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# Optical control after transfection of channelrhodopsin-2 recombinant adenovirus in visual cortical cells☆

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## Abstract

Channelrhodopsin-2 ectopically expressed in the retina can recover the response to blue light in genetically blind mice and rats, but is unable to restore visual function due to optic nerve or optic tract lesions. Long Evans rats at postnatal day 1 were used for primary culture of visual cortical cells, and 24 hours later, cells were transfected with recombinant adenovirus carrying channelrhodopsin-2 and green fluorescent protein genes. After 2–4 days of transfection, green fluorescence was visible in the cultured cells. Cells were stimulated with blue light (470 nm), and light-induced action potentials were recorded in patch-clamp experiments. Our findings indicate that channelrhodopsin-2-recombinant adenovirus transfection of primary cultured visual cortical cells can control the production of action potentials *via* blue light stimulation.

## Key Words

channelrhodopsin-2; recombinant adenovirus; visual cortex; blue light; action potentials; neural regeneration

## Abbreviations

ChR2, channelrhodopsin-2; MAP2, microtubule-associated protein 2

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## INTRODUCTION

Channelrhodopsin-2 (ChR2) is a light-sensitive cation channel isolated from the unicellular green alga, *Chlamydomonas reinhardtii* [1-4]. Genetically modified nerve cells expressing ChR2 are able to phototransduce light stimuli into electrical activity[5-8]. Light control of cellular activities is contact-independent, allows precise time- and space-modulation, and quantitative duplication. Thus, it has potentially broad applicability in the treatment of nervous system diseases[8-16]. To explore the repair of visual functions, the ChR2-carrying adeno-associated virus, injected into the vitreous humor of genetically blind RD1 mice and RCS rats, was used to transfect retinal neural cells. We found that visual evoked potential waveform activity was induced by flash stimulation at 470 nm (ChR2 sensitive wavelength), but no waveform was induced by flash stimulation at 580 nm (ChR2 insensitive wavelength). This was evidence that after ChR2 transfection, the originally non-photosensitive retinal neural cells could transduce optical stimuli into electrical signals, which were relayed to the brain, resulting in activity in the visual cortex[17-19]. ChR2 can be expressed without immunologically harmful reactions *in vivo*[20]. Two types of adeno-associated viruses carrying HaloR (light-driven chloride pump) and ChR2 were injected into the vitreous humor of blind adult RD1 mice.

Coexpression of ChR2/HaloR in retinal ganglion cells can produce ON, OFF, and even ON-OFF responses, depending on the wavelength of the light stimulus. However, the above experiments were confined to the retina, only examining visual functional recovery following retinal photoreceptor degeneration. Restoration of visual functions in the injured optic nerve and optic tract were not assessed.

This study aimed to express ChR2 in visual cortical cells by transfection with ChR2-recombinant adenovirus, and to examine the effects of light on these cells.

## RESULTS

### Growth of visual cortical neurons

After visual cortical neurons from healthy 1-day-old Long Evans newborn rats were cultured for 2 hours, cells began to adhere. At 24 hours, some necrotic cells were suspended in the medium. After the culture medium was completely changed, adherent cells varied in size, were oval-shaped, became transparent, and were refractive. The majority of these cells extended tiny protrusions. At 4 days, a large number of protrusions were visible and were interconnected with each other (Figure 1).

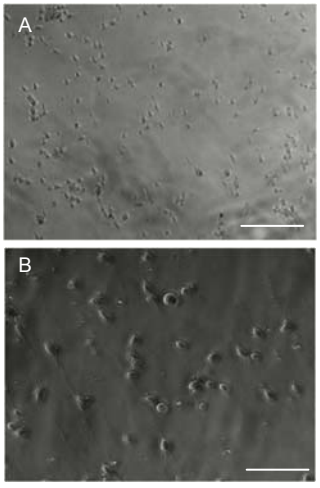


Figure 1 Primary cultured rat visual cortical neurons at 4 days (× 100 in A; × 200 in B).

A large number of slender processes are visible under the optical microscope, and they are interconnected with each other.

After 7–10 days of culture, pyramidal neurons increased in size, and had a smooth surface, a clear boundary, uniform cytoplasm, and a distinct contour. Neural cells survived for 1 month, and microtubule-associated protein 2 (MAP2)-positive cells accounted for more than 80% of total cells (Figure 2).

