Early Remodeling in an Inducible Animal Model of Retinal Degeneration


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Section Editor: Dr. Richard Weinberg

Financial Support: Grants (BT/PR6410/Med/14/801/2005 and BT/PR6615/Med/14/857/2005) to NKD from the Department of Biotechnology, Ministry of Science and Technology, Government of India.
List of Abbreviations:

1. BCA: Bicinchoninic Acid
2. BSA: Bovine Serum Albumin
3. DAPI: 4', 6-Diamidino-2-Phenylindole
4. Dil: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
5. EDTA: Ethylene Diamine Tetraacetic Acid
6. FITC: Fluorescein Isothiocyanate
7. GCL: Ganglion Cell Layer
8. GFAP: Glial Fibrillary Acidic Protein
9. INL: Inner Nuclear Layer
10. IPL: Inner Plexiform Layer
11. kDA: Kilo Dalton
12. MNU: N-Methyl-N-Nitrosourea
13. NFL: Nerve Fiber Layer
14. ONL: Outer Nuclear Layer
15. OPL: Outer Plexiform Layer
16. PBS: Phosphate Buffered Saline
17. PKC: Protein Kinase C
18. PNA: Peanut Agglutinin
19. PSD-95: Postsynaptic Density 95
20. RP: Retinitis Pigmentosa
21. SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ABSTRACT

Photoreceptor degeneration is followed by significant morphological changes in the second-order retinal neurons in humans and in several genetic animal models. However, it is not clear whether similar changes occur when photoreceptor degeneration is induced non-genetically, raising the question whether these changes are a general effect of deafferentation independent of the cause of degeneration. We addressed this by inducing selective photoreceptor degeneration with N-methyl-N-nitrosourea (MNU) and studying its effects on inner retinal neurons in a mouse for up to three months, using immunocytochemistry and iontophoretic labeling. To develop objective measures of photoreceptor degeneration and of retinal remodeling, we measured several retinal proteins using immunoblot analysis, and quantified gross visual ability of the animal in a Visual Cliff test. The MNU-induced progressive degeneration of rods and cones was associated with declining levels of postsynaptic density-95 protein in the retina, and with deteriorating visual performance of the animal. Müller glial cells showed enhanced reactivity for glial fibrillary acidic protein as demonstrated by immunocytochemistry, which also reflected in increased levels of the protein as demonstrated by immunoblotting. Horizontal cells and rod bipolar cells progressively lost their dendritic processes, which correlated with a slight decline in the levels of calbindin and protein kinase C respectively. Horizontal cell axons, immunoreactive for nonphosphorylated neurofilaments, showed sprouting into the Inner Nuclear Layer. Ganglion cells and their synaptic inputs, probed by immunolocalizing β-III-tubulin, neurofilaments, bassoon and synaptophysin, appeared to be unaffected. These results demonstrate that MNU-induced photoreceptor degeneration leads to retinal remodeling similar to that observed in genetic models, suggesting that the remodeling does not depend on the etiopathology that underlies photoreceptor degeneration.
Keywords: N-methyl-N-nitrosourea; MNU; Retinal Remodeling; Photoreceptor Degeneration; Mouse
Retinal degenerative diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are characterized by photoreceptor degeneration, and are among the leading causes of blindness (Margalit and Sadda, 2003; Hartong et al., 2006). In humans and in several genetic animal models the loss of photoreceptors is followed by significant morphological changes in the inner retinal neurons, including dendritic retraction and neuritic sprouting in bipolar and horizontal cells (Milam et al., 1998; Fariss et al., 2000; Strettoi and Pignatelli, 2000, Park et al., 2001; Strettoi et al., 2002, 2003; Jones et al., 2003; Marc et al., 2003; Pignatelli et al., 2004; Cuenca et al., 2005; Jones and Marc, 2005; Gargini et al., 2007; Sullivan et al., 2007; Barhoum et al., 2008).

It has been suggested that these secondary changes are similar to those observed after deafferentation in other parts of the brain, and are a consequence of photoreceptor loss irrespective of the underlying cause (Jones et al., 2003; Marc et al., 2003). This hypothesis is supported by the finding that the photoreceptor degeneration leads to similar morphological changes in various genetic models whether the degeneration is early and fast, or delayed and slow (Strettoi et al., 2002, 2003; Jones et al., 2003; Pignatelli et al., 2004; Gargini et al., 2007; Barhoum et al., 2008). However, in these animal models a genetic mutation is present since conception, which could potentially affect retinal cells even before the onset of photoreceptor degeneration, raising the question whether the secondary changes are simply the result of photoreceptor loss or are developmental defects, or both. For example, in a transgenic pig with rhodopsin gene mutation the cone-driven inner retinal function is reported to be defective even when the cones themselves are normal (Banin et al., 1999). Furthermore, morphological remodeling in bipolar and horizontal cells has not been unequivocally demonstrated in various non-genetic (inducible) animal models (Peichl and Bolz, 1984; Fisher and Lewis, 2003; Linberg
et al., 2006; Marc et al., 2008; Liang et al., 2008). In the present study, we asked whether bipolar and horizontal cells undergo morphological remodeling when selective photoreceptor degeneration is induced non-genetically. Several inducible models are available that can be produced by physical insults, such as exposure to strong light and intravitreal insertion of iron particles, or chemical agents, such as iodoacetic acid, sodium iodate, cobalt chloride, L-ornithine chloride and N-methyl-N-nitrosourea (MNU) (Noëll et al., 1966; Wang et al., 1998; Hafezi et al., 2000; Maeda et al., 1998; Kiuchi et al., 2002; Tsubura et al., 2003; Hara et al., 2006; Liang et al., 2008). In the MNU model, a single systemic injection of the drug results in selective and progressive degeneration of photoreceptors in a variety of animals, including non-human primates (Herrold, 1967; Nambu et al., 1997; Hafezi et al., 2000; Yoshizawa et al., 2000; Tsubura et al., 2003; Uehara et al., 2006; Miki et al., 2007; Chen and Nathans, 2007; Wan et al., 2008). An alkylating agent, MNU causes apoptotic death of photoreceptors in a dose-specific manner through the formation of 7-methoxyguanosine adducts, and the cells can be rescued by several inhibitors of apoptosis (Ogino et al., 1993; Yoshizawa et al., 1999, 2000; Tsubura et al., 2003; Moriguchi et al., 2003; Uehara et al., 2006). The MNU-induced photoreceptor loss is associated with Müller cell proliferation, macrophage infiltration and migration of retinal pigment epithelial cells (Nakajima et al., 1996; Taomoto et al., 1998; Tsubura et al., 2003).

