

## Original Article

# Upregulated expression of N-methyl-D-aspartate receptor 1 and nitric oxide synthase during form-deprivation myopia in guinea pigs

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**Abstract:** This study aimed to investigate the expression of N-methyl-D-aspartate receptor 1 (NMDAR1) and neuronal constitutive nitric oxide synthase (ncNOS) during form-deprivation myopia (FDM). FDM models were established in guinea pigs with facemasks. NMDAR1 expression in the retina was detected by immunohistochemistry and Western blot analysis. ncNOS mRNA expression was examined by in situ hybridization. cGMP content was measured by radioimmunoassay. In control group, NMDAR1 and ncNOS were expressed in binocular retinas, and there was no significant difference in NMDAR1 and ncNOS expression and cGMP content between the two eyes. However, NMDAR1 and ncNOS expression and cGMP content in the retina of FDM eyes were significantly higher than that of contralateral untreated eyes. Furthermore, ncNOS mRNA level and cGMP content was highly correlated. In conclusion, FDM upregulates the expression of NMDAR1 and ncNOS and increases cGMP content in the retina. NMDAR1/NO-cGMP pathway may contribute to abnormal visual signals during myopic progression.

**Keywords:** Retina, myopia, NMDAR1, nitric oxide, cGMP

## Introduction

The occurrence and development of myopia are sophisticated processes, which are closely related to the development and early experience of vision [1]. During vision development, form deprivation induces a variety of neurotransmitters and factors in the retina, leading to the occurrence of myopia through particular signal transduction pathways [2, 3]. However, the mechanism by which signaling pathway regulates myopia progression remains controversial.

Glutamate, synthesized from glucose and other precursors, is an important excitatory neurotransmitter in the retina and plays a key role in retinal synaptic circuitry [4]. In physiological conditions, glutamate is released into the synaptic cleft and binds to the receptors in post-synaptic membrane to exert effect. However, glutamate causes excitotoxicity in pathological conditions [5]. Previous studies demonstrated that glutamate produced excitotoxicity via bind-

ing with N-methyl-D-aspartate receptor (NMDAR) and activating the nitric oxide-cyclic guanine monophosphate (NO-cGMP) pathway, and led to a series of pathophysiologic changes of the retina [6]. NMDAR, an important ionotropic glutamate receptor, is a heterotetramer composed of NMDAR1 and NMDAR2. NMDAR1 is the indispensable component for the activation of receptors, and glutamate is a potent endogenous excitatory ligand [7]. The activation of NMDAR1 by glutamate increases Ca<sup>2+</sup> influx and release into the cytoplasm, resulting in the activation of nitric oxide synthase (NOS) and the synthesis of nitric oxide (NO). NO subsequently activates guanylate cyclase (GC) and increases the synthesis of cGMP, which mediates important physiological function such as the conductance of ion channels [8].

There is circumstantial evidence that NMDAR1 plays a critical role in vision development, neural survival and synaptic plasticity [9]. Manta et al. demonstrated the developmental and visual experience-dependent regulation of NMDAR1

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splicing in rat retina [10]. The change of NMDAR1 expression in the visual cortex might underlie amblyopia development [11, 12]. In addition, NMDAR antagonists suppressed FDM in chicks, suggesting a prominent role for NMDA receptors in FDM and ocular growth-control [13]. However, it is unclear whether NMDAR1 regulates myopia progression and the molecular mechanisms by which NMDAR1 regulates myopia remain to be defined.

In this study, we established form-deprivation myopia (FDM) model in guinea pigs by noninvasive facemasks, and used this model to investigate the potential role of NMDAR1/NO-cGMP pathway in the regulation of myopic progression.

### Materials and methods

#### *Animal studies*

Guinea pigs (160-200 g, 3-week old) were provided by the Experimental Animal Center of Central South University. Guinea pigs were placed in a clean environment, with free access to food and water under a natural day/light cycle, and allowed to acclimate for a week before being used in this study. All procedures were performed in accordance with the guidelines of the Committee on Animals of the Central South University.

