

# Expression of Early Growth Responsive Gene-1 in The Visual Cortex of Monocular Form Deprivation Amblyopic Kittens

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## Research Article

**Keywords:** Early growth responsive gene-1 (Egr-1), Amblyopia, Form deprivation, Visual cortex, Kitten

**Posted Date:** October 1st, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-877490/v1>

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**Version of Record:** A version of this preprint was published at BMC Ophthalmology on November 15th, 2021. See the published version at <https://doi.org/10.1186/s12886-021-02161-5>.

# Abstract

## Purpose

The present study compared the expression of early growth responsive gene-1 (Egr-1) in visual cortex between amblyopia kittens and normal kittens, and to explore the role of Egr-1 in the pathogenesis of amblyopia.

## Methods

A total of 20 healthy kittens were randomly divided into deprivation group and control group with 10 kittens in each group. Raised in natural light, and cover the right eye of the deprived kittens with a black opaque covering cloth. Pattern visual evoked potentials (PVEP) were measured before and at the 1st, 3rd and 5th week after covering in all kittens. After the last PVEP test, all kittens were killed. The expression of Egr-1 in the visual cortex of the two groups was compared by immunohistochemistry and in situ hybridization.

## Results

PVEP detection showed that at age of 6 and 8 weeks, the P100 wave latency in the right eye of deprivation group was higher than that in the left eye of deprivation group ( $P < 0.05$ ) and the right eye of control group ( $P < 0.05$ ), while the amplitude decreased ( $P < 0.05$ ). The number of positive cells ( $P < 0.05$ ) and mean optical density ( $P < 0.05$ ) of Egr-1 protein expression in visual cortex of 8-week-old deprivation group were lower than those of normal group, as well as the number ( $P < 0.05$ ) and mean optical density of Egr-1 mRNA-positive cells ( $P < 0.05$ ).

## Conclusions

Monocular form deprivation amblyopia can lead to the decrease of Egr-1 protein and mRNA expression in visual cortex, and then promote the occurrence and development of amblyopia.

## Background

Amblyopia is one of the important diseases causing visual loss of children in the world, and its incidence rate in Asia is 1.09%<sup>1</sup>. In recent years, with the in-depth research on the pathogenesis of amblyopia in molecular biology, neurobiology and other disciplines<sup>2</sup>, relevant studies have confirmed that the basic basis of amblyopia treatment lies in the existence of visual plasticity during the sensitive period of visual development<sup>3</sup>. The plasticity mechanism of visual development is related to many neurotransmitters, but

the specific pathogenesis of amblyopia has not been fully elucidated, and the changes of critical period and plasticity of amblyopia can not be explained in detail at the molecular level<sup>4-6</sup>.

Synapse is currently considered as the most critical link in amblyopia, and its plasticity can be divided into long-term potentiation (LTP) and long-term depression (LTD) according to time<sup>7,8</sup>. As a member of the Egr family of Immediate-early genes (IEGs), early growth responsive gene-1 (Egr-1) acts as a transcription factor encoding zinc finger structure. The increase of Egr-1 expression is associated with synaptic plasticity, memory consolidation, LTP induction and learning<sup>9,10</sup>. At the same time, immediate early genes also have the effect of coupling short-term signals with long-term changes<sup>11</sup>. However, there has been no study on the correlation between the expression of Egr-1 and amblyopia. Therefore, we examined changes in Egr-1 in the visual cortex in amblyopia to investigate the significance of this body in the pathogenesis of amblyopia and provide theoretical support for the occurrence and development of amblyopia.

## Methods

### Animals

We used 20 healthy 3-week-old kittens weighing between 240g and 350g, regardless of coat color and gender. The examination reveals no opacity of refractive medium or obvious abnormality of fundus, and the refractive error was +1.25 ~ +3.25D. All kittens were kept in a room with a room with plenty of natural light and a temperature of  $24 \pm 1^\circ\text{C}$ . Up to the age of 5 weeks, all kittens were fed milk powder and water 6 times a day as they were unable to feed on their own. After 5 weeks of age, the kitten has fed solid food and drank water four times a day. The kittens were provided by the Experimental Animal Center of North Sichuan Medical College. The study was supervised by the Experimental Animal Ethics Committee of North Sichuan Medical College and it has been performed according to the ARRIVE guidelines.

