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# Dose-dependent and combined effects of N-methyl-D-aspartate receptor antagonist MK-801 and nitric oxide synthase inhibitor nitro-L-arginine on the survival of retinal ganglion cells in adult hamsters<sup>☆</sup>

Yaoyu Li<sup>1</sup>, An'an Yang<sup>2</sup>, Tingting Zhu<sup>1</sup>, Zhao Liu<sup>2</sup>, Siwei You<sup>2</sup>, Kwok-Fai So<sup>3</sup>

<sup>1</sup>Department of Ophthalmology, General Hospital of Beijing Military Region, Beijing 100700, China

<sup>2</sup>Institute of Neurosciences, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China

<sup>3</sup>Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China

## Abstract

This study investigated the effects of daily intraperitoneal injections of N-methyl-D-aspartate receptor antagonist MK-801 and nitric oxide synthase inhibitor nitro-L-arginine (L-NA) on the survival of retinal ganglion cells (RGCs) at 1 and 2 weeks after unilateral optic nerve transection in adult hamsters. The left optic nerves of all animals were transected intraorbitally 1 mm from the optic disc and RGCs were retrogradely labeled with Fluorogold before they received different daily dosages of single MK-801 or L-NA as well as daily combinational treatments of these two chemicals. All experimental and control animals survived for 1 or 2 weeks after optic nerve transection. Our results revealed that the mean numbers of surviving RGCs increased and then decreased when the dosage of MK-801 (1.0, 3.0 and 4.5 mg/kg) and L-NA (1.5, 3.0, 4.5 and 6.0 mg/kg) increased at both 1 and 2 weeks survival time points. Daily combinational use of 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA lead to a highest RGC number that was even higher than the sum of the RGC numbers in 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA subgroups at 2 weeks. These findings indicated that both MK-801 and L-NA can protect axotomized RGCs in a dose-dependent manner and combinational treatment of these chemicals possesses a potentiative and protective effect.

**Key Words:** retinal ganglion cells; optic nerve transection; N-methyl-D-aspartate receptor; nitric oxide synthase; neuronal survival; hamster

**Abbreviations:** RGCs, retinal ganglion cells; ON, optic nerve; NO, nitric oxide; NOS, nitric oxide synthase; NMDA, N-methyl-D-aspartate

Yaoyu Li<sup>☆</sup>, Ph.D., Professor, Department of Ophthalmology, General Hospital of Beijing Military Region, Beijing 100700, China

Yaoyu Li and An'an Yang contributed equally to this work.

Corresponding author: Siwei You, Professor, Doctoral supervisor, Institute of Neurosciences, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China; Kwok-Fai So, Ph.D., Chair professor, Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China  
yousiwei@fmmu.edu.cn; hrmaskf@hkucc.hku.hk

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## INTRODUCTION

The excitatory amino acid glutamate is one of the major excitatory transmitters in the central nervous system, and its interactions with specific membrane receptors are responsible for many neurobiological functions. Glutamate has been implicated in delayed brain injury after cerebral ischemia and hypoglycemia, and traumatic brain injury also caused a marked elevation in extracellular glutamate adjacent to the trauma site. The effect of excitotoxic insult is caused by an increased formation of nitric oxide (NO)<sup>[1-2]</sup> catalysed by nitric oxide synthase (NOS) which is induced by excess calcium influx *via* N-methyl-D-aspartate (NMDA) subtype of glutamate receptor operated channels. NO released from injured neurons results in a direct massive neuronal death<sup>[3]</sup>. The stimulation of glutamate receptors by excitatory amino acids and released NO has been linked to neuronal degeneration and toxicity. The

