(+)-Pentazocine Reduces NMDA-Induced Murine Retinal Ganglion Cell Death Through a σR1-Dependent Mechanism

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Purpose. To evaluate, in vivo, the effects of the sigma-1 receptor (σR1) agonist, (+)-pentazocine, on N-methyl-D-aspartate (NMDA)-mediated retinal excitotoxicity.

Methods. Intravitreal NMDA injections were performed in C57BL/6J mice (wild type [WT]) and σR1$^{-/-}$ (σR1 knockout [KO]) mice. Fellow eyes were injected with phosphate-buffered saline (PBS). An experimental cohort of WT and σR1 KO mice was administered (+)-pentazocine by intraperitoneal injection, and untreated animals served as controls. Retinas derived from mice were flat-mounted and labeled for retinal ganglion cells (RGCs). The number of RGCs was compared between NMDA and PBS-injected eyes for all groups. Apoptosis was assessed using TUNEL assay. Levels of extracellular-signal–regulated kinases (ERK1/2) were analyzed by Western blot.

Results. N-methyl-D-aspartate induced a significant increase in TUNEL-positive nuclei and a dose-dependent loss of RGCs. Mice deficient in σR1 showed greater RGC loss (≈80%) than WT animals (≈50%). (+)-Pentazocine treatment promoted neuronal survival, and this effect was prevented by deletion of σR1. (+)-Pentazocine treatment resulted in enhanced activation of ERK at the 6-hour time point following NMDA injection. The (+)-pentazocine–induced ERK activation was diminished in σR1 KO mice.

Conclusions. Targeting σR1 activation prevented RGC death while enhancing activation of the mitogen-activated protein kinase (MAPK), ERK1/2. Sigma-1 receptor is a promising therapeutic target for retinal neurodegenerative diseases.

Keywords: sigma-1 receptor, NMDA, excitotoxicity, neuroprotection

The sigma-1 receptor (σR1) is a ligand-operated transmembrane chaperone protein expressed throughout the central nervous system and retina. Recent studies have described neuroprotective effects of ligands for σR1 in animal models of diabetic retinopathy and in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, and stroke.[1–6] In addition, evaluations of primary retinal ganglion cell (RGC) cultures have found σR1 activation to be neuroprotective.[7,8] Therefore, ligands for σR1 are strong candidates for treatment of retinal disorders that affect RGCs, including glaucoma and ischemia-related conditions such as vessel occlusion and diabetic retinopathy.

Literature indicates that σR1 is a multitasking protein involved in a broad range of cellular functions, and the mechanism of σR1-mediated neuroprotection is likely multifactorial.[9,10] Recent work shows that σR1 activation can rescue neuronal damage by affecting inflammatory responses and by activating protective mitogen-activated protein kinase (MAPK) signaling pathways.[8,11] An additional means of σR1-mediated neuroprotection is through effects on calcium homeostasis including inhibition of voltage-gated calcium channel (VGCC) activity.[12,13] There are conflicting reports, however, regarding the effects of σR1 stimulation on a key contributor to intracellular calcium levels, the N-methyl-D-aspartate (NMDA) receptor. Within the retina, one study demonstrated σR1 agonist–mediated inhibition of NMDA-induced calcium influx.[14] However, studies using brain-derived neurons indicate that σR1 activation potentiates, rather than inhibits, intracellular calcium influx through the NMDA receptor.[15,16] Furthermore, studies using cultured cerebral cortical neurons suggest that σR1 ligands interact directly with NMDA receptors, independent of the σR1.[17]

Sigma-1 receptor ligands may offer a novel therapeutic option for neuroprotection of the retina and optic nerve. N-methyl-D-aspartate receptor–mediated excitotoxicity is thought to be an important contributor to RGC death in the types of retinal disorders that could be treated using σR1 ligands.[18,19] Therefore, an understanding of the effects of σR1 ligands on NMDA-mediated retinal toxicity is key to evaluating their treatment potential and their mechanisms of action. The purpose of this study was to assess whether σR1 stimulation results in RGC protection using intravitreal NMDA treatment as the method for generating neurotoxicity. We found that the σR1 agonist, (+)-pentazocine, reduced RGC death when administered prior to and following NMDA injection. The protective effects of (+)-pentazocine were nullified in the absence of σR1. In addition, (+)-pentazocine treatment resulted in acute enhancement of phosphorylated extracellular regulated kinase (ERK1/2) levels within the retina. The results indicate that σR1 activation protects against excitotoxicity-induced

