Evaluation of focal photoreceptor degeneration using a multi-focal ERG

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Research article

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Abstract

Background

Photoreceptor degenerative diseases such as retinitis pigmentosa and age-related macular degeneration gradually proceed to photoreceptor degeneration. In animal models of photoreceptor degeneration, it is difficult to evaluate the loss of function in the retina, especially in a focal lesion.

Methods

To induce local lesion in rat retina, one microliter of N-Methyl-N-Nitrosourea (MNU) solution was injected subretinally into a temporal site of the retina. A week after the injection, multifocal electroretinogram (mfERG) was recorded to presume the degenerated site. Based on the data from mfERG, optical coherence tomography (OCT) was performed. After the OCT, eyes were fixed with 4% paraformaldehyde and the flat-mounted retinas were stained with peanut agglutinin (PNA) and wheat germ agglutinin (WGA). Following observation under a fluorescence microscope, the retina was embedded into an OCT compound and cryo-sections were obtained.

Results

The decreased responses in some divisions of the retina were detected in the recording of mf-ERGs. Most of the decreased responses were localized in the temporal part of the retina corresponding to the MNU injection site. The OCT images at the area recorded a lower response of the data from mfERG and showed the severe destruction of photoreceptors; however, a well-organized retinal structure was observed in other parts of the retina. PNA and WGA staining also showed a decreased number of cones and rods in the degenerated area.

Conclusions

Subretinal injection of MNU induced the local photoreceptor degeneration without effects on other retinal layers. mfERGs could detect the focal lesion, and it might be a very helpful indication to perform OCT. These methods would help the evaluation of the effects of regenerative medicine in rodents.

Background

Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are among the major retinal disorders that cause severe visual dysfunction (https://www.nei.nih.gov/learn-about-eye-health/eye-conditions-and-diseases). In RP, many gene mutations, most of them related to the phototransduction pathways (https://sph.uth.edu/retnet/), have been identified. Generally, in the early phase of RP, it starts with the degeneration of rod cells and followed by gradual degeneration of cone cells.[1, 2] On the other

hand, in AMD, cone-rod dystrophy and macular dystrophy, starts with the degeneration of cone cells.[3] However, the development and the pathological conditions of these diseases are varied in independent patients even in patients who have the same mutation. Various types of methods and equipment to diagnose the retinal disorders have been used in the clinical examinations. Fundus photography and optical coherence tomography (OCT) are useful for getting the information about histological changes. Functional loss in the retina can be evaluated using electroretinograms (ERGs) including multifocal ERGs.[4]

Various animal models have been used for evaluating the effects of drugs and gene therapies on these diseases. The rd and rds mice[5] that have a mutation of cGMP-dependent phosphodiesterase and peripherin gene, respectively, are well known as spontaneous models for RP. In the rat model, Royal College Of Surgeons (RCS) rats[6] as a spontaneous model and S334ter and P23H rats [7] with the mutation of the rhodopsin gene have been established as the transgenic lines. Light-damaged [8, 9] and chemical-induced retinal degeneration models [10, 11] were also used for the drug screenings. Thus, there are various types of models with retinal degenerations. On the other hand, the evaluation methods for retinal degeneration are similar to human clinical examinations such as fundus photography, OCT, and ERG. Recently, various regenerative therapies such as stem cells [12], iPS cells [13] and gene therapy [14, 15] have been explored and in case of iPS transplantation studies, the area that cells were transplanted in is strictly limited. However, in the case of rodent models, it is difficult to evaluate the local retinal dysfunction. Development of the methods to investigate focal retinal function in specific rodent models is needed.

In this study, we induced local photoreceptor degeneration in a rat by subretinal injection of N-methyl-N-nitrosourea (MNU) and tried to evaluate the focal retinal dysfunction by using an OCT and a multifocal ERG (mfERG) system. The recorded amplitudes of mf-ERGs in the temporal side corresponding to the injection site were decreased, and the local photoreceptor degeneration was clearly shown by the OCT. These methodologies would be useful for evaluating the photoreceptor degeneration in animal models.