#### *Establishment of a FDM model*

FDM model in guinea pigs was established as described previously [14]. Guinea pigs were randomly divided into 3 groups (n = 20): Group I: untreated control (free of FDM); Group II: FDM induced for two weeks; Group III: FDM induced for three weeks. Animal in FDM groups wore a facemask that covered the right eye and the left eyes were contralateral untreated eyes. The facemasks were made and worn as previously described [15]. Briefly, the heads of guinea pigs were covered by the latex balloons, leaving the left eye, nose, mouth and ears exposed to the air. The facemasks were held in place around the head and the mouth of the animals.

#### *Measurement of refraction and axial length*

The measurement of refraction was performed before the experiment, 2 and 3 weeks after the

experiment. One hour before the measurement, 0.25% tropicamide was dropped into the eyes to palsy the ciliary muscle. Retinoscopy was performed by the optometrist in double-blind manner in the dark room. Axial length was measured by the A-scan ultrasound (AVISIO Echograph class I-Type Bat; Quantel Medical, France) while the animals were lightly anaesthetized with 1% halothane oxygen.

#### *Preparation of specimens*

Guinea pigs were euthanized after the measurement of refraction and axial length. Both eyes were enucleated quickly, cut into halves along the equator and cut into halves once again along the optic nerve, with the removal of the vitreous and anterior. In each group, ten experimental specimens were randomly selected and fixed by 10% formaldehyde for paraffin sections. Another half retina was stripped carefully, weighted and stored in the liquid nitrogen for further use.

#### *Immunohistochemistry*

The tissue samples of posterior retina were fixed, dehydrated, embedded and cut into 5  $\mu$ m sections. The sections were incubated overnight at 4°C with polyclonal mouse anti-NMDAR1 antibody (1:1000 dilutions, Catalogue No. AB1516, Chemicon, USA), then with HRP labeled goat anti-mouse IgG (1:3000 dilutions, Zymed, San Francisco, CA, USA) at room temperature for 1 h, followed by incubation with 3,3'-diaminobenzidine (DAB). Finally, the stained slides were counterstained with Hematoxylin and Eosin (HE). The cells with brown granules in the cytoplasm were considered as positively stained cells. Blinded assessment of immunohistochemistry staining of NMDAR1 was scored as (-), (-/+), (+), (++) and (+++), according to the staining intensity and the percentage of positively stained cells.

#### *Western blot analysis*

The retinal tissues were lysed in ice cold lysis buffer containing 10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 50 mM NaF, and protease inhibitor cocktail. The lysates were centrifuged for 15 min at 14,000 rpm at 4°C. The supernatant was collected and the protein concentration was determined by using a MicroBCA protein assay kit

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**Table 1.** Change of refraction in the FDM guinea pigs

Group	I	II	III	F
Right eye	+2.732±1.313**	-1.584±1.328**	-3.501±1.169**	64.33
Left eye	+2.703±1.266*	+2.610±1.214*	+1.983±1.054*	2.27
t	0.64	7.26	11.89	

Group I: untreated control (free of FDM); Group II: FDM induced for two weeks; Group III: FDM induced for three weeks. Data are expressed as mean ± SD (n=20). \*P=0.000, \*\*P=0.000.

**Table 2.** Change of axial length in the FDM guinea pigs (mm)

Group	I	II	III	F
Right eye	7.427±0.057**	7.894±0.076**	8.264±0.075**	169.53
Left eye	7.397±0.049*	7.543±0.065*	7.604±0.074*	20.35
t	1.478	11.257	16.071	

Group I: untreated control (free of FDM); Group II: FDM induced for two weeks; Group III: FDM induced for three weeks. Data are expressed as mean ± SD (n=20). \*P=0.000, \*\*P=0.000.

(Beyotime Biotechnology, China). 50 µg proteins were separated by a 10% denatured SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with polyclonal mouse anti-rat NMDAR1 antibody, followed by incubation with HRP labeled goat anti-mouse IgG. The bands were developed with DAB. The quantitative analysis of NMDAR1 expression was performed by normalizing to β-actin using the image software ImagJ 1.36.

