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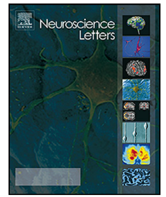

Research article

The neural pathway for lacrimal gland tear secretion in New Zealand White rabbits

Jiaojiao Wang, Rongyi Zhou, Weiping Gao*

Nanjing University of Traditional Chinese Medicine, Xianlin road no. 138, Qixia District, Nanjing City, Jiangsu Province 210023, China

HIGHLIGHTS

- Lacrimal gland tear secretion in rabbits is connected with the frontal cortex, the field CA1 of hippocampus, the paraventricular nucleus of hypothalamus, the pons, and the medulla oblongata.
- The neural pathway that regulates tear secretion from the lacrimal gland proceeds from the PVN to the superior olivary complex of the pons to the SSN, and finally to the lacrimal gland.
- This research provides a new idea for the study of the pathogenesis of dry eye.

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ABSTRACT

Objective: We investigated the neural pathway for tear secretion from the lacrimal gland of New Zealand White rabbits.
Methods: Nine healthy adult New Zealand White rabbits were randomly divided into three experimental groups, namely, an irritant-stimulated group, a non-stimulated group, and a saline-stimulated group. Sanitized dry cotton swabs with menthol were used to wipe both of the rabbits' eyelids in the irritant-stimulated group, and the non-stimulated group and saline- stimulated group were compared as controls. The animals in the three groups were killed 2 h later and the expressions of c-Fos in the frontal cortex, hippocampus, hypothalamus, pons, and medulla oblongata of the rabbits were detected using immunofluorescence labeling. According to the distribution of c-Fos protein expression, 12 healthy adult New Zealand rabbits were similarly divided into three groups for retrograde tract tracing via pseudorabies virus (PRV) injection into the lacrimal gland. Immunofluorescence labeling was used to analyze PRV-infected neurons in the brains of rabbits after survival for 30 h, 38 h, and 46 h.
Results: The most c-Fos-positive immunolabeled cells were observed in the menthol-stimulated group, whereas fewer c-Fos-positive immunolabeled cells were observed in the saline-stimulated group.The non-treated group showed the least c-Fos-positive immunolabeled cells. At 30 h after PRV injection, PRV-positive neurons were found only in the superior salivary nucleus of the pons (SSN). At 38 h, PRV-infected neurons were observed in the lateral nucleus of the superior olive (LSO) and the medial nucleus of the superior olive (MSO). At 46 h, PRV-infected neurons were found in the nucleus of the trapezoid body (Tz) and the hypothalamic paraventricular nucleus (PVN), and their distributions were dense in the LSO and MSO.
Conclusions: Menthol-induced c-Fos protein expression and PRV-mediated tract tracing suggest that in New Zealand White rabbits, the neural pathway that regulates tear secretion from the lacrimal gland proceeds from the PVN to the superior olivary complex of the pons to the SSN and finally to the lacrimal gland.

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Abbreviations: PRV, pseudorabies virus; SSN, superior salivary nucleus; LSO, lateral nucleus of the superior olive; MSO, medial nucleus of the superior olive; Tz, trapezoid body; PVN, hypothalamic paraventricular nucleus; CNS, central nervous system; PBS, phosphate buffer solution; SEU, Southeast University.

* Corresponding author.
E-mail addresses: wjjnzy@126.com (J. Wang), zhourongyitcm@sina.com (R. Zhou), gaoweiping1@126.com (W. Gao).

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1. Introduction

Dry eye is an ocular characterized by the inability to produce a tear film of sufficient quantity and quality to allow for adequate lubrication and protection of the corneal surface [1,2]. This condition may accompany diseases that are characterized by ocular surface tissue lesions [3]. To our knowledge, tear secretion is regulated by lacrimal gland dominated neuromodulation [4]. Dry eye has closely relationship to the central nervous system (CNS) [5,6]. The lack of a tear reflex is a common cause of dry eye and electrical stimulation of the lacrimal gland or afferent nerves may be used as a treatment for dry eye disease [7]. Moreover, an abnormal ocular surface and lacrimal nerve reflex pathway can reduce tear secretion and lead to dry eye. The tearing response to irritation of the ocular surface is a reflex that occurs via direct afferent nerve stimulation of an efferent pathway that acts on the lacrimal gland. The efferent pathway that regulates lacrimal gland tear secretion is known and a previous study reported that menthol increased tearing [8]. Therefore, sanitized dry cotton swabs with menthol were used to wipe the eyelids of rabbits to increase tears during this study.

By using c-Fos immunohistochemistry, acutely activated neurons can be readily detected within the CNS [9]. The present study assessed the expression of the immediate early gene c-Fos to identify brain areas that are active during eye irritation in rabbits. Retrograde transynaptic viral tracers are a powerful tool for identifying neuronal networks.The previous study identified a complete eyeblink premotor pathway, deep cerebellar interconnectivity, and specific neurons responsible for the generation of eyeblink responses [10]. However, the neural pathway for lacrimal gland tear secretion of New Zealand White rabbits is not clear. Therefore, pseudorabies virus (PRV) was injected into the lacrimal gland and detected via immunofluorescence labeling for retrograde tracing of the relevant efferent neural circuitry.