Since the MNU model has not been investigated specifically for retinal remodeling, we evaluated qualitative changes in several inner retinal cell types for up to three months following the initiation of photoreceptor loss. We also quantified the remodeling with immunoblot analysis of several proteins that are known to be expressed in inner retinal cells. Similarly, we quantified photoreceptor degeneration by measuring a photoreceptor-specific protein and by evaluating
gross visual ability of the animal in a Visual Cliff test. A part of this work has been published as a meeting abstract (Dhingra et al., 2007).

**EXPERIMENTAL PROCEDURES**

*Animals, drug administration and tissue preparation*

All experiments were approved by the Institutional Animal Ethics Committee of the National Brain Research Centre, India. The animals were maintained on a 12:12 hour light: dark cycle with an average ambient light of 25 -37 μW/cm² (170 - 250 Lux; measured with IL1400 photometer, International Lights Inc., Peabody, USA). An adult mouse (C57BL/6, male, 8-12 weeks, bred locally) was given a single intraperitoneal injection of MNU (60-65 mg/kg; Sigma-Aldrich or Chemservice, West Chester, USA; Tsubura et al., 2003). Control animal received physiological saline containing 0.05% acetic acid. Eyes were removed after cervical dislocation at 1, 2, 3, 5, 7, 14, 21, 28, 60 or 90 days after the injection, and processed for cryosectioning (number of animals at each stage = n = 2) or protein extraction (n=3). For cryosectioning, an eyeball was given a small incision, pre-fixed in 4% paraformaldehyde for 10 minutes at 4°C and hemisected, followed by post-fixation of the posterior eyecup in 4% paraformaldehyde for 45-60 minutes at 4°C. The eyecup was then immersed in 30% sucrose overnight at 4°C and radially sectioned at 10 μm thickness in a cryostat (model CM3050S, Leica, Wetzlar, Germany). Sections from midperipheral retina (0.88 ± 0.03 mm from the center; mean ± SE) were employed for immunocytochemical experiments. For protein extraction, an eyeball was hemisected, and retina was removed in ice-cold phosphate buffered saline (PBS) containing 10 mM EDTA (pH 8.0).
Antibody Characterization

All antibodies used here were obtained commercially (Table 1), and were characterized by the manufacturer (also confirmed in our laboratory in several cases). In mouse retina, where various cells and synapses are identifiably organized in distinct layers, the antibodies used here produced characteristic staining patterns.

The recoverin antibody, which detects a single band of 26 kDa (manufacturer’s data), is known to label photoreceptors and some cone bipolar cells (Milam et al., 1993; Haverkamp and Wässle, 2000). That the recoverin antibody stained all photoreceptors in the outer nuclear layer (ONL; see Fig. 1G) and some bipolar cells in mouse retina (not illustrated) confirmed the antibody specificity.

The rhodopsin antibody, which detects a 36 kDa monomer with no known reactivity to other proteins, labels the rod outer segments in all tested mammalian retinas (manufacturer’s data; Molday and MacKenzie, 1983; Li et al, 1995). Immunocytochemistry showed a strong labeling of the outer segments and weak labeling of the inner segments and the cell bodies of rods (see Fig. 2G; Fariss et al., 2000), confirming the antibody specificity.

The bassoon antibody, which detects a ~400 kDa protein (additional bands between 97 and 400 kDa from proteolytic bassoon degradation products may also be detected; manufacturer’s data), is known to label synaptic ribbons in outer plexiform layer (OPL) and conventional synapses in inner plexiform layer (IPL; Brandstätter et al., 1999). Usage by the manufacturer of rat brain tissue extract (LYT-RB100) as positive control, and a characteristic
horse-shoe shaped labeling of synaptic ribbons in the OPL and punctate staining in the IPL in mouse retina (see Fig. 1M and 7A) confirmed the antibody specificity.

The *synaptophysin* antibody, which detects a single band of 38 kDa (manufacturer’s data), is known to label all vesicular synaptic terminals in OPL and IPL (Dhingra et al., 1997). A characteristic punctate staining in the OPL and the IPL confirmed the antibody specificity in mouse retina (see Figs. 1Q and 7D). We also confirmed that the antibody detects a ~38 kDa protein in mouse retina extract (not illustrated).

The *PSD-95* antibody, which detects a ~100 kDa band (additional bands of 80 kDa and 50 kDa also detected in rat and mouse; manufacturer’s data), is known to label rod and cone terminals in mouse retina (Haverkamp et al., 2003). Usage by the manufacturer of rat brain tissue extract (LYT-RB100) and mouse brain extract (LYT-MB100) as positive controls, and detection of a ~100 kDa band in mouse retina extract (see Fig. 1Y) demonstrated the antibody specificity. Furthermore, immunocytochemistry showed a characteristic labeling in the OPL consistent with photoreceptor terminals (see Fig. 1U).

The *glial fibrillary acidic protein* (*GFAP*) antibody is known to label Müller glial cells in retina (Huxlin et al., 1995). The cross immunoelectrophoresis data from the manufacturer reports no reaction with human plasma and cow serum, but a distinct GFAP precipitate with cow brain extract. A characteristic staining pattern in mouse retina (see Fig. 4A) and detection of a single ~50 kDa band in mouse retina extract (see Fig. 4G; see also Immunoblotting below) confirmed the antibody specificity.

