Int J Clin Exp Med 2017;10(6):9073-9080
www.ijcem.com /ISSN:1940-5901/IJCEM0045616

Original Article

Coexistence and mutual regulation between tyrosine hydroxylase and calcitonin gene-related peptide in facial nucleus of rats regulate the neuronal activities of afferent and efferent neuro fibers

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Received December 2, 2016; Accepted April 28, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: To explore the coexist meaning of tyrosine hydroxylase (TH) and calcitonin gene-related peptide (CGRP) in facial nucleus, immunohistochemistry and immunofluorescence staining were used to detect whether there is TH expression in facial nucleus and whether there has coexist phenomenon between TH and CGRP. The mRNA levels and protein expression values of TH and CGRP in facial nerve cells were determined before and after transfection with siRNA-TH and shRNA-TH. There were many TH and CGRP double-label neuronal somas in ventrolateral subnuclear, dorsolateral subnucleus and dorsal intermediate subnucleus of facial nucleus. The nerve fibers packed around and connected with each other like a net. The mRNA levels and protein expression values of TH and CGRP decreased after facial nerve cells transfected with siRNA-TH, while the levels and values increased after transfection with shRNA-TH. In conclusion, TH and CGRP had coexistence phenomenon in facial nucleus. They regulate the neuronal activities of afferent and efferent neuro fibers collectively.

Keywords: Tyrosine hydroxylase (TH), calcitonin gene-related peptide (CGRP), facial nucleus, nerve fiber

Introduction

Hemifacial spasm (HFS) is a disease, of which the muscles often move unilaterally and involuntarily [1]. The characteristic of HFS is that muscles have tonic and clonic contractions which is related with ipsilateral facial nerve [2]. It is different from the involuntary facial movements caused by other reasons, and all those will result in social embarrassment and influence quality of life [3]. Treatments for HFS are often lack of specificity due to the unkown etiology. Previous studies showed there may exist abnormal transmission and/or ectopicexcitation [4, 5]. Morphology study also found there exists evident axonal degeneration and demyelination [6]. However, the causes are still unknown.

Pathophysiological mechanisms leading to these conditions have long been debated: ectopic excitation-ephaptic transmission at the root entry zone, and increased excitability of the facial nucleus are two candidate mechanisms for HFS [7]. In recent years, "theory of nuclei" has been paid more and more attention. Studies showed calcitonin gene-related peptide (CGRP) is widely exist in esthesioneure and participates in a variety of regulatory mechanism [8, 9]. CGRP is richly expressed in facial nucleus and has a strong nerve conduction adjustment [10]. Tyrosine hydroxylase (TH) plays an extensive and important role in regulating motor neurons [11]. It may regulate the afferent and efferent nerve fiber of facial nerve nucleus. TH and CGRP are coexist with acetyl choline (ACH) in facial nucleus, which illustrates the two neuro-

modulators interact with acetylcholine in facial nerve pathway [12].

In this study, we used immunohistochemistry and immunofluorescence staining to mark whether facial nucleus has the expression of TH and whether coexistence phenomenon is exist between TH and CGRP. The mRNA levels and protein expression values of TH and CGRP in facial nerve cells were determined before and after transfection with siRNA-TH and shRNA-TH.

Material and methods

Rat models

Sixteen healthy adult male SD rats (250-300 g; Animal Center of Shandong University) were used for all experiments. They were acclimatized at a 12 h light: 12 h dark cycle and fed a standard diet and tap water *ad libitum* for 1 week before the experiments commenced. The experiments were carried out in accordance with the Animal Experimentation Committee Regulation. Rats were randomly divided into two groups: HFS model group (Facial nerve at the right side was exposed under the operating microscope after rats were deeply anesthetized with 40 mg/kg pentobarbital sodium by ear vein injection. Block rubber was placed at the distal facial nerve which extended from stylomastoid foramen to fix rats) and the Normal group (without any further processing).

Facial nerve tissues were collected from the normal rats and HFS rats. After isolation, the tissues were stored at -80°C.