selective non-competitive NMDA receptor antagonist dizocilpine maleate (MK-801) and the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) could exert neuroprotective actions in experimentally induced ischemia<sup>[4-5]</sup>. In the visual system, the excitotoxicity induced by glutamate has been thought to be one of the major reasons for the death of retinal ganglion cells (RGCs) caused by glaucoma and retinal ischemia. However, the effects of glutamate are not clear in traumatic optic nerve (ON) injury since activation/inactivation of retinal metabotropic glutamate receptors does not play an important role for the initiation and execution of secondary RGC loss after ON transection and NMDA lesion in the adult rat<sup>[6]</sup>. Whether NMDA antagonists can decrease RGC death remains controversial<sup>[7-10]</sup>, and effects of NO/NOS on the survival of axotomized RGCs are still inconclusive<sup>[11-14]</sup>. Moreover, no studies have ever been reported to examine dose-dependent and combined effects of a NMDA antagonist and NOS inhibitor on the

survival of RGCs after traumatic ON injury. In this study we examined dose-dependent and combined effects of MK-801 and nitro-L-arginine (L-NA) on RGC survival after ON transection (Table 1).

## RESULTS

### Fluorogold-labeled RGCs and glial cells

RGCs of all sizes were labeled with fluorogold. They had large oval somas and very few processes could be seen with the exception of the proximal part of some primary dendrites. Due to uptake of the dye from dead RGCs, fluorogold-labeled glial cells appeared in the retina 1 week after ON transection. Glial cells had smaller somas and irregular cell borders, and their short and branched processes could be seen clearly. When RGC number decreased with the survival time, the number of glial cells increased (Figures 1 and 2).

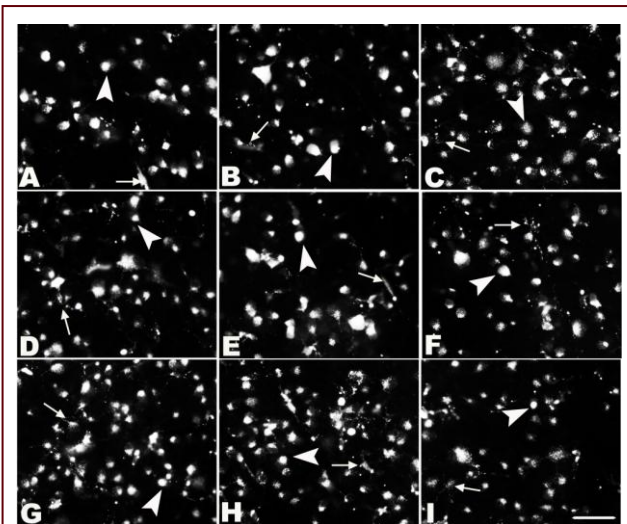


Figure 1 Microphotographs of Fluorogold-labeled surviving retinal ganglion cells 1 week after optic nerve transection in control (A), 1.0 mg/kg MK-801 (B), 3.0 mg/kg MK-801 (C), 4.5 mg/kg MK-801 (D), 1.5 mg/kg nitro-L-arginine (L-NA) (E), 3.0 mg/kg L-NA (F), 4.5 mg/kg L-NA (G), 6.0 mg/kg L-NA (H), combinational 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA (I) groups. Retinal ganglion cells have large circular cell bodies with clear boundaries (arrow heads). A few glial cells with small irregular bodies and short branched-processes are also visible in the retina (arrows). Scale bar: 100  $\mu$ m.

### Surviving RGCs

When compared with the mean numbers of Fluorogold-labeled surviving RGCs at 1 week in control group, no significant difference ( $P > 0.05$ ) in the RGC number could be observed in 1.0 mg/kg MK-801 subgroup. Similar RGC numbers ( $P > 0.05$ ) in 3.0 and 4.5 mg/kg MK-801 subgroups were significantly higher ( $P = 0.001$ ) than those in control group and 1.0 mg/kg MK-801 subgroup at 1 week (Figure 3). When survival time extended to 2 weeks, the RGC numbers in 3.0 and 4.5 mg/kg subgroups remained significantly higher than those in 1.0 mg/kg subgroup and control group ( $P < 0.001$ ). The

highest RGC numbers at 1 and 2 weeks were obtained in 3.0 mg/kg MK-801 subgroup (Figure 4 and Table 1).