### Animals model establishment

The Kittens were divided into a control group (n = 10) and a monocular deprivation group (n = 10) by random number table methods. The right eye of all deprived kittens was covered with black opaque eye mask to ensure the formation of amblyopia. At the 1st, 3rd and 5th week after covering, the Pattern Visual Evoked Potential (PVEP) test was used to measure each eye of kittens in the control group and the monocular deprivation group. According to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020), we euthanize all kittens after the last PVEP test. The visual cortex was separated and the expression of Egr-1 was detected by immunohistochemistry and in situ hybridization. (Fig. 1)

### PVEP detection

The Kittens were intraperitoneally injected with one percent pentobarbital sodium (35 mg/kg). The refractive error of the kittens was detected by static retinoscopy and corrected accordingly with lenses.

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During the PVEP test on all kittens, three animal electrode needles (RL-1223000030-RC-D, Roland Consult Stasche Finger GmbH) were inserted respectively in the following ways: the blue positive pole inserted the middle of the forehead, the red negative pole inserted the middle of the occiput, and the black ground pole inserted the subcutaneous back of the ear tip in the same direction as the measured eye. Position the kitten on a platform 40cm away from the display screen and position the head so that the center of the rear pole of the retina is level with the center of the computer screen. PVEP (RETI-port/scan 21, Roland Consult Stasche Finger GmbH) measurement parameters were adjusted to checkerboard flip stimulation, 0.3 cpd mode, and the time frequency was 1 Hz, a contrast of 97%, sampling time of 300ms and superimposed 64 times. Measurements of each eye were repeated three times to obtain an average.

(Fig. 1)

## Immunohistochemical detection

Paraffin sections were dewaxed to water and placed in 3% hydrogen peroxide solution and phosphate buffer saline (pH 7.4) (Boster Biological Technology Co., Ltd., China, AR0030) in turn to block endogenous peroxidase. The slices were placed in a repair box containing citric acid (pH 6.0) (Boster Biological Technology Co., Ltd., China, AR0024) antigen repair buffer for antigen repair. The tissue was then evenly covered with 5% BSA blocking solution in the culture dish for serum blocking. Followed by the addition of the first antibody (Egr-1) (Beijing Biosynthesis Biotechnology Co., Ltd., China, BS-1076R), second antibody (Biotin Conjugated goat anti-rabbit IgG) and strept avidin biotin complex (SABC) (Boster Biological Technology Co., Ltd., China, SA1022). DAB (Boster Biological Technology Co., Ltd., China, AR1022) was used to show colour, and positive results ranged from yellow to brownish yellow. The nucleus of haematoxylin staining (Beijing Solarbio Science & Technology Co., Ltd., China, Ltd, G1080) was blue and sealed via dehydration. Chemiluminescence was measured using image analysis by Image-Pro Plus.

## In situ hybridization

Paraffin sections were dewaxed in water and boiled in repair solution for 10min. After natural cooling, digested with protease K (20 $\mu$ l/ml) at 37°C for 30 min. Then 3% methanol-hydrogen peroxide was added, and the slide was placed in phosphate buffer saline (pH 7.4) (Boster Biological Technology Co., Ltd., China, AR0033) to block endogenous peroxidase. After pre-hybridization, Egr-1 mRNA probe (5'-GAGGAGATGATGCTGCTGAGC $\nabla$ CGGGGCT-3'; 5'-GC T TGC ACTCAGTCGGGCTC CAGGAG -3'; 5'-C T TTCTC CAGCAC $\nabla$  T G $\nabla$ AT T GCT -3) hybridization solution containing the probe was added (Boster Biological Technology Co., Ltd., China, MK1748) at concentration of 20 $\mu$ l. Hybridization was conducted at 37°C in an incubator overnight, and then the hybridization solution was washed away. BSA blocking solution was then added, followed by a drop of mouse anti-digoxigenin-labelled peroxidase (Boster Biological Technology Co., Ltd., China, MK1748). DAB (Boster Biological Technology Co., Ltd., China, AR1022) was used to show colour, and positive results ranged from yellow to brownish yellow. The nucleus of haematoxylin staining (Beijing Solarbio Science & Technology Co., Ltd., China, Ltd, G1080) was blue and sealed via dehydration. Chemiluminescence was measured using image analysis by Image-Pro Plus.

