A novel calpain inhibitor, SNJ-1945, reduces murine retinal cell death \textit{in vitro} and \textit{in vivo}

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Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OGD, oxygen-glucose deprivation; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC-5, retinal ganglion cell line; SNJ-1945, ((1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 5-methoxy-3-oxapentyl ester; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling;

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Abstract

We examined whether SNJ-1945, a new orally available calpain inhibitor, might reduce retinal cell death in vivo and/or in vitro. Retinal cell damage was induced in vivo in mice by intravitreal injection of N-methyl-D-aspartate (NMDA), and SNJ-1945 was intraperitoneally or orally administered twice. NMDA-induced calpain activity (measured as the cleaved products of α-spectrin) and its substrate, p35 (a neuron-specific activator for cyclin-dependent kinase 5), in the retina were examined by immunoblotting. In RGC-5 (a rat retinal ganglion cell-line) cell culture, cell damage was induced by a 4-h oxygen-glucose deprivation (OGD) treatment followed by 18-h reoxygenation. In mouse retinas, SNJ-1945 (30 or 100 mg/kg, i.p., 100 and 200 mg/kg, p.o.) significantly inhibited the cell loss in the ganglion cell layer (GCL) and the thinning of the inner plexiform layer induced by NMDA. Furthermore, the number of TUNEL-positive cells was significantly reduced in the GCL and inner nuclear layer of retinas treated with SNJ-1945 as compared vehicle-treated retinas 24 h after NMDA injection. Levels of cleaved α-spectrin products increased and p35 decreased, 6 h after NMDA injection or later, and their effects were attenuated by SNJ-1945. In vitro, SNJ-1945 (10 and 100 µM) inhibited the OGD stress-induced reduction in cell viability. In conclusion, SNJ-1945 may afford valuable neuroprotection against retinal diseases, since it was effective against retinal damage both in vitro and in vivo. Our results also indicate that calpain activation and subsequent p35 degradation may be involved in the mechanisms
underlying retinal cell death.
Introduction

Retinal ganglion cell (RGC) death is a common feature of many ophthalmic disorders, including glaucoma, optic neuropathies, and various retinovascular diseases (diabetic retinopathy and retinal vein occlusions). Retinal ganglion cells are extremely sensitive to the effects of both glutamate and its analog N-methyl-D-aspartate (NMDA), both of which produce a dose-dependent cell loss in vivo and in vitro. As well, glutamate toxicity has been implicated in the pathophysiology of glaucoma (Dreyer, 1998). Activation of Ca$^{2+}$-permeable NMDA receptors, and the subsequent neuronal Ca$^{2+}$-overloading, have been shown to mediate glutamate-induced neuron death (Choi, 1994). Induction of the Ca$^{2+}$-activated neutral cysteine protease, μ-calpain, by micromolar concentrations of Ca$^{2+}$ occurs early in excitotoxic neuron death (Siman et al., 1989; Faddis et al., 1997). Like the caspases, μ-calpain cleaves a variety of cytoskeletal proteins, enzymes, and transcription factors, and could also interfere with the proteolytic activity of the caspases (Croall and DeMartino, 1991).

The calpain family is represented by 15 genes in mammals, and is comprised of two major isozymes, (namely, μ- and m-calpain) which have been well-characterized (Saido et al., 1994; Evans and Turner, 2007). These isozymes are expressed ubiquitously, and they differ primarily in their Ca$^{2+}$ requirement for half-maximum activation [3 – 50 µM for μ-calpain (calpain-1) and 400 – 800 µM for m-calpain (calpain-2)] (Goll et al., 2003).
Calpain-mediated proteolysis has been observed in several pathological conditions, including Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease (Saito et al., 1993; Grynspan et al., 1997; Kim et al., 2001; Dufty et al., 2007). Calpain is also apparently activated during the apoptosis of both PC12 cells (Ray et al., 2000) and cerebellar granule cells (Nath et al., 1996a), as well as during the neuronal apoptosis induced by rat spinal cord injury (Ray et al., 1999). Chiu et al. (2005) reported that, in rats, intravitreal injection of NMDA increases µ-calpain immunoreactivity in RGC layer cells and in the inner nuclear layer, and that simultaneous injection of a calpain inhibitor reduces both cell death and the number of TdT-mediated biotin-dUTP nick end labeling (TUNEL)-positive cells observed following a NMDA injection. Calpains share endogenous substrates (such as α-spectrin) with caspases, suggesting that they may play similar, or overlapping, roles in cell death, but the requirement for calpains in different forms of neuronal cell death is not well understood (Nath et al., 1996b). The functional contributions made by calpains to neuronal cell death still require clarification.