### Morphology of rat visual cortical neurons after adenoviral transfection

After 24 hours of primary culture, the rat visual cortical cells were divided into three groups: normal control (no viral transfection, normal culture), negative control (transfection of virus carrying green fluorescent protein), and virus transfection (transfection of virus carrying ChR2-GFP), with 30 wells in each group. Green fluorescence was visible in the negative control group and virus transfection group at 24 hours after transfection (Figure 3), and fluorescence was significantly increased at 3–4 days. In these two groups, half of all cells exhibited green fluorescence.

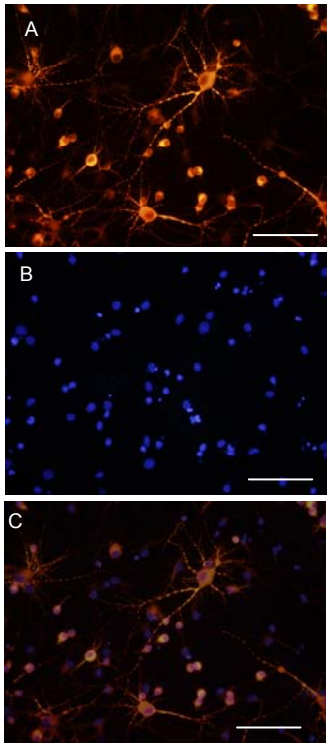


Figure 2 Morphology and identification of rat visual cortical neurons primary cultured for 8 days (immunocytochemistry; × 400).

(A) Anti-microtubule-associated protein 2 immunolabeled neurons (red).

(B) DAPI stained neuronal nuclei (blue).

(C) Fusion image showing that the proportion of neurons is greater than 80%.

### Bioelectrical properties of visual cortical neurons after transfection

Patch-clamp recordings showed that the three different groups of cultured visual cortical neurons had similar resting membrane potentials (65.1 ± 8.4 mV, 65.7 ± 9.0 mV and 64.7 ± 8.7 mV, respectively) with no significant difference between them. In the whole-cell mode, cultured cells in all three groups showed typical inward sodium currents and outward potassium currents

(Figure 4A).

In current clamp mode, membrane potential was defined as −60 mV, and the cultured visual cortical neurons were stimulated by blue light at 470 nm, $1.6 \times 10^{18}$ photons $cm^{-2}/s$. There was no light-induced inward current recorded in the normal control group. In the negative control group, neither the fluorescent cells (20 cells) nor the non-fluorescent cells (10 cells) displayed light-induced inward currents. After virus transfection, light-induced action potentials were recorded in cells displaying green fluorescence (24 cells) (Figure 4B), while non-fluorescent cells did not respond to light (6 cells).

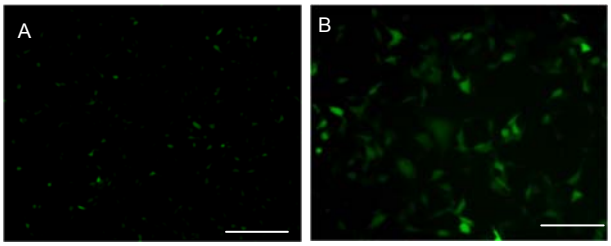


Figure 3 Morphology of visual cortical cells 2 days after channelrhodopsin-2/green fluorescent protein recombinant adenovirus transfection (immunofluorescence).

The cultured visual cortical cells exhibit green fluorescence: (A) Low magnification (scale bar: 150 μm); (B) high magnification (scale bar: 75 μm).

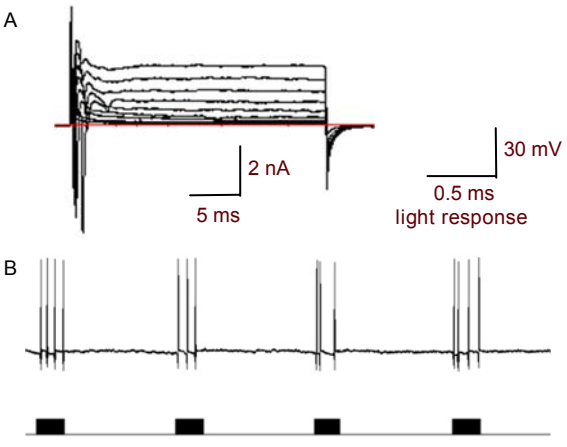


Figure 4 Whole-cell patch-clamp recording of cultured visual cortical neurons.

(A) Whole-cell currents in cultured nerve cells were recorded with voltage clamp, at stimulation voltages of −80 mV to 30 mV, step 10 mV, and duration 30 ms.

(B) After virus transfection, visual cortical nerve cells expressing green fluorescent protein were stimulated with blue light at 470 nm, strength of $1.6 \times 10^{18}$ photons $cm^{-2}/s$; action potentials were recorded using patch clamp.

