·Basic Research·

Influences of levodopa on expression of N-methyl-D-aspartate receptor-1-subunit in the visual cortex of monocular deprivation rats

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Abstract

• AIM: Many studies have demonstrated N-methyl-D-aspartate receptor-1-subunit (NMDAR1) is associated with amblyopia. The effectiveness of levodopa in improving the visual function of the children with amblyopia has also been proved. But the mechanism is undefined. Our study was to explore the possible mechanism.

• METHODS: Sixty 14-day-old healthy SD rats were randomly divided into 4 groups, including normal group, monocular deprivation group, levodopa group and normal saline group, 15 rats each. We sutured all the rats' unilateral eyelids except normal group to establish the monocular deprivation animal model and raise them in normal sunlight till 45-day-old. NMDAR1 was detected in the visual cortex with immunohistochemistry methods, Western Blot and Real time PCR. LD and NS groups were gavaged with levodopa (40mg/kg) and normal saline for 28 days respectively. NMDAR1 was also detected with the methods above.

• RESULTS: NMDAR1 in the visual cortex of MD group was less than that of normal group. NMDAR1 in the visual cortex of LD group was more than that of NS group.

• CONCLUSION: NMDAR1 is associated with the plasticity of visual development. Levodopa may influence the expression of NMDAR1 and improve visual function, and its target may lie in the visual cortex.

• KEYWORDS: levodopa; N-methyl-D-aspartate receptor; monocular deprivation

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INTRODUCTION

Amblyopia is caused by abnormal visual experience in the period of visual development .It is visual acuity ≤ 0.8 and can not be corrected[1]. According to statistics, there are about 10 million visually impaired patients with amblyopia in China[2]. The incidence of amblyopia is closely related to visual development plasticity in sensitive period. Many studies indicate that glutamate and its receptors especially N-methyl-D-aspartate receptor-1-subunit (NMDAR1) play an important role in the pathogenesis of amblyopia[3].

Levodopa is a precursor of dopamine, which passes through the blood-brain barrier and plays a role by converting into dopamine. Dopamine takes part in the visual cortex development in sensitive period. Dopamine and catecholamine can release the cortex inhibition of the amblyopic eye to improve visual function. Gottlob and Stangler-Zuschrott [4] found that visual acuity, contrast sensitivity and dark spots of patients with amblyopia get better after taking levodopa. Currently, levodopa has been widely used in clinic and has achieved primary success, but its specific mechanisms remain unclear. This study detected the NMDAR1 expression in the visual cortex of monocular deprivation rats before and after taking levodopa in order to provide some meaningful experimental data for the molecular mechanism of levodopa treating amblyopia.

MATERIALS AND METHODS

Materials

Animal model establishment and grouping Sixty healthy SD rats at 2 week (offered and reared by Animal Department of China Medical University) were divided into 4 groups randomly, 15 in normal group, 15 in monocular deprivation(MD) group, 15 in normal saline(NS) group and 15 in levodopa (LD) group. In MD, NS and LD group, rats were anesthetized by peritoneal injection of 5% chloral hydrate (3mL/kg), pelage surrounding palpebral margin sniped, sterilized with Iodophors, skin and palpebral cartilages sniped 1.0mm from superior and inferior palpebral margin, the oculo dextro eyelids were sutured closed in 2-3 needles using interrupted suture with 6-0 suture silk to set up monocular deprivation animal model.

Superior and inferior palpebral margins adhered after agglutination of the tresis vulnus. There was no treatment on the normal group. Rats in each group were reared in the same natural illumination environment to 45 days.

Methods

Intervention and treatment, detection index and methods of animals Rats were executed by decapitation at 45 days in normal and MD group, the expression of protein and mRNA of NMDAR1 in the left visual cortex was detected by immunohistochemistry, real time fluorescent quantitive PCR and protein immune imprinting technique. From 46 days on, rats received intragastric administration with 40mg/kg levodopa and normal saline of the same volume, once a day for 28 days in LD and NS group. Then rats were executed by decapitation, the expression of protein and mRNA of NMDAR1 in the left visual cortex was detected by immunohistochemistry, real time fluorescent quantitation PCR and protein immune imprinting technique.

