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# Contribution of calpains to photoreceptor cell death in *N*-methyl-*N*-nitrosourea-treated rats ☆

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**Abstract**

The purpose of the present study was to determine if proteolysis by the calcium-dependent enzyme calpains (EC 3.4.22.17) contributed to retinal cell death in a rat model of photoreceptor degeneration induced by intraperitoneal injection of *N*-methyl-*N*-nitrosourea (MNU). Retinal degeneration was evaluated by H&E staining, and cell death was determined by TUNEL assay. Total calcium in retina was measured by atomic absorption spectrophotometry. Activation of calpains was determined by casein zymography and immunoblotting. Proteolysis of α-spectrin and p35 (regulator of Cdk5) were evaluated by immunoblotting. Calpain inhibitor SNJ-1945 was orally administrated to MNU-treated rats to test drug efficacy. MNU decreased the thickness of photoreceptor cell layer, composed of the outer nuclear layer (ONL) and outer segment (OS). Numerous cells in the ONL showed positive TUNEL staining. Total calcium was increased in retina after MNU. Activation of calpains and calpain-specific proteolysis of α-spectrin were observed after MNU injection. Oral administration of SNJ-1945 to MNU-treated rats showed a significant protective effect against photoreceptor cell loss, confirming involvement of calpains in photoreceptor degeneration. Conversion of p35 to p25 was well correlated with calpain activation, suggesting prolonged activation of Cdk5/p25 as a possible downstream mechanism for MNU-induced photoreceptor cell death. SNJ-1945 reduced photoreceptor cells death, even though MNU is one of the most severe models of photoreceptor cell degeneration. Oral calpain inhibitor SNJ-1945 may be a candidate for testing as a medication against retinal degeneration in retinitis pigmentosa.


*Keywords:* Calpain; Calcium; α-Spectrin; Cdk5/p25; SNJ-1945; MNU; Retinitis pigmentosa

## Introduction

Retinitis pigmentosa (RP) affects approximately 1.5 million people worldwide (Berson, 1993). RP is a group of hereditary retinal dystrophies, characterized by the early onset of night blindness followed by a progressive loss of the visual field. RP is phenotypically and genetically very heterogeneous (van Soest et al., 1999). More than 25 gene mutations have been associated with RP. The primary defect underlying RP is malfunctioning of the rod photoreceptor cells, followed by apoptotic degeneration (Li and Milam, 1995). Many models show photoreceptor degeneration, including retinal degeneration (*rd*) mice and the Royal College of Surgeons (RCS) rats (Pierce, 2001). *rd* mice contain a defect in the β subunit of rod cGMP phosphodiesterase (β-PDE). RCS rats have a deletion in the receptor tyrosine kinase, mertk. Mutations of both genes have been detected in patients with RP.

*N*-methyl-*N*-nitrosourea (MNU) causes DNA methylation (Christmann and Kaina, 2000), and it has been used extensively to study retinal photoreceptor cell degeneration in a variety of animals (Nakajima et al., 1996; Yuge et al., 1996; Taomoto et al., 1998). MNU-induced photoreceptor degeneration is characterized by apoptosis. Apoptosis is induced by the caspase family of cysteine proteinases. Inhibition of caspases protected structures and functional properties of photoreceptors (Yoshizawa et al., 2000; Petrin et al., 2003).

☆ Calpains in photoreceptor cell death.

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Calpains (EC 3.4.22.17) are also known to be activated during cell death in many types of cells (Utz and Anderson, 2000; Wang, 2000). Calpains are cytoplasmic, calcium-activated, cysteine proteases, comprising a superfamily of 15 genes in mammals (Branca, 2004). Calpains 1 (μ-calpain) and 2 (m-calpain) are the best characterized and are ubiquitously expressed in the animal kingdom. Enzymatic activities of calpains 1 and 2 are regulated by an endogenous inhibitory protein calpastatin and calcium (Saido et al., 1992). Uncontrolled and prolonged calpain-mediated proteolysis has been suggested in the pathogenesis of neuronal cell death, such as in Alzheimer's and Parkinson's diseases (Nixon, 2003). Calpain activation was also observed in hereditary models associated with photoreceptor degeneration in WBN/Kob rats and *rd* mice (Azuma et al., 2004; Doonan et al., 2005). Calpains were also involved in calcium-induced cell death in a murine photoreceptor-derived cell line (Sanvicens et al., 2004; Gomez-Vicente et al., 2005).

Calpains have a large number of substrates, including signaling molecules, membrane proteins, intracellular enzymes, and structural proteins (Goll et al., 2003). However, most of these substrates were identified using *in vitro* cleavage site analysis and do not necessarily reflect calpain target proteins *in vivo*. Identification of intracellular calpain substrates during cell injury and death is needed to understand the role of calpains in photoreceptor degeneration. For example, the cytoskeletal protein α-spectrin is a well-known, sensitive substrate of calpain. Proteolysis of α-spectrin has been suggested in the pathogenesis of neuronal cell death in brain ischemia (Yokota et al., 2003). α-Spectrin supports the neuronal structures and tethers synaptic vesicles to the active zone of the synaptic plasma membrane (Sikorski et al., 2000). α-Spectrin breakdown products (SBDPs) have been used as biochemical markers for calpain activation in central neurons and cultured neuroblastoma cells (Saido et al., 1993; Nath et al., 1996). Our previous studies show that α-spectrin was proteolyzed *in vivo* and *in vitro* retinal degeneration models (Sakamoto et al., 2000; Tamada et al., 2002; Azuma et al., 2004; Oka et al., 2006). In mammalian retina, α-spectrin is ubiquitously localized in both of inner and outer retina (Isayama et al., 1991), suggesting that calpain-induced SBDPs are useful markers in the pathogenesis of photoreceptor cell death.

