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Expression of nitric oxide synthase in the retina of monocular deprivation amblyopia rats

Q.-M. WANG, X.-Y. ZHAO, Z. WANG, X.-B. YANG

Department of Ophthalmology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan City, Hubei Province, China

Abstract. – OBJECTIVE: Amblyopia or lazy eye is a common visual problem affecting children that cannot correct with lenses. Nitric oxide synthase (NOS) is a critical enzyme that regulates the activity of nitric oxide (NO), a key signaling molecule with multiple roles in many tissues. Among its many activities, NOS has been proposed to be required for normal eye development and altered NOS expression can lead to perturbations in eye development and vision.

MATERIALS AND METHODS: To examine the potential role of neuronal NOS (nNOS) in vision loss, we generated a model of monocular deprivation amblyopia in rats. After suturing one eye, we examined several parameters of neural activity and nNOS expression in the retina 7, 14 and 28 days later.

RESULTS: We found the rapid and progressive loss of neural activity in the retina of sutured eyes compared to non-treated and control eyes. The sutured eyes also showed decreased expression of nNOS at the protein and mRNA levels, indicating a strong correlation between nNOS expression and retina activity.

CONCLUSIONS: These data suggest a potential role for nNOS activity in vision loss, opening potential avenues for therapeutic intervention.

Key Words:
Amblyopia, Nitric oxide synthase, Visual evoked potential, Rat, Eye suture.

Introduction

Amblyopia or lazy eye is a common visual disability in children with an incidence of 2-2.5%[1]. The pathogenesis can be divided into peripheral and central amblyopia[2]. Peripheral amblyopia is due to a damaged optic pathway upstream of the retina, expressed as a refractive error in which the line of sight is blocked. Central amblyopia is mainly due to the repression of visual development in the visual cortex in the brain. The most common clinical symptoms include strabismus and anisometropia (unequal focus between the eyes). The treatment of amblyopia is most effective in young children, with best results obtained in 3-4 years olds. Amblyopia becomes harder to correct after the age of 12 due to the development of visual maturity[3]. Studies suggest that nitric oxide synthase (NOS) is an important enzyme that affects visual development[4]. NOS plays an important role by promoting optic nerve development and regulating eye blood circulation. This study aims to analyze the expression and distribution of neuronal NOS (nNOS) in rat retina in a monocular model of amblyopia. Understanding the relationship between NOS and sight alteration can provide a reference for future clinical interventions.

Materials and Methods

Animal Care and Monocular Deprivation Amblyopia Model

Two-week-old Wistar rats of both sexes and same brood with weight about 50 ± 5 g were provided by the Animal Center of Shanghai. The animals received normal diet and were kept in 12 h light/dark cycles. We gave the animals one week to adapt to the new environment before we started the surgery. Animals were randomized into control and monocular deprivation amblyopia groups with 15 rats in each group. To prepare the monocular deprivation amblyopia model, we trimmed the hair around one eyelid, applied local disinfectant, and cut the lateral palpebral margin 1-1.5 mm from the inner canthus to the outer canthus. Then, we sutured the skin and the subcutaneous layer of the eyelids with 5-0 silk thread to close the deprived (experimental) eye. After surgery, we applied local disinfectant again. We checked the sutured eye every morning and night for light leakage and those animals showing leakage were excluded from the experiment. The other

Corresponding Author: Qiming Wang, MD; e-mail: wangqiming160713@163.com

eye was left intact as the untreated (normal) eye. We also had a control group of rats in which no eye was treated. Then, we measured the pattern visual evoked potential of the bilateral retina of the treated animals and one retina of the control group (randomized) 7, 14, and 28 days after closing the eyes. After this, we euthanized the rats by cervical dislocation and the retinas were rapidly dissected and put on ice for histological, mRNA, and protein studies.