Methods

Animals

All animal experiments were conducted following the guidelines of the Animal Experiment Committee of Iwate University, Japan. Wistar rats were obtained from CLEA Japan (Tokyo) and they were housed in a 12-h/12-h light/dark cycle with access to water ad libitum. Male 6–12 months old wistar rats (n = 16) were used in this study.

Induction Of Focal Photoreceptor Degeneration

N-Methyl-N-Nitrosourea (MNU: Sigma-Aldrich, Tokyo) solution was freshly prepared with dimethyl sulfoxide immediately before use and stored at 4 °C in the dark. The final concentration of MNU solution was adjusted to 0.3 g/ml. Rats were anesthetized by intramuscular injection of 45 mg/kg ketamine and 4.5 mg/kg xylazine. Then, with an operating microscope, an incision was made in the conjunctiva to expose the sclera. One microliter of NMU solution was subretinally injected through the sclera by using an automatic syringe (Neurosyringe AC, ACrux inc., Morioka) with a 32-gauge needle (Hamilton Company, Reno, NV). As a control, the same volume of vehicle was applied to the contralateral eye.

Multi-focal ERG

Seven days after the subretinal injection, multifocal ERGs were recorded. For recording multifocal ERGs, mREC system (ACrux Inc., Morioka) attached to PuREC (Mayo Corp, Aichi) was used (Fig. 1 and Supplementary video). Under anesthetized condition as the same with taking OCT, the rat was placed on the recording tray. The distance from the screen to the eye was set to 19 cm, corresponding to 45 degrees of the viewing angle. The electrodes were attached to the tail, under the tongue and cornea. The recording of mfERG was performed with the setting at 37 retinal divisions.

Optical Coherence Tomography (OCT)

Under the anesthetized condition, their pupils were dilated with tropicamide (Midrin-P, Santen Co., Ltd., Osaka, Japan). A purified sodium hyaluronate (Hyalein Mini ophthalmic solution Santen Co., Ltd., Osaka, Japan) was applied to the eye, to avoid dryness during the procedure of OCT. Image acquisition of 1.1 mm length of the rat retina including the optic disk was performed using the line scan mode on an OCT imaging device equipped with a special ordered lens (RS-3000, NIDEK Co., Ltd., Aichi, Japan).

WGA- And PNA-staining Of Retinal Wholemount And Cryo-section

To evaluate the loss of cones and rods, the staining with peanut agglutinin (PNA), preferentially binds to cone photoreceptors, and wheat germ agglutinin (WGA), binds to rods, were performed.[16] After euthanasia with carbon dioxide, rat eyes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C and retinal whole mounts were made following washing steps with PBS. The specimens were incubated with 3% Bovine serum albumin (BSA) solution including 0.3% TritonX-100 in PBS for 60 minutes at room temperature and then treated with fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA; 1 µg/ml, Vector Laboratories, Tokyo) and Rhodamine-conjugated wheat germ agglutinin (WGA; 5 µg/ml, Vector Laboratories) overnight at 4 °C. After washing with PBS 3 times, the retinal specimen was mounted on a slide using a mounting medium (VECTASHIELD, Vector Laboratories). The retinal wholemount was observed with a fluorescence microscope (AxioVision; Zeiss, Tokyo).

After taking photographs, the retinal specimen was washed with PBS, immersed in 10%, 20%, and 30% sucrose in PBS sequentially, and then embedded in an OCT compound (Sakura Finetek, Tokyo). Ten micrometers of cryo-sections were obtained and observed using the fluorescence microscope.

Statistical analysis

Statistical analyses for in vitro experiments were performed using GraphPad Prism (MDF, Tokyo). The statistical method used was Tukey’s multiple comparison test, Dunnett's multiple comparison test, and Student’s t-test.

Results

Recordings of mfERGs

Retinal dysfunction induced by the subretinal injection of MNU was clearly shown in the 3-D view in mfERGs (Fig. 2A). A higher amplitude around the centre of the retina was recorded compared to the peripheral retina. After the injection of MNU, the center of the retina still had a higher amplitude; however, the decreased responses in the temporal side were recorded. When the highest and lowest response from the pre-recorded value and the value of the post-MNU injection were compared, a significant difference was observed in between the lowest responses though the highest responses were not detected (Fig. 2B).