Immunohistochemical staining

Rats were deeply anesthetized with pentobarbital sodium (40 mg/kg), and perfusion was conducted by intubating tube through the left ventricular to aorta. 200 ml normal saline was used to wash blood and 500 ml phosphate buffer containing 4% triformol was used for fixation. After perfusion, rats were decollated, skull was stripped, brain stem was removed and 4% triformol was used to fix for 12 h. Then samples were placed in 30% sucrose phosphate buffer (pH 7.4, 4°C) for overnight.

According to the brain stereotaxic atlas of rats [13], frozen section on facial nucleus was conducted. The thickness of each piece was 10 mm and pieces were selected every one piece. We obtained 4 groups and they were: TH immunohistochemical staining group, CGRP immunohistochemical staining group, TH control group and CGRP control group. There were 8 samples in each group and immunohistochemical staining was performed. Carved samples were put in 0.01MPBS TTBS (pH 7.4) to wash for 3 times with each for 3 min, and then put in methanol and hydrogen peroxide solution at room temperature for 10 min. Samples were washed again with 0.01MPBS TTBS and sealed in serum, and cultivated at 37°C for 30 min. Then they were put in primary antibodies to cultivate at 4°C for 72 h (Rabbit Anti-rat CGRP polyclonal antibody 1:400; Mouse anti rat TH monoclonal antibody 1:300). After cultivation, samples were taken out and put in 0.01MPBS TTBS to wash. After washing, biotinylation Goat Anti-Mouse IgG (Wuhan Boshide Ltd) and biotinylation horse anti rabbit IgG (Beijing Zhongshan Jinqiao Ltd; dilution at 1:100) were used for reaction at 37°C for 30 min. SABC was used to reactive samples at 37°C for 30 min and washed for 3 times with each time of 5 min. DAB kit was used for color reaction and optical microscope was used to take pictures.

Immunofluorescence staining

Rats were deeply anesthetized with pentobarbital sodium (40 mg/kg), and perfusion was conducted by intubating tube through the left ventricular to aorta. 200 ml normal saline was used to wash blood and 500 ml phosphate buffer containing 4% triformol was used for fixation. After perfusion, rats were decollated, skull was stripped, brain stem was removed and 4% triformol was used to fix for 12 h. Then samples were placed in 30% sucrose phosphate buffer (pH 7.4, 4°C) for overnight.

Facial nucleus was freezing sliced with each piece of 10 μm. Pieces were selected every one slice and divide into 2 groups with each group of 8 pieces (TH fluorescent immunohistochemistry; CGRP fluorescent immunohistochemistry; the controls). Samples were pasted and sealed in serum at 37°C to cultivate for 30 min. Filter paper was used to dry blocking serum and antibody diluent was added to dilute primary antibodies mixed liquor (Rabbit Anti-rat CGRP polyclonal antibody 1:400; Mouse Anti-rat TH monoclonal antibody 1:300). Samples were cultivated in humidified box at 4°C for 72 h and

washed with 0.01MPBS (pH 7.4) for 3 times with each time of 3 min. FITC was added to sign Goat Anti-Mouse IgG (Wuhan Boshide Ltd; dilution at 1:100) and cultivated in dark place at 37°C for 30 min. After washing for 3 times with each time of 3 min, Cy3 was added to sign Goat anti Rabbit IgG (Wuhan Boshide Ltd; dilution at 1:100) and cultivated in dark place at 37°C for 30 min. We used glycerin to seal pieces and fluorescence microscope to take pictures.

Cell culture

Facial nerve cell lines PC12 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were kept in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) in humidified air containing 5% CO_2 at 37°C. Dulbecco's Modified Eagle's Medium contained 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% penicillin/streptomycin.

Quantitative real-time reverse transcription-PCR

RNA was obtained by mirVana miRNA isolation kit which was producted at Austin in TX. Trizol was put into the kit and shake well. The solution was transferred into 1.5 mL tubes using chloroform and centrifuged at 12000×g for 15 min. Supernate was put into EP tubes again with isopropanol and centrifuged, and the precipitate was kept. Precipitate was treated with ethonal and DEPC was used to dissolve the precipitate. NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) was applied to determine RNA concentration. GAPDH gene was used as the control. The expression level was normalized using U6 small nuclear RNA by the $2^{-\Delta Ct}$ method. The ΔCt values were normalized to GAPDH level.

