytoin was reported recently in guinea pig brain [38]. We used the 1° MCs to confirm that the σ R binding we were studying was specific to type 1 (σ R1). In these studies the binding of [3 H](+)-3-PPP was examined in the absence and presence of 50 μ M 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 is specific to σ R1.

The ability to analyze σ R1 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. While it was not the focus of this paper to investigate exhaustively the regulation of σ R1, it is important to ascertain whether binding activity is likely to be altered when retinal cells are subjected to stress. To this end we performed preliminary studies in rMC-1 cells and analyzed σ R1 binding activity when cells were exposed to either NO or ROS donors. These stressors were selected because, in diabetic retinopathy, NO [39-42] and oxidative stress [43] have been implicated in the pathogenesis of the disease. The cells were exposed to NO donors: SNAP, SNOG, SIN-1 or to ROS donors: H_2O_2 , X/XO for 6 h following which the binding activity of σ R1 was assayed. Treatment of rMC-1 cells with all three NO donors resulted in marked increase in binding activity, with the greatest effect observed when cells were incubated with SIN-1. There was an approximate 3-fold increase in binding activity after 6 h exposure to this NO donor. In the experiments using ROS donors, the effects were similarly profound with a 3-fold increase in binding activity

following 6 h exposure to H₂O₂ and nearly 4-fold increase in the presence of X/XO. The findings were observed in three independent experiments.

Discussion

In this study, we examined σ R1 expression in isolated retinal cell types and characterized comprehensively σ R1 binding activity in these cells. We used molecular techniques and demonstrated that σ R1 is present in 1°MCs isolated from the mouse retina. The location of the σ R1 appeared to be perinuclear. We explored this question using LSCM analysis with antibodies that recognize the nuclear and ER membranes. Our studies demonstrated that in 1°MCs, σ R1 is localized to the both sites. σ R1 to the ER was consistent with the studies by Hyashi and Su [29] who localized σ R1 to the ER of oligodendrocytes. They did not report localization to the nuclear membrane; however they did not use antibody markers to explore this question, so it is not known whether oligodendrocytes might also place σ R1 on the nuclear membrane.

Previous studies of σ R in retina have demonstrated σ R1 binding activity; however these studies used whole retina from large models (bovine) [21, 22] and did not attempt to study the σ R1 binding activities of individual retinal cell types. If we are to postulate a role for σ Rs in mediating neuroprotection in retinal cells, it is essential to be able to quantify the σ R1 binding activity in the isolated cells. We were interested in σ R1 binding activity in both ganglion cells and in Müller cells. Ganglion cells die in several retinal diseases [44-46], and we predict that activation of the σ R1 may be beneficial to their survival; Müller cells play a key role in neuronal survival [30] and may activate σ R1 during stressful episodes providing protection for adjacent neurons. In the present studies, we exploited the retinal ganglion cell line, RGC-5 to study σ R1 binding activity in neurons. While isolation of ganglion cells from mouse retina is possible [35], these cells are terminally differentiated making it difficult to obtain sufficient numbers of these neurons to carry out σ R1 binding assays. For studies of σ R1 in Müller cells, we used the rMC-1 cell line and complemented the data using 1°MCs isolated from mouse. Given the plethora of genetic defects that cause retinal abnormalities in commercially available strains of mice, the ability to study σ R1 expression and activity in 1°MC cultures affords an excellent opportunity to dissect σ R1 activity and expression in Müller cells of various mouse models of retinal disease.

Our studies of σ R1 binding characteristics in Müller cells showed that the affinity of σ R1 in 1°MCs is comparable to the two retinal cell lines, RGC-5 and rMC-1. In all three cases, σ R1 bound its ligand, (+)-pentazocine, with great affinity. The density of receptors as indicated by the B_{max} value is similar between the 1°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, while the 1°MCs seemed slightly less sensitive to these inhibitors. Nonetheless, the binding in these 1°MCs was inhibited by 75-80% 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 being studied in these retinal cells is indeed mediated by the type 1 sigma receptor.