A novel calpain inhibitor, SNJ-1945, has been shown to have good aqueous solubility, good plasma exposure, and good CNS penetration in both rats and monkeys (Oka et al., 2006b; Shirasaki et al., 2006). Furthermore, administration of SNJ-1945 at 100 mg/kg, i.p. for 14 days produced no obvious toxicity signs or abnormalities in rats (Oka et al., 2006b). To date, there have been no reports on the efficacy of this inhibitor against retinal ganglion...
cell damage. The present study was therefore designed to examine whether SNJ-1945 might inhibit (a) the \textit{in vivo} retinal neuronal death induced by NMDA in mice and/or (b) the \textit{in vitro} RGC-5 cell damage induced by ischemia-reoxygenation.
Methods

Materials. Dulbecco’s modified Eagles’s medium (D-MEM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). SNJ-1945, ((1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 5-methoxy-3-oxapentyl ester, was a gift from Senju Pharmaceutical Co. Ltd. (Kobe, Japan). Isoflurane was acquired from Nissan Kagaku (Tokyo, Japan), and fetal bovine serum (FBS) was obtained from Valeant (Costa Mesa, CA, USA).

In vivo NMDA-induced Retinal Damage. All experiments were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male adult ddY mice (closed colony albino mice) weighing 35–45g (Japan SLC, Hamamatsu, Japan) were used for these experiments, and were kept under controlled lighting conditions (12 h light : 12 h dark). Anesthesia was induced using 3.0% isoflurane and maintained using 1.5% isoflurane in 70% N₂O and 30% O₂, delivered via an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). The body temperature was maintained between 37.0 and 37.5°C with the aid of a heating pad and heating lamp. Retinal damage was induced by injection (2 µl/eye) of
NMDA (Sigma-Aldrich) at 2.5 mM dissolved in 0.01 M phosphate-buffered saline (PBS).

This was injected into the vitreous body of the left eye under the anesthesia described above.

One drop of levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) as an antibacterial solution was applied topically to the treated eye after the intravitreal injection. SNJ-1945 (10, 30, or 100 mg/kg for i.p.), (100, or 200 mg/kg for p.o. by gavage), or an identical volume (10 ml/kg) of vehicle (0.5% sodium carboxymethyl cellulose) was administered at 30 min before and at 6 h after the NMDA injection.

**Histological Analysis of Mouse Retina.** Seven days after the NMDA injection, eyeballs were enucleated for histological analysis. The mice were euthanized with overdose of sodium pentobarbital (80 mg/kg, i.p. or higher). Each eye was then enucleated and kept immersed for at least 24 h at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 3 μm), cut through the optic disc of each eye, were prepared using standard histological procedures, then stained with hematoxylin and eosin. Retinal damage was evaluated as described previously (Shimazawa et al., 2007), with three sections from each eye being used for the morphometric analysis. Light-microscope images were photographed, and the cell counts in the ganglion cell layer (GCL) at a distance between 350 and 650 μm from the optic disc were measured on the photographs in a masked fashion, by a single observer (S.S.). Data from three sections
(selected randomly from the six sections) were averaged for each eye, and the values
obtained were used to evaluate the cell count in the GCL.