Other proteins could also be substrates for calpains in photoreceptor cells. In vertebrate outer segments, the two intracellular messengers cGMP and calcium regulate visual transduction and adaptation. Levels of intracellular calcium in photoreceptors are regulated by influx through cGMP-gated calcium channels and efflux through $Na^+/Ca^{2+},K^+$exchangers (McNaughton, 1995). Some $Na^+/Ca^{2+}$exchangers are cleaved by calpain, causing calcium overload and excitotoxicity in neuronal cells (Bano et al., 2005).

The photoreceptor cGMP-gated calcium channel is regulated by the concentration of cGMP, which is degraded by phosphodiesterase 6 (PDE6). PDE6 is composed of two homologous catalytic αβ subunits and two identical regulatory γ subunits. PDE6 is activated by removal of γ subunits from the $\alpha\beta\gamma_2$ complex after stimulation by upstream signals initiated by illuminated rhodopsin (Stryer, 1986). In *rd* mice, photoreceptor cell death is thought to be due to defects in the β subunit of PDE (β-PDE) causing lack of PDE6 activity, elevated cGMP, and sustained influx of calcium via the cGMP-gated channel (Yau and Baylor, 1989). Mutations in the gene for the β subunit of PDE (PDE6B) occur in some cases of human recessive RP (McLaughlin et al., 1993; Lolley, 1994).

Normally, the γ subunit of PDE6 is phosphorylated by cyclin-dependent kinase 5 (Cdk5). Phosphorylated γ subunits strongly bind to αβ subunits, leading to PDE6 inactivation (Matsuura et al., 2000). Cdk5 requires the regulatory subunit p35 for activity (Lew et al., 1994; Tsai et al., 1994). p35 is converted to p25 by calpains, leading to prolonged activation and mislocalization of Cdk5 found in neural degeneration in Alzheimer's disease (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000). This suggests that conversion of p35 to p25 by calpains could also lead to inactivation of PDE6 and calcium influx in photoreceptor cells. Further, p25 may be a useful marker for relating calpain activity to photoreceptor cell death.

The purpose of the present experiment was to determine if calpain-induced proteolysis of photoreceptor proteins contributed to retinal degeneration in MNU-treated rats.

## Materials and methods

### *Experimental animals*

Female Sprague–Dawley rats at 7 weeks of age were used as previously reported (Yoshizawa et al., 1999). The rats were obtained from Charles River (Yokohama, Japan) and were maintained at room temperature on a 12-h light/dark schedule, with ad libitum access to food and water. All experimental animals were handled in accordance with the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) and according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### *MNU-induced retinal degeneration model*

The *N*-methyl-*N*-nitrosourea (MNU)-induced retinal degeneration model was produced according to the methods of Yoshizawa et al. (1999). One percent MNU (Nacalai Tesque, Kyoto, Japan) was dissolved in ice-cold physiological saline containing 0.05% acetic acid just before use. Rats received an intraperitoneal injection of MNU as a single dose of 60 mg/kg body weight. For histological examination, protein analysis, and calcium measurement, rats were asphyxiated with $CO_2$ gas at several time points after MNU injection, and the eyes were immediately enucleated after death.

### *Drug administration*

SNJ-1945, ((1S)-1-((((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl)-3-methylbutyl) carbamic acid 5-methoxy-3-oxapentyl ester (Shirasaki et al., 2005),

was suspended at 5% (W/V) in distilled water containing 0.5% carboxymethyl cellulose. A suspension of SNJ-1945 was orally administrated at 100 or 200 mg/kg within 30 min after MNU injection and thereafter once daily at the same dose. The control group received the same volume of vehicle solution as the 200 mg/kg group but without SNJ-1945. Seven days after MNU injection, the animals were euthanized and H&E-stained retinal sections were prepared described as below.

*Hematoxylin and eosin (H&E) staining*

Enucleated globes were immersed in 10% neutralized formaldehyde containing 2.5% glutaraldehyde, oriented by using the posterior ciliary arteries visible on the back of the globe, hemisected through the optic nerve along the superior-inferior meridian (Szczesny et al., 1995), kept overnight, and then by dehydrated and embedded in paraffin. Three-micron sections of the retina through the optic disk were stained with H&E. The retinal sections were observed with an optical microscope (OLYMPUS BX60) and then digitalized using a CCD camera on a personal computer with the aid of a Studio-Lite software (Pixera, Osaka, Japan). To assess the effect of SNJ-1945 in photoreceptor cell death, the thickness of photoreceptor layer, composed of outer nuclear layer (ONL) and outer segment (OS), were measured at 2 sites 0.5 mm apart from the center of the optic disk with Image-Pro Plus 5.0 software (Media Cybernetics, Silver Spring, MD), using a masking procedure. The data from each section were expressed as the average thickness at 2 sites. The thickness of inner nuclear layer (INL) was also measured using the same methods. To eliminate variability of thicknesses caused by the cutting angle on different sections, the thickness of photoreceptor layer was normalized to that of the neighboring INL because the INL was not affected by MNU (Nakajima et al., 1996).