### *In situ hybridization*

ncNOS mRNA was detected by using ncNOS ISH Detection Kit (Boster Biotech, Wuhan, China). The retinal sections were deparaffinized, rehydrated, digested with diluted pepsin and fixed with 4% paraformaldehyde. Subsequently, the sections were prehybridized for 4 h with hybridization buffer followed by hybridization with digoxigenin-labelled probe for ncNOS (2 µg/ml in hybridization buffer) at 37°C overnight. Next the sections were rinsed with 2x saline-sodium citrate (SSC) buffer twice (5 min each time), then with 0.5x SSC for 15 min, and with 0.2x SSC for 15 min. The sections were blocked with normal goat serum and incubated with biotinylated mouse anti-digoxin antibody (1:1,000, Sigma), followed by incubation with streptavidin-peroxidase and DAB. The stained slides were counterstained with HE. Finally, the sections were mounted under coverslips for the observation under conventional microscope. The cells with brown granules in

the cytoplasm were considered as positively stained cells. For negative controls, the control probe provided by the kit was used instead of the probe for ncNOS for the hybridization. Five randomly chosen visual fields were observed on each section. The images were scanned and the gray scales were analyzed with color morphometric image system (CMIS).

### *Radioimmunoassay (RIA)*

The cGMP content was determined by RIA as follows. Briefly, the retinal tissues were homogenized in 50 mM acetate buffer (pH 4.75). Then the homogenate was mixed with 2 ml dehydrated

alcohol and the supernatant was collected after the centrifugation. The supernatant was dried in the oven at 60°C and the cGMP content was determined by using cGMP kit (Shanghai University of Traditional Chinese Medicine) following the manufacturer's protocol.

### *Statistical analysis*

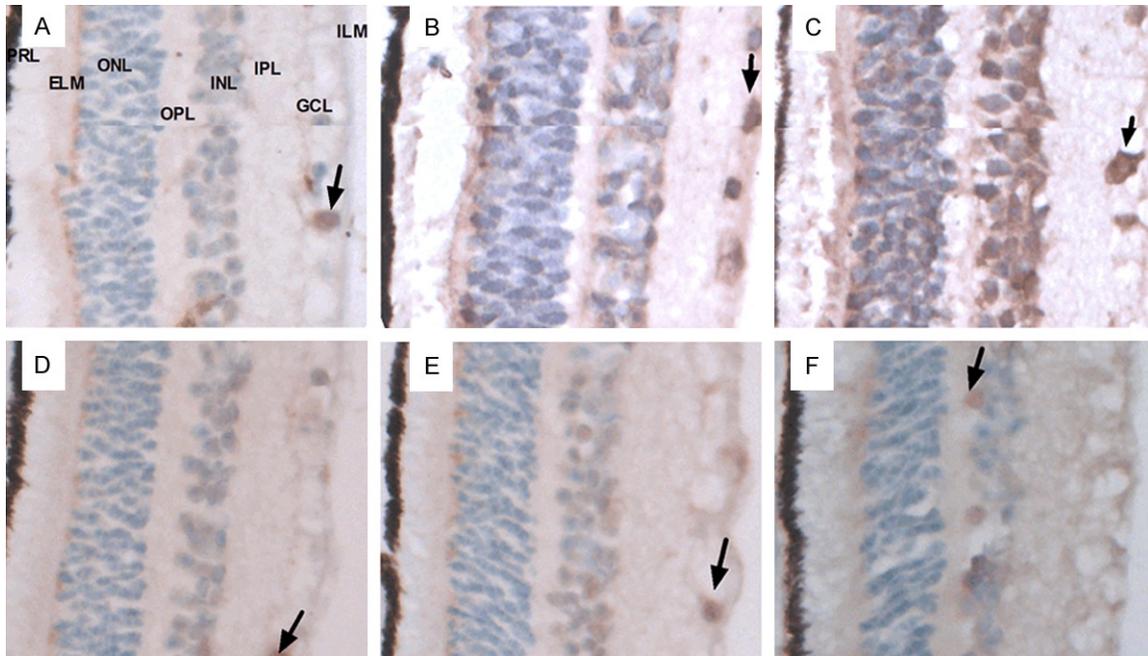
The comparison between two groups was analyzed by Student's unpaired t test. The comparison between the left and right eyes in the same groups was analyzed by Student's paired t test. One-way ANOVA with Newman-Keuls post-test were used to compare the means of three groups. The liner correlation analysis was performed to investigate the relationship between the cGMP and ncNOS expression. P value of <0.05 was considered statistically significant.