**TUNEL Staining.** TUNEL staining was performed according to the manufacturer’s
protocols (In Situ Cell Death Detection Kit; Roche Biochemicals, Mannheim, Germany) to
determine the extent of retinal cell death induced by NMDA. The mice were euthanized
with deep anesthesia 24 h after intravitreous injection of NMDA at 5 nmol/eye. The eyes
were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 48 h in 25%
sucrose with PBS. The eyes were then embedded in a supporting medium for frozen-tissue
specimens (OCT compound; Tissue-Tek, IL, USA). Retinal sections were cut at 10-µm
thick on a cryostat at -25°C, and stored at -80°C until staining. After washing twice with
PBS, sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme
at 37°C for 1 h. The sections were washed 3 times in PBS for 1 min at room temperature.
After washing twice with PBS, fluorescence images were photographed, and the labeled cell
counts in the ganglion cell layer (GCL) and inner nuclear layer (INL), at a distance between
375 and 625 µm from the optic disc, were obtained for two areas of the retina. The number
of TUNEL-positive cells was averaged for these two areas, and the value plotted as the
number of TUNEL-positive cells in GCL and INL, respectively.
Immunoblotting. Retinas were lysed using a cell-lysis buffer [RIPA buffer (R0278; Sigma-Aldrich) with protease (P8340; Sigma-Aldrich) and phosphatase inhibitor cocktails (P2850 and P5726; Sigma-Aldrich), and 1 mM EDTA]. Lysates were solubilized in SDS-sample buffer, separated on 4-20% SDS-polyacrylamide gradient gels, and transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). Transfers were blocked for 1 h at room temperature with 5% Blocking One-P (Nakarai Tesque, Inc., Kyoto, Japan) in 10 mM Tris-buffered saline with 0.05% Tween 20 (TBS-T), then incubated overnight at 4°C with the primary antibody. The transfers were then rinsed with TBS-T and incubated for 1 h at room temperature in horseradish peroxidase goat anti-rabbit or goat anti-mouse (Pierce, Rockford, IL, USA) diluted 1:2000. The immunoblots were developed using chemiluminescence (Super Signal® West Femto Maximum Sensitivity Substrate; Pierce), and visualized with the aid of a digital imaging system (FAS-1000; Toyobo Co. LTD, Osaka, Japan). The primary antibodies used were as follows: mouse anti-α-spectrin (clone AA6; Millipore, Billerica, MA, USA), rabbit anti-p35 (clone C64B10, Cell Signaling, MA, USA), and mouse anti-β-actin (Sigma-Aldrich).

Immunostainings. At various times after intravitreal injection of NMDA, eyes were processed as in the TUNEL staining, and finally prepared 10-µm-thick cryostat sections. Subsequently, the sections were processed for immunofluorescent staining using a rabbit
monoclonal antibody against p35 (1:200 dilution, Cell Signaling). After incubation, the primary antibody for 2 h at 37°C, the sections were incubated with Alexa Fluor-488-conjugated secondary antibody (1:8000 dilution; Molecular Probes, Eugene, OR) for 30 min at 37°C, mounted with a coverslip, and observed under an epifluorescence microscope (Power BX50; OLYMPUS, Tokyo, Japan).

**RGC-5 culture.** Cultures of RGC-5 were maintained in D-MEM (low glucose; 1 mg/ml) supplemented with 10% FBS, 100 U/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika Kaisha Ltd.) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The RGC-5 cells were passaged by trypsinization every 3 days, as in a previous report (Krishnamoorthy et al., 2001).

**Oxygen-glucose Deprivation (OGD)-induced Cell Death in RGC-5 Cell Cultures.**

RGC-5 cells were plated at a density of 1000 cells/well in 96-well culture plates (#3072; Becton Dickinson, Franklin Lakes, NJ, USA) with low-glucose D-MEM with 10% FBS. Twenty-four hours later, to induce OGD-stress, the cells were washed with D-MEM without either high glucose or FBS, and then placed in the same medium in a hypoxic-incubator (94% N2, 5% CO2, and 1% O2) for 4 h. At the end of the OGD period, glucose solution and FBS were added to give final concentrations of 4.5 mg/ml (high glucose) and 1%, respectively,
and the cultures were put back in the incubator for an additional 18 h at the regular atmospheric oxygen level (reoxygenation). In control group, RGC-5 was replaced to D-MEM with high glucose (4.5g/ml) and 1% FBS, and then placed in an aerobic-incubator (95% air, 5% CO\textsubscript{2}) for 22 h. SNJ-1945 (10 and 100 µM) or vehicle was added to the culture after the above replacement of the culture medium but immediately before the hypoxic treatment. Cell viability was measured at the end of the reoxygenation period. Cell viability was assessed following immersion in 10% WST-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] with an electron carrier, 1-Methoxy PMS (1-Methoxy-5-methylphenazinium, methylsulfate) (Cell Counting Kit-8, Dojin Kagaku, Kumamoto, Japan) for 3 h at 37°C, and absorbance was recorded at 450 nm (Jiang et al., 2004). WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in cell culture medium. The amount of formazan produced is directly proportional to the number of living cells. This absorbance is expressed as a percentage of that in control cells, after subtraction of background absorbance.