## DISCUSSION

Compared with albino rats, such as Sprague-Dawley or Wistar rats, the Long Evans rats used here are more suitable for neurological and ophthalmological research[23-24]. In this study, we used primary cultures of visual cortical neurons from 1-day-old Long Evans rats. At this stage, the central nervous system is largely developed, visual cortical regions can be accurately located, and central neurons can be successfully cultured *in vitro*[25-26]. The cells were cultured in serum-free culture medium, avoiding the cell growth promoting effect of serum, which requires the use of cytotoxic cytosine arabinoside to suppress the growth of glial cells[27-29].

The E1 deleted adenovirus type 5 vector used in this study is stable, the viral genome cannot rearrange, the inserted exogenous gene is stable, has a fragment capacity of 7.5 kb, and a titer of $10^{11}$ PFU/mL. Unlike a slow virus, adenovirus carrying exogenous DNA cannot integrate into the host genome, and has no toxicity[30-31]. We observed green fluorescence in cells in the negative control and virus transfection groups, which indicated that adenovirus had successfully transfected cultured visual cortical cells. While the whole-cell patch-clamp recordings showed that there was no significant difference in the resting membrane potentials between the virus transfection groups (negative control and virus transfection) and the non-transfection group (normal control), typical inward sodium currents and outward potassium currents were observed in all three groups. This result is evidence that recombinant adenovirus transfection has no impact on normal electrophysiological functions in cultured visual cortical cells.

Only after virus transfection were blue light-induced action potentials recorded in cells exhibiting green fluorescence. We conclude that only ChR2-expressing cells can respond to blue light. A number of *in vivo* animal experiments have confirmed that light control of nerve cells can be achieved through ChR2 expression[5-7]. Only after the adeno-associated virus, injected into the vitreous humor, had induced retinal ganglion cells to express ChR2, could blue light of 470 nm induce cells to generate action potentials in patch-clamp experiments[17-19, 21-22]. Using electroporation technology and transgenic animals, certain neurons were targeted to express ChR2. This enabled light-mediated induction of action potentials, thereby affecting activity and behavior[32-34]. In summary, we demonstrate that adenovirus can elicit ChR2 expression in cultured visual cortical cells as well, and that blue light can modulate cellular activity. Our work should lay the foundation for

the application of gene technology using channelrhodopsins in studies of the visual cortex and in visual rehabilitation research.

## MATERIALS AND METHODS

### Design

An *in vitro* controlled cytological experiment.

### Time and setting

Experiments were performed from March 2010 to October 2011 in the Department of Ophthalmology, Southwest Hospital of the Third Military Medical University of Chinese PLA, China.

### Materials

#### *Animals*

Long Evans newborn rats of clean grade, aged 1 day, males and females, weighing 3–5 g, offspring of Wistar female rats and wild male gray rats, were purchased from the Experimental Animal Center, Research Institute of Surgery, Daping Hospital of the Third Military Medical University of Chinese PLA (permit No. SCXK (Yu) 20020003). All experimental procedures involving animals was in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[35].

#### *ChR2 virus*

ChR2 coding sequence (GenBank Accession No. AF461397) was cloned into GFP-tracing Adenovirus Shuttle Plasmid pSB291 (Shanghai, China), forming a transfer vector pSB291-ChR2-GFP. The vector, together with adenovirus, was used to package plasmid pBHG lox ΔE1,3 Cre (Shanghai, China), which were cotransfected into HEK293 cells, to obtain ChR2 recombinant adenovirus[36].

### Methods

#### *Culture plate pretreatment*

The wells of a 24-well culture plate, containing 1 cm × 1 cm coverslips, were treated with 100 μL of 10% polylysine (Sigma, St. Louis, MO, USA) for 10 minutes. The solution was then discarded, and the wells were dried at room temperature prior to use for culture.

#### *Primary culture of rat visual cortical neurons*

All rats were decapitated under anesthesia, and the heads were immersed in 75% ethanol for 5 minutes for disinfection. The visual cortex was excised, and the cerebral pia mater was stripped under a stereomicroscope. Specimens were cut into 1 mm × 1 mm × 1 mm pieces and digested with 0.25% trypsin (Hyclone, Logan, Utah, USA) at 37°C for 30 minutes. The digestion was terminated with DMEM/F12 medium containing 10% calf serum (Shanghai Langton, Shanghai, China). Cells were triturated with a pipet into a single cell suspension, and 1 mL of suspension was inoculated into each well of a poly-L-lysine-coated 24-well culture plate. Cells were cultured at a density of $2–4 \times 10^5/cm^2$, in 37°C, 5% $CO_2$ for 24 hours. Then culture medium was changed to maintenance medium 1, composed of Neurobasal A (Gibco, Gaithersburg, MD, USA), B27 supplement (Invitrogen, Carlsbad, CA, USA), GlutaMAX (Invitrogen), antibiotic/antimycotic (Invitrogen) and basic fibroblast growth factor (Invitrogen), and then half replenished every 2 days with maintenance medium 2, which included Neurobasal A (Gibco), B27 supplement (Invitrogen), antibiotic/antimycotic (Invitrogen) and basic fibroblast growth factor (Invitrogen).