Optical Coherence Tomography

All the OCT images shown in Fig. 3 were of the same eye as known from the vessel travelling. The OCT image of the centre in the retina including the optic nerve clearly showed a normal retinal structure even after the MNU injection (Fig. 3A, B, C). On the other hand, the photoreceptor degeneration was observed in the temporal part of the retina that corresponded to the MNU injection site (Fig. 3D, E, F). Besides, the OCT image near the centre in the same scanning image (Fig. 3E) showed photoreceptors which survived (Fig. 3G). These results indicated that the area of photoreceptor degeneration induced by the subretinal injection of MNU was limited around the injection site. There was no difference in retinal thickness around the optic nerve in between the normal retina without MNU-injection and MNU-injected retina. However, in the MNU-injected site, the retinal thickness was significantly decreased (Fig. 3H).

WGA And PNA-staining Of Retinal Wholemount And Cryo-section

A wholemount retina showed that PNA and WGA-staining uniformly near the optic nerve (Fig. 4A) but not at the site of subretinal injection of MNU (Fig. 4B). The vertical sections also showed that cones and rods corresponding to PNA and WGA-staining were kept around the optic nerve (Fig. 4C). However, in the

peripheral retina expected as the injection site, PNA and WGA-staining were poor and we also observed the decreased number of the photoreceptor cells (Fig. 4D).

Discussion

Retinitis Pigmentosa and AMD are representative of retinal diseases, which lead to severe visual dysfunction. In the case of AMD, the retinal lesion is often limited, and it does not lead to complete blindness. However, there is no appropriate rodent model with a focal lesion in the retina, especially, photoreceptor degenerations, and no easier method to evaluate the visual function with a focal lesion in rodents. We reported here that the newly developed mfERG system could detect the loss of visual function induced by the focal lesion of the retina.

To induce the local lesion in the retina, we used the subretinal injection of MNU. It has been reported that the systemic application of MNU caused the photoreceptor degeneration by extensive oxidative stress [17] and the intravitreal injection made unilateral photoreceptor degenerations in mice.[18] Unilateral retinal degeneration is useful as an experimental model in primates. However, degenerated area reaches to the whole retinal photoreceptors and the photoreceptor degeneration induced by the systemic application of MNU proceed from central to peripheral [19], this differs from the usual occurrence in patients with a genetic mutation. In addition, it is difficult to induce the focal lesion by systemic or intravitreous injection. We previously reported the experimental model of focal photoreceptor degeneration in rabbits.[20] Intravitreous injection of the appropriate concentration of sodium nitroprusside induced photoreceptor degeneration in the whole retina without the damage of the inner retina. On the other hand, use of the toxic light condition with verteporfin (Novartis AG, Bülach, Switzerland) a photosensitizing dye that is used clinically, without any adverse effects, for patients with subfoveal choroidal neovascularization (CNV), caused the photoreceptor degeneration within the light-exposed area. The latter method is thought to be useful for creating a focal lesion. However, this is difficult to apply in case of rodents as it needs exposure to a focal toxic light.

Recordings of mfERGs showed a decreased response after the subretinal injection of MNU. The 3-D view indicated that the responses of the central retina were higher than those of the peripheral retina (Fig. 2A). The macula has a lot of cones in humans, does not exist in rodents. However, the population of the cones in rodent's retina is also high in the centre of the retina.[21] The responses of the central retina might include the blind spot brought by the optic nerve because the rat eye was set at the centre of the screen to record the mfERGs. However, we could not detect the blind spot on the 3D-view of mfERG. If an ocular axial length assumes 7 mm in rat, the stimulus area is estimated to be 38.465 mm^2. This stimulus area is divided into 37 divisions and a division corresponds to 1.04 mm^2. The division might be too rough to detect the blind spot. Therefore, the blind spot by the optic nerve might be averaged by the high population of cones around the optic nerve. The strongest point of our mfERG system is to record easily, on the other hand, the weak point is that the recorded area does not always correspond to the same area if you rearrange a rat body setting on the recording tray. After the subretinal injection of MNU, the

decreased response at the temporal area was observed (Fig. 2A). The different part of the retina that corresponding to the higher or lower responses in the mfERG also showed a different pattern of PNA and WGA-staining (Fig. 4) and clearly showed the loss of rods and cones.