**Statistical Analysis.** Data are presented as the means ± S.E.M. Statistical comparisons were made, using a two-tailed Student’s \( t \)-test or Dunnett’s test, by means of STAT VIEW version 5.0 (SAS Institute Inc., Cary, NC). \( P < 0.05 \) was considered to indicate statistical
significance.
Results

Effect of SNJ-1945 on Calpain Activation in Mouse Retina after NMDA Injection

To examine calpain activation in the retina after intravitreal injection of NMDA, the cleaved products (145kDa/150kDa) of α-spectrin, which is proteolyzed by activated calpain, were measured using an immunoblotting procedure (Fig. 1A and 1B). As shown in Fig. 1A, intact α-spectrin (represented at 280 kDa) was observed in the vehicle-treated normal retina, while NMDA treatment resulted in the appearance of proteolysis products of α-spectrin. Quantitative analysis of band density showed significant increases of the cleaved product (at 150kDa) of α-spectrin (proteolyzed by caspase-3 and calpain) and a new cleaved product (at 145 kDa) of α-spectrin (proteolyzed by calpain) at 6 and 12 h after NMDA injection. The levels of these products were reduced to control levels by 24h (Fig. 1B). SNJ-1945 inhibited the increases of both of these two cleaved products (145kDa/150kDa) at 6 h after NMDA injection (Fig. 1C), with its effect being significant at both dosages of 30 and 100 mg/kg, i.p (Fig. 1D).

Effects of SNJ-1945 administered intraperitoneally against NMDA-induced Retinal Damage in Mice

Intravitreal injection of NMDA at 5 nmol/eye decreased both the cell count in the GCL and the thickness of IPL in the retina (versus the non-treated normal retina) (Fig. 2A, 2B, 2F,
2G or Fig. 3A, 3B, 3E, 3F). In the normal retina, the cell number in the GCL and the thickness of IPL were 107.0 ± 3.8 cells/mm and 22.7 ± 1.3 µm, respectively (n = 6).

Intravitreal injection of NMDA significantly reduced the cell number in the GCL (to 38.4 ± 2.2 cells/mm, n = 8) and thickness of IPL (to 11.5 ± 0.7 µm, n = 8). Treatment with SNJ-1945 at 30, and 100 mg/kg, i.p. significantly suppressed the decreases in GCL cell-count and IPL thickness induced by NMDA (Fig. 2).

Effects of SNJ-1945 administered orally against NMDA-induced Retinal Damage in Mice

Effects of SNJ-1945, when it was orally administered, on NMDA-induced retinal cell death were evaluated. Treatment with SNJ-1945 at 100 and 200 mg/kg, p.o. suppressed the decreases in GCL cell-count and IPL thickness induced by NMDA, with significant effects seen at 100 (GCL cell count only) and 200 mg/kg (GCL count and IPL thickness) dosages (Fig. 3).

NMDA-induced Expression of TUNEL-positive Cells

TUNEL-positive cells were observed in both GCL and the inner part of the inner nuclear layer (INL) at 24 h after NMDA injection (Fig. 4B). In contrast, TUNEL-positive cells were almost absent from the untreated retinas (Fig. 4A). SNJ-1945 (30 mg/kg, i.p. and 200
mg/kg, p.o.) significantly reduced the number of TUNEL-positive cells in GCL and INL (versus the vehicle-treated retina) (Fig. 4B to 4E). In the present study, SNJ-1945 showed protective effects by both i.p. and p.o. administrations on NMDA-induced retinal cell death. Therefore, the following studies used intraperitoneal administration of SNJ-1945.

**Effect of SNJ-1945 on p35 Degradation in Mouse Retina after NMDA Injection**

Proteolytic cleavage of p35 by calpain produces p25 upon neurotoxic insult, resulting in prolonged activation and mislocalization of Cdk5 with p25. Accumulation of p25 is found in neurodegenerative diseases such as Alzheimer’s disease (Patrick et al., 1999). Therefore, we focused on the p35 changes after NMDA injection. Representative p35-immunofluorescence images in mouse retinal-cross sections, at 6 and 24 h after NMDA injection, are shown in Fig. 5A to 5C. In non-treated normal retinas, p35 protein was ubiquitously expressed (Fig. 5A), and it was notably highly expressed along the some endfeets of Müllar cells, and outer plexiform layer (OPL). Furthermore, it was localized at cell periphery in cells of GCL and inner nuclear layer (INL), but not in outer nuclear layer (ONL). As shown in Fig. 5B, p35 fluorescence intensities in the GCL and the INL were reduced 6 h after NMDA injection, but no apparent changes were seen in the endfeets of Müllar cells and ONL. By 24 h after injection, the localization in the GCL was apparently changed as some of the cells in the GCL showed a condensed distribution (Fig. 5C).
shown in Fig. 5D, p35 protein was detected as a 35 kDa single band immunoblots from of the
vehicle-treated normal retinas. Intravitreal injection of NMDA significantly decreased p35
at 6 h, and this decrease persisted to the 24 h sampling time (Fig. 5E). SNJ-1945 at 30
mg/kg, i.p. significantly inhibited the decrease in the p35 at 6 h after NMDA injection (Fig.
5F, G).