SPSS 25.0 statistical software was used. The data are expressed as means  $\pm$  standard deviation ( $\pm s$ ), using one-way analysis of variance (SNK) and two independent samples  $t$ -tests. The P100 waves of each eye in the control group and the deprivation group were analyzed by one-way ANOVA (SNK), and the comparison between any two groups was performed by two independent sample  $t$ -test. The results of immunohistochemistry and in situ hybridization were analyzed by independent samples  $t$ -test.

## Results

### P100 wave of PVEP

The PVEP examination showed that the main wave image of kitten PVEP showed N75-P100-N135 complex wave, which was composed of two positive waves and one negative wave, showing an "M" shape (Fig. 2). At the age of 3 weeks, there was no statistical difference in latency ( $F = 0.254$ ,  $P = 0.778$ ) and amplitude ( $F = 0.009$ ,  $P = 0.990$ ) of P100 wave between right eye and left eye of deprivation group and right eye of control group.

At the age of 6 weeks, the latency ( $F = 26.768$ ,  $P < 0.001$ ) and amplitude ( $F = 12.142$ ,  $P < 0.001$ ) of P100 wave were statistically different among the three groups; the latency of P100 wave in the right eye of deprivation group was higher than that in the left eye of deprivation group ( $P = 0.0002$ ) and the right eye of control group ( $P = 0.0004$ ). The amplitude of the right eye of the deprivation group was lower than that of the left eye of the deprivation group ( $P < 0.001$ ) and the right eye of the control group ( $P < 0.0001$ ).

At the age of 8 weeks, the latency of P100 wave in the right eye of deprivation group was higher than that in the left eye of deprivation group ( $P < 0.0001$ ) and the right eye of control group ( $P < 0.0001$ ). The amplitude of the right eye of the deprivation group was lower than that of the left eye of the deprivation group ( $P = 0.0001$ ) and the right eye of the control group ( $P = 0.0001$ ).

Therefore, at the age of 6 weeks, monocular form deprivation amblyopia has formed in the right eye of deprived kittens (Fig. 3, Table 1–2). (relevant data is available at <https://figshare.com/s/b237d9430f77928923df>).

Table 1  
P100 amplitude in each group. ( $\bar{x} \pm S, V$ )

$\mu$					
Time (weeks)	right eye of the deprivation group	left eye of the deprivation group	right eye of the control group	<i>F</i>	<i>P</i>
3	4.958 ± 0.646	4.923 ± 0.515	4.917 ± 0.839	0.009	0.990
4	6.383 ± 0.771	7.039 ± 0.771	7.266 ± 0.778*	3.749	0.037
6	7.036 ± 0.834	8.712 ± 0.748*	8.834 ± 0.995*	12.142	< 0.001
8	7.538 ± 0.921	9.430 ± 0.705*	9.354 ± 0.691*	17.008	< 0.001
<i>F</i>	17.634	88.407	51.491		
<i>P</i>	< 0.001	< 0.001	< 0.001		
*Compared with the right eye of the deprivation group, there was a difference ( $P < 0.05$ ).					

Table 2  
P100 latency in each group. ( $\bar{x} \pm S, ms$ )

Time (weeks)	right eye of the deprivation group	left eye of the deprivation group	right eye of the control group	<i>F</i>	<i>P</i>
3	120.20 ± 3.19	121.00 ± 3.66	121.30 ± 3.29	0.254	0.778
4	114.50 ± 3.11	112.50 ± 2.73	112.50 ± 3.17	1.326	0.282
6	109.80 ± 2.64	101.50 ± 2.73*	101.90 ± 2.77*	26.768	< 0.001
8	107.20 ± 3.19	97.10 ± 3.18*	95.00 ± 3.85*	32.772	< 0.001
<i>F</i>	31.827	109.499	111.537		
<i>P</i>	< 0.001	< 0.001	< 0.001		
*Compared with the right eye of the deprivation group, there was a difference ( $P < 0.05$ ).					

## Immunohistochemical

Four visual fields were randomly selected from each slice for statistical analysis. All of the sections showed Egr-1 protein expression in the cytoplasm, which was brown-yellow, and the nucleus was blue. At 8 weeks of age, the mean optical density of positive cells in the control group was higher than that in the

deprivation group ( $P < 0.001$ ). The number of positive cells in the control group was greater than that in the deprivation group ( $P < 0.001$ ) (Fig. 4, Table 3). (relevant data is available at <https://figshare.com/s/b237d9430f77928923df>).