The *calbindin* antibody is known to label horizontal cells and a subset of amacrine and ganglion cells in mouse retina (Haverkamp and Wässle, 2000). The manufacturer’s data reports 10% cross-reactivity with *calretinin*. That the antibody stained the laterally extending horizontal
cells near the OPL (see Fig. 5A) and some cells in the inner nuclear layer (INL) and ganglion cell layer (GCL) (not illustrated) in mouse retina, and that it detected a single ~28 kDa band in mouse retina extract (see Fig. 5H) confirmed the antibody specificity.

The SMI-32 antibody, which detects 180 kDa and 200 kDa bands (manufacturers data), is known to label a subset of large ganglion cells and their axons (Coombs et al., 2006), and horizontal cell axonal processes in mouse retina (Peichl and Gonzalez-Soriano, 1994; Haverkamp and Wässle, 2000). The SMI-32 has been characterized by phosphorylating the tail domain of the neurofilament-H which eliminates the SMI-32 binding (Sternberger and Sternberger, 1983; Nixon et al., 1994). A characteristic labeling of horizontal cell processes in the OPL (see Fig. 5J; arrowhead), ganglion cell axons in the nerve fiber layer (NFL; see Fig. 7G) and a subset of large ganglion cells in flatmounted mouse retina (not illustrated) confirmed the antibody specificity.

The PKC\(\alpha\) antibody, which detects a single band of ~80 kDa (manufacturers data), is known to label rod bipolar cells in mouse retina (Haverkamp et al., 2003). Usage by the manufacturer of Jurkat whole cell lysate as positive control, a characteristic staining of rod bipolar cells in the inner nuclear layer (INL) and their large terminals in inner part of the IPL in mouse retina (see Fig. 6A), and that the antibody detected a single band of ~80 kDa in mouse retina extract (see Fig. 6H) confirmed the antibody specificity.

The \(\beta\)-III-tubulin antibody, which detects a ~50 kDa band (manufacturers data), is known to label the retinal ganglion cells and their processes in IPL in mouse retina (Robinson and Madison, 2004). The manufacturers data reports that this antibody is well characterized and highly reactive to neuron-specific class III \(\beta\)-tubulin. A distinct staining of ganglion cells in GCL
and their processes in IPL (see Fig. 7J), and detection of a single 50 kDa band in mouse retina extract (not illustrated) confirmed the antibody specificity.

**Labeling of Cones with Peanut Agglutinin**

Peanut agglutinin (PNA), a 110 kDa plant lectin is known to bind specifically to interphotoreceptor matrix that ensheaths the cone outer and inner segments, and the cone pedicles (Johnson et al., 1986; Krishnamoorthy et al., 2008). The labeling of cones with PNA was carried out along with immunolabeling for rods with *rhodopsin* antibody (see below) to identify the photoreceptors that remained after MNU treatment. After blocking with 3% normal horse serum containing 1% BSA in PBS for 45-60 minutes at room temperature the retinal sections were incubated overnight with anti-*rhodopsin* (Table 1) at 4°C. After 3 x 5-minute wash with PBS the sections were incubated in a mixture of anti-mouse secondary antibody conjugated with Texas Red and 0.00125% PNA conjugated with fluorescein (Vector Laboratories, Burlingame, USA) for 1 hour in a dark, humidified chamber. The sections were again washed 3 x 5-minutes with PBS, mounted using Vectashield containing 4', 6-diamidino-2-phenylindole (DAPI) and imaged with an upright fluorescent microscope (AxioImager.Z1 with ApoTome grid projection system; Carl Zeiss, Gottingen, Germany).

**Immunocytochemistry**

The retinal sections were first incubated in a blocking buffer (3% normal horse or goat serum, 1% BSA, 0.3% Triton X-100 in PBS) in a dark humidified chamber for 1 hour at room temperature followed by incubation with the primary antibody (Table 1) overnight at 4°C. The sections were then washed 3 x 5-minutes with PBS and incubated with either anti-mouse or anti-
rabbit secondary antibody conjugated with FITC or Texas Red for one hour at room temperature. The sections were washed again 3 x 5-minutes with PBS, mounted using Vectashield containing DAPI or propidium iodide (Vector Laboratories, Burlingame, USA) and imaged with an upright fluorescent microscope (Axioplan-2 or AxioImager.Z1 with ApoTome grid projection system; both Carl Zeiss, Gottingen, Germany) or a confocal microscope (LSM 510Meta; Carl Zeiss). The levels in the images were adjusted for display purpose using Adobe Photoshop (version 6; Adobe Systems).

**Iontophoretic Labeling**

Several rod bipolar cells were iontophoretically labeled with DiI in normal and in MNU-treated retinas (Dhingra and Smith, 2004). Isolated retina pieces were flattened with photoreceptors facing up on a membrane filter (pore size 0.22 μm; Millipore, USA), mounted on the stage of a McIlwain tissue chopper (Vibratome, USA) and cut into 200 μm thick slices. The slice was placed in a recording chamber on the stage of an upright microscope (Olympus, Japan) and perfused with carboxygenated Ame’s medium at 3-4 ml/minute. A rod bipolar cell soma, tentatively identified by its presence in the outer part of INL, was approached with a DiI-filled (1% in absolute alcohol; Molecular Probes, USA) borosilicate glass electrode (tip resistance 70-120 MΩ) under visual control. A square-wave pulse (200-500 pA, 2 Hz) was applied for approximately one minute to iontophoretically label the selected cell with DiI. The labeled cells that showed characteristic rod bipolar cell morphology were imaged in the confocal microscope.
**Immunoblotting**