Effect of SNJ-1945 on RGC-5 Cell Damage Induced in vitro by Oxygen-glucose Deprivation

Calcium overload shares a common pathway for retinal cell death through
NMDA-receptor activation and ischemia, and its increase in intracellular space can activate
calpain. To examine the direct effect of SNJ-1945 against retinal cell death without
affecting the pharmacokinetic profile of the test drug or blood flow, we examined its effect on
the OGD-induced RGC-5 death, a mimic of in vitro ischemia model. Representative
fluorescence stainings of nuclei made using Hoechst 33342 dye are shown in Figures 6A to
6C. Non-treated control cells displayed normal nuclear morphology. (Fig. 6A)
OGD-stress led to condensation and fragmentation of nuclei (Fig. 6B), while treatment with
SNJ-1945 at 10 µM reduced the extent of these morphological changes (Fig. 6C). In the
cell-viability assay (performed using WST-8), OGD-stress reduced cell viability by over 50%
(Fig. 6D). SNJ-1945 at 10 and 100 µM significantly inhibited this decrease in cell viability.
Discussion

In the present study, we demonstrated that systemic administration of SNJ-1945, a calpain inhibitor, reduced NMDA-induced retinal neuronal death in mice in vivo and OGD-induced RGC-5 cell damage in vitro.

Activation of the NMDA receptor leads to an intracellular Ca\textsuperscript{2+} influx, and subsequently to an activation of Ca\textsuperscript{2+}-dependent enzymes (such as calpain) that may be detrimental to cell viability. Chiu et al. (2005) reported that \( \mu \)-calpain, but not m-calpain, was increased in retinal extracts obtained 6 – 12 h after intravitreal injection of NMDA, and that intensely immunopositive cells were co-localized with TUNEL-positive cells. Furthermore, retinal ischemia-reperfusion has been reported to increase the proteolysis of \( \alpha \)-spectrin, a biochemical indicator of calpain activation, in the rat retina preceding the induced retinal morphological damage (viz. cell loss in GCL and IPL thinning). Furthermore, a calpain inhibitor, SJA6017, has been shown to protect against the cell loss in GCL seen after ischemia-reperfusion (Sakamoto et al., 2000). In the present study, cleaved products of \( \alpha \)-spectrin (represented as 145kDa and 150kDa molecular weight proteins) were increased at 6 and 12 h after NMDA injection, and their increases were diminished at 24 h.

Administrations of the calpain inhibitor, SNJ-1945, either orally or intraperitoneally, not only inhibited this production of cleaved \( \alpha \)-spectrins, but caused an attenuation of the NMDA-induced retinal damage. These findings suggest that calpain activation may be
involved in the retinal damage observed after NMDA-receptor activation or retinal ischemic insults. NMDA has been reported to induce apoptotic cell death in GCL and inner part of INL (Lam et al., 1999). Approximately 60% displaced amacrine cells of total cells exist in GCL and the remainder is retinal ganglion cells (RGCs) of mice, and amacrine cells are mostly located at the inner part of INL (Jeon et al., 1998). In this study, NMDA also increased the number of TUNEL-positive cells in GCL and inner part of INL, and SNJ-1945 inhibited their increases in both regions. On the other hand, the cells in the GCL counted in HE staining include not only RGCs but also displaced amacrine cells. Although we did not determine the difference between RGCs and displaced amacrine cells in the protective effects of SNJ-1945, SNJ-1945 protected the presumed amacrine cell death in the inner part of INL. Accordingly, these findings suggest that SNJ-1945 can protect cell death against RGCs and amacrine cells. However, further studies will be needed to provide direct evidence in GCL.

RGC death is a common feature of many ophthalmic disorders (such as glaucoma, and central retinal artery or vein occlusion), and RGCs are susceptible to glutamate or ischemia. In previous studies on rat and primate retinal organotypic cultures, hypoxia (95% N₂/5% CO₂ without glucose) induced activation of calpains and proteolysis of their substrates, and these effects could be inhibited by a calpain-specific inhibitor (SJA6017), resulting in a prevention of cell death (Tamada et al., 2002; Nakajima et al., 2006). In the present study, RGC-5 cells clearly underwent cell death 24 h after oxygen-glucose deprivation (OGD; 94% N₂/1%
Treatment with SNJ-1945 clearly protected against this cell death. As shown here, OGD-stress induces RGC-5 cell death that is accompanied by nuclear condensation. This is the first study to demonstrate that a calpain inhibitor exerts a protective effect against OGD stress-induced RGC-5 cell death in vitro. We have recently observed that these effects could be abolished by chelation of extracellular Ca\(^{2+}\) (using EDTA-Na) or by use of an inhibitor of the Na\(^+/Ca\(^{2+}\) exchanger (Inokuchi et al., 2009).