Conclusions

The present study suggested a useful method to evaluate the focal lesion in the retina by using a combination of the OCT and newly developed mfERG system and to induce a focal lesion by the subretinal injection of MNU. To compare the maximum and the minimum responses from the pre-injection and that from the post-injection was a good way to detect the focal lesion. The degenerated area could be estimated from the results of mf-ERGs, that might be a very helpful indication to performe the OCT. The methodologies including MNU-induced focal lesion would contribute the evaluation on the effects of regenerative medicine such as the iPS transplantation and gene therapies in rodents.

Abbreviations

RP: Retinitis pigmentosa, AMD: age-related macular degeneration, mfERG: multifocal ERG, MNU: N-methyl-N-nitrosourea, PNA: peanut agglutinin, WGA: wheat germ agglutinin,

Declarations

Ethics approval and consent to participate

The animal care strictly conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Iwate University Guidelines for Animals in Research. All protocols (No. A201801-1) were reviewed and approved by the Animal Experiment Committee of Iwate University, Japan.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

KT, TY, and YI performed on mfERG recordings. ES stained the sections with PNA and WGA. TK analyzed the data. HT designed the whole experiments and wrote the manuscript. All authors read and approved the final manuscript.

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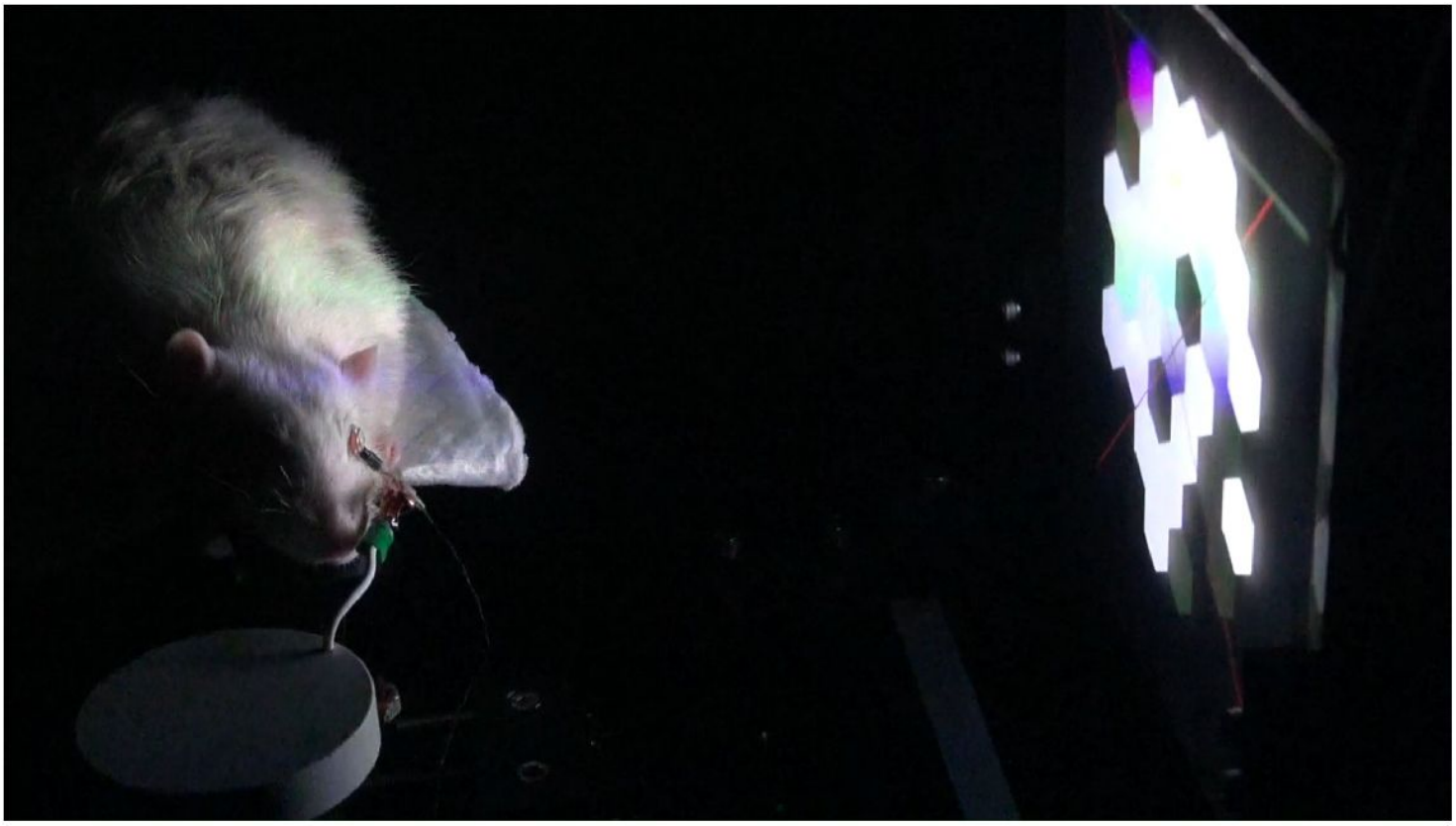
Figures