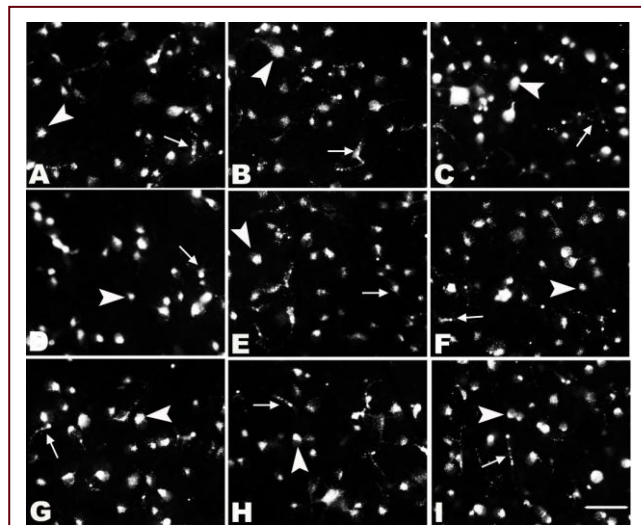


Figure 2 Microphotographs of Fluorogold-labeled surviving retinal ganglion cells (arrow heads) 2 weeks after optic nerve transection in control (A), 1.0 mg/kg MK-801 (B), 3.0 mg/kg MK-801 (C), 4.5 mg/kg MK-801 (D), 1.5 mg/kg nitro-L-arginine (L-NA) (E), 3.0 mg/kg L-NA (F), 4.5 mg/kg L-NA (G), 6.0 mg/kg L-NA (H), combinational 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA (I) groups. A few glial cells with small irregular bodies and short branched processes are also visible in the retina (arrows). Scale bar: 100  $\mu$ m.

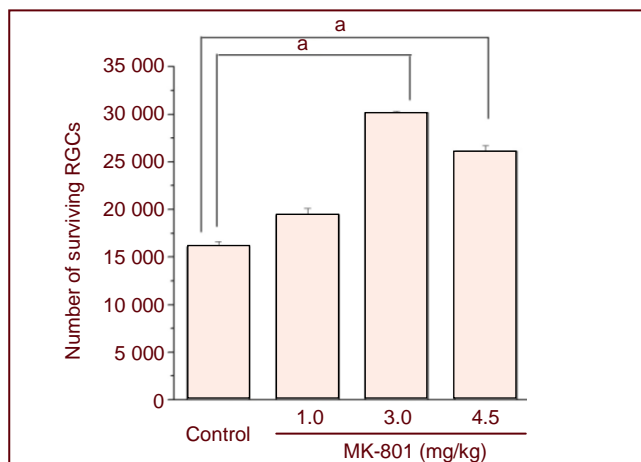


Figure 3 The effect of MK-801 on the survival of axotomized retinal ganglion cells (RGCs) at 1 week after MK-801 injection. The number of surviving RGCs increased markedly when 3.0 mg/kg MK-801 was injected. Although the number of RGCs decreased slightly when the dosage of 4.5 mg/kg was given, the number of RGCs was still significantly higher than that in control and 1.0 mg/kg MK-801-injected animals ( $^aP < 0.001$ ). The data are represented by mean  $\pm$  SEM, and analyzed using one-way analysis of variance.

The RGC numbers were elevated with increased dosage of L-NA at 1 week. The highest RGC number in 4.5 mg/kg L-NA subgroup was then followed by a slightly lower but similar RGC number in 6.0 mg/kg L-NA

subgroup ( $P > 0.05$ ). All these 4 numbers were significantly higher ( $P < 0.001$ ) than those in control group (Figure 5). Although there was no significant difference ( $P > 0.05$ ) in the RGC numbers between 1.5 mg/kg L-NA subgroup and control group at 2 weeks, increased dosage of L-NA at 3.0, 4.5 and 6.0 mg/kg resulted in significantly higher RGC numbers ( $P = 0.0001$ ), with the highest RGC number also in 4.5 mg/kg L-NA subgroup (Table 1 and Figure 6).