Table 3  
Egr-1 immunohistochemistry of visual cortex of 8-weeks-old kittens.

Group	Mean optical density of positive	Positive cell number
Control group	0.014365 ± 0.005558	51.28 ± 15.99
Deprivation group	0.006403 ± 0.002859	31.85 ± 14.14
<i>t</i>	-8.057	-5.754
<i>P</i>	< 0.001	< 0.001

## In situ hybridization

Four visual fields were randomly selected from each slice for statistical analysis. All of the sections showed Egr-1 mRNA expression in the nucleus, which was brown-yellow, and the nucleus was blue. At 8 weeks of age, the mean optical density of positive cells in the control group was higher than that in the deprivation group ( $P < 0.001$ ). The number of positive cells in the control group was greater than that in the deprivation group ( $P < 0.001$ ) (Fig. 5, Table 4). (relevant data is available at <https://figshare.com/s/b237d9430f77928923df>).

Table 4  
Egr-1 in situ hybridization of visual cortex of 8-weeks-old kittens.

Group	Mean optical density of positive	Positive cell number
Control group	0.022897 ± 0.004059	80.58 ± 11.25
Deprivation group	0.015012 ± 0.005543	66.88 ± 14.62
<i>t</i>	-7.258	-4.697
<i>P</i>	< 0.001	< 0.001

## Discussion

Form deprivation amblyopia is caused by congenital cataract, ptosis, corneal leukoplakia and other reasons in infancy, which makes light unable or abnormal to enter the eye and deprives the macula of the opportunity to receive normal light stimulation<sup>12-14</sup>. The reduction of physiological stimulation causes macular dysplasia or stagnation during development<sup>13</sup>. For kittens, the effect of form deprivation on their

visual acuity reached its peak from postnatal to 4 weeks old. After 12 weeks old, form deprivation caused by covering could hardly interfere with their visual acuity development<sup>15</sup>. Therefore, we selected 3-week-old kittens to establish amblyopia models by monocular form deprivation. At present, the classic method of modeling form deprivation amblyopia is upper eyelid suture<sup>16-18</sup>.

In the past, our team used the the Suture Covering Method<sup>19</sup>, that is, using black covering cloth instead of eyelid suture to model amblyopia. The continuous observation of PVEP changes in experimental animals was realized by suture while preventing eye injury caused by eyelid suture. This method can effectively block the light from covering the eyes of animals in all directions and successfully establish amblyopia animal models. However, during the modeling period, some kittens still had head movements, which led to light passing through the side of the covering cloth. In order to improve this situation, this experiment increased the diameter of the covering cloth in the original covering method, and made a black opaque covering ring with a certain height in the center of the covering cloth to further enhance the covering efficiency.

In this experiment, checkerboard flip stimulation was used to detect PVEP, and the wave images of PVEP in kittens were recorded, which were composed of two positive waves and one negative wave, showing an "M" shape. By comparing the amplitude and incubation period of P100 wave between different eyes at the same time and the same eye at different times, we found that the amplitude of P100 wave decreased and the latency of P100 wave increased in the right eye of deprivation group compared with the left eye of deprivation group and the right eye of control group. This is similar to the previous amblyopia modeling results by other schemes<sup>20,21</sup>. The results of this study suggest that the improved covering method is feasible and effective. The animal model of monocular form deprivation amblyopia can be successfully established by the the Suture Covering Method, and the dynamic detection of eye physiological parameters of experimental animals can be realized.