Furthermore, exposing RGC-5 cells in culture to ionomycin (a calcium ionophore) or interferon-\(\gamma\) increases intracellular Ca\(^{2+}\) influx, and calpeptin (a calpain-specific inhibitor) attenuates both proteolytic activities and apoptosis (Das et al., 2006). These findings support the idea that calpain activation may be involved in the RGC cell death seen under hypoxic conditions.

Activated calpain hydrolyzes a wide range of protein substrates including those involved in cytoskeletal structure (\(\alpha\)-spectrin, \(\alpha\)-actinin, paxillin, etc.). Cytoskeletal degradation in turn induces disruption of cellular architecture (Evans and Turner, 2007). Furthermore, proteolysis by calpain can also degrade some apoptosis-related proteins such as caspase-3 (Blomgren et al., 2001), apoptosis-inducing factor (AIF) (Polster et al., 2005), and p35 (Lee et al., 2000). Consequently, calpain can also be involved in the regulation of programmed cell death. In our preliminary study, caspase-3 was hydrolyzed to a 30 kDa protein, a calpain-cleaved product, in mouse retinas at the early stages after intravitreal injection of
However, no AIF cleavage products were detected (Shimazawa et al., unpublished data). On the other hand, significant effects were seen for p35 in response to NMDA injection. The p35 protein is a neuron-specific activator of cyclin-dependent kinase 5 (Cdk5), and its association with Cdk5 is critical for kinase activation. Cdk5 is a proline-directed serine/threonine kinase that is implicated in the regulation of neuronal migration, neuronal survival, and synaptic functions. Recently, Cdk5 has been reported to possibly exert a key role in promoting neuronal survival by regulating (i) Akt activity through the neuregulin/PI3-kinase signaling pathway (Li et al., 2003) and (ii) Bcl-2 activation, with phosphorylation by Cdk5 as the antiapoptotic effect (Cheung et al., 2008). Proteolytic cleavage of p35 by calpain produces p25 upon neurotoxic insult, resulting in prolonged activation and mislocalization of Cdk5 with p25. Accumulation of p25 is found in neurodegenerative diseases such as Alzheimer’s disease (Patrick et al., 1999). Interestingly, subcellular localization of p35 is changed by proteolytic cleavage to p25: p35 is localized at the cell periphery bound for plasma membrane, and the cleaved p25 by calpain is enriched in nuclear and perinuclear regions of the cells (Patrick et al., 1999). In the present study, p35 expressed in cells of GCL and INL in normal retina was predominantly localized at the cell periphery, but p35 fluorescence was decreased at 6 h after NMDA treatment. Furthermore, 24 h after NMDA injection, p35 in some cells of the GCL showed smaller shape and condensed staining. Therefore, the alteration in subcellular localization with Cdk5 may
promote its functional changes. On the other hand, the level of p35 was persistently decreased in mouse retinas after NMDA injection, and SNJ-1945 prevented the degradation of p35. However, the cleaved p25 protein was not detected in retinal extracts obtained from 6 to 24 h after NMDA injection in this study. Previously, Oka et al. (2006a) reported that transient ocular hypertension for 120 min and reperfusion induced a persistent decrease in p35 during 7 days and a transient increase in p25 at an early stage (4 h) after the ischemic insult, and its increase returned to normal level by 12 h. Collectively, the immunofluorescence in retinal cross-section is considered to show p35, but not p25, at 6 and 24 h after NMDA injection. Furthermore, some condensed immunopositive cells in GCL at 24 h after NMDA are considered to result from morphological changes accompanied by cell death, but not nuclear translocation of p25. These findings suggest that p35/Cdk5 may be critical for the maintenance of retinal cell survival, and that p35 degradation induced by calpain activation may induce apoptotic cell death.

Overall, SNJ-1945 has good water-solubility (0.65 mg/ml in water) (Shirasaki et al., 2006), good penetration to blood and retina after oral administration (Shirasaki et al., 2005), and a good safety profile (Oka et al., 2006b). Moreover, SNJ-1945 exerts protective effects against retinal damage upon systemic administration (Oka et al., 2006b; Oka et al., 2007). This is the first study to demonstrate that a calpain inhibitor can, when given systemically, have the protective effects against NMDA-induced mouse retinal damage in vivo. Based on
these findings, SNJ-1945 may prove to be a useful candidate drug for neuroprotective interventions against retinal diseases. However, in the present study we administered SNJ-1945 before NMDA injection. Therefore, further studies are needed to clarify its therapeutic time windows after NMDA injection.