To the retina isolated for protein extraction, we added 100 µl of a buffer containing 50 mM Tris (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovandate, 2 % SDS and a cocktail of protease inhibitors (Complete Protease Inhibitor Cocktail; Roche Applied Science, Mannheim, Germany), and placed it on ice for 20 minutes. Extracts were then sonicated and centrifuged at 15°C for 15 minutes at 12000× g. The supernatant was collected and protein concentration was measured by BCA method (BCA kit, Sigma-Aldrich, Bangalore, India). A 10 - 20 µg aliquot of protein per sample was fractionated by SDS-PAGE and electroblotted to activated polyvinylidene fluoride membrane (MDI Membrane Technologies, Ambala, India). The blot was incubated at room temperature for 1 hour in a blocking solution (5% BSA prepared in Tris buffered saline with 0.1% Tween-20, pH 7.4), followed by overnight incubation at 4°C in the primary antibody diluted in the blocking solution. The primary antibodies included mouse anti-**postsynaptic density** (*PSD-95*; 1:3000; Stressgen), rabbit anti-**calbindin** (1:8000; Swant), mouse anti-**protein kinase C alpha** (*PKCα*; 1:2000; Santa Cruz), rabbit anti-**GFAP** (1:4000, Dako), mouse anti-**synaptophysin** (1:4000; Sigma-Aldrich), mouse anti-β-**III-tubulin** (1:3000; Sigma-Aldrich) and mouse anti-β-**tubulin** (1:3000; Sigma-Aldrich).

The blot was then probed with an appropriate horseradish peroxidase-conjugated secondary antibody (Vector Laboratories), and developed by enhanced chemiluminescence method (GE Healthcare or Millipore). The immunoreactive bands were detected either directly with Chemi Genius Bioimaging system (Syngene, Cambridge, UK) or using a photographic film (Amersham Hyperfilm ECL, GE Healthcare). Densitometric analysis was performed with ImageJ software (National Institutes of Health, USA). After the background subtraction, the signal for
each protein was normalized with the signal for the loading control, $\beta$-tubulin. The data (integrated density) was expressed as percentage change in the levels of various proteins for the MNU injected animals from the controls, where the statistical comparison was made with unpaired, one-tailed t-test.

For GFAP, while the normal control showed a single band at ~50 kDa, the MNU-treated animals expressed an additional band at 45 kDa which was also included in the analysis. Multiple GFAP bands have been detected earlier in different tissues and species, which have been considered to represent different isoforms or degradation products of GFAP (Newcombe et al., 1986; Marcus and Easter, 1995).

**Visual Cliff Test**

A Visual Cliff test was employed to evaluate gross visual ability, including visual acuity and depth perception of the animals (Fox, 1965; Crawley, 1999; Krishnamoorthy et al., 2008). The custom apparatus comprised an open-top box (75 cm x 75 cm x 15 cm) made of clear panels of polymethylmethacrylate. The central 30 cm x 30 cm was covered from outside with a gray and white checkerboard (each square 1 cm x 1 cm) pattern. The box was raised one meter above the ground to give the animal a sense of height, while the edge of the checkerboard created the edge of a “cliff”. To minimize the reflections, the room was uniformly illuminated with several downward-facing lamps on the room walls at about 60 cm above the ground. Since the clear base of the apparatus extended beyond the edge of the checkerboard, the cliff was only virtual, and the animal did not actually fall. The test was initiated by placing an animal with its whiskers trimmed on the checkerboard, followed by video-recording (DCR-HC96E, Sony, Tokyo, Japan) its behavior from above for 1-2 minutes in bright light condition (illuminance of 70 $\mu$W/cm$^2$).
The video clips were analyzed offline with AnyMaze software (Stoelting Co., Wood Dale, USA) to measure the amount of time the animal spent on or off the checkerboard, using the center of the animal’s body as the reference point. The apparatus was divided in 3 zones (see Fig. 3A). The central zone included the checkerboard and a 2 cm area surrounding it. The additional 2 cm was included because normal animals frequently stretched their body over the cliff edge to assess the area outside the checkerboard without actually venturing out. The middle zone comprised the region outside the central zone, except a 4 cm border. This 4 cm peripheral zone, which equaled approximately the width of the animal, was designed to exclude the time spent in this zone because sometimes a normal animal used tactile cues from the apparatus walls, and moved only in this zone. When the animal spent two-third of the total time in the peripheral zone, the video recording was discarded (7 of 121 recordings), and when more than half of the recordings for an animal did not pass this criterion, the animal was excluded from analysis (3 of 22 animals). The percent time an animal spent on the checkerboard, computed as the ratio of time spent in central zone to the time spent in central and middle zones, was taken to reflect the visual ability, and thus the functional status of photoreceptors. The statistical comparison between MNU-treated and control animals was made with unpaired, one-tailed t-test. In addition, the entire track of the animal movement was recorded.

RESULTS

Selective and progressive loss of photoreceptors and their terminals

As expected, a single systemic injection of MNU resulted in selective and progressive loss of photoreceptors (Fig. 1). Photoreceptor cell bodies, detected with a nuclear stain (DAPI) or with antibody to recoverin, disappeared nearly completely within a week, although a few were present
in random retinal locations (with slightly higher probability in peripheral retina) even three months after the MNU treatment (Figs. 1A – 1L). The number of cells in the INL and GCL did not appear to change (Figs. 1A – 1F).

Using an antibody to bassoon, synaptophysin or PSD-95, we asked how photoreceptor terminals responded to the MNU treatment. We found that most of the photoreceptor terminals disintegrated and disappeared within a week after the MNU treatment (Figs. 1M – 1X). The levels of PSD-95, measured with immunoblotting and densitometric analysis, also declined progressively by approximately 80% during the first week, and this decrease was maintained during the following three weeks (Figs. 1Y, 1Z).