With the development of amblyopia research in recent years, many neurotransmitters involved in the plasticity mechanism of visual development, such as AMPA receptor and its GluR2 subunit, NEP1-40, synaptophysin, cholinergic neurons, growth-associated protein-43, GABA, cPKC-r, NMDAR, NGR-1, etc<sup>22-27</sup>, are considered to be related to amblyopia, but the relationship between the critical period of amblyopia and its plasticity changes cannot be completely explained at the molecular level<sup>27,28</sup>. As a class of proto-oncogenes encoding transcription factors, immediate early genes have the effect of coupling short-term signals with long-term changes, mainly including C-fos, Egr family and Arc, among which many transcription factors are regulated by visual activities<sup>29,30</sup>. Among them, there is sufficient evidence to show the association between C-fos and amblyopia<sup>17,31</sup>. The transcription factor of Egr-1 in the Egr family of immediate early genes is essential in the changes of visual cortex plasticity<sup>32</sup>. Egr-1 is a transcription factor encoding zinc finger structure, and its expression increases during synaptic plasticity, memory consolidation, LTP induction and learning<sup>7,8</sup>. Cytoskeleton related gene Arc, as one of the target genes of Egr-1, is an effector molecule induced by synaptic activity and plays an important role in late

conditions, Egr-1 can regulate the transcription of late activity-

dependent Arc gene in hippocampus CA1 region, and the immediate early gene Arc can connect the change pattern of neural activity and synaptic plasticity because of its pluripotent and fine tuning system, thus optimizing the information storage of nervous system<sup>32</sup>. In addition, previous studies on amblyopic animal models have found that there are structural changes in ganglion cells, lateral geniculate body and visual cortex of amblyopic animals, accompanied by a decrease in synaptic density, which will lead to further changes in their functions<sup>2,33,34</sup>.

Immunohistochemistry and in situ hybridization were used to compare and analyze the expression of Egr-1 protein in visual cortex of 8-week-old amblyopia kittens and normal kittens. The results of this experiment suggest that during the critical period of visual development, due to the unequal input of binocular vision, the number and morphology of visual cortex cells may change, resulting in the decrease of Egr-1 protein and mRNA expression, which further affects the normal physiological function of visual cortex and affects visual development, thus promoting the occurrence and development of amblyopia.

The balance of excitation and depression at axonal level of visual cortex is the condition of maintaining normal development and function of visual cortex, and it is also an important factor affecting the plasticity of visual system<sup>35</sup>. The plasticity of nerve is mainly manifested in the plasticity of synaptic structure and function<sup>36,37</sup>. The occurrence of amblyopia is related to synaptic plasticity changes, and long-term changes will occur in the process of amblyopia. Egr-1 and Arc genes are closely related to long-term changes of synapses. At present, most studies also show that perceptual learning can improve the visual function of amblyopia patients<sup>5</sup>. Perceptual learning can effectively restore the visual function through repeated visual stimulation and visual experience with supervision and feedback<sup>38-40</sup>. Researchers found that its mechanism may be related to the balance of excitement and depression related to plasticity, and Egr-1 and Arc play an important role in learning and memory of humans and animals<sup>41,42</sup>. This study not only confirmed the correlation between Egr-1 protein and mRNA expression in visual cortex and amblyopia, but also indirectly verified the correlation between perceptual learning and amblyopia.

## Conclusions

To sum up, the animal model of amblyopia can be established and the dynamic measurement of eyeball parameters can be realized by using the method of ocular covering. Based on this method, the expression of Egr-1 in visual cortex decreased significantly in amblyopia animal model. This experiment speculates that Egr-1 plays an important role in visual development. This study provides a new idea and direction for further exploring how monocular form deprivation regulates visual cortex neurons, and for treating amblyopia and deeply understanding its pathogenesis.

## Abbreviations

Egr-1

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early growth responsive gene-1; LTP:long-term potentiation; LTD:long-term depression; IEGs:immediate-early genes; PVEP:Pattern Visual Evoked Potential

## Declarations

### Acknowledgements

Not applicable.

### Author contributions

Yunchun Zou participated in the design of the experiment, analysed the data, and modified the manuscript. Haobo Fan and Ying Wang participated in creating the animal model, specimen collection, experimental manipulation, data collection and analysis, and manuscript writing. Liyuan Yang, Xiuping Tang and Weiqi Song participated in creating the animal model, PVEP testing, specimen collection and experimental operation. All authors read and approved the final manuscript.

### Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article Nanchong Science and Technology Project (No.18SHZ0386), and the Health Department of Sichuan Province (No. S18034). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. Or all relevant datasets related to the study can be found in the specified database.