In conclusion, we found that the calpain inhibitor, SNJ-1945, protects against both NMDA-induced retinal damage and ischemia/reoxygenation-induced RGC-5 cell death. This suggests that calpain activation and subsequent p35 degradation may be involved in the mechanisms underlying the retinal cell death occurring in many ocular diseases.
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Footnotes

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Legends for Figures

Fig. 1. Effect of SNJ-1945 on calpain activation in mouse retina after intravitreal injection of NMDA.

(A) Representative immunoblots showing proteolysis of α-spectrin in mouse retinal extracts at 6, 12, and 24 h after NMDA injection. (B) Quantitative analysis of the band density of the cleaved products at 145 and 150 kDa of α-spectrin (280 kDa). Each column represents the mean ± S.E.M. (n = 6). *, p < 0.05; **, p < 0.01 versus control group. (C) Representative immunoblots showing proteolysis of α-spectrin in mouse retinal extracts at 6 h after NMDA injection with or without treatment with SNJ-1945. SNJ-1945 (at 30 and 100 mg/kg i.p.) or an identical volume (10 ml/kg) of vehicle (0.5% sodium carboxymethyl cellulose) was administered 30 min before intravitreal injection of NMDA (5 nmol/eye).

(D) Quantitative analysis of the band density at 145kDa/150kDa (proteolyzed from α-spectrin at 280 kDa). Each column represents the mean ± S.E.M. (n = 5-6). ###, p < 0.01 versus control. *, p < 0.05 versus NMDA plus vehicle-treated group.

Fig. 2. Effects of SNJ-1945 administered intraperitoneally on NMDA-induced retinal damage in mice

Representative photographs showing non-treated normal retina (A), NMDA-injected retina treated with vehicle (B), and NMDA-injected retinas treated with SNJ-1945 at 10 mg/kg (C),
at 30 mg/kg (D), and at 100 mg/kg (E). SNJ-1945 (at 10, 30, and 100 mg/kg i.p.) or an identical volume (10 ml/kg) of vehicle (0.5% sodium carboxymethyl cellulose) was administered 30 min before and at 6 h after intravitreal injection of NMDA (5 nmol/eye). Scale bar represents 25 μm. Quantitative analysis of cell number in GCL (F) and thickness of IPL (G) at 7 days after NMDA injection. Each column represents the mean ± S.E.M. (n = 4-8). ##, p < 0.01 versus control. *, p < 0.05; **, p<0.01 versus NMDA plus vehicle-treated group.

**Fig. 3.** Effects of SNJ-1945 administered orally on NMDA-induced retinal damage in mice. Representative photographs showing non-treated normal retina (A), NMDA-injected retina treated with vehicle (B), and NMDA-injected retinas treated with SNJ-1945 at 100 mg/kg (C), and at 200 mg/kg (D). SNJ-1945 (at 100, and 200 mg/kg p.o.) or an identical volume (10 ml/kg) of vehicle (0.5% sodium carboxymethyl cellulose) was administered 30 min before and at 6 h after intravitreal injection of NMDA (5 nmol/eye). Scale bar represents 25 μm. Quantitative analysis of cell number in GCL (E) and thickness of IPL (F) at 7 days after NMDA injection. Each column represents the mean ± S.E.M. (n = 8 or 10). ##, p < 0.01 versus control. *, p < 0.05; **, p<0.01 versus NMDA plus vehicle-treated group.

**Fig. 4.** Effect of SNJ-1945 on expression of TUNEL-positive cells at 24 h after intravitreal
injection of NMDA.

Representative photographs of TUNEL staining showing non-treated normal retina (A), NMDA-injected retina treated with vehicle (B), and NMDA-injected retinas treated with SNJ-1945 at 30 mg/kg, i.p. (C), and at 200 mg/kg, p.o. (D). SNJ-1945 (at 30 mg/kg, i.p., and 200 mg/kg p.o.) or an identical volume (10 ml/kg, p.o.) of vehicle (0.5% sodium carboxymethyl cellulose) was administered 30 min before and at 6 h after intravitreal injection of NMDA (5 nmol/eye). Scale bar represents 25 μm. (E) Quantitative analysis of cell number of TUNEL-positive cells in GCL (open column) and INL (closed column) at 24 h after NMDA injection. Each column represents the mean ± S.E.M. (n = 7 or 8). ##, p < 0.01 versus control. *, p<0.05; **, p<0.01 versus NMDA plus vehicle-treated group.