Western blotting

Total proteins from the cells were extracted by ice-cold RIPA lysis buffer supplemented with 1 mM proteinase inhibitor PMSF (Sigma, St. Louis, MO, USA). The protein concentration was quantified with a BCA assay kit (Beyotime, Shanghai, China). Equal amounts of protein was separated by 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF, Millipore, Bedford, MA, USA) membrane, and then blocked with 5% non-fat milk in Tris-buffered saline. The membranes were incubated with primary antibodies, mouse antihuman monoclonal TH and CGRP antibody (Santa Cruz Biotechnology, CA, USA) and mouse anti-human monoclonal GAPDH antibody (Santa Cruz Biotechnology, CA, USA), at 4°C overnight. The membranes were washed and subsequently probed with secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1/4000 dilution for 1 h at room temperature. Proteins were visualized with chemiluminescent detection system (ECL; Beyotime). GAPDH was used as internal control.

Cell transfection

RNA interference (RNAi) is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. To investigate the role of TH on CGRP, we used a RNAi-based strategy (c-TH-siRNA) to specifically silence TH expression and overexpression of TH was performed with c-TH-shRNA. The siRNA used to downregulate TH expression targeted the following sequence in TH mRNA: 5'-GGC UAC GUC CAG GAG CGC ACC-3'. The sense strand of this molecule, TH siRNA, was 5'-P GGC UAC GUC CAG CGC ACC-3' and the antisense strand was 5'-P U GCG CUC CUG GAC GUA GCC UU-3'. ShRNA was produced in vitro using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates were designed such that they contained T7 promoter sequences at the 5' end. ShRNA transcripts subjected to in vitro Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained from Dharmacon, Inc. Cells were transfected with indicated amounts of siRNA and shRNA using standard calcium phosphate procedures at 50%~70% confluence in 6-well plates.

Statistical analysis

Statistical analyses were performed using SPSSs (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD). Student's *t*-test or one-way ANOVA test was performed to determine significant differ-