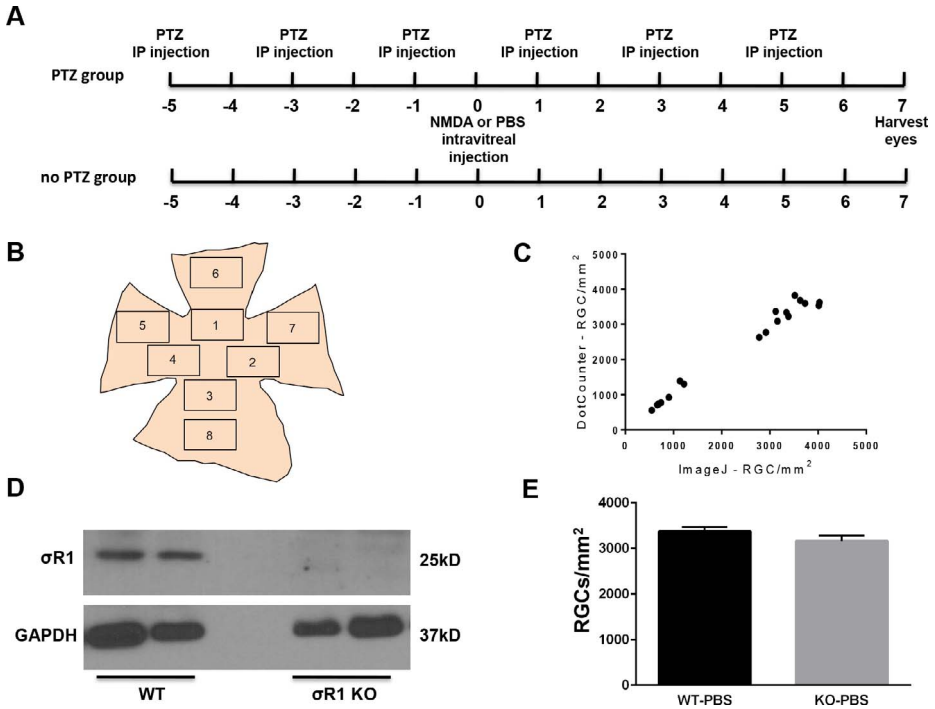

Figure 1. Time line of pentazocine (PTZ) treatment and quantification of RGC densities. (**A**) Time line showing administration of (+)-PTZ (0.5 mg/kg) intraperitoneally three times per week for 1 week prior to and 1 week post NMDA intravitreal injection in (+)-PTZ group and no treatment in the control (no PTZ) group. (**B**) Retinal flat mount showing location of eight micrographs taken to assess numbers of Brn3a positive RGCs. Images were taken with a 10× objective and covered an area of 0.59 mm^2. (**C**) Validation of Brn3a semiautomated counting method shows a strong correlation between RGC densities obtained using the Dotcounter method versus blinded, manual counts. (**D**) Western blot showing expression of σR1 protein in WT (C57BL/6J) retina but not in σR1 KO retina. (**E**) Retingal ganglion cell densities showed no significant difference between PBS intravitreal injected WT and σR1 KO retinas. Wild type retinas had 3374 ± 217 RGCs/mm^2 and KO retinas had 3160 ± 267 RGCs/mm^2 at 7 days post PBS injection, $n = 5$.

retinal neurodegeneration through a mechanism that involves enhancement of ERK1/2 activation.

Methods

Animals and Injection

Experiments requiring animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 72 male C57BL/6J mice (wild type [WT]) 3- to 5-months-old (The Jackson Laboratory, Bar Harbor, ME, USA) and 54 sigma receptor 1 knockout mice (σR1 KO) 3- to 5-months-old (see Wang et al.[20]) were kept under controlled lighting conditions (12-hour light/12-hour dark). A subset of WT and σR1 KO mice received intraperitoneal (IP) injection of (+)-pentazocine (Sigma-Aldrich Corp., St. Louis, MO, USA) 0.5 mg/kg every other day for three treatments prior to NMDA injection and three treatments after NMDA injection (Fig. 1A). The (+)-pentazocine used in these experiments was dissolved initially in DMSO and diluted with 0.01 M PBS for a final dosage of 0.5 mg/kg. For intravitreal injections, mice were deeply anesthetized with a single IP injection of ketamine (80 mg/kg) and xylazine (12 mg/kg; Sigma-Aldrich Corp.). Retinal damage was induced by the intravitreal injection of NMDA (1 μL/eye, dissolved at 7.5 mM, 10 mM, and 20 mM in 0.01 M phosphate-buffered saline [PBS] at pH 7.4; Sigma-Aldrich Corp.). Intravitreal injection was performed using a 33-gauge needle (Hamilton, Reno, NV, USA) into the vitreous body of the right eye. Sham controlled eyes (left eyes) were intravitreally injected with 1 μL 0.01 M PBS. N-methyl-D-aspartate–injected mice were euthanized at 6 hours, 24 hours, or 7 days post injection.

Quantification of RGCs

To evaluate RGC number, retinal flat mounts were prepared. At 7 days post NMDA injection, eyes were enucleated and fixed overnight in 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS at 4°C. Retinas were dissected and incubated in 0.3% Triton X-100 in PBS for 1 hour at room temperature, followed by blocking with 10% donkey serum in 0.3% Triton X-100 (Sigma-Aldrich Corp.) in PBS 1 hour at room temperature. Then retinas were incubated in Brn-3a antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 10% donkey serum in 0.1% Triton X-100 in PBS at 4°C overnight. Next day, retinas were washed and incubated in Invitrogen Alexa Fluor 555 (1:1000; Thermo Scientific, Waltham, MA, USA) 2 hours at room temperature. Finally, retinas were thoroughly washed in PBS and mounted with Aqua-polymount (Polysciences, Warrington, PA, USA). Retina flat mounts were observed by immunofluorescence using a Zeiss Axioplan2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with AxioVision Program (version 4.6.3) and a high-resolution microscopy camera. For each flat mount, eight separate images were taken across the central and peripheral retina (Fig. 1B). The field of view for each image covered an area of 0.59 mm^2. For counting, electronic images were uploaded into a Web-based application from MedTrack Solutions LLC (Dotcounter). The Brn3a-positive RGCs were counted semiautomatically using the Dotcounter algorithm. Trained investigators manually checked Dotcounter results for each image, removing false-positives and adding missed cells. Approximately 16,000 cells from eight images were counted for each control retina.