### Ethics approval

The study that has been performed according to the ARRIVE guidelines was approved by the Medical Ethics Committee of North Sichuan Medical College and supervised throughout the process.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

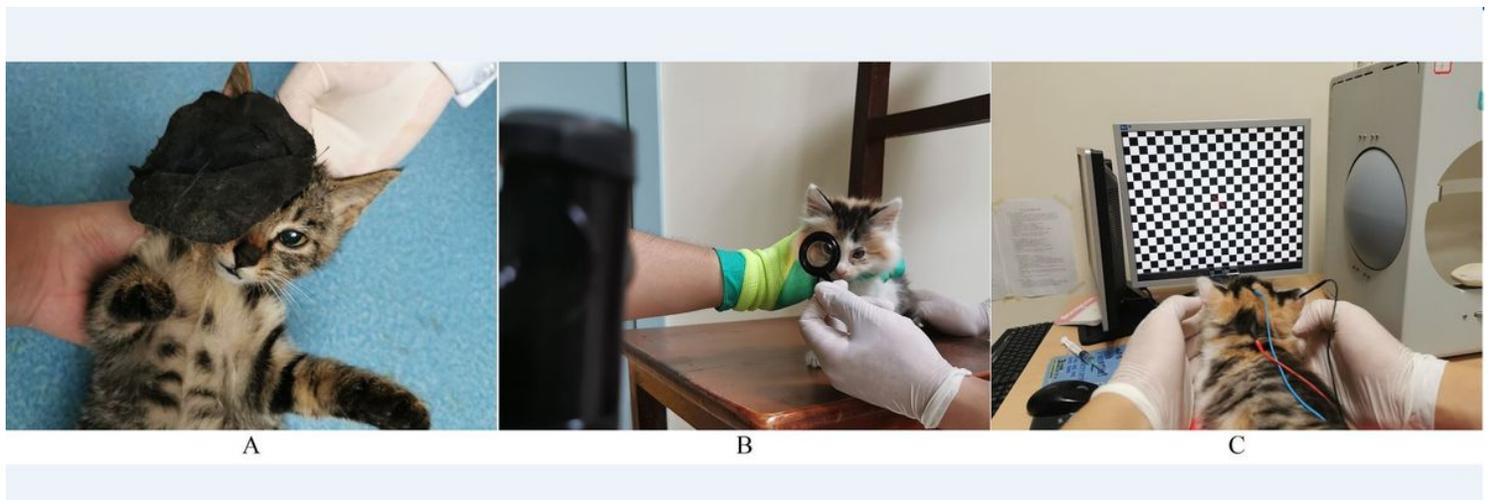
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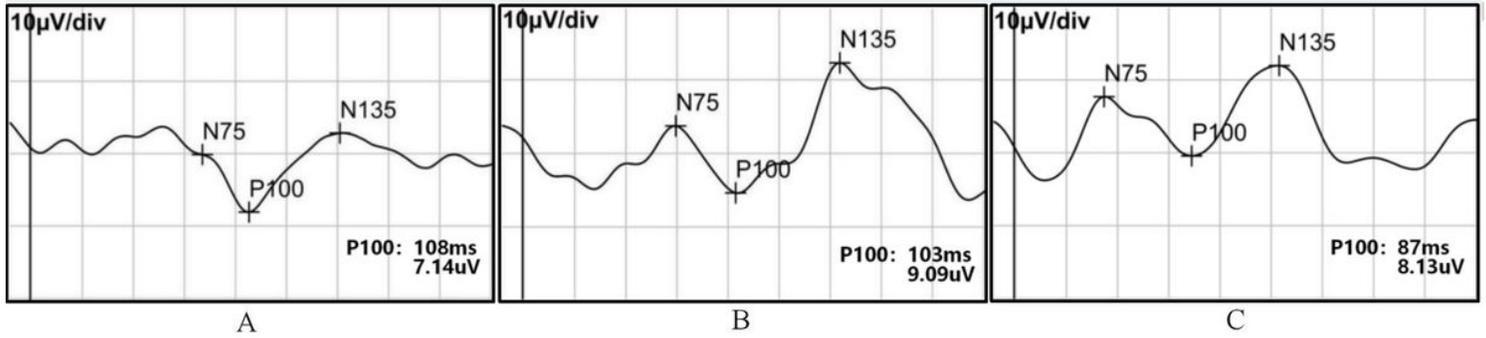
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## Figures