#### *Transfection of adenovirus carrying ChR2-GFP*

After visual cortical cells were cultured for 24 hours, the normal control group was cultured with maintenance medium 1, with no virus transfection. The negative control group was cultured in maintenance medium 1 with virus carrying GFP, while the virus transfection group was cultured in maintenance medium 1 with virus carrying ChR2-GFP. Each well of the two transfection groups was treated with 1 μL of virus at a titer of $7.9 \times 10^9$ PFU/mL. The medium was half replenished with maintenance medium 2 every 2 days. At 3–5 days, cells displaying green fluorescence were irradiated with blue light at 470 nm, and light-evoked responses were examined using patch-clamp recording.

#### *Immunohistochemical identification of rat visual cortical neurons*

Anti-MAP2 antibody is a neural cell-specific antibody that can be used as a neuron-specific marker[37-39]. Immunocytochemical labeling was performed as follows: After 8 days of culture, cells were isolated, rinsed with PBS twice for 5 minutes each, fixed with 10% acetic acid and 90% ethanol for 20 minutes at room temperature, rinsed with PBS three times for 5 minutes each, permeabilized using 5 mM Lysine + 0.3% Triton X-100 + 100 mL PBS for 5 minutes, blocked with donkey serum for 2 hours, incubated with mouse anti-MAP2 monoclonal antibody (Abcam, Cambridge, CB, UK; 1:800) in a wet box at 4°C overnight, rinsed with PBS three times for 5 minutes each, incubated with CY3-labeled sheep anti-mouse IgG (AP124C, Millipore Chemicon, Billerica, MA, USA; 1:500) in a wet box at 37°C for 50 minutes, and rinsed with PBS three times for

5 minutes each. Specimens were then dehydrated, cleared and mounted. Under the fluorescence microscope (Leica MZ10F, Oskar Barnack, Solms, Germany), positive cells were labeled red.

### *Patch-clamp recording of resting membrane action potential changes in rat visual cortical neurons*

The recordings were performed in Hank's solution, consisting of NaCl, 138 mM; $NaHCO_3$, 1 mM; $Na_2HPO_4$, 0.3 mM; KCl, 5 mM; $KH_2PO_4$, 0.3 mM; $CaCl_2$, 1.25 mM; $MgSO_4$, 0.5 mM; $MgCl_2$, 0.5 mM; HEPES-NaOH, 5 mM; and glucose, 22.2 mM; pH 7.2–7.4, adjusted with 0.3 N NaOH. The electrode was filled with electrode solution, consisting of K-gluconate, 133 mM; KCl, 7 mM; $MgCl_2$, 4 mM; EGTA, 0.1 mM; HEPES, 10 mM; Na-GTP, 0.5 mM; and $Na^+$-ATP, 2 mM; pH 7.2–7.4, adjusted with KOH. The electrode impedance was 5–10 MΩ at 22°C. Data were collected on a MultiClamp 700A (Axon Instruments, New York, NY, USA) using pClamp 10.1 software (Axon Instruments). The inward and outward currents were recorded in voltage clamp mode. Cells were irradiated with blue light at 470 nm, intensity of $1.6 \times 10^{18}$ photons $cm^{-2}/s$, and action potentials were recorded in current clamp mode.

### *Statistical analysis*

Data were analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). The mean values of samples in each group were tested for homogeneity of variance using the Levene method. Resting membrane potentials were compared with one-way analysis of variance, and two groups were compared with the least significant difference *t*-test. $P < 0.05$ was considered statistically significant.

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**Author contributions:** Junping Yao had full access to data integration and analysis, and drafted the manuscript. Wensheng Hou was responsible for study concept and design, and validated the study. Hao Wang participated in cell culture and virus transfection experiments. Hui Liu performed visual cortical cell culture experiments. Chuanhuang Weng was in charge of the patch clamp experiment. Zhengqin Yin was in charge of funds and supervised the study.

**Conflicts of interest:** None declared.

**Ethical approval:** All experimental procedures using animals complied with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China.

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