## Results

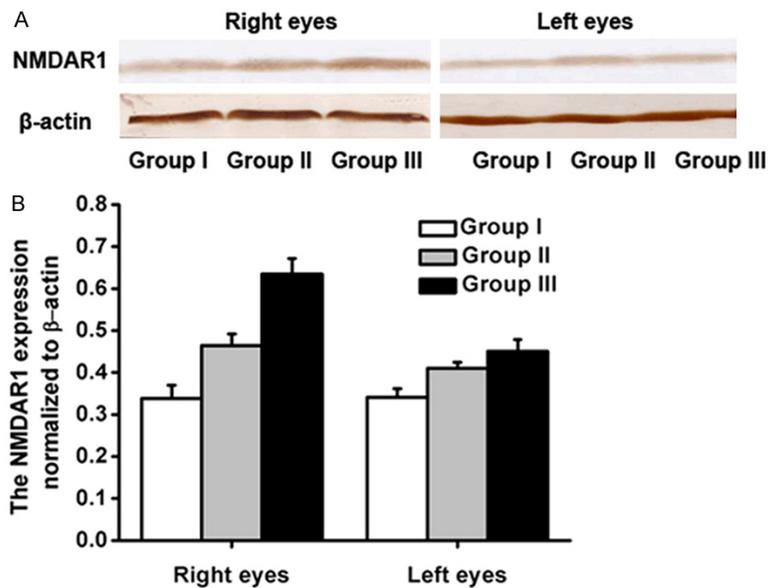
### *Establishment of the FDM model*

The successful establishment of FDM model in guinea pigs was demonstrated by the measurement of refraction and axial length. As shown in **Table 1**, there was no significant difference in refraction between the two eyes in group I (P>0.05). However, in the right eyes, the refraction was lower in group II and group III than in group I (P<0.001). Furthermore, the refraction was lower in the right eyes than in the left eyes in group II and III (P<0.001). The axial length

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**Figure 1.** Immunohistochemistry analysis of NMDAR1 expression in the retina of FDM eyes (right eyes) and untreated eyes (left eyes) in guinea pigs. A. The right eyes of group I; B. The right eyes of group II; C. The right eyes of group III; D. The left eyes of group I; E. The left eyes of group II; F. The left eyes of group III. Magnification: 200 ×. The arrows indicate typical strongly stained areas.