The value of the present work is that it forms a scaffold upon which to study the regulation of σ R1 activity in isolated retinal cells. We now have substantial baseline data for two retinal cell lines and 1°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 σ R1 binding characteristics in isolated retinal cells. While not exploring this comprehensively in the present study, we performed preliminary experiments using NO donors and ROS donors to determine the effects on σ R1 binding. Our studies showed that oxidative stress led to increased σ R1 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 σ R1 to translocate from its typical location at the nuclear and ER membranes to other cellular sites, such as the plasma membrane.

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Fig. 1. Analysis of $\sigma R1$ expression in retinal cell lines and in primary mouse Müller cells (1°MCs) (A) Verification of the purity of the 1°MCs used in these studies. The left panel shows a phase contrast image of cells grown for 3 days in culture and the middle two panels show positive immunostaining for CRALBP (red fluorescence) and vimentin (green fluorescence). The right panel shows 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 $\sigma R1$ mRNA in rat ganglion (RGC-5) and Müller (rMC-1) cell lines (512 bp) and mouse 1°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 1°MC showing detection of $\sigma R1$ ($M_r \sim 27$ kD) and β -actin ($M_r \sim 50$ kD). (D) Cell lines or 1°MCs were cultured as described in the text and subjected to immunocytochemistry to detect $\sigma R1$ followed by a Cy-3 conjugated secondary antibody (red staining); DAPI was used to label nuclei (blue). $\sigma R1$ was detected in the cell lines (RGC-5, rMC-1) and in 1°MCs. Calibration bar = 15 μ m.

Fig. 2. Subcellular localization of σ R1 in 1°MCs

Müller cells were isolated from mouse retina and cultured as described in the text. They were subjected to double-labeling immunocytochemical analysis using a polyclonal antibody specific for σ R1 (red fluorescence) and monoclonal antibodies (green fluorescence) that label the nuclear membrane (lamin-A) or the endoplasmic reticulum (PDI). Optical sectioning by confocal microscopy detected co-localization of σ R1 with lamin-A (merged image) and with PDI (merged image). In the merged images, the orange signal is detected when the red and green fluorescence overlap indicative of colocalization. Calibration bar = 15 μ m.

Fig. 3. Saturation kinetics of $\sigma R1$ binding in membrane preparations

The two retinal cell lines (RGC-5 and rMC-1) and 1° MCs were cultured as described, membranes prepared and the binding of [^3H](+)-pentazocine over a broad concentration range measured in (A) RGC-5 cells; (B) rMC-1 cells, and (C) 1° MCs. Values are the mean $\pm$ SE for two independent experiments performed in triplicate. *Inset*, Scatchard plot.

Fig. 4. Inhibition of [^3H](+)-pentazocine binding by σR1 ligands

RGC-5, rMC-1 and 1°MCs were cultured as described, membranes prepared and the inhibition of the binding of [^3H](+)-pentazocine was measured using several known ligands for σR1 . (A) RGC-5 cells; (B) rMC-1 cells, and (C) 1°MCs. Data are shown as relative percent of control binding (100%) measured in the absence of ligands. Values are the mean $\pm$ 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, $p < 0.01$).

Fig. 5. Allosteric effects of phenytoin on the binding of [^3H]-3-PPP

The rMC-1 cell line was cultured and membranes prepared as described. The allosteric effect on [^3H]-3-PPP binding activity in the absence (Control) or presence of phenytoin (DPH) was measured after a 90 min incubation as described. Values are mean $\pm$ SE for two experiments performed in triplicate. (*Significantly different from control, $p < 0.01$).

Fig. 6. Regulation of σ R1 binding activity by NO and ROS donors

The rMC-1 cells were cultured as described and were treated for 6 h with nitric oxide (NO) donors: 3-nitroso-N-acetylpenicillamine (SNAP, 250 μ M), S-nitrosoglutathione (SNOG, 250 μ M), or 3-morpholinosydnonimine (SIN-1, 100 μ M) or with reactive species (ROS) donors: hydrogen peroxide (H₂O₂, 0.00025%, W/W) solution or xanthine/xanthine oxidase (X/XO, 25 μ M/10 mU/ml) following which the binding activity of σ R1 was assayed. Values are mean $\pm$ SE for two experiments performed in triplicate. (*Significantly different from control, $p < 0.01$).