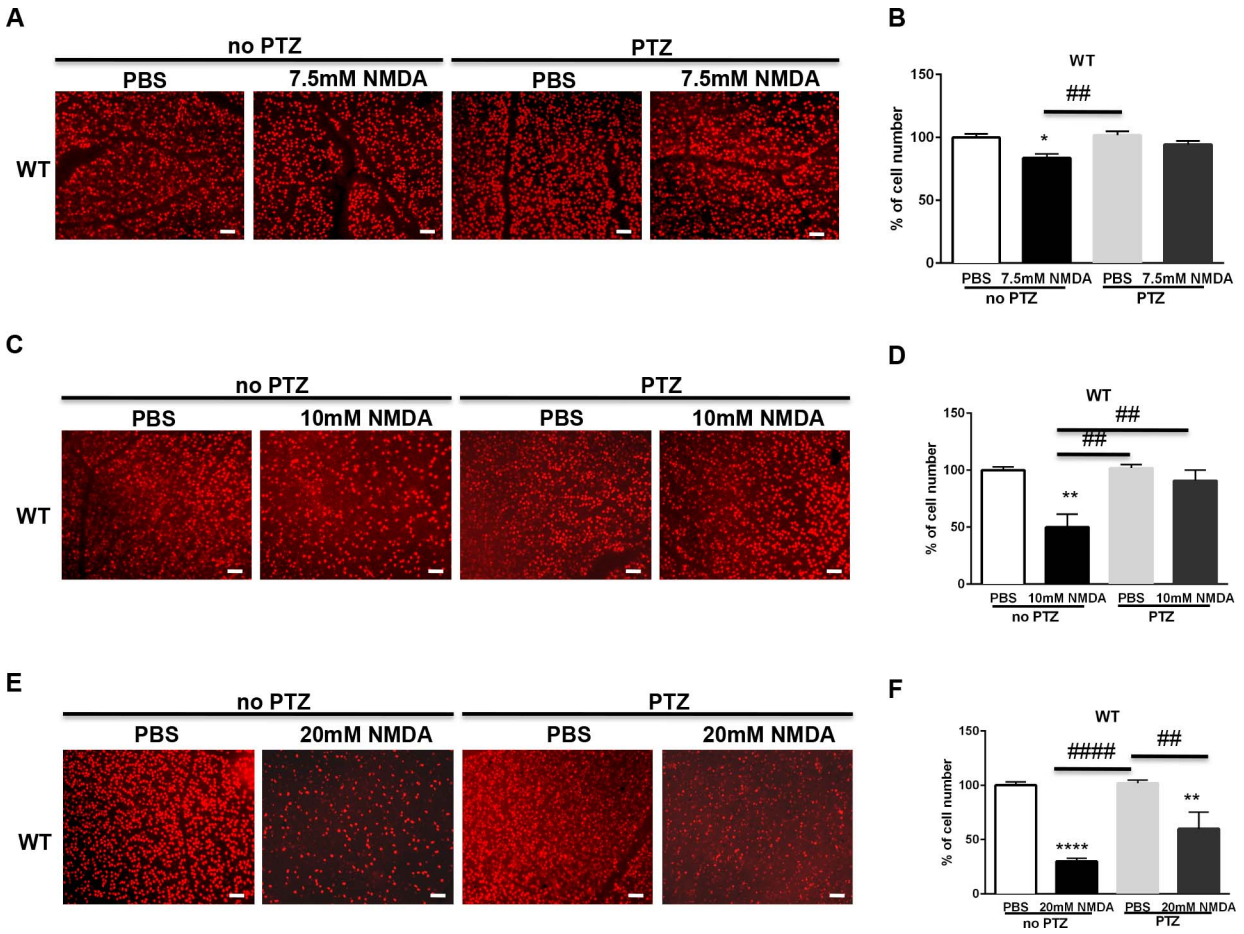

FIGURE 2. Effects of intraperitoneally-administered (+)-pentazocine (PTZ) on NMDA-induced retinal damage in WT (C57BL/6J) mice. Pentazocine (0.5 mg/kg) was administered intraperitoneally three times per week for 1 week prior to and 1 week post NMDA injection. (**A**) Representative micrographs of retina flat mounts from WT mice given 1-μL intravitreal injection of PBS (vehicle) or 7.5 mM NMDA. (**B**) Quantitative analysis of cell number in each field of retina flat mount in WT mice at 7 days after NMDA injection normalized to no PTZ, PBS injected controls. (**C**) Representative micrographs of retina flat mounts from WT mice given 1-μL intravitreal injection of PBS (vehicle) or 10 mM NMDA. (**D**) Quantitative analysis of cell number in each field of retina flat mount in WT mice at 7 days after NMDA injection normalized to no PTZ, PBS injected controls. (**E**) Representative micrographs of retina flat mounts from WT mice given 1-μL intravitreal injection of PBS (vehicle) or 20 mM NMDA. (**F**) Quantitative analysis of cell number in each field of retina flat mount in WT mice at 7 days after NMDA injection normalized to no PTZ, PBS injected controls. *Scale bars*: 50 μm. Significantly different from control $^{*}P < 0.05$. Significantly different between groups $\#\#P < 0.01$, $\#\#\#\#P < 0.0001$, $n = 4$–11 in each treatment group.

Validation of Dotcounter Semiautomated Counting Method

The Dotcounter semiautomated computer-assisted counting method was validated by performing a manual blinded count of Brn3a positive RGCs from a sample of 16 randomly selected images across all treatment groups. Manual counts were performed with the aid of ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).[21] Dotcounter results plotted against ImageJ counts showed good correlation with a Pearson correlation coefficient of $r = 0.989$, $P < 0.0001$.

TUNEL Assay

At 24 hours after NMDA injection, eyes were enucleated and frozen in Tissue-Tek Optimum Cutting Temperature (OCT) compound (Sakura Finetek, Tokyo, Japan). Retinal cryosections (10 μm thick) were subjected to a TUNEL assay (ApopTAG Fluorescein In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA, USA), according to the manufacturer's protocol. DAPI counterstaining was used to label all nuclei. Sections were viewed with the fluorescence microscope equipped as described earlier. TUNEL positive (green fluorescing) cells in the ganglion cell layer (GCL) and inner nuclear layer (INL) were quantified using ImageJ software.[21]

Western Blot Analysis

At 6 hours, 24 hours, or 7 days post NMDA injection, retinas were removed from the eyes and sonicated and lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich Corp.). The lysates were centrifuged at 14,000*g* for 15 minutes. Protein concentration of the supernatant was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Thermo Scientific). The membrane was blocked with 5% nonfat milk in Tris-buffered saline, 0.05% Tween 20 (TBST) for 1 hour at room temperature, then incubated overnight at 4°C with primary antibody. After three washes in TBST, the membrane was incubated for 1 hour with