Because some photoreceptors seemed to escape the MNU effect even after three months, we asked if the surviving cells were rods or cones, or both. Using rhodopsin immunoreactivity and labeling with PNA (Blanks et al., 1993; Li et al., 1995; Krishnamoorthy et al., 2008) we found that the remnant photoreceptors comprised both rods and cones (Fig. 2). In normal retina, rhodopsin was primarily localized in the zone containing rod outer segments, but a weak labeling of the rod inner segments and cell bodies was also observed, as reported earlier (Fig. 2G; Fariss et al., 2000). The number of rhodopsin-immunoreactive cells declined progressively after the MNU treatment (Figs. 2H-2L). However, even at three months after the MNU treatment, a few disintegrated rods were occasionally observed (Fig. 2L). PNA labeled the outer segment, the inner segment and the synaptic terminals of cones in normal retina (Fig. 2M). These structures disintegrated progressively after the MNU treatment (Figs 2N-2R). However, as in the case of
rods, a few cones were observed even at three months after the MNU treatment (Fig. 2R). We found that all of the imaged retinal areas containing remnant photoreceptors comprised both rods and cones (Fig. 2), suggesting that MNU affected both rods and cones with similar sensitivity.

<Figure 2 about here>

**Deterioration of gross visual behavior in visual cliff test**

An MNU-treated or a control animal was placed on the checkerboard in the visual cliff apparatus and allowed to move freely for 1-2 minutes. The video recording was done one day before (-1), and on 1, 2, 5, 7, 14, 21 and 28 days after the injection. Typically, a normal control animal “hesitated” at the cliff edge and did not cross it, while an MNU-injected animal moved in the entire area (Fig. 3A), as reported previously (Krishnamoorthy et al., 2008). The experimental animals (Fig. 3B, *filled circles*) spent 97% ± 2% (mean ± SE; n=13) in the central zone before the MNU treatment. This value progressively decreased after the MNU treatment to 64% ± 7 % (n=13) on day 1 and to 31% ± 6% (n=8) on day 7 (Fig. 3B). There was no significant change subsequently for up to at least day 28 (data not illustrated). On the other hand, the control animals (Fig. 3B, *empty circles*) did not show any significant change over 7 days of testing. Although statistically insignificant, there was progressive decline in the proportion of time the control animal spent in the central zone: 98 ± 2% (n=6) on day -1 that reduced to 76 ± 15% (n=3) on day 7, which could reflect gradual learning by the animal that crossing the cliff edge was safe.

<Figure 3 about here>
Increased Müller cell reactivity and formation of glial seal

Müller glial cells express GFAP within inner half of normal retina, extending radially from GCL to OPL (Fig. 4A). However, retinas of MNU-injected mouse showed enhanced GFAP expression in the hypertrophied Müller cells which now also appeared prominently in the ONL (Fig. 4C, arrowhead). During the first week when photoreceptors were progressively lost and the ONL thickness decreased, the Müller cell processes in the ONL appeared to retract towards inner retina (Figs. 4C – 4E). This resulted in formation of a “glial seal”, which covered the remaining retina by day 28 (Fig. 4F). The glial seal remained unchanged for at least next two months (data not illustrated). The immunoblot analysis showed that GFAP levels increased by more than 90% within a week, and remained approximately 70% higher than the controls during the 4 weeks after the MNU treatment (Fig. 4G, 4H). The sharp peak at day 5 was not significantly different from the values on the following days, and probably represents variability in the data.

<Figure 4 about here>

Remodeling of horizontal cell processes and rod bipolar cell dendrites

To study the effects of MNU-induced photoreceptor loss on second-order retinal neurons and their processes, we immunostained horizontal cells and rod bipolar cells for calbindin and PKCα respectively, and horizontal cell axons for nonphosphorylated neurofilaments (SMI-32). In the control animal, the calbindin was expressed in horizontal cell somata and their fine, bushy dendrites as tiny puncta (Fig. 5A). However, in MNU-treated animals, the labeling with anti-calbindin highlighted a progressive retraction of the dendritic processes which almost completely disappeared within a week (Figs. 5B – 5E). The immunoblot analysis showed that calbindin
levels were approximately 25% lower than the controls during the first week after the MNU injection, but gradually recovered to the control levels during the next 3 weeks (Figs. 5H, 5I). However, the changes in calbindin levels were not statistically significant.

<Figure 5 about here>

The horizontal cell axonal processes, immunostained with SMI-32, which normally make a dense network in the OPL (Fig. 5J, arrowhead), also appeared in the INL after the MNU treatment (Figs. 5K – 5P, arrows), implying axonal sprouting. However, the frequency of finding these sprouts declined progressively, particularly after one week of MNU treatment. The axonal network in the OPL also became progressively scanty and sparse. In fact, the horizontal cell axonal processes both in the OPL and the INL were absent in most areas at 90 days, and when present, they were accompanied by at least some remnant photoreceptors in the same area (data not illustrated).

Rod bipolar cells, identified by PKCα immunostaining, also showed progressive retraction of dendrites after MNU treatment (Figs. 6A – 6G). The dense PKC-immunopositive dendrites of rod bipolar cells in the OPL which were evident in normal retina (Fig. 6A, arrowhead) progressively retracted in the MNU-treated retinas and disappeared completely in about 4 weeks (Fig. 6F, arrowheads). Progressively disappearing dendrites of rod bipolar cells was further confirmed by DiI labeling (Fig. 6H-6J). The immunoblot analysis showed that, similar to calbindin, the levels of PKCα were approximately 25% lower than the controls within two days after the MNU treatment, but gradually recovered to control levels by one week (Figs. 6H, 6I). However, the changes in the PKCα levels were not statistically significant.
Effects of MNU on ganglion cells and their synaptic inputs

*Bassoon*, apart from its expression in ribbon synapses in the OPL, is also expressed in conventional synapses in the IPL. The staining pattern of *bassoon* in the IPL of MNU-treated animal was indistinguishable from the control animal (Figs. 7A – 7C).