*TUNEL staining*

To determine cell death 1 day after MNU injection, cleavage of DNA was visualized *in situ* by labeling the free 3′-OH ends of DNA with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL). Sections for TUNEL staining were prepared as for H&E staining described above, except 10% neutralized formaldehyde was used for fixation. TUNEL staining was performed using an *in situ* apoptosis detection kit (Wako, Osaka, Japan). Fragmented DNA was labeled with TdT and biotin-dUTP. The streptavidin–HRP/DAB system was used to visualize the labeling.

*Preparation of retinal proteins from MNU-treated rats*

The retinas from right and left eyes of each rat were pooled, except in the 7-day MNU group where 4 eyes from 2 rats were pooled. The pools were homogenized in 150 μL buffer, containing 20 mM Tris (pH 7.5), 5 mM EGTA, 5 mM EDTA, and 2 mM dithioerythritol (DTE). The soluble proteins were obtained by centrifugation at 13000×*g* for 20 min at 4°C. Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL) with bovine serum albumin as the standard. All steps were performed at 4°C.

*Casein zymography*

Casein zymography was performed using the method of Raser et al. (1995). Eight percent acrylamide gels, co-polymerized with 0.1% casein (Tefco, Tokyo, Japan), were pre-run with buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 1 mM EGTA, and 1 mM dithiothreitol (DTT) for 15 min at 4°C. Thirty micrograms of retinal soluble proteins was then loaded and electrophoresed. After electrophoresis, the gels were incubated overnight at room temperature in buffer containing 20 mM Tris (pH 7.4), 2 mM $CaCl_2$, and 10 mM DTT, with gentle shaking. Gels were stained with Coomassie brilliant blue. Calpains 1 and 2 were separated on the non-denaturing zymogram gels on the basis of differences in isoelectric points. Digital images of the zymography gels were inverted to visualize caseinolysis as dark bands caused by calpains. To eliminate variability of staining between individual gels, the staining intensity of the bands determined by densitometric image analysis for calpains 1 and 2 was normalized to the intensity of calpains 1 and 2 from rats before MNU treatment run on the same gel.

*Immunoblot analysis*

SDS–PAGE of soluble proteins (10 μg/lane) was performed on 10% Bis–Tris gels (NuPAGE; Invitrogen, Carlsbad, CA) for calpain 2, 12% gels for cyclin-dependent kinase 5 (Cdk5) and p35, and 4–12% gels for α-spectrin with the MOPs buffer system (Invitrogen). This system included a molecular weight marker (MagicMark XP; Invitrogen). Immunoblots were performed by electrotransferring proteins from NuPAGE gels onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) using an ice-cooling unit, according to standard procedures using NuPAGE transfer (Invitrogen). Antibody sources and dilutions were as follows: rabbit polyclonal antibodies against calpain 2 (Ma et al., 2001) 1:250, Cdk5 (sc-173; Santa Cruz Biotechnology, Santa Cruz, CA) (Kusakawa et al., 2000; Ubeda et al., 2004) 1:1,000, p35 (sc-820; Santa Cruz) (Kusakawa et al., 2000; Ubeda et al., 2004) 1:250, calpain-specific 150 kDa α-spectrin breakdown product (Saido et al., 1993) 1:100, and mouse monoclonal antibody against α-spectrin (nonerythroid) (clone AA6; Affiniti Research Product, Exeter, UK) (Nath et al., 1996; Shi et al., 2000) 1:1,000. Immunoreactivity was visualized with alkaline phosphatase conjugated to secondary antibodies and BCIP/NBT (AP conjugate substrate kit; Bio-Rad, Hercules, CA), or with horseradish peroxidase conjugated to secondary antibodies and chemiluminescence (SuperSignal West Femto; Pierce). The staining intensity of the bands for proteolyzed α-spectrin at 145 kDa and p25 was determined by use of densitometric image analysis with ImageJ 1.33 software (NIH, Bethesda, MD). To eliminate variability of staining

between individual gels, the densities of bands for truncated fragments were normalized to the density of their parent proteins in rats before MNU treatment and run on the same gel.

*Calcium determinations*

To obtain sufficient starting material for calcium determination, retinas from 4 eyes were pooled. Dry weights of retinas were measured following heating at 100°C for 16 h. The retinas were then digested overnight in 0.2 mL of concentrated HCl with gentle agitation at room temperature, 1.8 mL water were added, and calcium content was measured by atomic absorption spectrophotometry (Polarized Zeeman Atomic Absorption Spectrophotometer model Z-8100; Hitachi, Tokyo, Japan). Total calcium content in the retinas was expressed as meq/kg retina dry weight.