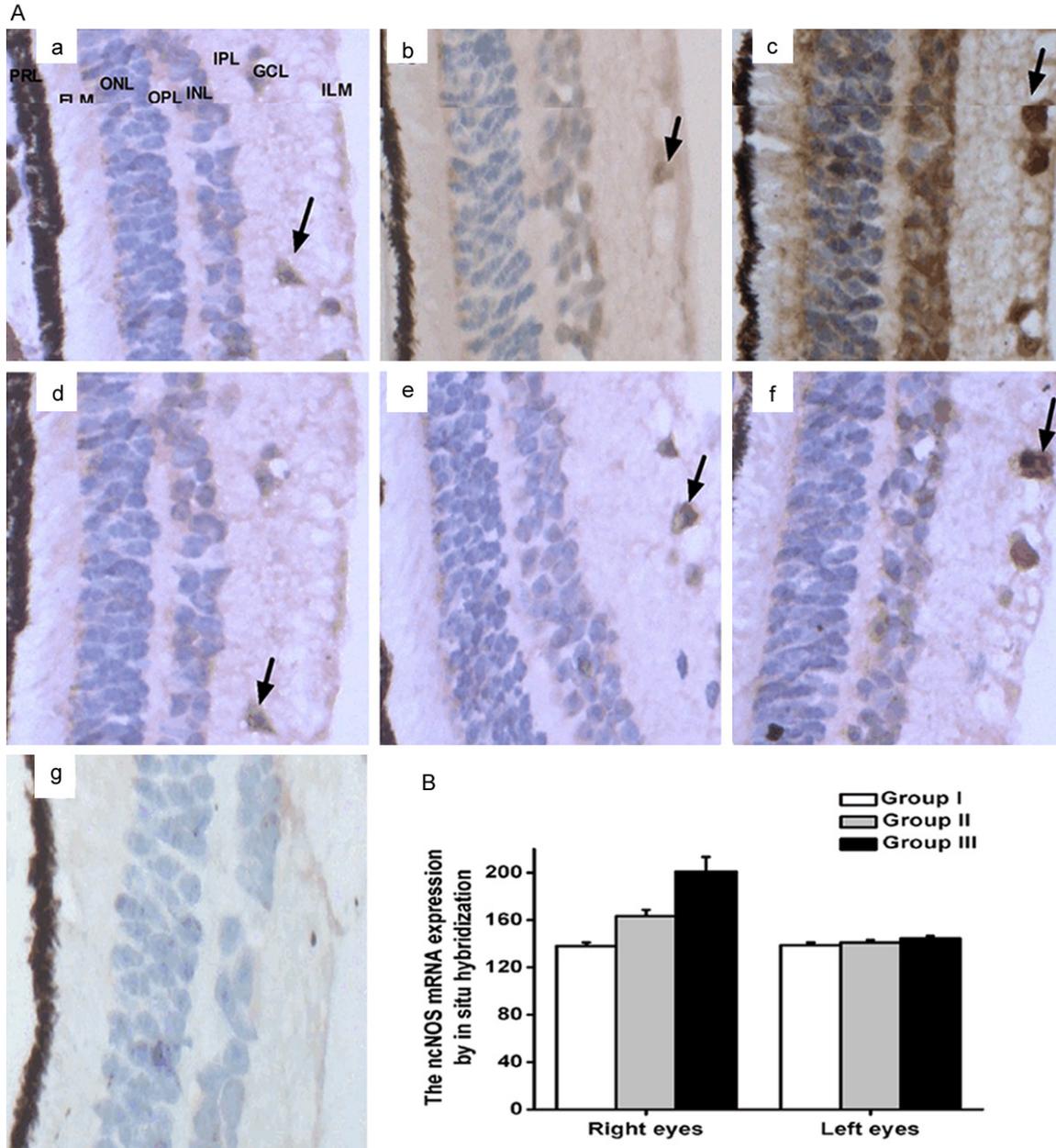
**Figure 2.** Western blot analysis of NMDAR1 expression in the retina of FDM eyes (right eyes) and untreated eyes (left eyes) in guinea pigs. A. Shown were representative blots. B. The quantitative analysis of NMDAR1 protein level by normalizing against  $\beta$ -actin. Data were expressed as mean  $\pm$  SD (n=10). NMDAR1 protein level was significantly higher in right eyes of group II and III than that in group I.

exhibited similar change to the refraction. **Table 2** showed that there was no significant differ-

ence in axial length between the two eyes in group I ( $P > 0.05$ ). However, in the right eyes, the axial length was longer in group II and group III than in group I ( $P < 0.001$ ). Furthermore, the axial length was longer in the right eyes than in the left eyes in group II and III ( $P < 0.001$ ). In summary, after form deprivation, the refraction became lower and axial length became longer, suggesting that FDM model is successfully established in guinea pigs.

### *Form-deprivation induces the expression of NMDAR1 in the retina*

NMDAR1 expression in the retina was detected by immunohistochemistry. Weak expression of NMDAR1 (pale brown) in inner nuclear layer (INL), ganglion cell layer (GCL) and external limiting membrane (ELM) was observed in group I, and



**Figure 3.** In situ hybridization analysis of ncNOS mRNA expression in the retina of FDM eyes (right eyes) and untreated eyes (left eyes) in guinea pigs. A. a. The right eyes of group I; b. The right eyes of group II; c. The right eyes of group III; d. The left eyes of group I; e. The left eyes of group II; f. The left eyes of group III. g. Negative control with the control probe. B. The quantitative analysis of relative ncNOS mRNA level based on OD value. Data were expressed as mean  $\pm$  SD (n=10). Magnification: 200  $\times$ . The arrows indicate typical strongly stained areas.