*Synaptophysin* is a synaptic vesicle protein that is expressed in all chemical synapses in retina, including the bipolar and amacrine cell inputs to the ganglion cells. Similar to *bassoon*, the pattern of *synaptophysin* expression in the IPL did not appear to change after MNU treatment (Figs. 7D – 7F).

The antibody to nonphosphorylated neurofilament (*SMI-32*) labeled a subset of large retinal ganglion cells in a flatmounted mouse retina (not illustrated). In vertical retinal sections, *SMI-32* stained the ganglion cell axons in the NFL (Fig. 7G). We did not find any qualitative difference in the *SMI-32* immuoreactivity between the control and MNU-treated animal for up to 90 days after the treatment (Figs. 7G – 7I, data illustrated only up to 14 days).

*β-III-tubulin* is expressed in retinal ganglion cells and their dendritic processes in the IPL. The pattern of *β-III-tubulin* immunoreactivity in the MNU-treated animal was similar to that in the control animal (Figs. 7J – 7L). We also found no significant change in the levels of *β-III-tubulin* in MNU-treated animals for up to 28 days after the treatment (n=1; data not illustrated).
DISCUSSION

Retinal remodeling comprises a series of changes in the inner retinal neurons, which are known to follow photoreceptor degeneration in humans and in several genetic animal models. These changes include Müller cell hypertrophy, loss of dendritic processes in the OPL, neuritic sprouting, formation of new connections and cell migration. Because these changes are similar to the ones observed after loss of sensory inputs in other parts of the mature CNS, they are considered a general sequel of deafferentation (Jones et al., 2003; Marc et al., 2003). If so, degeneration of photoreceptors induced by non-genetic factors should also result in similar retinal remodeling. However, the literature on retinal remodeling in inducible models of retinal degeneration is inconclusive.

In a model where photoreceptor degeneration was induced by systemic injection of iodoacetic acid, the bipolar and horizontal cells were reported not to undergo remodeling for up to 6 months (Liang et al., 2008). However, the extent of photoreceptor degeneration in this model varied considerably - among animals, between eyes and within retina (Liang et al., 2008). Moreover, the earliest time point after toxin administration when retinas were analyzed was two weeks, which raises the possibility that horizontal cell axons may have sprouted initially, but were missed when examined at two weeks. In another model where photoreceptor degeneration was induced with exposure to strong light, “extreme” remodeling was demonstrated, including extensive migration of Müller cells and inner retinal neurons into the choroid (Marc et al., 2008). However, it is not clear whether bipolar and horizontal cells in this model undergo the kind of morphological remodeling that has been demonstrated in genetic models (Wasowicz et al., 2002; Marc et al., 2008). In contrast, in the inducible models produced by retinal detachment or kainic acid injection, neuritic sprouting in the second-order retinal neurons has been reported even
though the photoreceptors in these models are either intact or only partially degenerated (Peichl and Bolz, 1984; Fisher and Lewis, 2003; Linberg et al., 2006). From these data, it remains unclear whether the photoreceptor loss of a non-genetic origin causes morphological remodeling in the second-order retinal neurons.

In the present study, we induced a selective and progressive photoreceptor degeneration with MNU and found the time course of photoreceptor degeneration as previously reported (Nambu et al., 1997). This degeneration is different in its etiopathogenesis from that in genetic animal models and in human disease, but they all lead to apoptosis-mediated cell death. The retinal changes that follow the photoreceptor degeneration were also broadly similar: 1) the Müller cells showed enhanced reactivity which was accompanied by formation of glial seal, 2) the rod bipolar and horizontal cells progressively lost their dendrites, and 3) horizontal cell axons showed sprouting (Fariss et al., 2000; Strettoi and Pignatelli, 2000; Park et al., 2001; Marc et al., 2003; Gargini et al., 2007; Barhoum et al., 2008). These data establish that the morphological remodeling in the inner retinal neurons is a consequence of photoreceptor loss independent of the underlying cause, and that mature inner retinal neurons demonstrate remarkable plasticity even in the absence of a genetic mutation.

There were also some interesting differences between the MNU-induced remodeling and that observed in the genetic models or in human. For example, we did not observe any migration of retinal cells, and the remnant retina appeared relatively preserved. Since cell migration is typically observed at a relatively late stage when the remnant retinal cells also start to degenerate (Marc et al., 2003), it is possible that similar changes also occur in the MNU model at a later stage. We also did not find any sprouting of rod bipolar cells into the OPL, which has been reported in AMD patients (Sullivan et al., 2007), perhaps reflecting the very fast and widespread
degeneration of photoreceptors in the MNU model. Our finding that horizontal cell axonal sprouting occurs in the MNU model is similar to the several genetic models reported earlier (Strettoi et al., 2003; Pignatelli et al., 2004; Gargini et al., 2007). However, we also find that the probability of finding the horizontal axons in the OPL and the sprouting in the INL declined progressively, particularly after one week of MNU treatment. It is not clear if other models also show a similar temporal change. It is possible that since horizontal cell axonal network becomes progressively loose and sparse after the photoreceptor degeneration (Strettoi and Pignatelli, 2000), their frequency of occurrence in a radial retinal section becomes proportionately lower. The relatively fast degeneration of photoreceptors in the MNU model could also lead to relatively early disappearance of horizontal cell axons and their sprouting. This hypothesis is consistent with our finding that the axonal processes and sprouts were observed only in areas where at least some photoreceptors remained.