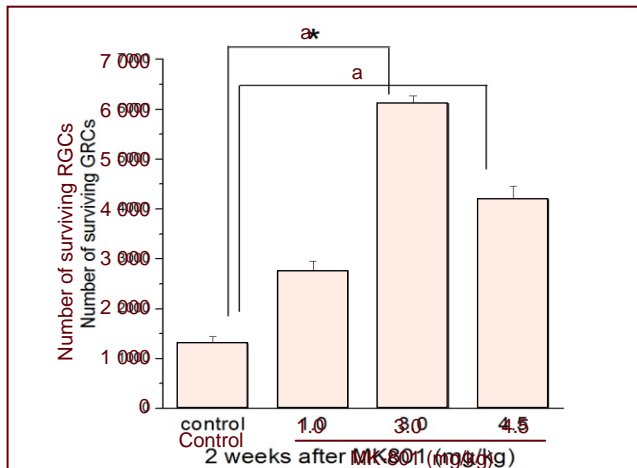


Figure 4 The effect of MK-801 on the survival of retinal ganglion cells (RGCs) at 2 weeks after MK-801 injection. The number of surviving RGCs was significantly increased with treatment of a higher dosage of 3.0 or 4.5 mg/kg when compared to that in control animals. The highest number of surviving RGCs was also obtained with daily injection of 3.0 mg/kg MK-801 for this time point ( $^aP < 0.001$ ). The data are represented by mean  $\pm$  SEM, and analyzed using one-way analysis of variance.

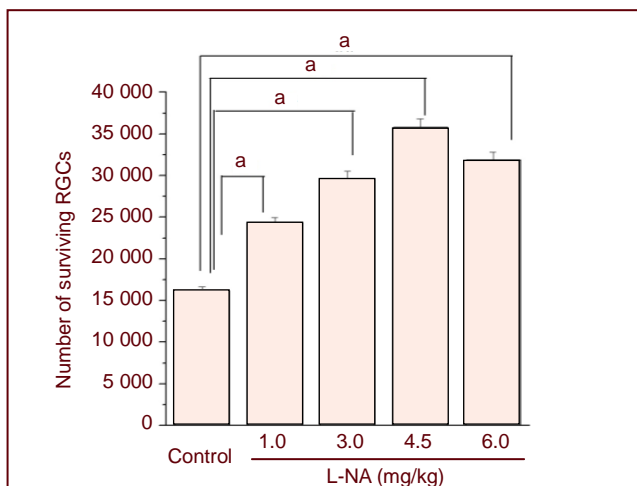


Figure 5 The effect of nitro-L-arginine (L-NA) on the survival of retinal ganglion cells (RGCs) at 1 week after L-NA injection. The highest number of RGCs was found at 4.5 mg/kg and all the RGCs numbers in the experimental subgroups were significantly higher than those in the control animals ( $^aP < 0.001$ ). The data are represented by mean  $\pm$  SEM, and analyzed using one-way analysis of variance.

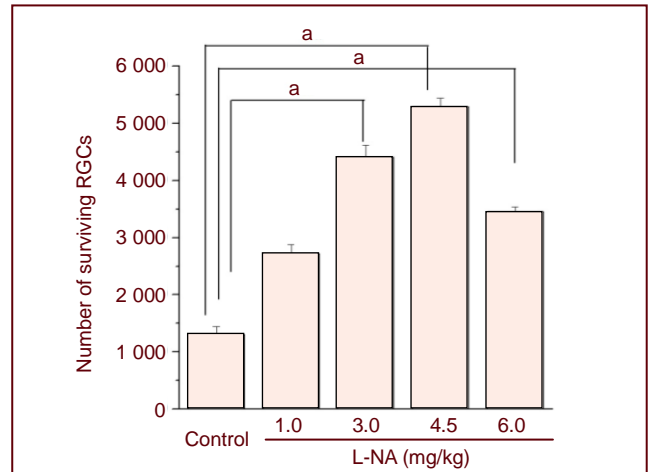


Figure 6 The effect of nitro-L-arginine (L-NA) on the survival of retinal ganglion cells (RGCs) at 2 weeks after L-NA injection. There was no significant difference in the RGCs number between 1.5 mg/kg L-NA subgroup and control group. Increased dosages of L-NA at 3.0, 4.5 or 6.0 mg/kg resulted in increased RGCs numbers with the highest number of RGCs also being at 4.5 mg/kg ( $^aP < 0.001$ ). The data are represented by mean  $\pm$  SEM, and analyzed using one-way analysis of variance.