Pattern Visual Evoked Potential Detection

For measuring eye function we used instruments for brain stereotaxis (RWD Life Technology Co., Shenzhen) and nerve evoked potential WOND2000 (Medical Information Industry Co., Guangzhou). We injected rats with 4% chloral hydrate intraperitoneally (1 ml/100 g). To open the pupil and induce the contraction of the palpebral conjunctiva, we administered two drops of Compound Tropicamide eye drops (AR, batch number 20120506, Double-Crane modern medical technology, Beijing, China). We fixed and properly adjusted the collimation axis to make sure that the light spot of the retina was parallel to the center of the visual screen. P-VEP test standard: pattern reversal stimulation settings for stimulus frequency 4.00, pattern checkerboard, graph size 8 x 6, full field of vision. The amplifier is provided with an upper limit frequency of 500 Hz, the lower limit of the frequency of 1 Hz. Record electrode Oz; reference electrode Fpz; sampling time of 300 ms; 128 times of superposition; 50 micro-amplitude. Recording electrodes were placed 1.5 cm above rats' occipital. The reference electrode was inserted into the middle of forehead brow. The vertical distance between the retina and the screen was 57 cm when stimulated. The impedance of the recording electrode and the reference electrode was $\leq$ 5Ω.

Immunofluorescence

We used Pap Pen (ZLI-9305, Zhongshan Company, Beijing) to mark the area of the tissue to stain. We washed the retinas with 0.01M PBS for 10 min three times, blocked in 6% normal donkey serum (SP-072-VX10, ImmunoReagents, Raleigh, NC, USA), and incubated overnight in a wet box at 4°C. The next day, we placed the slices in the CO2 incubator at 37°C for rewarming for 1 h, discarded the block buffer, added rabbit anti-NOS antibody (N7155, 1:10000; Sigma-Aldrich, St. Louis, MO, USA), and incubated overnight at 4˚C. Then, we washed with 0.01M PBS for 10 min 3 times, added Alexa-Fluor-488 donkey anti-rabbit IgG (H+L) (711-545-152, 1:200; Jackson ImmunoResearch, West Grove, PA, USA), and incubated at room temperature for 2 h. Finally, we washed with 0.01M PBS for 10 min 3 times, and sealed the section. We imaged several layers for the stained retinas in a laser scanning microscopy (Olympus fv1000, Tokyo, Japan), including the outer nuclear layer (ONL), the inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and the ganglion cell layer (GCL). We analyzed localization and percentage of nNOS-positive expression.

RT-PCR

Total RNA (Invitrogen, Carlsbad, CA, USA) was extracted by Trizol. cDNA was constructed by reverse transcription (Fermentas, Burlington, Ontario, Canada) after testing concentration, purity, and integrity of RNA. We designed primers for nNOS in Primer Premier 5.0 after verifying the sequence in NCBI GeneBank (NM001160110.1, Bethesda, MD, USA). The nNOS and GAPDH (internal control) primers were synthetized by Shengong Industrial (Shanghai). nNOS (162 bp): Fw-5'-ACACTCTTGACCCTCCACCC-3', Rv-5'-CCCCAGCATCCCTACTCCCA-3',. Internal reference, GAPDH (146 bp): Fw-5'-GAGCTGAACGGGAAACTCAC-3', Rv-5'-GGTCTGGGATGGAAACTGTG-3'. PCR conditions: 94°C for 3 min, 94°C for 45 sec, 57°C for 45 sec, 72°C for 45 sec, for 35 cycles. The results are shown following the $2^{-\Delta\Delta}$Ct method. Real-time PCR was done in a FTC-3000 (Biotech Funglyn, Shanghai, China) using a Fluorescent PCR Kit from Fermentas (Burlington, Ontario, Canada).