Our qualitative analyses of the ganglion cells and their synaptic inputs suggested that they are not affected by MNU or by the photoreceptor loss. This is consistent with previous observations that ganglion cells remain morphologically and functionally intact in RP patients and in animal models, and can be exploited in designing new therapeutic strategies (Humayun et al., 1996; Nakajima et al., 1996; Santos et al., 1997; Coffey et al., 2002; Meyer et al., 2006; MacLaren et al., 2006; Margolis et al., 2008). However, since we studied the MNU-induced remodeling only up to three months, late changes cannot be ruled out. The MNU model, which has been employed to understand cellular and molecular mechanisms underlying retinal degeneration and to test experimental treatments (Nambu et al., 1997, 1998; Yoshizawa et al., 1999, 2000; Miki et al., 2007; Chen and Nathans, 2007; Wan et al., 2008), offers several advantages, 1) it is cost-effective and easy to generate, 2) it causes rapid photoreceptor
degeneration and retinal remodeling, 3) it allows control over the onset time and the severity of degeneration (Nambu et al., 1998; Chen and Nathans, 2007), and 4) it can be produced in a variety of animals, including non-human primates (Tsubura et al., 2003).

One of the major goals of developing an animal model is to test therapeutic strategies for the disease it represents. The efficacy of an experimental treatment can be evaluated by quantifying the disease-induced changes and how the treatment can reverse them. To that end, we have quantified the photoreceptor degeneration by measuring a specific photoreceptor protein, PSD-95, and a photoreceptor-dependent visual behavior. Other clinical methods, such as electroretinography, are also available to evaluate retinal function (Kiuchi et al., 2003). However, the Visual Cliff test is more comprehensive in terms of measuring an animal’s gross ability to see and to perform a behavioral task which is the ultimate yardstick for evaluating the efficacy of a treatment protocol. In addition, we have measured the levels of several other proteins in an attempt to quantify retinal remodeling. Although the list of cell-specific markers used here is not exhaustive, these measures would serve as reference list with which the post-treatment values could be compared.

For PSD-95 and GFAP, the results from immunocytochemistry and immunoblotting were consistent with each other. However, for PKCα and calbindin, the immunocytochemical results (retraction of dendrites in rod bipolar and horizontal cells) were unpredictable from the immunoblotting results (insignificant change in the protein levels). This is likely because the horizontal cell dendrites express only a small proportion of the protein present in the entire cell. Furthermore, because calbindin is also expressed in a subset of ganglion cells and amacrine cells (Haverkamp and Wässle, 2000), the horizontal cell dendrites express even smaller proportion of the calbindin present in the entire retina.
In summary, we show here for the first time that non-genetically induced photoreceptor degeneration leads to retraction of rod bipolar and horizontal cell dendrites and sprouting of horizontal cell axons. In addition, we provide two quantitative measures, behavioral performance in a visual task and relative amount of certain key retinal proteins, which can be useful not only to study the effects of photoreceptor degeneration, but also to monitor the outcome of potential therapies.
Acknowledgements

We thank Soumya Iyengar for providing synaptophysin antibody and Pankaj Seth for GFAP antibody; Shiv K. Sharma, Ellora Sen and Chinmoyee Maharana for their advice in immunoblotting experiments; Sonia Baloni for her assistance in some of the immunocytochemistry experiments; Stanley Jose and Kailash Tiwari for their assistance in initial behavioral recordings and analysis; Ellora Sen, Shiv K. Sharma and Robert G. Smith for their useful comments on the manuscript.
References


FIGURE LEGENDS

Figure 1. A single systemic injection of MNU resulted in selective and progressive loss of photoreceptors.

A - X) Midperipheral retinal sections of mouse, stained with DAPI (A - F) or anti-recoverin antibody (G - L) show selective and progressive decline in the number of photoreceptors (arrow in A and G) in ONL. A similar decline in the number of photoreceptor terminals, immunostained with anti-bassoon (M – P; horseshoe-shaped structures in OPL, arrowhead in M), anti-synaptophysin (Q – T; bright puncta in OPL, arrowhead in Q) or anti-PSD-95 (U – X; brightly stained cone pedicles and rod spherules in OPL, arrowhead in U) was also observed. Eccentricity (mean ± SE) = 1.0 ± 0.1 mm (recoverin); 0.9 ± 0.1 mm (bassoon); 0.6 ± 0.1 mm (synaptophysin); 1.1 ± 0.1 mm (PSD-95). Pointers and the antibody under study are shown only for controls (extreme left panels) but also apply to other panels in the row. Scale bar: 10 μm (shown in extreme right panel but also applies to other panels in the row). Same labeling plan applies to all other figures containing images.

Y) Representative immunoblots for PSD-95 and β-tubulin for control (c) and up to 28 days after MNU injection.

Z) Western data is expressed as ratio of integrated band density for PSD-95 to β-tubulin and presented for MNU-treated animals as percentage change from normal control (n=3 for days 1 and 7; n=2 for others). * p<0.05; ** p<0.01 (one-tailed t-test).

(n)d = n days after MNU injection, ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = Ganglion cell layer
Figure 2: MNU affected both rods and cones

A-F) DAPI-stained mid-peripheral retinal sections showing progressive decline in the number of photoreceptors. Eccentricity = 0.9 to 1.3 mm.

G-L) Same sections as in the left panel, immunostained for rhodopsin showing progressively degenerating rods. Rhodopsin was localized mainly in the outer segments (arrow), but the inner segment (thin arrow) and cell body (arrowhead) are also weakly stained. Note the presence of a few disintegrated rods even three months after the MNU treatment.

M-R) Same sections as in left panel, stained with PNA-fluorescein showing progressively degenerating cones. PNA labeled the cone outer segment (arrow), inner segment (thin arrow) and synaptic terminals (arrowhead). Note the presence of a few disintegrated cones even three months after the MNU treatment. Scale bar: 10 µm.

Figure 3: Visual behavior of animals in a Visual Cliff test deteriorated progressively after MNU treatment.