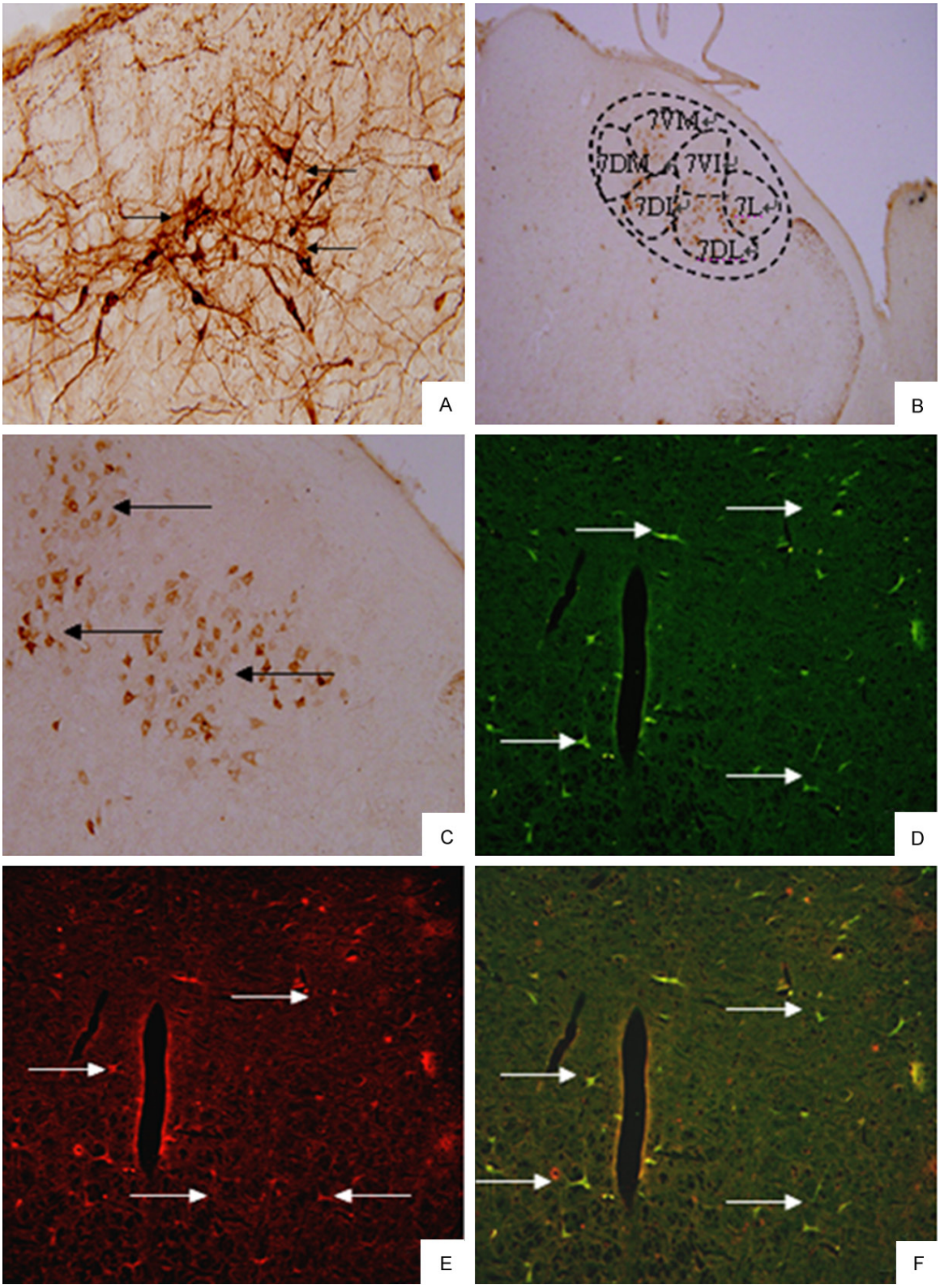

Figure 1. A. The expression of TH immune positive neurons in ventral intermediate subnucleus (200×). Indicated by the arrows were the dense nerve net formed by TH positive nerve fiber. B. Distribution of CGRP immune positive neurons in facial nucleus. C. Expression of CGRP immune positive neurons in dorsolateral subnucleus, dorsomedial subnucleus and ventromedial subnucleus (100×). Indicated by the arrows were dense and aligned CGRP positive neurons. Cells presented fusiformis, oval, triangle and other shapes. Cytoplasm and fiber presented claybank. Cy-

toplasm was deeply stained and brown granules can be seen. D. Distribution of TH immunofluorescence positive neurons in facial nucleus. Indicated by the arrows were green marked TH positive neurons. The cells were large with soma of 12~14 μm (100×). E. Distribution of CGRP immunofluorescence positive neurons in facial nucleus (100×). Indicated by the arrows were red marked CGRP positive neurons and cells were small with soma of 6~8 μm. F. Expression of TH and CGRP immunofluorescence double-marked positive neurons in ventrolateral subnuclear, dorsolateral subnucleus and intermediate subnucleus (100×). Indicated by the arrows were net-link nerve fibers among TH and CGRP immunofluorescence double-marked positive neurons.