The RGC numbers in combination group were significantly higher than those in control groups ( $P < 0.001$ ) at 1 and 2 weeks (Figure 7).

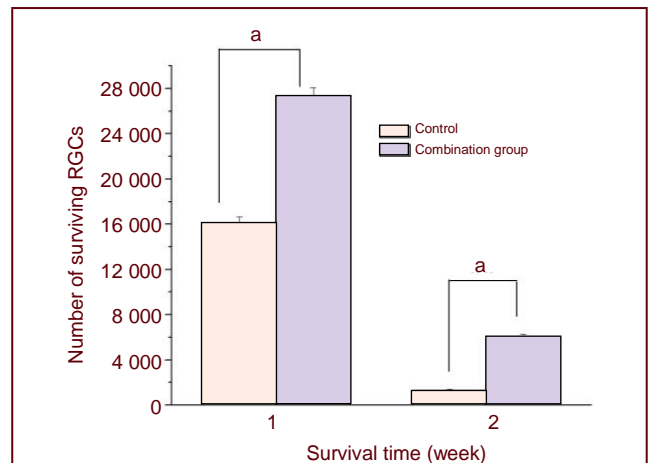
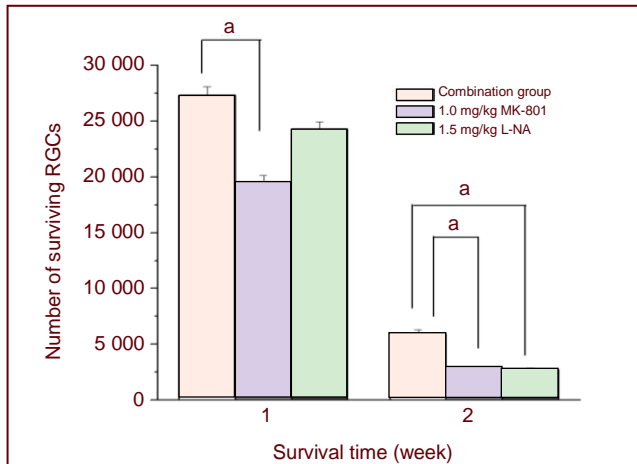


Figure 7 The effect of combinational use of MK-801 and nitro-L-arginine on the retinal ganglion cells (RGCs) survival at 1 and 2 weeks after injection. Significantly higher RGCs numbers were revealed in combination group at both time points ( $^aP < 0.001$ ). The data are represented by mean  $\pm$  SEM, and analyzed using one-way analysis of variance.

When compared to those in 1.0 mg/kg MK-801 subgroup and 1.5 mg/kg L-NA subgroup at 1 week, significant difference was only detected between combination group and 1.0 mg/kg MK-801 subgroup ( $P < 0.001$ ). At 2 weeks, the RGC number in combination group was significantly higher ( $P = 0.0004$ ) than those in both 1.0 mg/kg MK-801 subgroup and 1.5 mg/kg L-NA subgroup.

Moreover, the effect of the combinational treatment of MK-801 and L-NA was potentiated in comparison with the added effects of MK-801 or L-NA alone because the RGC number in combination group at 2 weeks (5 900) was higher than the added number of 2 749 in 1.0 mg/kg MK-801 subgroup and 2 696 in 1.5 mg/kg L-NA subgroup (Figure 8 and Table 1).



**Figure 8** The effect of MK-801, nitro-L-arginine (L-NA) alone or their combination on the retinal ganglion cells (RGCs) survival at 1 and 2 weeks. The RGCs numbers in combination group at both time points were significantly higher than those in 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA subgroups ( $^aP < 0.001$ ). The data are represented by mean  $\pm$  SEM, and analyzed using one-way analysis of variance.