2. Methods

2.1. Animals and groups

Rabbits purchased from the Animal Center of the Medical College of Southeast University(SEU) were used during this experiment. The experiment protocol was approved by the Animal Experiment Ethics Committee of SEU. Healthy adult male and female New Zealand White rabbits weighing 2.0–2.5 kg and aged 4–5 months were provided by the Animal Center of the Medical College of SEU. One week before the experiment began, the experimental animals were housed in cages in a quiet room at room temperature ($22 \pm 2\,^{\circ}$C) under normal conditions during a 12:12-h light:dark cycle with, water and standard feed provided ad libitum.

2.2. Instruments and reagents

Frozen tissue sections were cut using a Leica cryostat (Wetzlar, Germany). Optical microscopy imaging was performed using an Olympus microscope (Tokyo, Japan). A decoloring table (Medical Instrument Factory, Jiangsu, China), Nanofil NF34BV-2 microsyringe (WPI, Sarasota, FL, USA), rat polyclonal antibody against c-Fos (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal antibody against PRV (Abcam, Cambridge, UK), and goat anti-rat Alexa Fluor 488-conjugated antibody and goat anti-rabbit Alexa Fluor594-conjugated antibody, (Invitrogen, Carlsbad, CA, USA) were used. In addition, normal goat serum (Jinqiao Co., Beijing, China), closed 25% urethane (Shanghai National Medicine Group Chemical Reagent Co., Ltd.,Shanghai, China), paraformaldehyde (Kelon Chemical Reagent Co.,Chengdu, China), phosphate buffer (Wuhan Boster Biotechnology Co.,Wuhan, China), and a peppermint camphor mixture (mentholatum, China Pharmaceutical Co., Ltd.,Zhongshan, China) were used during this study.

2.3. Experimental design

Nine healthy adult New Zealand White rabbits were randomly divided into three groups, namely, an irritant-stimulated group, a non-stimulated group, and a saline-stimulated group. For the irritant stimulated group, sanitized dry cotton swabs doused in menthol were used to wipe both of the rabbits' eyelids. For the saline-stimulated group, sanitized dry cotton swabs doused with saline were used to wipe both of the rabbits' eyelids. The non-stimulated group of rabbits was left untreated. The expression of c-Fos was detected 2 h later [11]. Based on the pattern of c-Fos protein expression, an additional 12 New Zealand White rabbits were randomly divided into three survival time groups ($n = 4$ per group) following PRV injection into the lacrimal gland (i.e., 30-h, 38-h, and 46-h survival time groups).

2.4. Tissue preparation

After treatment as indicated above, the groups of experimental rabbits were administered 25% urethane at 4 ml/kg via the ear marginal vein. The hearts of the rabbits were exposed for perfusion where the left ventricle of the heart was punctured for intubation into the ascending aorta and the right atrium was cut. In this manner, perfusion proceeding through the left ventricle with approximately 750 ml saline at 37 °C was used for exsanguination and 1000 ml of 4% paraformaldehyde was to fix the tissue. After perfusion, brain tissue was excised, placed into 4% paraformaldehyde for 12 h, and then placed in a solution of 30% sucrose at 4 °C for approximately 3 d. After embedding the tissue in OCT, serial 30-μm sections of the brain were coronally cut at −20 °C on a cryostat, and collected into 0.01 mol/L of phosphate-buffered saline (PBS) with a pH of 7.4 for immunofluorescence histochemistry.

2.5. Immunofluorescence histochemistry

To perform immunofluorescence histochemistry, the tissue sections were placed in 3% hydrogen peroxide solution for 15 min and then washed in PBS three times (for 5 min each time). Next, the tissue was permeabilized via incubation in 0.4% Triton at 37 °C for 30 min, blocked in 10% normal sheep serum for 45 min at room temperature, and incubated in primary antibody at 4 °C overnight. After washing four times in PBS (for 10 min each time), the tissue was mounted onto slides. These slides were glycerin-sealed for fluorescence microscopy analysis.

3. Results

3.1. Expression and distribution of c-Fos immunopositive neurons

Rabbit brain tissue sections showed no c-Fos expression when the primary antibody was replaced by PBS. Moreover, few c-Fos immunopositive cells were observed in brains of the non-treated group of rabbit, some c-Fos immunopositive cells were observed in the saline-stimulated group of rabbits, and many c-Fos immunopositive cells were observed in the menthol-stimulated group of rabbits. Many cells expressing c-Fos protein were observed in the frontal cortex, on both sides of the third ventricle, i.e., the paraventricular nucleus (PVN) of the hypothalamus, and in the pons. Fewer cells expressing c-Fos protein were observed in the hippocampal CA1 area and in the spinal nucleus of trigeminal nerve in the medulla. This c-Fos expression appeared to be nuclear because

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