NMDAR1 expression showed no significant difference between the two eyes in group I (**Figure 1A** and **1D**). Notably, NMDAR1 expression level was higher in the right eyes of group II than in group I or the left eyes of group II (**Figure 1E**). In the right eyes of group II, NMDAR1 showed positive expression (brown) not only in INL and GCL, but also in inner plexiform layer (IPL),

nerve fiber layer (NFL) and ELM (**Figure 1B**). Furthermore, NMDAR1 expression in the right eyes of group III was strong (dark brown) (**Figure 1C**). NMDAR1 staining was the strongest in INL and GCL, and was obvious even in the photoreceptor layer (PRL) and outer plexiform layer (OPL). Western blot analysis of NMDAR1 expression showed similar results (**Figure 2**). In group

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**Table 3.** cGMP content in the FDM guinea pigs (pmol/ml)

Group	I	II	III	F
Right eye	5.42±1.71**	11.77±2.54**	17.85±2.07**	84.80
Left eye	5.34±1.23*	5.84±1.55*	5.70±1.73*	0.29
t	0.309	10.775	14.151	

Group I: untreated control (free of FDM); Group II: FDM induced for two weeks; Group III: FDM induced for three weeks. Data are expressed as mean ± SD (n=20).  $P=0.751$ , \*\* $P=0.000$ .

II and III, NMDAR1 protein expression was significantly enhanced in the right eyes than in the left eyes ( $P<0.01$ ). However, there was no significant difference in NMDAR1 protein expression between the two eyes in group I ( $P>0.05$ ). Taken together, these data demonstrate that form deprivation induces elevated expression of NMDAR1 in the retina.

### *Form deprivation upregulates ncNOS transcription in the retina*

The transcription of ncNOS in the retina was detected by in situ hybridization. The staining of ncNOS mRNA in INL and GCL in group I was pale brown (**Figure 3A**), and ncNOS mRNA level in both eyes showed no significant difference (**Figure 3B**). Furthermore, the staining of ncNOS mRNA in INL, GCL, IPL and NFL was light brown in group II, and ncNOS mRNA level in the right eyes was significantly higher than in the left eyes ( $P<0.01$ ). In group III, the staining of ncNOS mRNA in INL, GCL, IPL and NFL was brown, and ncNOS mRNA level in the right eyes was significantly higher than in the left eyes ( $P<0.01$ ). In the right eyes, ncNOS mRNA level was significantly higher in group II and III than in group I ( $P<0.01$ ). These results suggest that form deprivation upregulates ncNOS mRNA expression in the retina.

### *Form deprivation increases cGMP content in the retina*

cGMP content in the retina of guinea pigs was measured by RIA. We found no significant difference in cGMP content between the two eyes in group I ( $P>0.05$ , **Table 3**). However, in the right eyes, cGMP content was lower in group II and group III than in group I ( $P<0.001$ , **Table 3**). These data demonstrate that form deprivation increases cGMP content in the retina.

### *Correlation between ncNOS expression and cGMP content*

Finally, we performed correlation analysis between ncNOS expression and cGMP content. The results showed that ncNOS mRNA level was positively correlated with cGMP content (correlation coefficient = 0.94,  $P<0.001$ ) (**Figure 4**).

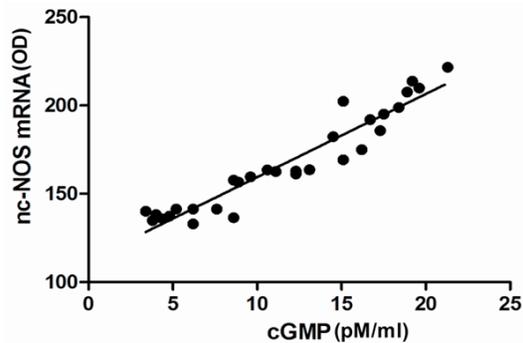
## Discussion

In this study, we focused on the changes in NMDAR1/NO-cGMP signaling pathway in the retina of guinea pigs with FDM. Our results showed that FDM could significantly upregulate the expression of NMDAR1, and increase ncNOS and cGMP levels.