Immunohistochemistry In normal and MD group, after rats were executed at 45 days, the left (opposite to the deprive-side) visual cortex was fixed with 4% paraformaldehyde, mineral wax embedding sliced, followed by deparaffinage with dimethyl benzene and hydration with sequential ethanol,washed with 0.01mol/L PBS(3× 5 minutes); 0.5% parenzyme was droped in sections until the tissue was completely covered, incubated for 30 minutes at 37℃; washed with 0.01mol/L PBS (3×5 minutes); incubated with 3% H_2O_2 for 15 minutes at room temperature to block endogenous peroxidase, washed with 0.01mol/L PBS (3× 5 minutes); incubated with normal goat serum for 15 minutes at room temperature, discarded the serum with no wash; incubated in wet cabinet overnight at 4℃ with diluted primary antibody in PBS (1:50), washed with 0.01mol/L PBS (3×5 minutes); incubated in wet cabinet for 30 minutes at 37℃ with 1:200 diluted secondary antibody, washed with 0.01mol/L PBS (3×5 minutes); incubated with HRP-avidin for 30 minutes at 37℃, washed with 0.01mol/L PBS (3×5 minutes); chromogenic reaction with DAB; counterstained with hematoxylin, differentiated; dehydrated with sequential ethanol, became transparent with dimethyl benzene, sealed with neutral gum prior to 400×microscopy. The expression of NMDAR1 was observed.

In LD and NS group, rats were executed at 75 days after intragastric administration, above-mentioned methods were used for detection of immunohistochemistry to observe the expression of NMDAR1.

Protein immune imprinting technique (**Western**) In normal and MD group, after rats were executed at 45 days, membrane protein extract agent A was added into the left (opposite to the deprive-side) visual cortex, put on ice, the tissue was shattered to unicell suspension by hand-hold homogenate machine, put on ice for 5 minutes. Then put into refrigerated centrifuge for 5 minutes at 700rpm，4℃, the supernatant was obtained, then 30 minutes at 14000rpm, 4℃，the supernatant was bloted to the limit. appropriate volume of membrane protein extract agent B with 1%PMSF was added, spallation on ice, vortex concussion every 10 minutes to ensure schizolysis in extenso, repeated for 3 times, centrifugalization for 30 minutes at 14000rpm, 4℃, the supernatant was obtained to extract cell membrane protein. Protein concentration was determined by Bicinchoninic acid (BCA) method. From each group, 30μg sample was obtained to separate protein sufficiently by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred the protein onto nitrocellulose membrane by wet-transfer method under 70V voltage at 4℃,transmembraned over night, blocked in confining liquid with 5% non-fat dry milk for 4 hours at 37℃, incubated the membrane with primary antibody NMDAR1 (1:1000) over night at 4℃，incubated the membrane with HRP labeled secondary antibody (1:10000) for 2 hours at room temperature, chromogenic reaction of polyvinylidene difluoride (PVDF)membrane was performed by ECL emit-light agent, exposed to film in dark room then visualized and fixed. β actin was inner reference. Gelpro32 gel image analysis software was applied to analyze extinction luminance value. The ratio of extinction luminance value of NMDAR1 and β actin in each group was regarded as the relative amount (M) of each sample，M value was recorded and statistical analyzed.

In LD and NS group, rats were executed at 75 days after intragastric administration, above-mentioned methods were used for detection of Western Blot, M value was recorded and statistical analyzed.

Real time fluorescent quantitive PCR In normal and MD group, after rats were executed at 45 days, the left (opposite to the deprive-side) visual cortex was obtained, RZ lysate was added in, misce bene, put at room temperature for 5 minutes，total RNA extraction kit was used to extract total RNA of the sample. TIANScript RT Kit(TIANScript cDNA first chain synthesis kit) was used to reversely transcribe the RNA sample obtained from the previous experiment for obtaining the corresponding cDNA, preserved at -20℃. Reverse transcription reaction system oligo (dT)151μL, dNTP (2.5mmol/L each)2μL，5×First- Strand Buffer 4μL，RNasin 0.5μL，TIANScript M-MLV 1μL (200U), homo-quality mRNA sample, ddH_2O was added in until total reaction system was 20μL. 20μL cDNA sample was obtained eventually, preserved at -20℃. PCR reaction system was set up, primer NMDAR1-F CGGTATCAGG AATGCGACTC,NMDAR1-RAGGAAAATCCCAGCTAC GAT,length of amplification fragment was 110bp；β-actin F ACGTTGACATCCGTAAAGAC, β-actinRGAAGGTGGA CAGTGAGGC, length of amplification fragment was