**Fig. 5.** Effect of SNJ-1945 on p35 protein levels in mouse retina after intravitreal injection of NMDA.

Representative photographs showing p35-immunofluorescence images in no-treated normal retina (A), NMDA-treated retinas at 6 h (B), and at 24 h (C). Arrows indicate cells in GCL. ONL: outer nuclear layer, OPL: outer plexiform layer. (D) Representative immunoblots showing p35 protein levels in mouse retinal extracts at 6, 12, and 24 h after NMDA injection. (E) Quantitative analysis of the band density at 35 kDa. Each column represents the mean ± S.E.M. (n = 3). **, p < 0.01 versus control group. (F) Representative immunoblots
showing p35 protein levels in mouse retinal extracts at 6 h after NMDA injection in animals treated or not treated with SNJ-1945. SNJ-1945 at 30 mg/kg i.p. or an identical volume (10 ml/kg) of vehicle (0.5% sodium carboxymethyl cellulose) was administered 30 min before intravitreal injection of NMDA (5 nmol/eye). (G) Quantitative analysis of the band density at 35 kDa. p35 reduction had the variability with a range of 50 to 80% reduction between each experiment (E, G). We used a semi-quantitative analysis by Western blotting for comparing p35 level among samples, and therefore, the variability may be due to the differences in transfer efficiency to blotting membranes and/or in something of an animal condition between each experiment. Each column represents the mean ± S.E.M. (n = 4 or 5). ##, p < 0.01 versus control. *, p < 0.05 versus NMDA plus vehicle-treated group.

Fig. 6. Effect of SNJ-1945 on RGC-5 cell damage induced by OGD stress in vitro

Representative fluorescence microscopy of Hoechst 33342 staining of RGC-5 after 4-h OGD-stress followed by 18-h reoxygenation. (A) Non-treated cells showed normal nuclear morphology. (B) OGD-stress induced neurotoxicity, with cells showing nuclear condensation (arrows). (C) Treatment with SNJ-1945 at 10 μM reduced the nuclear condensation induced by OGD-stress (arrow). Arrows indicate nuclear-condensated cells. Bar = 50 μm. (D) Cell damage was induced by 4-h OGD exposure followed by 18-h reoxygenation, and cell viability was measured by means of the tetrazolium salt (WST-8)
reduction test. SNJ-1945 (at 10 and 100 µM) or vehicle was applied simultaneously with OGD-stress exposure. Each column represents the mean ± S.E.M. (n = 8). ##, p < 0.01 versus control. **, p < 0.05 versus OGD-stress alone.
Fig. 1

A

NMDA

C 6 12 24 h

α-spectrin

β-actin

280 kDa

150 kDa

145 kDa

B

Cleaved spectrin/actin

1.8

1.6

1.4

1.2

1.0

0.8

0.6

0.4

0.2

0.0

Control 6 12 24 h

NMDA

C

NMDA

SNJ-1945

C V 30 100

α-spectrin

β-actin

280 kDa

150 kDa

145 kDa

D

Cleaved spectrin/actin

1.4

1.2

1.0

0.8

0.6

0.4

0.2

0.0

Control Vehicle 30 100 mg/kg, i.p.

SNJ-1945

NMDA

** * * * * *

### * *

** * * * * *
Fig. 2

**NMDA**

<table>
<thead>
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<th>SNJ-1945 (mg/kg, i.p. x 2)</th>
<th>Control</th>
<th>Vehicle</th>
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<tr>
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</table>

![Images A to E showing tissue samples with GCL and IPL labels](image)

**GCL and IPL**

**Cell numbers in GCL (mm)**

Control: 120, Vehicle: 80, 10: 60, 30: 40, 100: 30

**IPL thickness (μm)**

Control: 30, Vehicle: 25, 10: 20, 30: 15, 100: 10

SNJ-1945 compared to NMDA treatment.
NMDA

<table>
<thead>
<tr>
<th>SNJ-1945 (mg/kg, p.o. x 2)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>Vehicle</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>200</td>
</tr>
</tbody>
</table>

**Fig. 3**

E

![Graph showing cell numbers in GCL](image)

F

![Graph showing IPL thickness](image)
Fig. 6

Control  OGD  OGD +SNJ-1945 10 μM

A  B  C

D

Cell viability (% of control)

Control  Vehicle  10  100

μM

SNJ-1945

OGD