**Table 1** Numbers of Fluorogold-labeled retinal ganglion cells at two different survival time points in all control and experimental groups

Group	Survival time (week)	
	1	2
Normal without optic nerve cut	76 810 $\pm$ 3 644	
Control with optic nerve cut	16 121 $\pm$ 1 263	1 296 $\pm$ 367
1.0 mg/kg MK-801 treatment	19 444 $\pm$ 1 718	2 749 $\pm$ 504
3.0 mg/kg MK-801 treatment	30 145 $\pm$ 496	6 107 $\pm$ 412
4.5 mg/kg MK-801 treatment	26 065 $\pm$ 1 595	4 189 $\pm$ 674
1.5 mg/kg L-NA treatment	24 325 $\pm$ 1 624	2 713 $\pm$ 412
3.0 mg/kg L-NA treatment	29 531 $\pm$ 2 419	4 406 $\pm$ 529
4.5 mg/kg L-NA treatment	35 769 $\pm$ 2 503	5 287 $\pm$ 381
6.0 mg/kg L-NA treatment	31 843 $\pm$ 2 420	3 445 $\pm$ 238
Combinational use of 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA	27 319 $\pm$ 1927	5 900 $\pm$ 959

Examination of the right retina from hamsters of all groups indicated that daily injections of MK-801 and/or L-NA, and the long term labeling of the RGCs with fluorogold for at least 11 weeks did not affect the intact RGCs, and hence the results of the present study.

## DISCUSSION

Neuronal production of NO is triggered when glutamate binds to and activates a NMDA receptor on an adjacent

neuron. A channel in the receptor is therefore opened, admitting  $\text{Ca}^{2+}$  into the cell where it binds to calmodulin. The  $\text{Ca}^{2+}$ /calmodulin complex then binds to and activates NOS so that, in the presence of oxygen and 5,10-nicotinamide-adenine dinucleotide phosphate reductase, NOS converts L-arginine to L-citrulline and NO which is then released from the cell. It has been reported that NMDA-receptor blockade with the NMDA receptor antagonist MK-801 enhances cell apoptosis in the developing retina of the postnatal rat<sup>[4]</sup> and a noncompetitive NMDA receptor antagonist, Bis(7)-tacrine, can prevent glutamate-induced rat RGCs damage *in vitro* and *in vivo*<sup>[9]</sup>. However, MK-801 at 0–100  $\mu\text{M}$  can not significantly attenuate the  $\text{H}_2\text{O}_2$ -induced cytotoxicity<sup>[10]</sup>. We have demonstrated in the present study that daily intraperitoneal injections of MK-801 increased neuronal survival of RGCs following ON transection in a dose-dependent manner. At both 1 and 2 weeks, the numbers of surviving RGCs were not promoted significantly with the treatment of 1.0 mg/kg MK-801 when compared to control groups. However, RGC numbers increased significantly with a higher dosage of 3.0 or 4.5 mg/kg. Both the highest RGC numbers at 1 and 2 weeks were obtained with the treatment of MK-801 at 3.0 mg/kg.

It is also interesting that either increased or decreased NOS expression has been shown after neuronal injury<sup>[11–16]</sup>. NOS expression can also remain unchanged within the adult rodent retina up to 4 months following intraorbital ON transection<sup>[11, 17]</sup>. The finding that lesion-induced NOS within injured neurons is coincident with death of these neurons which suggests a role of NO in neuronal degeneration. If NO, produced by lesion-induced NOS, is responsible for the death of injured neurons, inhibition of NOS should therefore prevent the death of these neurons. However, although this hypothesis has been widely supported by a large number of studies using NOS inhibitors<sup>[18–19]</sup>, the effects of NO on neuronal survival can be either protective or destructive<sup>[18]</sup>. Such paradoxical phenomena may probably be interpreted as differences in distinct response to axonal injury among NOS-containing neuron groups. In two main lines of investigations, NOS expression is markedly augmented in spinal neurons after spinal root avulsion<sup>[20–21]</sup> or spinal cord transection<sup>[21]</sup> and in the hypothalamic supraoptic nuclei and paraventricular following hypophysectomy<sup>[19, 22]</sup>. Inhibition of NOS protected RGCs against glaucomatous damage<sup>[14]</sup>. L-N(6)-(1-iminoethyl)lysine 5-tetrazole amide, a prodrug of a selective inhibitor of inducible NOS, prevented the loss of retinal ganglion cells in eyes with chronic, moderately elevated intraocular pressure in an animal model of glaucoma<sup>[13]</sup>. Daily intraperitoneal injection of L-NA in the present study also retarded the degeneration of axotomized RGCs in a dose-dependent fashion. This protective effect may be achieved by the inhibition of NO production in adjacent amacrine cells with L-NA.