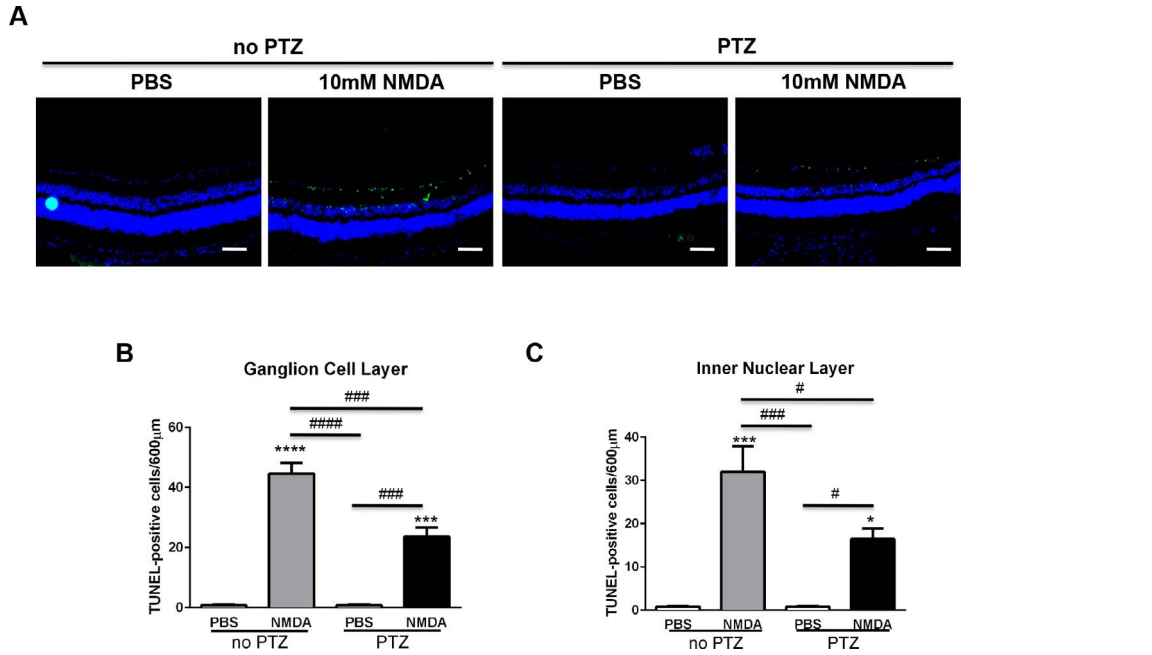

FIGURE 3. Neuronal apoptosis decreased in NMDA intravitreal injected mice treated with (+)-pentazocine (PTZ). (**A**) Representative images of TUNEL staining of cryosections of WT (C57BL/6J) retinas taken 24 hours after intravitreal injection of PBS or NMDA (10 mM) with or without PTZ treatment. *Scale bars*: 100 μm. (**B**) Quantitative analysis of TUNEL positive cells in GCL. (**C**) Quantitative analysis of TUNEL positive cells in INL. Pentazocine treatment in NMDA injected mice results in a significant 2-fold decrease in cell death compared to NMDA injected mice without PTZ treatment. Phosphate-buffered saline intravitreal injected mice had minimal cell death with or without PTZ treatment. Significantly different from control $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Significantly different between groups $\#P < 0.05$, $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$. $n = 3$–4 in each treatment group.

an appropriate HRP-conjugated secondary antibody at room temperature. Proteins were visualized by incubating with a SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and quantified by densitometry with ImageJ software.[21] Blots were stripped and reprobed for loading controls. Phosphorylated-ERK polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, and HRP-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology. Sigma-1 receptor rabbit polyclonal antibody was raised from a peptide sequence and generated in the Smith[22] laboratory.

Statistical Analysis

Data for RGC counting, Western blot, and TUNEL assay were analyzed using 1-way ANOVA followed by Tukey-Kramer post hoc test for multiple comparisons. Significance was set at $P < 0.05$ (Prism; GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Quantification of RGCs in WT and σR1 KO Mice

We wanted to determine whether the σR1 ligand, (+)-pentazocine, protects RGCs from NMDA-induced neurotoxicity. In addition, we wished to evaluate the role of σR1 in (+)-pentazocine-mediated effects. To accomplish these goals, we used cohorts of WT and σR1 KO mice. These animals were treated with IP injection of (+)-pentazocine (0.5 mg/kg per injection) for 1 week prior to and 1 week following intravitreal NMDA injection (Fig. 1A). The NMDA-injected, (+)-pentazocine-treated mice were compared with mice that received NMDA injection without (+)-pentazocine treatment (Fig. 1A). Survival of RGCs was systematically assessed using a computer-based, semiautomated counting method to quantify Brn3a-labeled RGCs in retinal flatmounts (Fig. 1B). Previous reports indicate that the Brn3a transcription factor is a reliable and efficient marker for identification and quantification of RGCs in control and optic nerve–injured retinas.[23,24] Validation of the computer-based method is shown in Figure 1C by the strong correlation of semiautomated counts with blinded manual counts (Pearson correlation coefficient $r = 0.989$, $P < 0.0001$).

We used the computer-assisted, semiautomated method to compare baseline Retinal ganglion cell densities in retinas derived from PBS-injected WT and σR1 KO animals. Although σR1 KO mice lacked the σR1 protein (Fig. 1D), the ganglion cell densities in σR1 KO mice were not significantly different from WT (Fig. 1E) after PBS injection. Retinal ganglion cell densities in WT (3374 RGC/mm^2) and in σR1 KO mice (3160 RGC/mm^2) are similar to Brn3a-labeled RGC densities reported by others.[24]

(+)-Pentazocine Promotes Survival of RGCs

In WT mice, we considered the ability of (+)-pentazocine to protect RGCs at three different levels of NMDA-induced neurotoxicity. We assessed RGC number from flat-mounted retinas 1 week following intravitreal NMDA injection at dosages of 7.5 mM, 10 mM, and 20 mM NMDA (Fig. 2). We found that dosages of 7.5 mM (Figs. 2A, 2B), 10 mM (Figs. 2C, 2D), and 20 mM NMDA (Figs. 2E, 2F) resulted in dose-dependent decreases in RGC number compared with control injection of PBS. Quantification of RGC numbers showed that treatment with (+)-pentazocine mitigated the decreases in RGC number at all NMDA dosages tested (Figs. 2B, 2D, 2F). The most significant improvement in RGC survival with (+)-