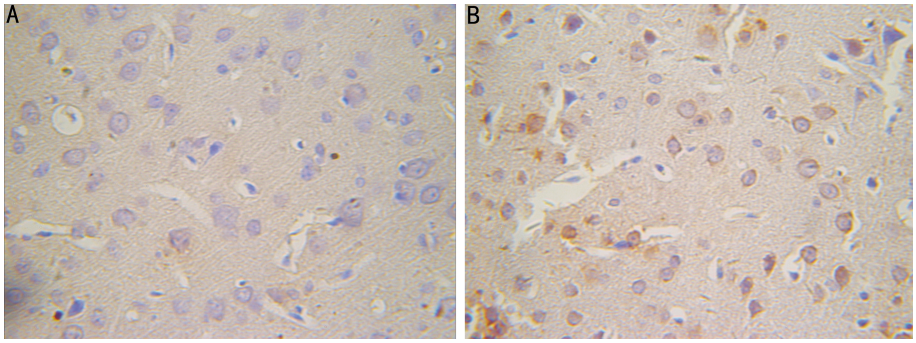

Figure 1 A: Monocular deprivation leads to reduction of NMDAR1 positive cells in the visual cortex of rats, mineral wax embedding slice of brain visual cortex, immunohistochemistry staining 400×; B: NMDAR1 positive cells in visual cortex of rats in normal group, mineral wax embedding slice of brain visual cortex, immunohistochemistry staining 400×.

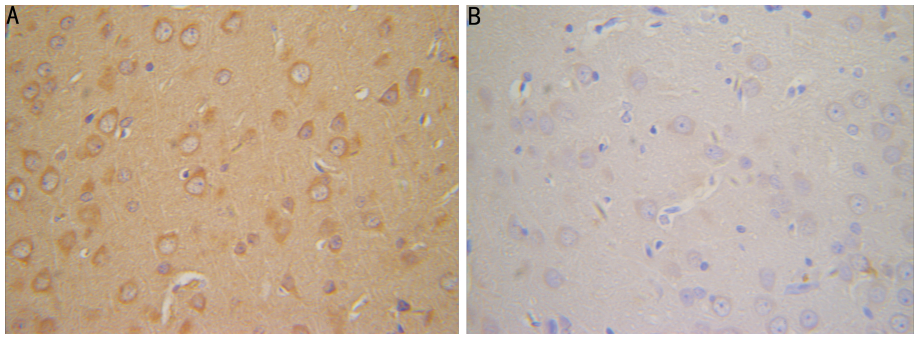

Figure 2 A: LD treatment caused increasing of NMDAR1 positive cells, immunohistochemistry staining 400×; B: NS treatment caused no marked change on NMDAR1 positive cells, immunohistochemistry staining 400×.

200bp. PCR reaction system 20μL, including cDNA template 2μL, up and down stream primer 1μ each, 2×Taq PCR Master-mix10μL, ddH_2O was added in to make up for 20μL. Amplification condition: pre-degeneration for 5min at 95℃, degeneration for 10 seconds at 95℃, renaturation for 20 seconds at 60℃, elongation for 30s at 72℃, up to 35 circulation, elongation for 5 minutes at 72℃ eventually. 5μL product was put in 1.5% agarose gel electrophoresis, then used gel imaging system to take a photograph for the gel. Real time fluorescent quantitive PCR, according to the PCR condition and laboratory apparatus integrated, the ultimately optimized fluorescent quantitive reaction system and reaction condition was: reaction system cDNA template 1μL, up and down stream primer (10μmol/L) 0.5μL each, SYBR GREEN mastermix 10μ,ddH_2O was added in to make up for 20μL; reaction condition pre-degeneration for 10 minutes at 95℃, degeneration for 10s at 95℃, renaturation for 20 seconds at 60℃, elongation for 30 seconds at 72℃, up to 40 circulation, elongation for 5 minutes at 4℃ eventually. Fluorescent quantitive analysis was made by using ExicyclerTM 96 fluorescent quantitation instrument of BIONEER corporation from Korea.

In LD and NS group, rats were executed at 75 days after intragastric administration, above-mentioned methods were used for detection of real time fluorescent quantitive PCR, data was recorded and statistical analyzed.