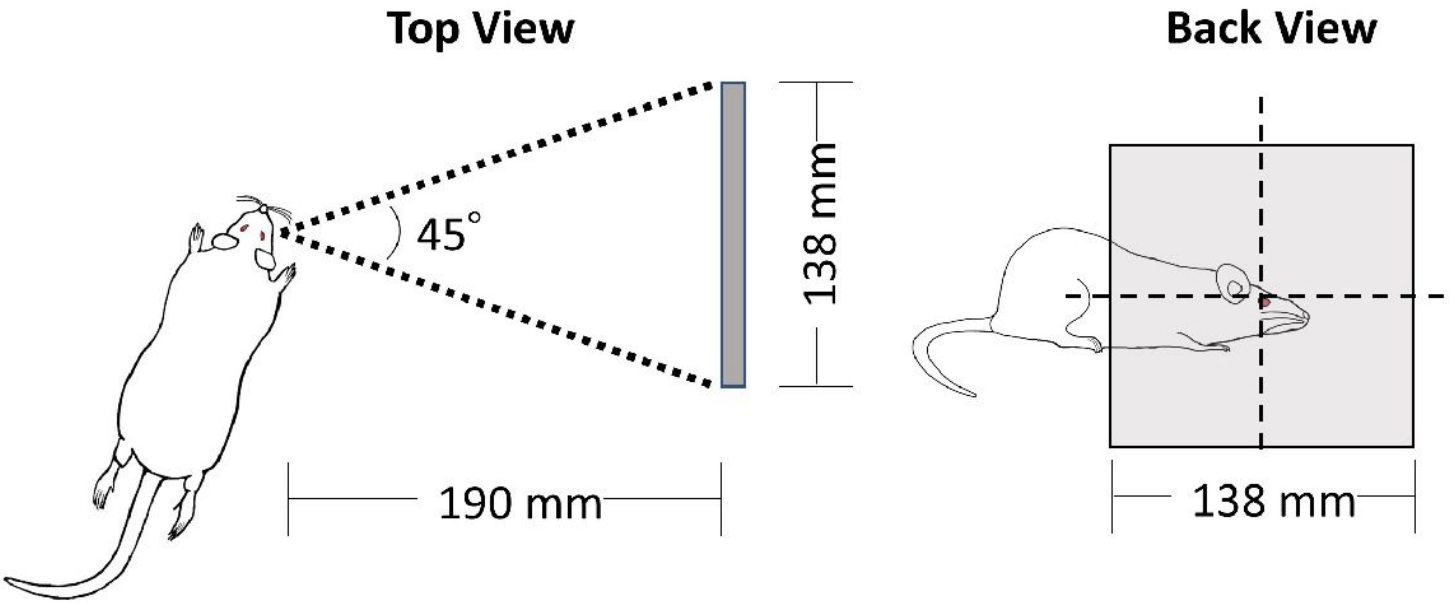


Figure 1

Overview of multifocal ERG system. Flashing of a hexagon is randomly presented on the screen divided into 37 areas. The screen was set at 190 mm of distance from the rat eye and the screen wide was 138 mm. The position of the eye was exactly set at the centre of the screen. The condition corresponded to the visual angle of 45 degrees.