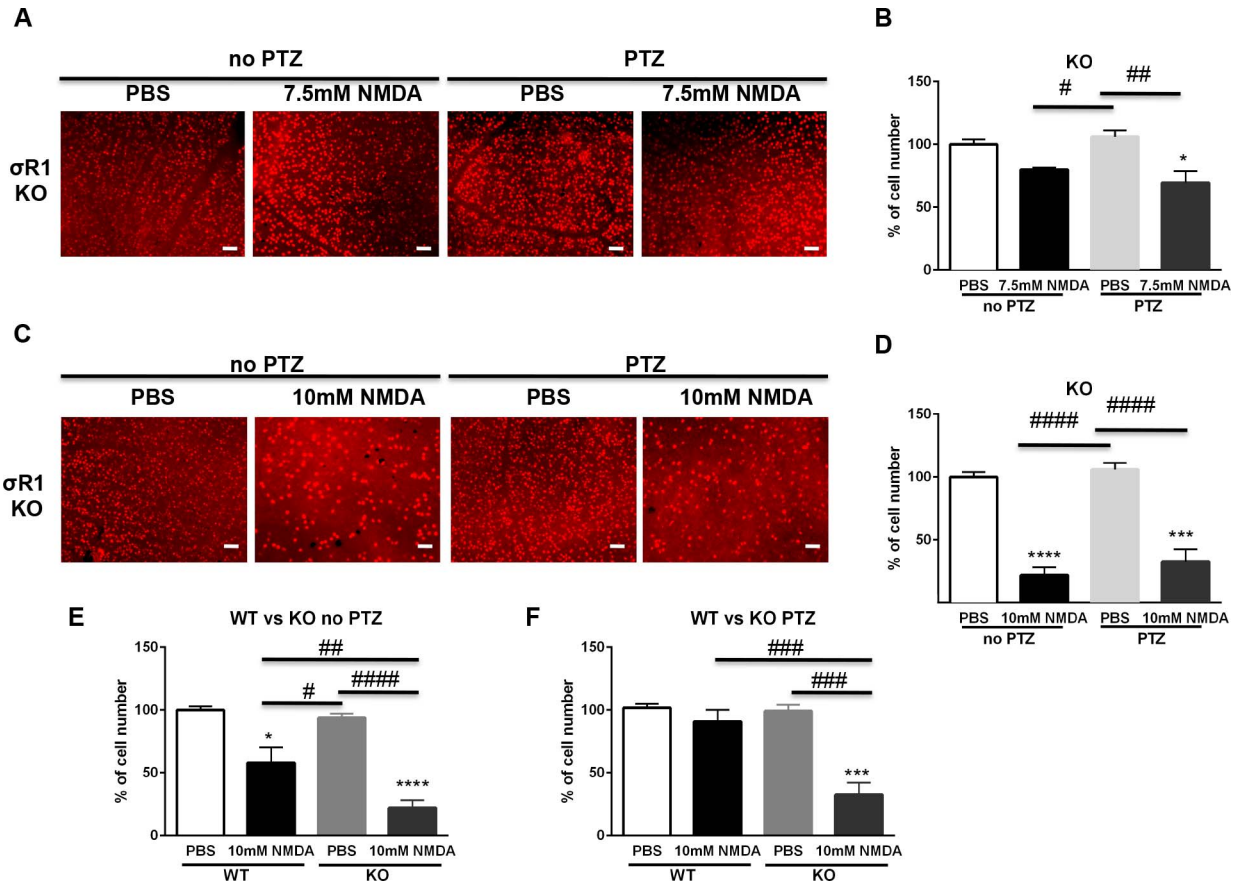

Figure 4. Effects of intraperitoneally-administered (+)-pentazocine (PTZ) on NMDA-induced retinal damage in σR1 KO mice. Pentazocine (0.5 mg/kg) was administered intraperitoneally three times per week for 1 week prior to and 1 week post NMDA injection. (**A**) Representative micrographs of retina flat mounts from σR1 KO mice given 1-μL intravitreal injection of PBS (vehicle) or 7.5 mM NMDA. (**B**) Quantitative analysis of cell number in each field of retina flat mount in σR1 KO mice at 7 days after NMDA injection normalized to no PTZ, PBS injected controls. (**C**) Representative micrographs of retina flat mounts from σR1 KO mice given 1-μL intravitreal injection of PBS (vehicle) or 10 mM NMDA. (**D**) Quantitative analysis of cell number in each field of retina flat mount in σR1 KO mice at 7 days after NMDA injection normalized to no PTZ, PBS injected controls. (**E**) Direct comparison of NMDA-induced retinal damage in WT and σR1 KO mice. Quantitative analysis of cell number in each field of retina flat mount in WT or σR1 KO mice at 7 days after 10 mM NMDA injection normalized to PBS injected WT mice without PTZ treatment. (**F**) Direct comparison of the effect of PTZ treatment on NMDA-induced retinal damage in WT or σR1 KO mice. Quantitative analysis of cell number in each field of retina flat mount in PTZ treated WT or σR1 KO mice at 7 days after 10 mM NMDA injection normalized to PTZ-treated, PBS-injected WT mice. *Scale bars*: 50 μm. Significantly different from control $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Significantly different between groups $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$. $n = 5$–13 in each treatment group.

pentazocine treatment was seen at the intermediate (10 mM) dosage of NMDA. At this dosage, treatment with (+)-pentazocine resulted in a significant 2-fold increase in RGC number compared with no treatment (Figs. 2C, 2D). These results suggest that (+)-pentazocine treatment protects RGCs against NMDA-induced toxicity and that this protection is most significant when the NMDA-mediated injury causes approximately 50% RGC loss.

To further evaluate whether (+)-pentazocine decreases the NMDA-mediated death of cells within the retina, we used the TUNEL assay on retinal cryosections. We evaluated cell death within retinas of WT mice 24 hours following intravitreal injection of 10 mM NMDA, with and without (+)-pentazocine treatment. Previous studies have shown that intravitreal injections of NMDA cause death of neurons within the retina.[25,26] Consistent with these reports, we found a significant increase in TUNEL-positive cells in both the GCL and INL of NMDA-injected retinas compared with PBS-injected controls (Figs. 3A–C). In contrast, retinas in the (+)-pentazocine-treatment group showed a significant 2-fold decrease in TUNEL-positive cells compared to the no (+)-pentazocine-treatment group in both the GCL and INL (Figs. 3A–C). These results suggest that under conditions of NMDA-mediated toxicity, treatment with (+)-pentazocine promotes cellular survival not just for RGCs but also for other cell types within the retina.