Western Blot

Retina homogenates were performed following the manufacturer's recommendations (KangChen Bio-Tech, Shanghai, China). We used the BCA protein concentration Kit (Beyotime Technology, Beijing, China) on an Elisa EXL 800 UV Bio-Tek Instrument to determine protein concentration. We run homogenates on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis on a Bio-Rad chamber (Hercules, CA, USA) (separation gel, 120V, electrophoresis for 2h). We transferred bands into a polyvinylidene fluoride (PVDF) membrane with a semi-dry Bio-Rad chamber (Mercules, CA, USA). We next blocked membranes in 5% skim milk in TBST at room temperature on a shaker

Table I. Comparison of pattern visual evoked potentials.

Groups		Affected eye	Normal eye	Control	F	*p*
P100 (ms)	7d	32.5±5.3	24.5±3.8	23.4±4.2	5.236	0.025
	14d	53.7±6.2	26.7±4.0	23.6±4.3	7.524	0.009
	28d	55.8±6.6	28.3±4.4	23.2±4.1	8.329	0.000
N45-P100 (μv)	7d	14.5±3.2	18.2±3.4	19.6±3.5	4.623	0.032
	14d	7.8±3.0	17.6±3.6	21.1±3.8	5.632	0.020
	28d	6.3±2.2	16.5±3.2	20.5±3.7	6.237	0.011

for 1 h, and rinsed with TBST buffer for 10 min 3 times. Then, we added rabbit anti-nNOS monoclonal antibody (1: 500, Cell Signaling Technology, Danvers, MA, USA) in a self-sealing bag and incubated at 4°C overnight followed by 2 h shaking at room temperature. Next, we rinsed with TBST buffer for 10 min 3 times, added 1:10,000 goat anti-rabbit secondary antibody (Li-Cor, lnc., Bad Homburg, Germany), and incubated for 30 min at room temperature. Finally, we analyzed the protein expression via computer imaging system (Odyssey, Bad Homburg, Germany).

Statistical Analysis

SPSS20.0 (SPSS Inc., Chicago, IL, USA) was used for statistics analysis. Values were shown as mean ± standard deviation. Comparison between groups was performed using single factor ANOVA analysis and LSD-t test for comparison between two and two groups. For comparisons within the group at different time points we used repeated measures of variance analysis. Differences were considered statistically significant if $p < 0.05$.

Results

Effect of Eye Closure on Visual Evoked Potentials

The chronaxie (the minimum amount of time needed to stimulate a nerve fiber) of the control group (P100) and the amplitude of N45-P100 had no change over the 14 days of the experiment (Table I). Among the rats subjected to surgery, the normal showed increased P100 and decrease of amplitude, although the difference was not significant ($p > 0.05$). In contrast, the affected eyes of treated rats showed elevated P100 that gradually increased with time. The amplitude was initially low and dramatically decreased in subsequent measurements. When comparing the values among the three groups, P100 was the highest on the affected side, whereas the amplitude was the lowest (Table I). These differences were statistically significant ($p < 0.05$) (Table I).

Effect of Eye Closure on nNOS Distribution in the Retina

In the control group, we found the nNOS-positive neurons mainly in the inner nuclear layer. We determined that these were amacrine cells dispersedly distributed. Some cells had rosary-like protrusions and were located near the inner plexiform layer. Protrusions were large and displayed many branches that connected into a network. A few cells were located in the ganglion cell layer. We found no significant difference between the normal eyes of treated rats and the eyes of control rats (Table II). In the affected eyes, the cell density of nNOS-positive cells was slightly lower (Table II). The affected eyes showed loss of retinal thickness and the fluorescence signal became progressively weaker.

Effect of Eye Closure on nNOS mRNA Expression

To conduct more quantitative studies on the dynamics of nNOS expression in the retina of the amblyopia rat model, we extracted total RNA and performed RT-PCR to amplify nNOS mRNA. The eyes of controls rats and the normal eyes of treated rats showed consistent expression levels of nNOS mRNA over time (Table III). However, the affected side exhibited lower levels of nNOS mRNA that decreased gradually over time (Table III).

Table II. Comparison of average fluorescence intensity of nNOS positive cells in retina.