NMDAR1 is considered to play a critical role in vision development, neural survival and synaptic plasticity. In guinea pig ganglion model, it was shown that OFF alpha and delta cells used NMDA receptors for encoding either the full contrast range (alpha) or only a high range (delta). However, contrast sensitivity depended substantially on NMDA receptors only in OFF alpha cells [16]. In this study, we found that NMDAR1 was expressed in the ONL, GCL and ELM of retinas of normal guinea pigs. The ELM is composed of Muller cells and photoreceptor synaptic connections, and mainly the serosa of Muller cells. These data suggest that NMDAR1 is expressed in the retinal ganglion cells, bipolar cells, Muller cells and amacrine cells. Our results are consistent with previous studies showing the weak expression of NMDAR1 in the retinal cells of on/off phenotype in the 9-day-old rat [17, 18]. In addition, we found that NMDAR1 expression was enhanced in the retina, specifically in the OPL and IPL, suggesting that NMDAR1 may play a role during experimentally induced myopia development.

NO-cGMP pathway is crucially involved in many pathological processes. NO is synthesized in the retina and acts as a key messenger to regulate retinal development, vision excitation, vision information transmission and FDM [19-21]. Nitric oxide synthase (NOS), including neuronal (ncNOS), endothelial (ecNOS) and cytokine-inducible (iNOS), is critical to the synthesis of NO. When glutamate stimulates the overexpression of NMDAR1 and the influx of  $Ca^{2+}$ , NOS is activated to synthesize NO, resulting in cGMP

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**Figure 4.** The correlation between cGMP content and ncNOS mRNA level. The correlation analysis showed the positive correlation between cGMP content and ncNOS mRNA level.  $R=0.94$ .

production. Park et al. reported that all three NOS isozymes were expressed in the retinal pigment epithelium, the photoreceptor layer and the choroid membrane, while ncNOS was only expressed in the inner layers of retina [22]. Our results showed that ncNOS could be detected in both ONL and GCL in the retina, and its expression was significantly upregulated accompanying the prolongation of FDM. Fujii et al. reported that form deprivation downregulated iNOS expression in chicken [23]. These data suggest that form deprivation reduces iNOS expression in the retinal outer layers, but induces ncNOS expression in the retinal inner layers. The changes of NOS mRNA expression may lead to reduce NO accumulation in the retinal outer layers and increased NO accumulation in the retinal inner layers. The differential expression of NOS may play a role in the development of myopia. Fujikado et al. injected L-NAME, a selective inhibitor of ncNOS, to the vitreous space in chicken and achieved the successful inhibition of the myopia occurrence, accompanied by reduced NOS production and amplitude of OP2 [24]. In this study, we found that the area of ncNOS expression was almost the same as that of NMDAR1 expression, suggesting that NO produced in retinal inner layers may mediate the neuroexcitotoxicity induced by the binding of glutamate to NMDAR1.

The effects of cGMP are regulated by cGMP-controlled ionic channels, which are expressed in rod cells, bipolar cells and some ganglion cells. After the exposure to light, the visual purple in the rod cells is activated and cGMP is hydrolyzed, resulting in membrane hyperpolar-

ization and decreased release of transmitters [25]. We found that cGMP content was significantly increased after FDM, which was related to the reduction of light stimulus after form deprivation. Furthermore, ncNOS expression is positively correlated to cGMP content. These data suggest that NO-cGMP signaling pathway may be involved in the development of FDM.

In summary, our data demonstrate that FDM could significantly upregulate the expression of NMDAR1 and ncNOS mRNA and increase cGMP content in the retina of guinea pigs. These results suggest that form deprivation induces the excessive release of glutamate in the retina and the overexpression of NMDAR1, resulting in the activation of ncNOS-cGMP pathway and the occurrence of myopia.

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### Disclosure of conflict of interest

None.

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