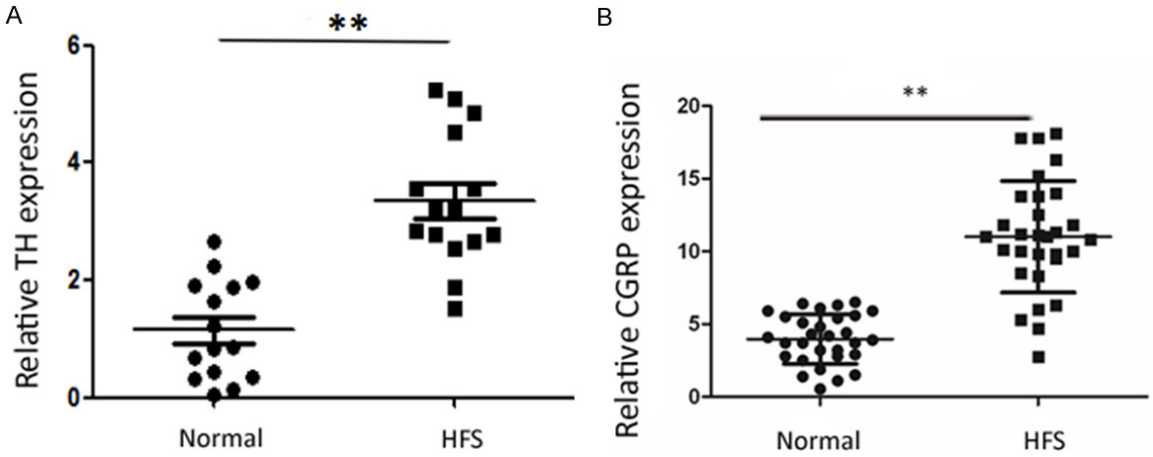

Figure 2. Relative mRNA levels of TH (A) and CGRP (B) in HFS tissues and the normal tissues. $^{**}P < 0.01$, compared with the control, the mRNA level of TH and CGRP in HFS group had significant difference.

ences. A $P < 0.05$ was considered statistically significant.

Results

Distribution of TH and CGRP

As shown in **Figure 1A-F**, there were abundant TH and CGRP double-label neuronal somas in ventrolateral subnucleus, dorsolateral subnucleus and intermediate subnucleus of facial nucleus. The endochylema of green marked TH positive neuron was densely stained, and cells were large with soma of 12-14 um. Most cells were long protuberant fusiform and oval. The protuberances were thick, long and had multi-level branches. The forms of cells were various and protuberances among cells linked like a net. We did not see evident staining in the negative control. Red marked CGRP positive neuronal somas presented triangle, roundness or polygon. The endochylema was densely stained and cells were small with soma of 6~8 μm. CGRP nerve fibers connected with each other closely and distributed like a net. Abundant of TH and CGRP double-label neuronal somas connected with each other via neurons and net-connected nerve fibers. Nerve fiber of TH and CGRP had significant branches and crosses.

The level of TH and CGRP

To explore the relationship between TH and CGRP, we determined the mRNA levels of TH and CGRP in facial nerve tissues of rats with HFS and the normal by qRT-PCR. Results showed the mRNA levels of TH and CGRP in normal group were low and the levels were high in HFS group (**Figure 2**). The protein expression values of TH and CGRP in facial nerve tissues of rats with HFS and the normal were also determined by western blotting. Results showed the protein expression values of both TH and CGRP in normal group were low, while the values in HFS group were high (**Figure 3**).

Verification

To explore the relationship between TH and CGRP, facial nerve cells were transfected with siRNA-TH to silence TH and shRNA-TH to enhance the level of TH. After transfection, we determined the mRNA levels of TH and CGRP with qRT-PCR and protein expression values by western blotting. Results showed the mRNA levels and protein expression value of CGRP

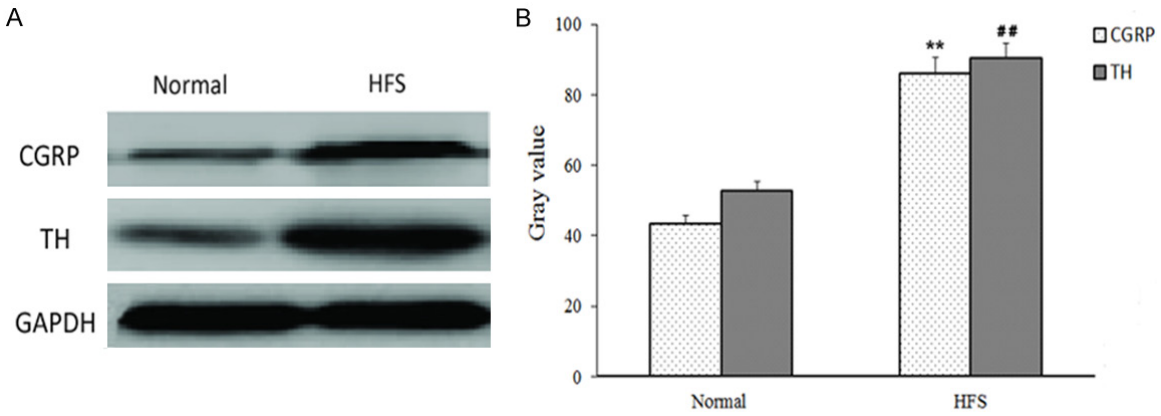

Figure 3. A. The protein expression values of TH and CGRP in HFS tissues and the normal tissues by western blotting. B. The gray value of TH and CGRP in HFS tissues and the normal tissues. $^{**}P < 0.01$, compared with the normal group, the gray value of CGRP in HFS group had significant difference. $^{\#\#}P < 0.01$, compared with the normal group, the gray value of TH in HFS group had significant difference.