*Incubation of retinal soluble protein with calcium*

Dissected retinas from rats were homogenized in buffer containing 50 mM Tris (pH 7.4), 1 mM EGTA, and 3 mM dithiothreitol (DTT). Soluble proteins were prepared by the same procedure as the above and were incubated at the final concentration of 3 μg/μL at 37°C. The reaction was initiated with 1.5 mM calcium to activate endogenous calpains and was stopped by the addition of excess EDTA. After incubation, proteolysis of α-spectrin was assessed by immunoblotting as described above. In some reactions, 100 μM calpain inhibitors or 3 mM EDTA were added to the reaction mixtures just prior to addition of calcium.

*Statistical analysis*

Statistical analysis of the data was performed by one-way ANOVA followed by Dunnett's *t* test (SAS statistical software; SAS Institute, Cary, NC).

## Results

*Histological changes in MNU-treated rats*

H&E staining of retinal sections showed no substantial differences in the retinal structures 6 h after MNU injection (Fig. 1A). The first changes observed 12 h after injection of MNU were expansion of extracellular space with vacuoles in the outer nuclear layer (ONL) and the outer segment (OS). By day 1, the thicknesses of the ONL and OS were slightly thinner than those in retinas from rats before MNU treatment (Fig. 1A, 1 day vs. Time 0). These changes became more severe over 7 days. Numerous positive TUNEL staining cells were also observed in the ONL one day after MNU treatment (Fig. 1B, 1 day), but not in any other layers. No TUNEL-stained cells were detected in retinas before MNU treatment (Fig. 1B, Time 0), and no other obvious structural changes were observed in the MNU rats. Thus, our MNU-treated rats comprise a valid model of photoreceptor cell degeneration.

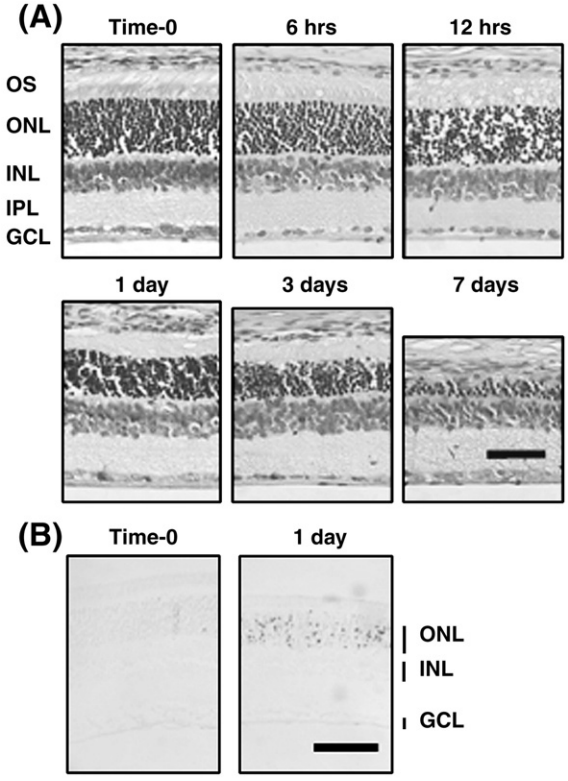


Fig. 1. (A) Representative photomicrographs of H&E-stained sections of retina from rats before and after MNU treatment, showing expansion of extracellular space in the ONL and OS followed by a decrease of thickness in both layers. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment. Scale bar is 50 μm. (B) Photomicrographs of TUNEL-stained sections from retina in rats before and after MNU treatment, showing positive TUNEL-stained cells in ONL 1 day after MNU injection. Histological analyses were performed on at least two samples at each time point with reproducible results.

*Retinal calcium in MNU-treated rats*

Total calcium in retinas from MNU-treated rats were significantly increased on day 1 and continuously increased over 7 days (Fig. 2). These data are relevant because calpains are activated by increased calcium.

*Proteolysis of signature calpain substrate*

*In vitro data*

In order to demonstrate that calcium could induce calpain-dependent proteolysis in retinal proteins, total soluble proteins from rat retinas were incubated *in vitro* with exogenous calcium. Proteolysis of the cytoskeletal protein α-spectrin was examined because α-spectrin is a sensitive substrate for calpains and α-spectrin proteolysis is also a marker of neural cell death (Nath et al., 1996). A band at 280 kDa representing intact α-spectrin was observed before addition of calcium (Fig. 3, initial). Thirty minutes after the activation of endogenous calpains by addition of calcium, proteolysis of α-spectrin was observed as SBDPs at 150 and 145 kDa (Fig. 3). The SBDP band at 150 kDa contains fragments produced by calpains and caspase-3, while the 145-kDa

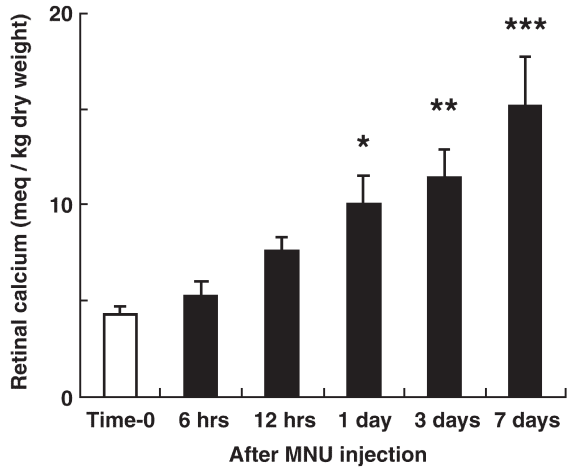


Fig. 2. Total calcium content in retinas from rats before MNU treatment (Time-0, open column), and from rats treated with MNU (solid column), showing a gradual increase in total calcium after MNU treatment. Data are expressed as means±SEM ($n=7$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ relative to Time-0.