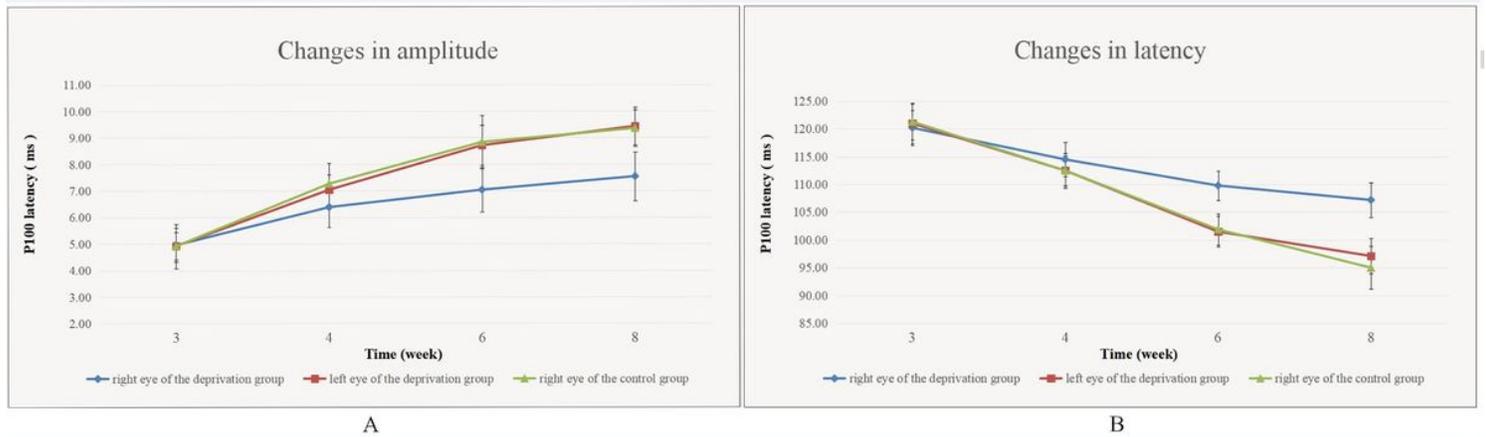
**Figure 1**

Establishment and examination of animal model. A: Cover the right eye of the kitten with a covering cloth B: Detection the diopter of the kitten with a band ophthalmoscope C: Schematic diagram of PVEP detection