Statistical Analysis M value for detection of Western was represented as mean±SD, t test method was used to analyze the expression of NMDAR1 in the visual cortex opposite to the deprive-side in normal and MD group, LD and NS group respectively. $2^{-\triangle\triangle CT}$ method was used to analyze the result of real time fluorescent quantitive detection. t test method was used to analyze the the expression of NMDAR1 in the visual cortex opposite to the deprive-side in normal and MD group, LD and NS group respectively.

RESULTS

Immunohistochemistry The visual cortex of rats from MD group and normal group were mineral wax embedding sliced, treated with primary antibody and secondary antibody prior to observation under 400×light microscope. NMDAR1 positive cells membrane showed buffy, most cells were round or oval, cell membrane thick stained, kytoplasm and nucleus thin stained. In MD group, NMDAR1 positive cells in the visual cortex of rats decreased, in normal group, NMDAR1 positive cells appeared in every visual field (Figure 1).

The visual cortex of MD rats from LD group and NS group were mineral wax embedding sliced, treated with primary antibody and secondary antibody prior to observation under 400×light microscope. In LD group, NMDAR1 positive cells increased in visual cortex of MD rats, while there was no marked change in NS group (Figure 2), showing that levodopa can reverse reduction of NMDAR1 expression originated by monocular deprivation.

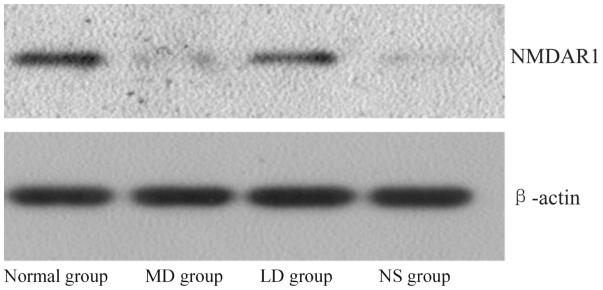

Figure 3 Protein expression of NMDAR1 by Western Blot in normal group, MD group, LD group and NS group.

Table 1 M value of protein expression of NMDAR1 in MD group, normal group, LD group and NS group (mean±s)

	n	M		*n*	M
MD group	5	0.18±0.02	LD group	5	0.44±0.04
Normal group	5	0.58±0.05	NS group	5	0.21±0.03
t		16.611			10.286
P		<0.05			<0.05

Western Blot Protein expression of NMDAR1 in the visual cortex of rats in MD group and normal group was detected by Western Blot. Results showed that M value of NMDAR1 was 0.18±0.02 in the visual cortex in MD group and was 0.58±0.05 in normal group, the difference between two groups showed statistical significance (t=16.611, P<0.05, Table 1, Figure 3). Protein expression of NMDAR1 in the visual cortex of MD rats in LD group and NS group was detected by Western Blot. Results showed that M value of NMDAR1 was 0.44±0.04 in the visual cortex in LD group and was 0.21±0.03 in NS group, the difference between two groups showed statistical significance (t=10.286, P<0.05, Table 1, Figure 3). The result of statistical analysis was coincident with the protein expression tendency observed by immunohistochemistry.

Real Time Fluorescent Quantitive PCR mRNA expression of NMDAR1 in the visual cortex of rats in MD group and normal group was further detected by Real Time PCR. Results showed that $2^{-\triangle\triangle CT}$ value of NMDAR1 expression was 0.39±0.02 in the visual cortex in MD group and was 0.98±0.09 in normal group, the difference between two groups showed statistical significance (t=14.310, P<0.05). Real Time PCR detection was carried out on the visual cortex of MD rats in LD group and NS group. Results showed that $2^{-\triangle\triangle CT}$ value of NMDAR1 expression was 6.12±0.12 in LD group and was 1.00±0.08 in NS group, the difference between two groups showed statistical significance (t=79.382, P<0.05, Table 2). The result showed that the change tendency of NMDAR1 mRNA expression was coincident with that of NMDAR1 protein expression.