Groups	7d	14d	28d
Affected eye	2.2±0.5	2.0±0.3	1.7±0.3
Normal eye	2.3±0.4	2.3±0.3	2.3±0.5
Control	2.4±0.3	2.3±0.4	2.4±0.5
F	4.123	4.532	5.427
p	0.036	0.032	0.026

Table III. Comparison of nNOS mRNA level.

Groups	7d	14d	28d
Affected eye	0.4218±0.1259	0.3639±0.1238	0.3254±0.1452
Normal eye	0.5523±0.1524	0.5624±0.1329	0.5429±0.1218
control	0.5634±0.1247	0.5784±0.1325	0.5429±0.1148
F	4.325	4.714	5.562
p	0.033	0.030	0.022

Table IV. Comparison of expression level of nNOS protein.

Groups	7d	14d	28d
Affected eye	0.42±0.12	0.36±0.13	0.32±0.14
Normal eye	0.51±0.15	0.52±0.13	0.52±0.12
control	0.52±0.13	0.53±0.14	0.53±0.12
F	4.218	4.636	5.348
p	0.035	0.032	0.024

Effect of Eye Closure on nNOS Protein Levels

We then performed semi-quantitative studies to determine the effects of eye closure on nNOS protein levels by western blot. For this, we homogenized the retinas of the different groups and immune-detected NOS in the membranes. The eyes of controls rats and the normal eyes of treated rats showed consistent expression levels of nNOS over time (Table IV). In contrast, the affected side expressed lower levels of nNOS that decreased gradually with time (Table IV).

Discussion

NOS is widely expressed in endothelial cells, macrophages, neural phagocytic cells, and nerve cells. NOS has three isoforms: nNOS, endothelial NOS (eNOS), and inducible NOS (iNOS). Nitric oxide (NO) is an important messenger, with close ties to peripheral nerve injury and regeneration. The transfer of neural information is the main responsibility of nNOS[6]. Ientile et al[7] found that NOS activity was significantly increased in early chicken embryo development and then decreased at later stages before the development of the retina. Glutamate increased the activity of NOS, while the NMDA antagonist MK-801 reduced the NOS activity. The NOS-positive neurons in the visual cortex of the deprived eyes were pale and the ocular dominance columns of the deprived eyes shifted in a two-month-old monkey monocular deprivation model[8]. Additionally, changes of NOS in monocular deprived kittens in the lateral geniculate nucleus were positively correlated with visual deprivation time[9]. Huang et al[10] found that nNOS gene expression defects can affect the maturation of the visual process in rats, resulting in optic projection disorder. The critical window of monocular deprivation can cause reduced levels of NOS in the lateral geniculate body, which may be related to long-term loss of visual information stimulation to the input layer of the deprived eye, resulting in disuse atrophy. Also, NO produced by the retinal blood vessels can make the small artery rapid response to blood pressure and oxygen synthesis changes, regulating visual development through the regulation of blood circulation.

Pattern visual evoked potential (P-VEP) is an important index for early diagnosis of amblyopia and it is also an objective index to assess the degree of amblyopia[11]. The monocular suture method is simple to prepare, with reliable effect and high replication success rate, which has become a classic model to study visual development and plasticity[12]. In this paper, we report that the chronaxie (P100) of the closed eyes gradually increased with time, while the increase amplitude of N45-P100 decreased after 14 days. These results suggest that we established a successful model of amblyopia. Although we observed changes in the normal eyes of treated animals, there were no differences between the groups. This result may be related to the bilateral optic nerve regulation and the inhibitory effect of the affected eye on normal eye[13]. The number of nNOS-positive cells in the model group was significantly decreased, and the nNOS mRNA and protein level were gradually decreased with time. NO is an important neurotransmitter in the

process of formation, integration and transmission of visual development. It can transmit the activity information before and after synapse, which plays an important role in the generation and maintenance of long-term potentiation (LTP)[14] and take effect through MDA receptor-related T NO/cGMP pathway[15,16]. NOS is the only catalytic enzyme of endogenous NO and its level can indirectly reflect the level of NO. There is a closed correlation. In the developing process of the visual system, the proper connection between the retina and the visual center is achieved through the refinement process. This process is not only the critical period of visual development to reach the normal peak, but also a critical period of the most likely to form amblyopia[17]. NO, as a kind of retrograde messenger, is involved in the communication between the end of the synapses, and is involved in the modification and refinement of the synapse, which affects the visual center and the synaptic plasticity of the retina. NOS take part in this process by regulating the formation of NO[18].