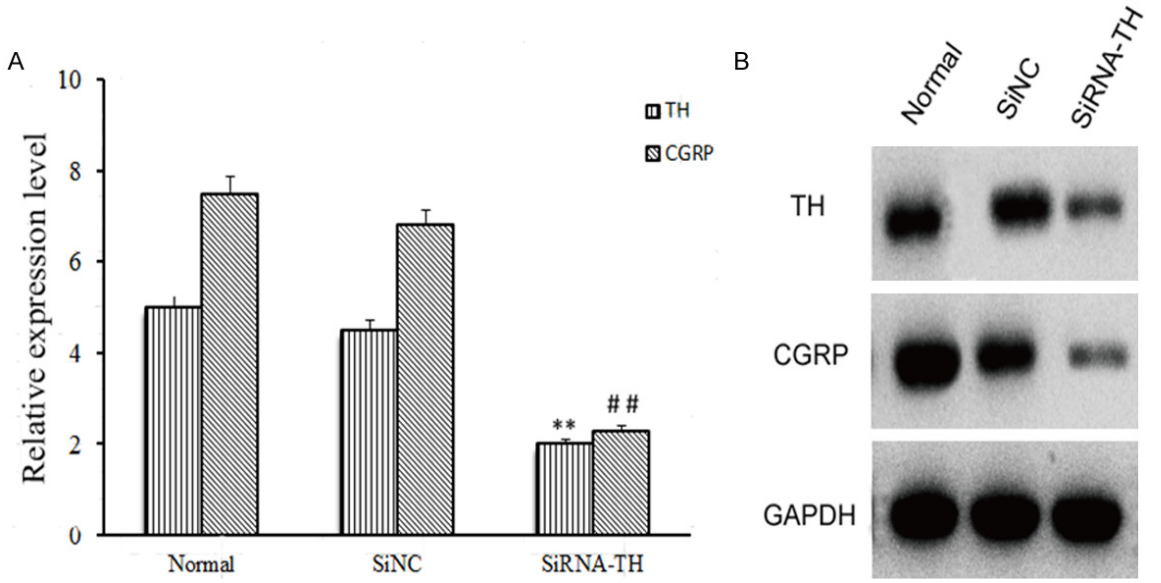

Figure 4. A. The relative expression levels of TH and CGRP in HFS cells before and after transfection with siRNA-TH. $^{**}P < 0.01$, compared with the normal and si-NC group, the mRNA level of TH in siRNA-TH group had significant difference. $^{\#\#}P < 0.01$, compared with the normal and si-NC group, the mRNA level of CGRP in siRNA-TH group had significant difference. B. The protein expression of TH and CGRP in HFS cells before and after transfection with siRNA-TH determined by western blotting.

decreased after transfection with siRNA-TH (**Figure 4A** and **4B**), while the level and value of CGRP increased after transfection with shRNA-TH (**Figure 5A** and **5B**). All those indicated TH and CGRP in rat facial nerve nucleus existed coexistence phenomenon.

Discussion

Hemifacial spasm (HFS) is defined as unilateral, involuntary, irregular clonic or tonic movement of muscles innervated by the seventh cranial nerve [14]. Its pathogenesis is unclear and HFS usually tends to aggravate gradually. It is frequently aggravated by fatigue, stress, anxiety or self-consciousness [15]. Facial optional movement may also induce or aggravate facial tic [16]. “The theory of facial nucleus” had been payed more attention now [17]. Facial nucleus is brainstem primary center and the constitution is complicated. Subnucleuses that constitute the facial nucleus are ventromedial subnucleus, dorsomedial subnucleus, dorsal in-