fragment is produced only by calpains (Nath et al., 1996). Two hours after incubation with calcium, intact α-spectrin at 280 kDa decreased and SBDPs at 150 and 145 kDa increased. No changes were observed when soluble proteins were incubated without calcium. Calpain inhibitors SNJ-1945 and SJA6017 almost completely inhibited proteolysis of α-spectrin (Fig. 3). As further confirmation that proteolysis was due to calcium, EDTA was added to the incubation mixtures. EDTA also inhibited the loss of intact α-spectrin and the increase of SBDPs (Fig. 3). EDTA also inhibited calpain activation as determined by zymography (data not shown). Thus, these data showed that the calpains obtained from the soluble proteins of rat retinas are potentially capable of being activated in vivo by increased retinal calcium.

*In vivo data*

As expected, the band at 280 kDa representing intact α-spectrin was also observed in retinas from rats before MNU treatment (Fig. 4A, Time 0). One day after MNU treatment, the proteolyzed α-spectrin band at 150 kDa became denser, and a new band of proteolyzed α-spectrin at 145 kDa appeared. Both fragments remained over 3 days and disappeared by 7 days.

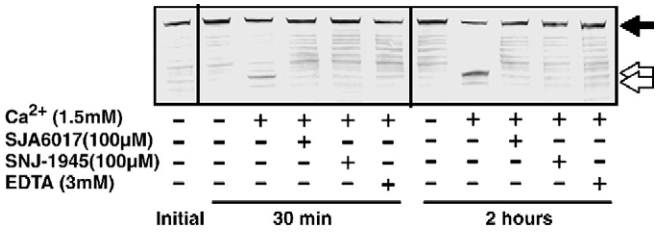


Fig. 3. Representative immunoblot showing proteolysis of α-spectrin in isolated total retinal soluble proteins incubated with exogenous calcium, and inhibition by EDTA or calpain inhibitors SJA6017 and SNJ-1945. Solid arrow indicates intact α-spectrin at 280 kDa and open arrows indicate proteolyzed α-spectrin fragments at 150 and 145 kDa, respectively.

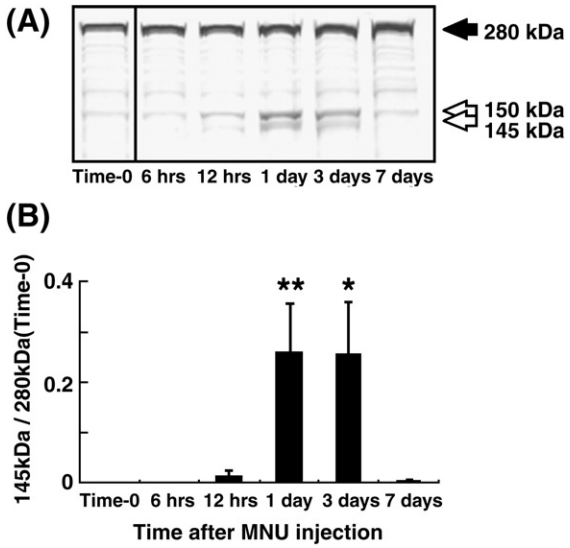


Fig. 4. (A) Immunoblot showing proteolysis of α-spectrin in retinal soluble proteins from rats treated with MNU. Solid arrow indicates the intact α-spectrin band at 280 kDa and open arrows indicate 150 and 145 kDa fragments. (B) Densitometric image analysis of calpain-specific α-spectrin breakdown product at 145 kDa, showing proteolysis of α-spectrin by activated calpain. Data are expressed as means±SEM ($n=5$). *$P<0.05$, **$P<0.01$ relative to Time-0.

Densitometric image analysis of calpain-specific, 145-kDa fragment was performed (Fig. 4B). The fragment at 145 kDa was dramatically increased 1 day after MNU treatment, maintained at a constant level over 3 days, and decreased at 7 days. Proteolysis of α-spectrin by calpains was also confirmed using an antibody that recognizes only the calpain-specific band in the SBDP proteins at 150 kDa (data not shown). The apparent "recovery" of α-spectrin 7 days after MNU treatment possibly resulted from the decrease of photoreceptor cells in the MNU-treated retinas (Fig. 1A, 7 days). Loss of MNU-damaged cells or repair of surviving cells in photoreceptor layer may have contributed to these results. Our results are consistent with the hypothesis that calpain-induced proteolysis was mainly localized in the ONL and OS, and did not occur in other layers.

*Effect of calpain inhibitor SNJ-1945 in MNU-treated rats*

Calpain inhibitor SNJ-1945 was tested in vivo to further demonstrate involvement of calpains in photoreceptor degeneration in rats treated with MNU. Histological observations showed that oral administration of 200 mg/kg/day SNJ-1945 caused a reduction in the severity of photoreceptor (ONL and OS) degeneration 7 days after MNU injection (Fig. 5A). Quantitative analysis confirmed that 200 mg/kg/day SNJ-1945 significantly inhibited the decrease in thickness of the photoreceptor layer 7 days after MNU injection; inhibition was 46% (Fig. 5B).