Conclusions

We showed that the expression of nNOS was down regulated in the retina of monocular deprived amblyopia rats, and the trend was consistent with the decrease of visual evoked potentials. Intervention on the expression of nNOS will become a potential target for treatment of amblyopia.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Autrata R, Krejčířová I, Griščíková L, Doležel Z. Refractive surgery in children with myopic anisometropia and amblyopia in comparison with conventional treatment by contact lenses. Cesk Slov Oftalmol 2016; 72: 12-19.
2) Olusanya BA, Ugalahi MO, Okoli CE, Baiyeroju AM. Refractive Amblyopia among children in Ibadan: the need for amblyopia screening programmes. Afr J Med Med Sci 2015; 44: 237-242.
3) Celik E, Çakir B, Turkoglu EB, Doğan E, Alagoz G. Evaluation of the retinal ganglion cell and choroidal thickness in young Turkish adults with hyperopic anisometropic amblyopia. Int Ophthalmol 2016; 36: 515-520.
4) Gao J, Zhang D, Gong S. Histochemical studies on the change of nitric oxide synthetase in lateral geniculate nucleus of monocular deprived kittens. Zhonghua Yan Ke Za Zhi 2002; 38: 476-479.
5) Vagge A, Nelson LB. Amblyopia update: new treatments. Curr Opin Ophthalmol 2016; 5: 14-15.
6) Liu S, Liu H. The advances of suppression in research of amblyopia. Zhonghua Yan Ke Za Zhi 2016; 52: 305-308.
7) Ientile R, Malecka B, Piceiurro V, Naso A, Pedale S, Macaione S. Nitric oxide synthase in chick embryo retina during development. FEBS Lett 1996; 371: 82-84.
8) Sandell JH. NADPH diaphorase histochemistry in the macaque striate cortex. J Comp Neurol 1986; 251: 388-397.
9) Gunluk AE, Bickford ME, Sherman SM. Rearing with monocular lid suture induces abnormal NADPH-diaphorase staining in the lateral geniculate nucleus of cats. J Comp Neurol 1994; 350: 215-228.
10) Huang PL, Lo EH. Genetic analysis of NOS isoforms using nNOS and eNOS knockout animals. Prog Brain Res 1998; 118: 13-25.
11) O'Hara MA. Instrument-based pediatric vision screening. Curr Opin Ophthalmol 2016; 25: 14-15.
12) Elflein HM. Amblyopia: Epidemiology, causes and risk factors. Ophthalmologe 2016; 113: 283-288.
13) Lagrèze WA. Amblyopia. Ophthalmologe 2016; 113: 280-282.
14) Sloper J. The other side of amblyopia. J AAPOS 2016; 20: 1.e1-13.
15) Pizzorusso T, Medini P, Landi S, Baldini S, Berardi N, Maffei L. Structural and functional recovery from early monocular deprivation in adult rats. Proc Natl Acad Sci USA 2006; 103: 8517-8522.
16) Jefferis JM, Connor AJ, Clarke MP. Amblyopia. Br Med J 2015; 351: h5811.
17) Zhao K, Kang X, Liu H, Wei Y. See the strabismus and amblyopia research development trend from the twelfth World Congress of strabismus. Zhonghua Yan Ke Za Zhi 2015; 51: 401-405.
18) Fresina M, Campos EC. A 1-year review of amblyopia and strabismus research. Asia Pac J Ophthalmol 2014; 3: 379-387.