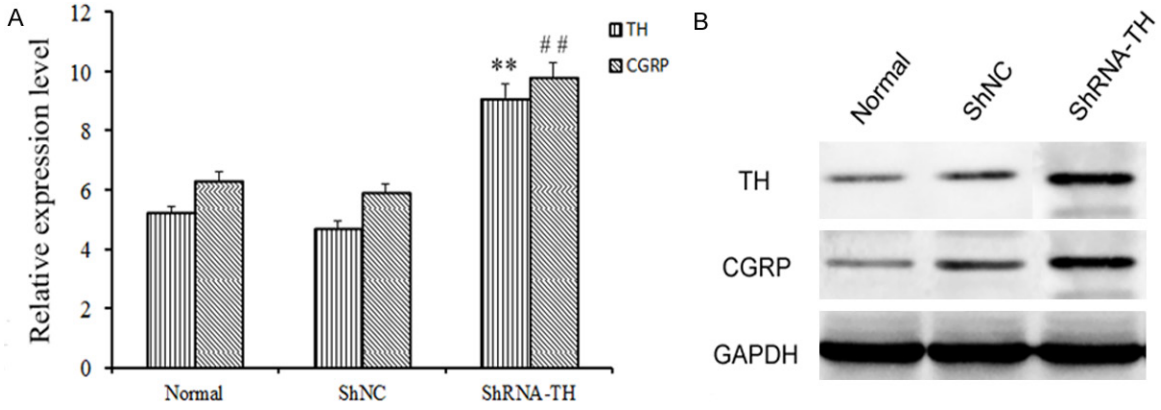

Figure 5. A. The relative expression levels of TH and CGRP in HFS cells before and after transfection with shRNA-TH. $^{**}P < 0.01$, compared with the normal and sh-NC group, the mRNA level of TH and CGRP in shRNA-TH group had significant difference. $^{\#\#}P < 0.01$, compared with the normal and sh-NC group, the mRNA level of CGRP in shRNA-TH group had significant difference. B. The protein expression of TH and CGRP in HFS cells before and after transfection with sHRNA-TH determined by western blotting.

termediate subnucleus, dorsolateral subnucleus and lateral subnucleus. Facial nucleus not only has extensive fiber link with other nucleus of brainstem, but also has complicated fiber link with other subnucleus [18]. Facial nucleus accepts afferent nerve fibers from a variety of sources and these nerve fibers control the movement and reflection of facial muscle and mimetic muscle [19]. There are many neuromodulators that participate in mediating and regulating the activity of afferent facial nucleus and efferent nerve fiber, including TH, CGRP and so on. They rely on the coexistence relationship of neurotransmitter to realize the mutual promotion or antagonism.

In this paper, we studied two hot neuromodulator TH and CGRP. As important neuromodulators in central nervous system, they have coexistence phenomenon with a variety of neurotransmitters and neuromodulators in neuron and other cells. The coexistence phenomenon of neurotransmitters indicated concomitant neurotransmitter or neuromodulator regulate collectively the efficiency of information transmission, and also strengthen or weaken the effect of corresponding neurotransmitters [20]. TH and CGRP coexist with classical neurotransmitter acetyl choline in facial nucleus, and play an important role in the connection of nerve fiber. Research showed TH and CGRP had coexistence phenomenon in spiral modiolar artery [21]. Our study showed there were many TH and CGRP double-label neuronal somas in ventrolateral subnuclear, dorsolateral subnucleus and dorsal intermediate subnucleus of facial nucleus. The nerve fibers packed around and connected with each other like net. We inferred that the two neuromodulator had combined action and regulated with each other in different subnucleus of facial nucleus. They not only play roles in classical neurotransmitters and regulate nerve conduction activity, but also they were adjusted by each other [22]. Results of qRT-PCR and western blotting showed the level of CGRP was decreased after transfection with siRNA-TH and increased after transfection with shRNA-TH in facial nerve cells. This verified that TH and CGRP had coexistence phenomenon.

In conclusion, TH and CGRP had coexistence phenomenon in facial nucleus, which indicated the two has the possibility of interaction and regulate each other. We concluded the two neuromodulators had combined action in different subnucleus of facial nucleus, and they regulate the neuronal activities of afferent and efferent neuro fibers collectively.

Acknowledgements

The study was supported by the National Natural Science Foundation of China (81170914/H1304).

Disclosure of conflict of interest

None.

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