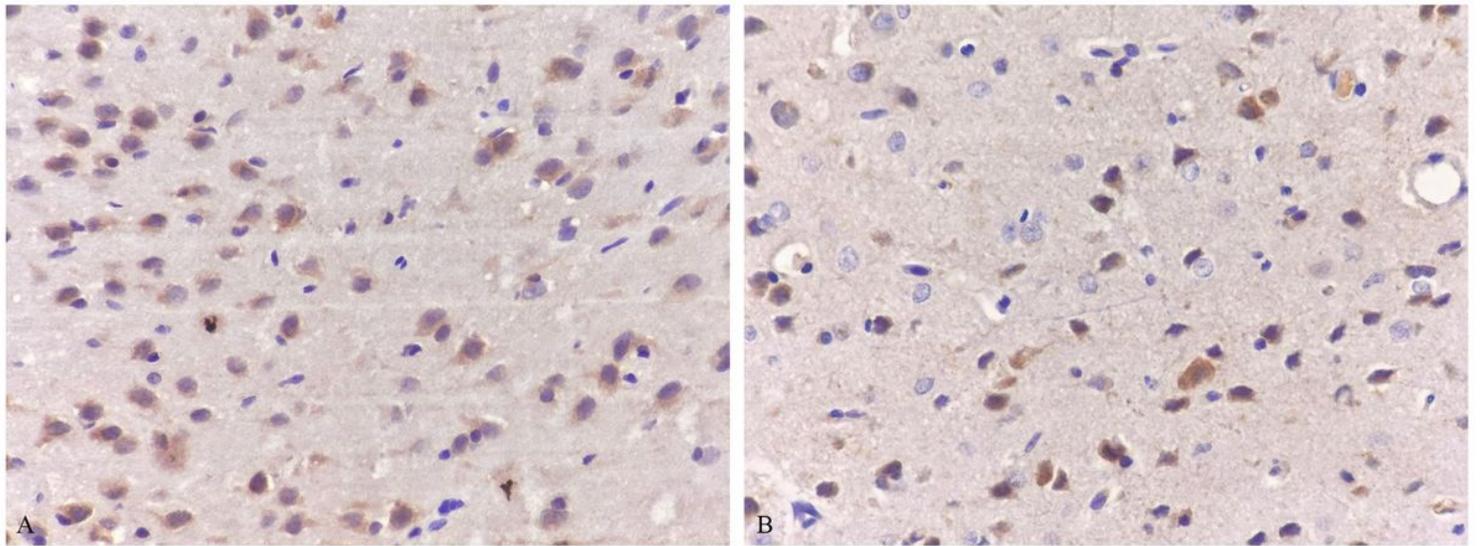
**Figure 2**

PVEP waveform of kittens in each group after covering for 5 weeks. A: Right eye in deprivation group; B: Left eye in deprivation group; C: Right eye of control group



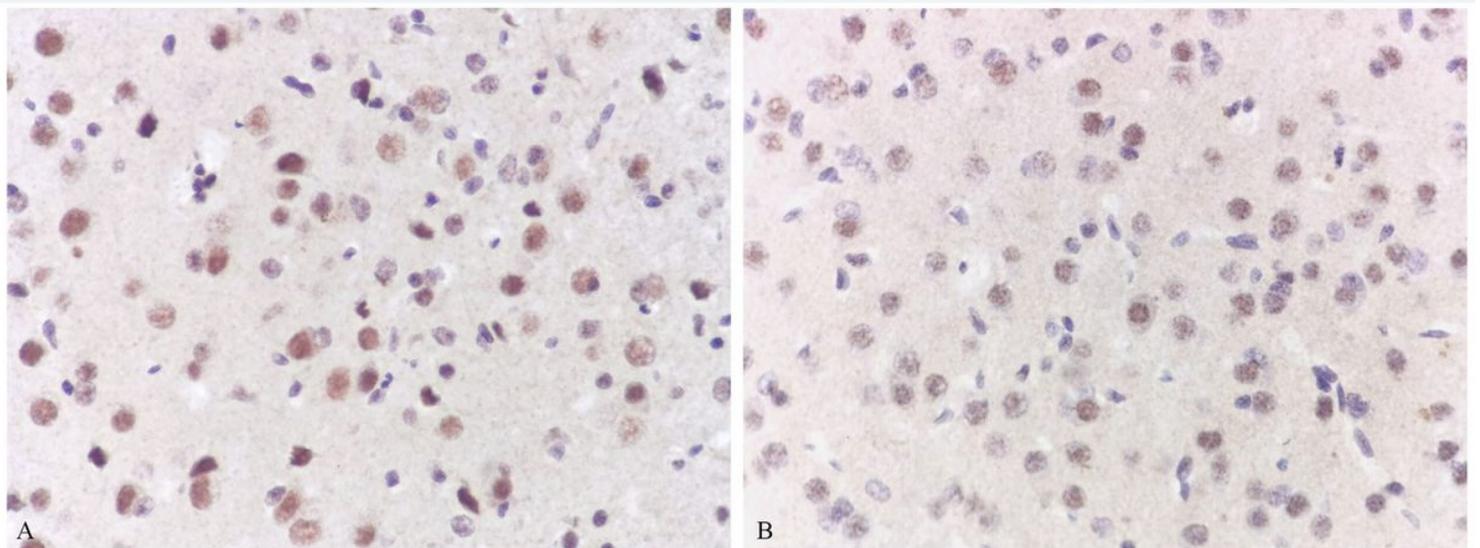
**Figure 3**

The latency and amplitude trend of P100 waves in 3-week-old to 8-week-old kittens. With increasing age, the amplitude of the P100 wave in the each eye of the control group and the deprivation group gradually increased (A), and the latency shortened gradually (B).



**Figure 4**

Immunohistochemical performance in visual cortex in each group (DAB X400). The protein of Egr-1 positive expression in the cytoplasm of neurons was brown-yellow. At the age of 8 weeks, there were more positive cells in the control group (A) and fewer in the deprivation group (B).



**Figure 5**

In situ hybridization performance in visual cortex in each group (DAB X400). The mRNA of Egr-1 positive expression in the nucleus of neurons was brown-yellow. At the age of 8 weeks, there were more positive cells in the control group (A) and fewer in the deprivation group (B).