Deletion of σR1 Expression Enhances RGC Loss and Prevents (+)-Pentazocine–Induced Protection From NMDA-Mediated RGC Toxicity

Next, we explored how deletion of σR1 impacts RGC survival in the face of NMDA-induced retinal toxicity and treatment with (+)-pentazocine. A group of σR1 KO mice were treated with IP injection of (+)-pentazocine followed by NMDA (7.5 mM or 10 mM) intravitreal injection using the same protocol as WT animals (Fig. 1A). Similar to experiments using WT mice, a comparison of RGC number was made between (+)-pentazocine-treated and untreated σR1 KO mice 7 days postintravitreal injection of 7.5 mM and 10 mM NMDA (Figs. 4A–D). The σR1

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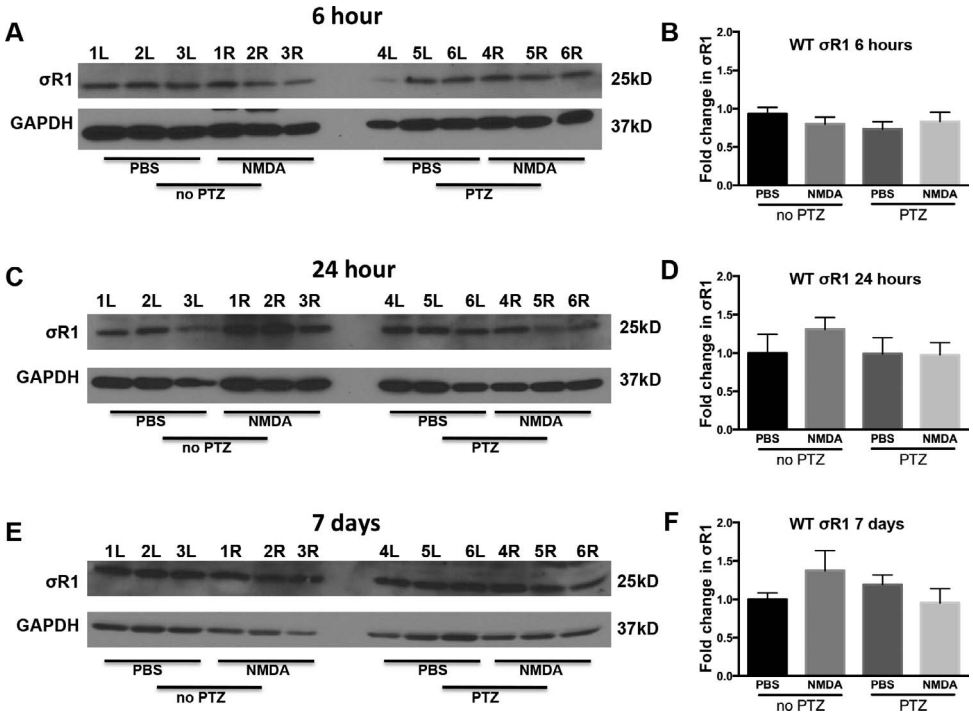

Figure 5. Sigma-1 receptor expression in NMDA intravitreal injected retina with or without (+)-pentazocine administration. Pentazocine (0.5 mg/kg) was administered intraperitoneally three times per week for 1 week prior to and 1 week post NMDA injection. For representative Western blots, lanes 1L, 2L, and 3L were retinas from three individual animals in which left eyes were injected with PBS. Lanes 1R, 2R, and 3R were retinas from three individual animals in which right eyes were injected with 10 mM NMDA. Lanes 4L, 5L, and 6L were retinas from three individual animals treated with PTZ in which left eyes were injected with PBS. Lanes 4R, 5R, and 6R were retinas from three individual animals treated with PTZ in which right eyes were injected with 10 mM NMDA. Western blots showed σR1 expression in (**A**) WT retinas 6 hours post NMDA injection, (**C**) WT retinas 24 hours post NMDA injection, and (**E**) WT retinas 7 days post NMDA injection. Quantitative analysis of σR1 expression was normalized to GAPDH as the internal control. Results are presented as fold change compared to σR1 expression in no PTZ, PBS control retinas at (**B**) 6 hours post NMDA injection ($n = 6$), (**D**) 24 hours post NMDA injection ($n = 3$), and (**F**) 7 days post NMDA injection ($n = 3$). The σR1 expression in retinal lysates was not significantly altered by NMDA intravitreal injection or PTZ treatment at any of the times tested.

KO mice showed an NMDA dose-dependent decrease in RGC number compared with control injection of PBS. In addition, the NMDA induced RGC death was greater in σR1 KO mice than their WT counterparts. At 7 days post 10 mM NMDA intravitreal injection, the σR1 KO mice showed 80% decrease in RGC number (Figs. 4C, 4D) compared with 50% decrease in WT animals (Figs. 2C, 2D). A direct comparison of RGC number between WT and σR1 KO retinas for untreated and pentazocine-treated animals is shown in Figures 4E and 4F. Through this analysis, we found that RGC loss was significantly greater in NMDA-injected KO animals compared with their WT counterparts for both untreated and pentazocine-treated cohorts (Figs. 4E, 4F). These results suggest that absence of σR1 exacerbates RGC loss following NMDA injection.

Our studies of σR1 KO mice led to a second interesting observation. In contrast to results for WT mice, retinas derived from mice that lacked σR1 but received (+)-pentazocine treatment showed no mitigation of the NMDA-induced RGC loss at either 7.5 mM or 10 mM NMDA when compared with untreated mice (Fig. 4). These results indicate that σR1 is necessary for (+)-pentazocine–mediated RGC protection from NMDA-induced injury.