*Calpain activation in rat retinas treated with MNU*

Casein zymography followed by densitometric image analysis of the lytic bands was performed to measure calpain

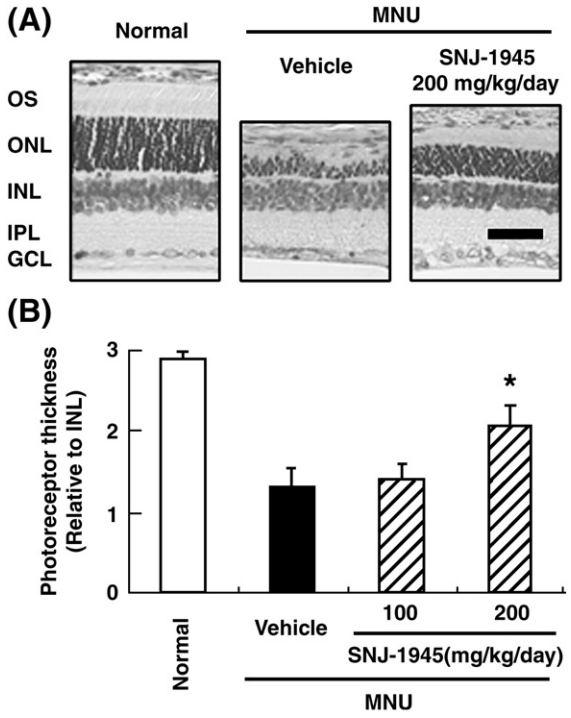


Fig. 5. (A) Photomicrographs of H&E-stained rat retinal sections showing partial protective effect of oral 200 mg SNJ-1945/kg/day against MNU-induced decrease in the thickness of the photoreceptor layer (ONL and OS) 7 days after MNU injection. Scale bar is 50 μm. (B) Decreased thickness of photoreceptor cells layer in retina from rat 7 days after MNU injection, and significant inhibitory effect by oral administration of SNJ-1945. Data are expressed as means±SEM ($n$=8–10). *$P$<0.05 relative to vehicle treatment.

activities in the retinas after MNU injection. The data were expressed as density of the band relative to time 0 (Fig. 6A). Calpain 1 showed significantly decreased caseinolysis on day 1 after MNU treatment, and this returned to normal by 3 days, possibly due to shedding of the damaged photoreceptor cells. Decreased calpain activity has been interpreted as indirect evidence of calpain activation due to autodegradation of calpain following its activation (Nakamura et al., 1999). Unexpectantly, the caseinolytic band for calpain 2 was increased 3 days after MNU treatment (Fig. 6B). To aid in the interpretation of these data, immunoblotting with a calpain 2 antibody was performed because an autolysis product at 43 kDa from the 80-kDa subunit of calpain 2 has been used as indirect marker of calpain 2 activation (Azuma et al., 1997). Three days after MNU treatment, we observed a new band at 43 kDa from the autolyzed 80 kDa subunit of calpain 2 (Fig. 6C, 3 days), suggesting calpain 2 was activated along with the calpain 1 activation as described above.

*Proteolysis of Cdk5 and p35 in MNU-treated rat retinas*

To test one possible mechanism for calpain-induced photoreceptor cell degeneration, Cdk5 and its regulator protein p35 were examined in MNU-treated rat retinas. Conversion of p35 to p25 by calpain causes prolonged activation of Cdk5 (Kusakawa et al., 2000; Nath et al., 2000). Cdk5 was observed in retinas from rats before MNU treatment, and Cdk5 did not change in retinas up to 7 days after injection of MNU (data not shown). p35 was observed in retinas from rats before MNU treatment (Fig. 7A, Time 0). In retinas from MNU-treated rats, a decrease in p35 and the appearance of p25 were observed (Fig. 7A). Semi-quantitative analysis of the immunoblots showed that p25 in the retina increased at 1 day after MNU treatment, peaked at 3 days, and was lowered at 7 days (Fig. 7B). Such data are consistent with our hypothesis that calpain-related, prolonged activation of Cdk5 and excessive phosphorylation are mechanisms for MNU photoreceptor cell death.

## Discussion

The major findings of the present study were that calpains could contribute to photoreceptor cell death in the ONL and OS from rats treated with MNU, and that oral administration of calpain inhibitor SNJ-1945 reduced cell death. These conclusions were based on increased total calcium, activation of calpains, appearance of calpain-specific proteolysis products, and inhibition of cell death by calpain inhibitor SNJ-1945.