In this experiment, RGC loss after ON transection was reduced, but not completely prevented, by NMDA receptor antagonist and NOS inhibitor. There are several explanations for this incomplete neuroprotective effect of NOS inhibitors: (1) the lesion-induced NOS was not completely inhibited by MK-801 and L-NA at the dose level used; (2) lesion was not the only stimulus to induce NOS/NO responsible for the death of injured RGCs; (3) the neurotoxicity of glutamate could not be overcome completely with MK-801; the antagonist of the NMDA subtype of glutamate receptors, as it has been reported recently that low doses of glutamate can activate  $\alpha$ -amino-3-diglydro-5-methyl-isoxazol-4-propionic acid-kainate receptors in cultured RGCs, which causes increases in intracellular calcium and decreases in cell survival<sup>[23]</sup>. Also, there might be other than NMDA and NOS death mechanisms activated after ON transection. The mechanisms for the protective effects of these NOS inhibitors might be both indirect, reducing the production of NO which is synthesized in and released from adjacent amacrine cells in either the inner nuclear layer or the ganglion cell layer, and direct, blocking the neurotoxicity of glutamate with MK-801 on injured RGCs. We have also shown that combinational injections of 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA, neither of the dosages are the optimal ones, enhanced the survival of RGCs 1 and 2 weeks after ON transection, suggesting the potentiative action of these two chemicals, which influence different phases of NO biosynthesis on the same biochemical pathway, on the survival of axotomized RGCs. NO synthesis can be inhibited by MK-801 as a NMDA antagonist or L-NA as a competitive NOS inhibitor, thus inhibiting NOS more effectively. Our findings indicated that both MK-801 and L-NA can protect axotomized RGCs in a dose-dependent manner and combinational treatment of these two chemicals possesses a potentiative and protective effect.

## MATERIALS AND METHODS

### Design

A randomized controlled animal experiment.

### Time and setting

This study was conducted in the Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong and Institute of Neurosciences, the Fourth Military Medical University from February to December, 2011.

### Materials

One hundred and fourteen adult male hamsters of 6–7 weeks old (*Mesocricetus auratus*, the Laboratory Animal Units of the University of Hong Kong and the Fourth Military Medical University) were divided into one control and three experimental groups (MK-801, L-NA and combination groups).

### Methods

#### Pre-labeling of RGCs and ON transection

All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight;

Ovation Pharmaceuticals, USA) for all surgical procedures. The RGCs of the animals were retrogradely labeled by applying small pieces of Gelfoam (Upjohn, USA) soaked in 5% fluorogold (Fluorochrome, USA) onto the surfaces of bilateral superior colliculi after the cortical tissues and pia overlying the superior colliculi was carefully removed. Six animals in control group were allowed to survive for 4 days after pre-labeling without ON transection. The left ONs of all other 108 animals were exposed 4 days later through a superior temporal intraorbital approach and transected intraorbitally 1 mm from the optic disc under an operating microscope (Olympus OME, Japan). Care was taken to avoid any damages to retinal blood supply from the ophthalmic artery<sup>[24]</sup>.