A) Track plot of an animal in visual cliff apparatus before and after MNU treatment. The schematic of the apparatus shows a central zone (inner dashed square) that included the central checkerboard (inner solid square) and a 2 cm region around it, a peripheral zone comprising a 4 cm border region (between outer solid and outer dashed squares), and a middle zone (between outer and inner dashed squares). The start and end of the track plot are indicated by a filled triangle and a square respectively. While a normal animal moved mostly within central zone (left panel), after MNU treatment it moved all over the apparatus showing no “hesitation” at the edge of the checkerboard.
B) The time an animal spent in the central zone was taken as a measure of the animal’s gross visual ability. Data were collected from 2 sets of animal: normal controls and MNU-treated. Arrow points to the day when MNU was injected in the treated group. The treated animals were also recorded one day before the injection (day -1) for comparison between and within groups. The proportion of time the MNU-treated animals spent in the central zone (mean ± SE; n=13 for day -1 and day 1, n=11 for 2 days, n=9 for 5 days, and n=8 for 7 days) declined considerably within one day of MNU injection, and kept declining over 7 days. The control animals also showed a slight decrease (mean ± SE; n = 6 for day -1 and day1, n=4 for 2 and 5 days, and n=3 for 7 days), probably attributable to their gradual learning, but this decrease was not statistically significant. *p<0.05; **p<0.01 (one-tailed t-test).

Figure 4. MNU treatment resulted in enhanced Müller cell reactivity

A – F) Compared to normal retina where GFAP expressed in Müller glial cells in inner half of retina (A), MNU-treated mouse retina showed enhanced expression across entire retinal thickness, including ONL (arrowhead in C). With progressive reduction in ONL thickness the glial cell processes retracted and made a “glial seal” that enclosed the remaining retinal cells in INL and GCL (arrowheads in F). The nuclei are stained with propidium iodide (red) for reference. Eccentricity (mean ± SE) = 0.8 ± 0.1 mm. Scale bar: 10 μm.

G) Representative immunoblots for GFAP and β-tubulin. Note that there is a single ~50 kDa band in control animal (c), but an additional 45 kDa band in most of the MNU-
treated animals (see Materials and Methods). Both bands were included in the analysis shown in H.

H) The immunoblot analysis of GFAP (n=3 for up to 7 days and n=2 for later days) showing 91% ± 24% (mean ± SE) increase in GFAP levels at 5 days after MNU treatment. The mean levels remained high (46% to 72% above control) from 7 days to 28 days. *p<0.05; ** p<0.01 (one-tailed t-test).

Figure 5. MNU treatment resulted in retraction of horizontal cell processes.

A – G) In normal retina (A), calbindin is expressed in horizontal cells and their fine dendritic processes (arrowhead) in the OPL. Note a considerable reduction in the number of horizontal cell dendritic processes within one day after MNU treatment (B) and their nearly complete disappearance by one week (E). Eccentricity (mean ± SE) = 1.0 ± 0.1 mm. Scale bar: 10 μm.

H) Representative immunoblots for calbindin and β-tubulin for control (c) and up to 28 days after MNU injection.

I) Immunoblot analysis showing a reduction of approximately 25% in calbindin levels of MNU-treated animals (mean ± SE; n=3 for up to 7 days and n=2 for others) during the first week, which subsequently recovered.

J – P) SMI-32 normally localizes the horizontal cell axonal processes in the OPL (arrowhead in J). However, in MNU treated animals (K – P) these axons sprouted (arrows) into the INL (defined by 2 bars shown at the right border in each panel). Although all the images shown here demonstrate sprouting, the frequency of occurrence of these sprouts declined progressively, particularly after one week, from
occasional at 14 days to rare at 90 days after the MNU treatment. Images in the top panel (A - G) are projections of six confocal planes of 1 μm each (except Fig. 5D which has 5 planes), whereas the images in the lower panel (J - P) are collapsed z-stacks (4 - 10 stacks of 1 μm each) taken with an upright fluorescent microscope (AxioImager.Z1 with ApoTome grid projection system; Carl Zeiss). Eccentricity (mean ± SE) = 0.6 ± 0.1 mm. Scale bar: 10 μm.

**Figure 6. MNU caused progressive retraction of rod bipolar cell dendrites.**

A – G) The rod bipolar cells in normal animal have dense dendrites in the OPL (arrowhead in A) while in the MNU-treated animal (B – G) they progressively lose their dendrites and become completely devoid of dendrites by 28 days (arrowheads in F). Eccentricity (mean ± SE) = 0.9 ± 0.1 mm. Scale bar: 10 μm.

H – J) Representative confocal microphotographs of rod bipolar cells labeled iontophoretically with DiI show progressive disappearance of dendrites over three weeks after the MNU injection. Horizontal bars in each panel denote the extent of INL and IPL. Scale bar (lower right corner in K): 10 μm.

K) Representative immunoblots for PKCa and β-tubulin for control (c) and up to 28 days after MNU injection.

L) The immunoblot analysis revealed only a slight effect of MNU on PKCa levels, but an interesting temporal pattern. The levels (mean ± SE; n=3 for 1, 2 and 7 days and n=2 for others) declined up to approximately 25% initially, but recovered to control levels in a week.
Figure 7. Effect of MNU on ganglion cells and their synaptic inputs

MNU treatment did not appear to affect the expression pattern of bassoon (A - C) and synaptophysin (D - F) in the IPL, of SMI-32 (G – I) in NFL, and of β-III-tubulin (J – L) in IPL and GCL. Images are projections of four confocal planes of 1 μm each.

Eccentricity (mean ± SE) = 0.6 ± 0.3 mm (bassoon); 0.6 ± 0.1 (synaptophysin); 0.5 ± 0.4 (SMI-32); 0.5 ± 0.4 (β-III-tubulin). Scale bar: 10 μm.
Table 1

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A  Representative track plot of an animal

Before MNU  7 days after MNU

B  

% Time in central zone (mean ± SE)

0  20  40  60  80  100

-1  0  1  2  3  4  5  6  7

Days after MNU

Control  MNU

* * * * * *

** ** ** ** **
A control ONL OPL INL IPL GCL

B 1d C 2d D 5d E 7d F 28d

GFAP

G

GAP β-Tubulin

c 1 5 7 14 21 28
Days after MNU

H

GFAP/β-Tubulin (% change)

Days after MNU

* * *