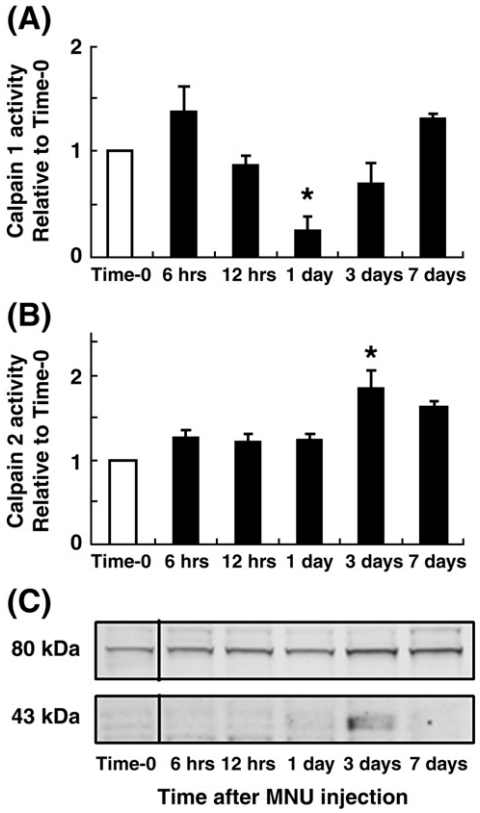


Fig. 6. (A) Densitometric image analysis of caseinolytic bands due to calpain 1 in retinal soluble proteins from rats treated with MNU, showing a decrease in calpain 1 activity after MNU treatment. Data are expressed as means±SEM ($n$=5). *$P$<0.01 relative to Time-0. (B) Densitometric image analysis of caseinolytic bands due to calpain 2, showing an increase in calpain 2 activity after MNU treatment. (C) Immunoblots for calpain 2 in retinal soluble proteins from rats treated with MNU, showing initially observed catalytic subunit at 80 kDa and a new autolyzed fragment at 43 kDa appearing 3 days after MNU treatment.

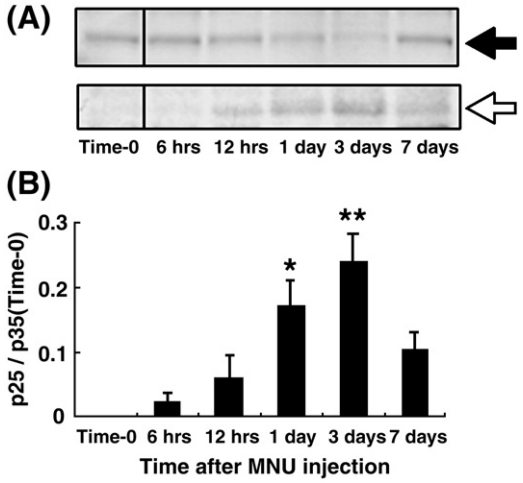


Fig. 7. (A) Immunoblots showing proteolysis of p35 and appearance of p25 in retinal soluble proteins from rats treated with MNU. (B) Densitometric image analysis of p25 shown in panel A, showing an increase in p25 after MNU treatment. Data are expressed as means±SEM ($n=3$). *$P<0.05$, **$P<0.01$ relative to Time 0.

*Elevated calcium*

Total calcium increased in retinas after MNU treatment. However, the present study does not show if increased calcium was compartmentalized in the cellular organelles, cytoplasm, extracellular space, or specific cells. MNU causes carbamoylation of the amino groups in membrane aminophospholipids and/or proteins (Yano et al., 1988), causing altered membrane fluidity and permeability. MNU also causes a decrease in reduced glutathione (GSH), which effectively scavenges free radicals and other reactive oxygen species (Cengiz et al., 1996; Wu et al., 2004). These abnormalities may have caused the initial increase in intracellular calcium in the present studies. Oxidative stress also elevated intracellular calcium in cultured photoreceptor cells (Sanvicens et al., 2004). Insults by MNU may be transient, since chemical half-life of MNU in plasma was only 5–15 min (Smith and Yielding, 1986). Increased calcium activates a multitude of signaling pathways including those involving calpains, causing Cdk5/p25 activation and proteolysis of α-spectrin. These changes may have lead to the sustained increase in calcium.

Increased calcium can further activate calpain 2 as well as calpain 1 shown in Fig. 6. Calpains 1 and 2 are activated in vitro by μM and mM levels of calcium, respectively. A direct relationship between increased calcium and activation of calpain was observed in retina until 3 days after MNU treatment. However, this relationship was not sustained 7 days after MNU treatment, where calpain activities returned to normal in the presence of continued high levels of calcium in the whole retina. The reason for this discrepancy is unknown. It may be caused by accumulation of calcium in the extracellular space due to destruction of the retina. Alternatively, Müller cells and the drastic infiltration of macrophages into the damaged photoreceptor cell layer may have phagocytized apoptotic photoreceptor cell bodies containing high levels of calcium (Nakajima et al., 1996).

*Calpain activation*

Casein zymograms for calpains 1 and 2 and immunoblots for calpain 2 confirmed that both calpain isoforms were activated in the retinas after MNU injection, although the activation of calpain 1 was earlier than that of calpain 2. These observations were similar to those seen in *in vivo* models of acute retinal degeneration, such as in rats with ischemia-reperfusion (Sakamoto et al., 2000) and with acute ocular hypertension (Oka et al., 2006). However, decreased caseinolytic activities were not observed in hereditary WBN/Kob rats, which showed retinal degeneration with age, although proteolysis of α-spectrin was observed (Azuma et al., 2004). The timing and level of calpain activation may be different in each model, and these factors may affect the progression rate of retinal degeneration. The present study using whole retinas does not show in which specific layer(s) calpains were activated, although ONL and OS were indirectly suggested. In situ calpain activation analyses may clarify this point. Calpains could also have been degraded by other proteases. However, activation of calpains in the present study was confirmed by observing of calpain-specific spectrin breakdown products.