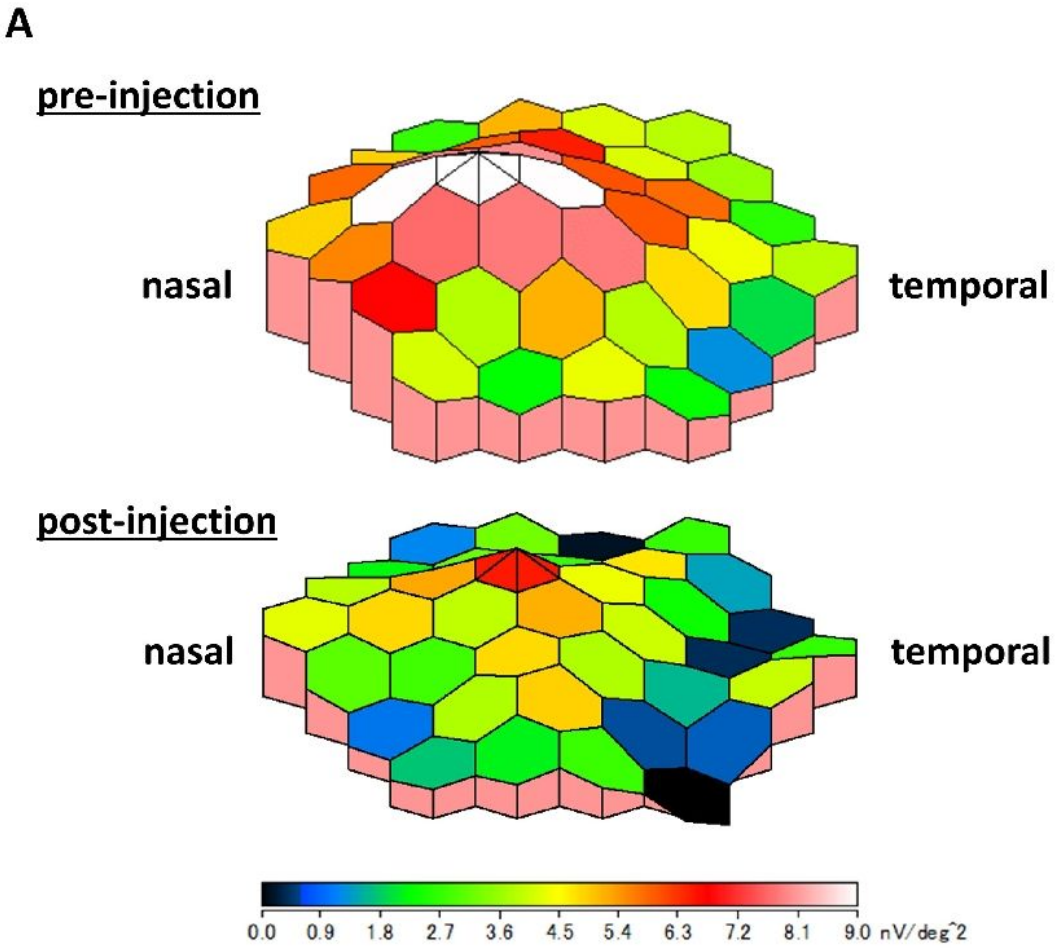


Figure 2

Recordings of mf-ERGs in rats received the subretinal injection of MNU. The subretinal injection of MNU was made at the temporal site. The 3-D views based on the recorded amplitudes clearly showed the differences between the pre- and the post-injection of MNU (A). The minimum response but not the maximum response after the MNU-injection significantly decreased (B). Each data shown as mean ± SEM (n=7, unpaired t-test, *p<0.001).