(+)-Pentazocine Treatment Does Not Change Retinal σR1 Levels

To determine if NMDA injections or (+)-pentazocine treatments altered levels of σR1 within the retina, we performed Western blot analysis of retinal lysates derived from WT experimental animals. We measured σR1 levels in (+)-pentazocine–treated and nontreated mice at 6 hours (Figs. 5A, 5B), 24 hours (Figs. 5C, 5D), and 7 days (Figs. 5E, 5F) post NMDA injection. No significant changes in σR1 levels were observed in NMDA-injected retinas compared with PBS injected control retinas. In addition, we found no significant change in retinal σR1 levels for (+)-pentazocine–treated versus nontreated mice (Fig. 5).

σR1 Activation With (+)-Pentazocine Increases Expression of Phospho-ERK1/2 During NMDA-Induced Retinal Injury

Previous in vitro studies have indicated that ERK1/2 activation is increased 6 hours following (+)-pentazocine treatment of purified RGCs.[8] In addition, the (+)-pentazocine–induced, increased ERK1/2 phosphorylation protects cultured RGCs from ischemia-like insult via σR1.[8] Given these previous studies, we evaluated whether (+)-pentazocine treatment affected the level of retinal ERK1/2 phosphorylation in our NMDA-injected animals, in vivo. We performed Western blot analysis of retinal lysates derived from individual retinas 6 hours following intravitreal NMDA injection. Figure 6A shows representative Western blot analysis derived from three individual retinas per treatment group. When comparing phospho-ERK levels between each retina, we observed some variability, even among those animals that received the control PBS treatment. A degree of variability is expected given that

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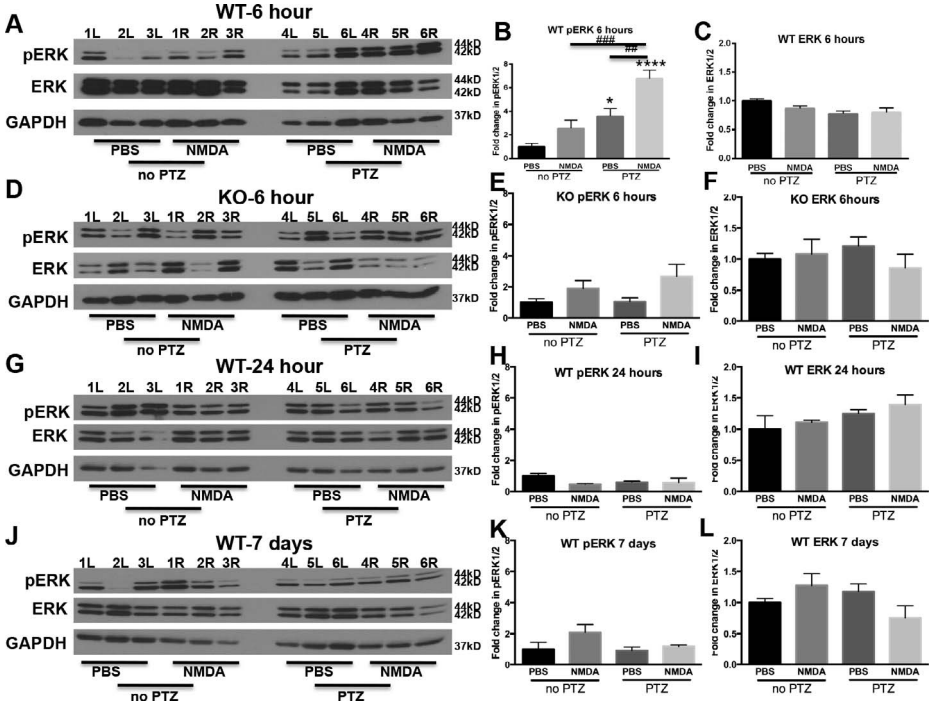

Figure 6. Phosphorylated-ERK (pERK) and ERK expression in NMDA intravitreal injected WT or σR1 KO mice retina with or without (+)-pentazocine (PTZ) treatment. Pentazocine (0.5 mg/kg) was administered intraperitoneally three times per week for 1 week prior to and 1 week post NMDA injection. For representative Western blots, lanes 1L, 2L, and 3L were retinas from three individual animals in which left eyes were injected with PBS. Lanes 1R, 2R, and 3R were retinas from three individual animals in which right eyes were injected with 10 mM NMDA. Lanes 4L, 5L, and 6L were retinas from three individual animals treated with PTZ in which left eyes were injected with PBS. Lanes 4R, 5R, and 6R were retinas from three individual animals treated with PTZ in which right eyes were injected with 10 mM NMDA. Western blots showed pERK and ERK expression in (**A**) WT mice retinas 6 hours post NMDA injection, (**D**) σR1 KO retinas 6 hours post NMDA injection, (**G**) WT retinas 24 hours post NMDA injection, and (**J**) WT retinas 7 days post NMDA injection. Quantitative analysis of pERK expression was normalized to GAPDH as the internal control. Results are presented as fold change compared to pERK expression in no PTZ, PBS control retinas in (**B**) WT mice retinas 6 hours post NMDA injection ($n = 6$), (**E**) σR1 KO retinas 6 hours post NMDA injection ($n = 3$), H) WT retinas 24 hours post NMDA injection ($n = 3$), and (**K**) WT retinas 7 days post NMDA injection ($n = 3$). Quantitative analysis of ERK expression was normalized to GAPDH. Results are presented as fold change compared to ERK expression in no PTZ, PBS control retinas in (**C**) WT mice retinas 6 hours post NMDA injection ($n = 6$), (**F**) σR1 KO retinas 6 hours post NMDA injection ($n = 6$), (**I**) WT retinas 24 hours post NMDA injection ($n = 3$), and (**L**) WT retinas 7 days post NMDA injection ($n = 3$). Significantly different from control $^{*}P < 0.05$, $^{****}P < 0.0001$. Significantly different between groups $\#\#P < 0.01$, $\#\#\#P < 0.001$. $n = 3$–6 in each treatment group.

phosphorylation is a highly labile posttranslational modification, and we are assessing phosphorylation levels using an in vivo model system.