Table 2 mRNA expression of NMDAR1 in MD group, normal group, LD group and NS group by Real time fluorescent quantitive PCR ($2^{-\triangle\triangle CT}$±S)

	n	$2^{-\triangle\triangle CT}$		*n*	$2^{-\triangle\triangle CT}$
M D group	5	0.39±0.02	LD group	5	6.12±0.12
Normal group	5	0.98±0.09	NS group	5	1.00±0.08
t		14.310			79.382
P		<0.05			<0.05

DISCUSSION

In recent years, studies have shown that glutamate and its receptors play a role in the eye diseases, such as deprivation amblyopia, strabismus, amblyopia, retinopathy, glaucoma, PVR and etc [5-9]. Visual development studies have shown that the human visual system is immature at birth and visual system development is a gradual adaption to environmental stimuli, as neural connections being established and neural functions being perfected. Visual experience can be adjusted according to stimuli from the environment,thus changes congenital neural synaptic connections, which is the plasticity of visual development. When we look into the anatomical, physiological and biochemical effects of monocular deprivation, the information-driven competition between the two eyes seems to be a key to the deprivation effect. Carmignoto and others first put forward the "NTs" theory based on visual plasticity of binocular competition . The hypothes is that during the critical period the OD shift of area 17 neurones towards the open eye in monocularly deprived animals is due to competition between the afferents from the two eyes for NGF implies that geniculo- cortical axonal terminals express the NGF receptor. NTs mediated with the primary visual cortex neurons axon contact, and ultimately the formation of ocular dominance columns [10]. Visual plastic period of normal rats is during 15 days to 45 days after birth, of which 14 to 15 days is the critical period of visual development . In this period, the eyelid suture will lead to deprivation amblyopia [11]. Monocular deprivation causes the asymmetry of visual signals transmission to the visual cortex.The signals from the deprived eye are reduced, leading to a series of change in the modality and function of visual cortex neurons.In these changes, glutamic acid and its receptors play an important role, especially NMDAR1 plays an important role [3].

NMDAR1 is a major subtype of glutamate receptors.It's a specific ion channel. It can improve the nerve cell's membrane permeability for Ca_2 ±by binding ligand. Ca_2 ± pours into the cell, activating Ca_2 ±dependent enzyme as a second messenger, causing intracellular physiological changes. NMDAR1 is related to long-term potentiation and long-term depression, learning, memory and synaptic plasticity. NMDAR1 plays an important role both in the visual cortex plasticity regulation during the critical and adult period , induced c-fos gene expression, and interaction with protein kinase C [12]. Yin and colleagues [13] found that NMDAR1 expression of strabismic amblyopia cats' visual

cortex neurons is less than that of normal cats.Shao Li-gong and etal [14] found that during sensitive period, strabismus and monocular deprivation cats' neurons in the visual cortex 17 area express less NMDAR1 than that of normals. This study had detected NMDAR1 in the visual cortex of monocular deprived rats during critical period.The result shows that NMDAR1 protein and mRNA expression in the visual cortex of monocular deprived rats is less than that of normal group, suggesting that the effect of monocular deprivation on the visual cortex of NMDAR1 expression lies in the upper level of transcription or transcription.

Dopamine is an important neurotransmitter in the central nervous system.There is a big amount of dopamine in human retinal amacrine cells. Studies have shown that visual sensitivity, color vision, vision, spatial signal and many other visual functions are affected by dopamine. Levodopa is a dopamine precursor that goes through the blood-brain barrier, then converts to dopamine. Leguire *et al* [15] made a comparisive study on the visually-impaired patients with oral levodopa and oral placebo and found that the improvement of visual acuity and contrast sensitivity were both better than that of the control group.Gottlob *et al*[16] found that both a single dose (200mg/50kg) and taking levodopa for 1 week (2mg/kg, 3 times daily) can improve visual function in adult patients with amblyopia. However, the specific mechanisms for the role of dopamine are not entirely clear. In this study, the expression of NMDAR1 in the rats' visual cortex was detected and compared between the LD group and NS group. The results showed that the level of NMDAR1 protein and mRNA expression in the rat visual cortex of the LD group were higher than that of the NS group. This suggests that the target site of dopamine to improve the visual function is likely lying in the parts of visual cortex. L-dopa can reverse the decreasing expression of NMDAR1 caused by deprivation in or above the level of transcription.The specific molecular mechanism of dopamine increasing the expression of NMDAR1 remains to be further studied in depth.

In summary, this study found that monocular deprivation in critical period could lead to a decrease of NMDAR1 expression in rats' visual cortex. NMDAR1 expression increased in rats given oral levodopa after monocular deprivation. The results suggested that NMDAR1 was related to the plasticity of visual development and levodopa might reverse the expression of NMDAR1 decreasing caused by monocular deprivation. This mechanism may be related to the improvement of visual function caused by levodopa, and its target may lie in the visual cortex.

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