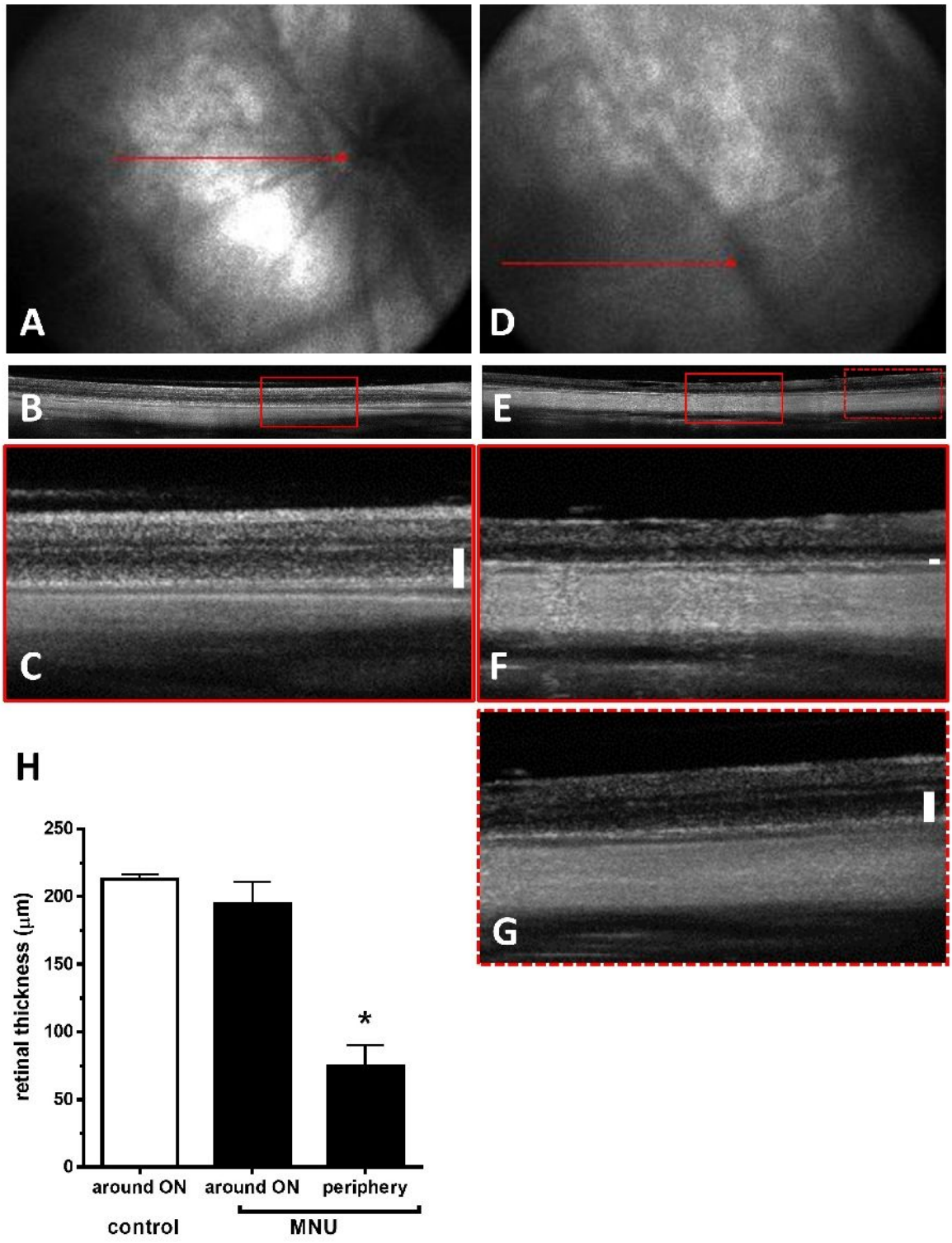


Figure 3

Typical OCT of the retina made a subretinal injection of MNU. Fundus image around the optic nerve (A). A red arrow indicated the site OCT scanning. The whole OCT images scanned in A and D (B, E). Red rectangle areas in B or E were magnified in C or F. Red rectangle with broken line was magnified in G. All OCT images were taken from the same retina. A white vertical bar in C, F and G indicated the photoreceptor layer. The thickness of retinas with (MNU) or without (control) subretinal injection of MNU was measured (H). Data were shown as mean ± SD (n=4, Tukey's test, *p<0.001).