After quantifying our results and accounting for interanimal variability, we compared ERK1/2 activation levels in animals that received (+)-pentazocine treatment for 1 week prior to the NMDA injection to those that did not receive (+)-pentazocine treatment. We observed a statistically significant difference (Figs. 6A–C). The animals that received (+)-pentazocine treatment showed significantly enhanced retinal ERK1/2 activation compared with no (+)-pentazocine treatment (Figs. 6A–C). The enhanced ERK1/2 activation was present only at the 6-hour time point following NMDA treatment and resolved by the 24-hour and 7-day time points following intravitreal NMDA (Figs. 6A–C, 6G–L).

To determine whether the (+)-pentazocine-mediated increase in ERK1/2 phosphorylation found at the 6-hour time point was dependent on σR1, we performed Western blot analysis of retinal lysates derived from $\sigma R1^{-/-}$ mice. These mice had received either (+)-pentazocine treatment or no treatment prior to NMDA injection, according to the same experimental conditions as used for WT animals. Retinas derived from (+)-pentazocine-treated mice lacking σR1 showed no significant change in the level of ERK1/2 phosphorylation compared with the no (+)-pentazocine treatment group (Figs. 6D–F).

Discussion

The first important finding of this in vivo study is that (+)-pentazocine, administered by IP injection, protects RGCs from NMDA-induced cell death, via a mechanism involving σR1.

Our results agree with previous studies using σR1 agonists. Smith et al.[1] found that IP injection of (+)-pentazocine into diabetic mice resulted in attenuated cell loss in the GCL of retinal sections. In addition, decreased retinal damage was observed in a model of ischemia-reperfusion injury in rats using the σR1 agonists, PRE-084 and N-methyladamantan-1-amine derivative [(−)-MR22].[27,28]

Studies that address the effect of σR1 agonists on the NMDA receptor are controversial.[15,16,29] Some previous studies support a direct interaction between (+)-pentazocine and the NMDA receptor, independent of σR1.[17] Our study is the first to show, in vivo, an absence of (+)-pentazocine–mediated RGC protection in σR1 KO mice. Therefore, it appears the neuroprotective properties of (+)-pentazocine are mediated through σR1 and are not the consequence of a direct effect of (+)-pentazocine on the NMDA receptor. This fills an important gap in our understanding of the mechanism of action for (+)-pentazocine–induced neuroprotection, at least relevant to excitotoxic conditions.

Our second important finding is that RGCs show increased susceptibility to NMDA-mediated toxicity in the absence of σR1. Mavlytov et al.[30] described accelerated RGC death in σR1

KO mice compared with WT mice under conditions of optic nerve crush. Together, these results indicate an endogenous role for σR1 in slowing down injury-induced or excitotoxicity-induced RGC degeneration.

Stimulation of σR1 likely protects neurons through a pleiotropic set of mechanisms. Proposed mechanisms for σR1-mediated neuroprotection include attenuation of calcium influx through NMDA or L-type Voltage Gated Calcium Channels.[9,12,14,31,32] In addition, other studies suggest that the neuroprotective effects of σR1 stimulation are due to modulation of ER or oxidative stress.[20,33] Our studies show that treatment with (+)-pentazocine increases ERK1/2 MAPK phosphorylation at the 6-hour time point following NMDA exposure.

Studies indicate that phospho-ERK1/2 expression increases in the retina during glaucomatous or ischemic insult, and increased ERK1/2 activation may be an endogenous retinal protective response.[34,35] Our finding of enhanced ERK1/2 activation in the NMDA-exposed retinas of (+)-pentazocin–treated animals is very exciting considering recent analyses. Mueller et al.[8] reported a σR1-dependent increase in ERK1/2 phosphorylation in (+)-pentazocine–treated primary RGC cultures following 6 hours of oxygen-glucose deprivation insult. In addition, a recent study by Moriguchi et al.[36] demonstrated that σR1 stimulation ameliorated cognitive impairment effects in olfactory bulbectomized mice through the activation of ERK. In our system, the pentazocine-mediated enhancement of ERK phosphorylation is a transient event, and it resolved at the 24-hour and 7-day time points. However, increased ERK activation may enact changes in gene expression that have long-lasting, prosurvival effects. Taken together, our studies and those of others offer support for involvement of the ERK signaling cascade within the mechanism of σR1-mediated neuroprotection. Our work provides the first in vivo report of increased ERK1/2 phosphorylation in the presence of the σR1 agonist, (+)-pentazocine.

The σR1 is expressed within neuronal and glial cell types throughout the central and peripheral nervous systems. The exact relationship between σR1 and the ERK signaling cascade may depend on cell type-specific factors. In fact, previous studies from our laboratory indicate that treatment of retina-derived microglia cultures with (+)-pentazocine results in a σR1-dependent decrease in ERK1/2 phosphorylation under conditions of exposure to inflammatory stimuli.[11] These results would seemingly conflict with the current findings of (+)-pentazocine–mediated increased ERK1/2 phosphorylation levels in NMDA-exposed retina. Other studies that assay ERK1/2 activation levels in σR1 agonist–treated hippocampal tissues, cortex-derived neuronal cultures, or mixed hippocampal and cortex-derived cultures also describe differing results, possibly dependent on the cell type analyzed.[36–38] Therefore, stimulation of σR1 with (+)-pentazocine might promote neuroprotection by simultaneously enhancing ERK1/2 phosphorylation in RGCs while decreasing ERK1/2 activation in glial cells such as microglia. These cell-type specific effects of (+)-pentazocine on ERK1/2 phosphorylation might enhance the endogenous neuroprotective response of RGCs while decreasing the neurotoxic effects of retinal microglia. However, future studies that address the relationship between σR1 and the ERK signaling cascade are needed.

In conclusion, our findings show that activation of σR1 is neuroprotective of RGCs in vivo, under conditions of excitotoxic stress. The additional finding that σR1 plays an endogenous role in protecting RGCs from injury further exemplifies the importance of σR1. These results support the growing body of evidence suggesting that σR1 ligands are strong candidates for treatment of retinal disorders that affect RGCs, including glaucoma, ischemia-related conditions such as vessel occlusion, and diabetic retinopathy.

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