### Treatments

One day before the left ON transection, 84 animals received intraperitoneal injections of different dosages of MK-801 (1.0, 3.0 or 4.5 mg/kg; ICN Pharmaceuticals, USA) and L-NA (1.5, 3.0, 4.5 or 6.0 mg/kg; Aldrich, USA) in sterile saline in MK-801 and L-NA groups ( $n = 12$  for each dosage) and survived for 1 or 2 weeks ( $n = 6$  for each time point). Another 12 animals in combination group received daily intraperitoneal injections of combinational 1.0 mg/kg MK-801 and 1.5 mg/kg L-NA until the animals were killed 1 or 2 weeks following ON transection ( $n = 6$  for each time point). Another 12 control animals received IP injections of the same volume of saline before they were killed 1 or 2 weeks after ON transection ( $n = 6$  for each time point).

### Counting of Fluorogold-labeled surviving RGCs

All animals were sacrificed with an overdose (100 mg/kg body weight) of anesthesia at the appropriate survival time. The left retinas were removed, post-fixed in 4% paraformaldehyde (Merck, Germany) in PBS (0.1 M, pH 7.4) for 1 hour, rinsed in PBS (0.1 M, pH 7.4) for 3 times, divided into superior nasal, superiotemporal, inferionasal and inferiotemporal quadrants with four radial cuts (Figure 9), flat-mounted on gelatin-coated slides in 30% glycerol (Merck, Germany) and eventually coverslipped.

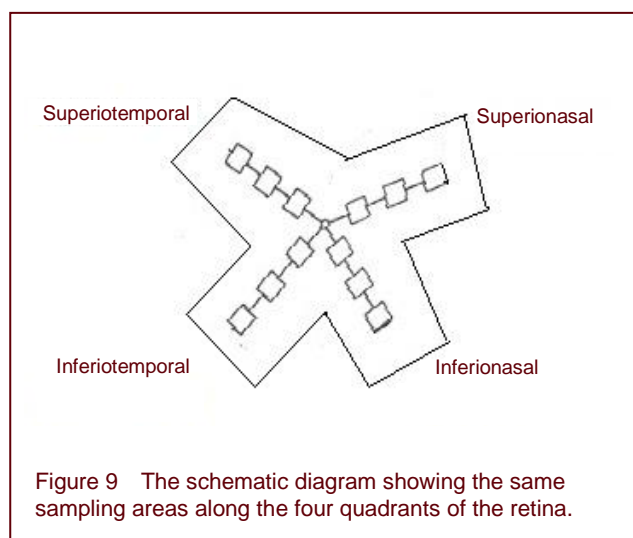


Figure 9 The schematic diagram showing the same sampling areas along the four quadrants of the retina.

The total populations of Fluorogold-labeled surviving RGCs in the retinas were estimated using a sampling technique<sup>[25]</sup>. In each retina, the number of RGCs was counted in 28–30 sample areas (each occupying 40 000  $\mu\text{m}^2$ ) at 7 to 8 different eccentricities from the optic disc in four different quadrants of the retina under a BX51 fluorescence microscope (Olympus, Japan) using a 355–425 nm ultraviolet filter. The data from all sample areas were pooled and the total number of RGCs counted was divided by the areas sampled, thus obtaining the estimated mean density of surviving RGCs. Finally, the estimated number of surviving RGCs in each retina was calculated by multiplying the mean density of RGCs with the area of the retina.

### Statistical Analysis

One-way analysis of variance was used to compare the mean numbers of surviving RGCs using SPSS (Version 11.0, USA). All data values were presented as Mean  $\pm$  SEM and differences were considered significant when  $P < 0.05$ .

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**Author contributions:** Yaoyu Li and An'an Yang performed the experiments. Tingting Zhu and Zhao Liu conducted the statistical analysis and completed all figures. Siwei You guided this study, wrote the manuscript and contributed to part of the fundings. Kwok-Fai So designed this study and was in charge of part of the fundings.

**Conflicts of interest:** None declared.

**Ethical approval:** Animal Care and Use Committees at the University of Hong Kong and the Fourth Military Medical University approved all animal protocols in this study.

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