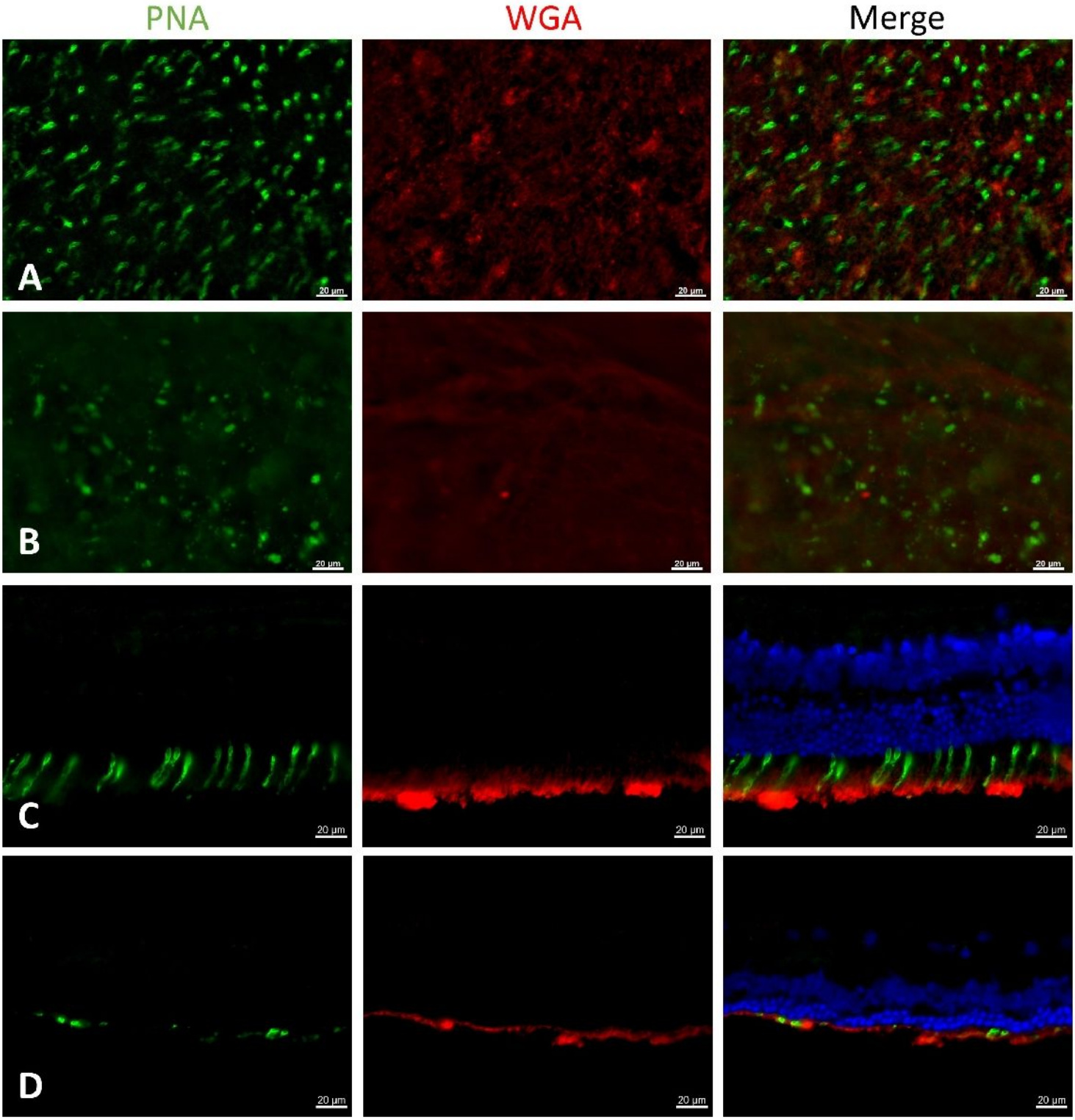


Figure 4

PNA- and WGA-staining of MNU-injected retina. PNA and WGA-staining were uniformly observed near the optic nerve (A). The part of the temporal area showed small staining of PNA, especially WGA (B). Cryo-vertical sections of the areas identified by the staining intensities were made. The areas near the optic nerve and the expected injection site of MNU were shown in (C) and (D), respectively.

Supplementary Files

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