In mammalian retina, α-spectrin is ubiquitously localized in both the inner and outer retina (Isayama et al., 1991). In present experiment, SBDPs at 145 and 150 kDa were observed in retinas after MNU treatment, and then they returned to normal levels, possibly due to shedding of degenerated cells. This proteolysis of α-spectrin was well correlated with maximum activation of calpain 1 at day 1 and calpain 2 at day 3. The 150-kDa fragments of α-spectrin are produced not only by calpain but also by caspase-3, while the 145-kDa band is produced only by calpain (Nath et al., 1996; Wang et al., 1996). In present study, the 145-kDa SBDP was observed in retina after MNU treatment, confirming activation of calpains.

*Role of conversion of p35 to p25 by calpains*

Cyclin-dependent kinase 5 (Cdk5) is a protein serine/threonine kinase requiring the regulatory subunit p35 for activity (Lew et al., 1994; Tsai et al., 1994). p35 is proteolyzed by calpains to p25, leading to prolonged activation and mislocalization of Cdk5 found in the neural degeneration of Alzheimer's disease (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000). Proteolysis of p35 was inhibited by a calpain inhibitor in a rat model of retinal degeneration (Tamada et al., 2005). In the present experiment, conversion of p35 to p25 was observed in retinas from rats treated with MNU. Production of p25 was well correlated with activation of calpains, suggesting prolonged activation of Cdk5 by p25. Since the γ subunit of PDE6 is a substrate for Cdk5 (Matsuura et al., 2000) and phosphorylated γ subunit leads to PDE6 inactivation, conversion of p35 to p25 by calpains may be an additional mechanism extending the photoreceptor damage induced by MNU. Of course, activated Cdk5 phosphorylates other proteins (Smith et al., 2001), such as microtubule-associated protein tau. Phosphorylated tau is a major antigenic component of paired

helical filaments (PHF), pathological hallmarks of Alzheimer's disease (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986). Decreased PDE activity has been reported in *rd* mice and human RP retinas, and direct measurements of PDE6 activity in MNU rats would be helpful in clarifying the role of conversion of p35 to p25 by calpain.

*Reduction of photoreceptor degeneration by calpain inhibitor SNJ-1945*

Oral administration of calpain inhibitor SNJ-1945 significantly protected against loss of photoreceptor cells in rats treated with MNU, confirming involvement of calpains in photoreceptor cell death. These are the first in vivo data showing that a calpain inhibitor can protect against photoreceptor cell death. These results also suggested that calpains were mainly active in ONL and OS, but not in other layers. Intravenous injection of a dipeptidyl aldehyde calpain inhibitor SJA6017, *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal also showed a protective effect against ganglion cell death in rats with ocular ischemia-reperfusion (Sakamoto et al., 2000). SJA6017 was also effective against cell death in cultured photoreceptor cells (Sharma and Rohrer, 2004). However, the bioavailability of oral SJA6017 is lower because an aldehyde group in SJA6017 may be rapidly metabolized (Nakamura and Inoue, 2002). SNJ-1945 was developed as novel calpain inhibitor to provide higher cellular permeability and higher metabolic stability (Shirasaki et al., 2005). Oral SNJ-1945 showed rapid penetration and long stability in retina. The concentration of SNJ-1945 in retina 4 h after a single oral administration of 10 mg/kg was exceeded the $IC_{50}$ against calpains 1 and 2 (Shirasaki et al., in press). Further, oral SNJ-1945 at 1000 mg/kg/day for 14 days produced no obvious toxic signs or abnormalities in rats (Yamaguchi, M., Senju Pharmaceutical Co., Ltd., personal communication). In present experiment, calpain inhibitors SNJ-1945 and SJA6017 also inhibited proteolysis of α-spectrin by calpains in retinal soluble proteins. These observations confirmed involvement of calpains and inhibition of photoreceptor cell death by oral SNJ-1945 in the rat.

To date, there is still no cure or effective therapy for the treatment of RP, although vitamin A supplementation has been shown to slow vision loss in RP patients (Berson et al., 1993). Therefore, understanding the detailed mechanism of photoreceptor cell death will lead to the development of more effective medications to prevent vision loss. The present experiment showed that calpain inhibitor SNJ-1945 can protect photoreceptor cell death in rat treated with MNU. In contrast, another calpain inhibitor ALLN had no suppressive effect against photoreceptor degeneration in *rd* mice despite inhibition of α-spectrin breakdown, suggesting involvement of multiple pathways in photoreceptor cell death (Doonan et al., 2005). The similarity between human RP and MNU model is that photoreceptors undergo cell death, although mechanisms causing photoreceptor cell death are different. However, calpain activation occurred with photoreceptor cell death in the *rd* mouse, which is thought to be an animal model for human RP (Paquet-Durand et al., 2006). Calpain inhibitors, such as SNJ-1945, may be candidate compounds for treatment of retinal degeneration such as RP.

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