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ORIGINAL ARTICLE

Exploration on the Underlying Mechanism of Female Predominance in Spasmodic Dysphonia: An Anatomical Study of Nodose Ganglion in Rats

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Abstract To study the gender differences of amount of neurons in the nodose ganglions of rats. Fourteen Sprague–Dawley rats (7 males and 7 females) were selected. Bilateral nodose ganglions were dissected and serial sections of nodose ganglion were cut in a cryostat, followed by Cresyl-violet staining for neurons. Eight to ten consecutive sections from mid-portion of each nodose ganglion sample, which represent the most neuron number per section, were counted and averaged. Gender difference in the amount of neurons in the nodose ganglions was compared. No gender difference of neuron numbers was found in either side of nodose ganglion ($p > 0.05$). However, average neuron number of nodose ganglions on the left side of male (654 ± 60) and female (616 ± 37) were significantly more than that on the right side of male (470 ± 22) and female (453 ± 40) respectively ($p < 0.05$). There is no gender difference in total neuron number of nodose ganglions between male and female rat. However, the neuron number in the left nodose ganglion is greater than that in the right one. The difference may be due to the fact that left and right nodose ganglion is receiving different visceral sensory impulses separately, which is associated with different physiological functions. Further work should be carried out with retrograde tracing on neurons of nodose ganglions in an animal model, which are directly related to laryngeal sensory transmission, in order to determine the gender difference in the neuron number and morphology related to laryngeal functions.

Keywords Nodose ganglion · Neuron · Gender difference · Animal model · Cell counting · Spasmodic dysphonia

Introduction

Spasmodic dysphonia is a voice disorder that is characterized by continuous and involuntary contraction of laryngeal muscles during speech. It is manifested by an abrupt voice interruption, a decrease in voice volume, hoarseness and change of breathing rate, which significantly impairs vocal communication. Little is known about the cause of spasmodic dysphonia so far. It was previously categorized as mental and psychological disorder [13]. Most scholars recently believe that this disease stems from neurodegenerative diseases [10], as classified by the National Institute of Neurological Disorders and Stroke (NINDS). In [24] reported a female predominance of 79 % (134 cases) out of a total population of 168 spasmodic dysphonia patients, which is consistent with previous reports [6].

There is little known about the neurological etiology of spasmodic dysphonia. In 2009 [26] reported two cases of spasmodic dysphonia in which the histopathology revealed that both patients had brainstem lesions, manifested by depigmentation and inflammation in substantia nigra, reticular formation, locus coeruleus, and more severely, nucleus of solitary tract (NST). NST receives neuronal impulses from

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ascending laryngeal sensory nerves stemming from nodose ganglion, which is mostly composed of sensory neurons. And peripheral process of nodose ganglion neurons are mainly located at laryngeal mucosa responsible for general visceral sensation. Abbruzzese and colleagues confirmed that sensory afferent system plays an essential role in the underlying mechanism of focal dystonia [1]. Malfunction of sensory afferent system leads to an abnormally greater transmission of sensory impulses into NST, accompanied by a reduction in sensation negative feedback. In hence strong and sustained descending impulses sent from central nervous system causes elevated tension and spasms in the innervated muscles. Spasmodic dysphonia has been identified as a disorder that occurs focally within the laryngeal area [18], while nodose ganglion is the most important structure in the laryngeal sensory transmission system. So far little is known about the role that nodose ganglia may play in the occurrence and progression of spasmodic dysphonia. In addition, it needs to be further elucidated whether there is a difference in the anatomical structure of nodose ganglions between male and female humans, and whether there is a correlation between female predominance in spasmodic dysphonia and the unique anatomical structure of female nodose ganglion.

This study examined the anatomical structure of rat nodose ganglion and compared total neuron number of such ganglion between female and male rats, as a basis for human-related research. We hypothesize that more nodose ganglion neurons will be present in female than in male, which may correlate with female predominance of human spasmodic dysphonia.

Materials and Methods

All experimental Sprague–Dawley rats were used by following the animal use protocol in accordance with National Institutes of Health (NIH) animal care and use guidelines. We used as few rats as possible and ensure all animals experience a minimum of pain. A total of 14 young adult rats (7 males and 7 females) were used with the weight between 250 and 400 g. Phenobarbital (190 mg/kg) was applied to deeply anesthetize the experimental rat. The animal head was immobilized followed by cardiac perfusion with 4 % paraformaldehyde (PFA) prepared in phosphate buffer and precooled to 4 °C. Bilateral nodose ganglions with residual vagus nerves were dissected from neck incision, incubated in 4 % PFA overnight, and embedded with 20 % agarose. Samples were subsequently incubated in 4 % PFA overnight then transferred into 30 % sucrose solution till they sank to the bottom of the container. Samples were fast frozen by isopentane (pre-cooled with dry ice) and stored at −80 °C till dissection.

Serial cryosections of 16 μm thickness were cut at −20 °C following tissue sample embedding. Sections were washed in 1× phosphate buffered solution and mounted to 1 % gelatin-pretreated microscopy slides. Then, the sections were stained with cresyl-violet, dehydrated and cover slipped. 8–10 consecutive slices were chosen from the middle portion of each tissue sample, which represent the most neuron counting per slice. Neurons were observed under the microscope (Neurolucida system). Only cells with clear staining and complete edge were counted with Photoshop and numbers were averaged. Cryosection selection, cell counting and imaging were all performed as blind experiments by one of our researchers.

Data Analysis

Data were presented as mean ± standard deviation. ANOVA test was used to compare the gender and side difference in nodose ganglion neuron number to determine the significant differences at $p < 0.05$.

Results

The average number of neurons from the left nodose ganglion was 654 ± 60 in male rats and 616 ± 37 in female rats. The average number of neurons from the right nodose ganglion was 470 ± 22 in male rats compared with 453 ± 40 in female rats. There was no significant difference in average number of neurons between the male and female rats ($p > 0.05$).

However, in the same gender, the average number of neurons from left nodose ganglion is significantly greater than that from right nodose ganglion ($p < 0.05$, see Fig. 1 for details). Figure 2 represented Cresyl-violet staining of nodose ganglion neurons. The left nodose ganglion contains significantly more neurons than the right one.

Discussion

The first case of spasmodic dysphonia was discovered and reported by Truabe in 1871. The disease has three different types: adductor spasmodic dysphonia, abductor spasmodic dysphonia and mixed spasmodic dysphonia, based on the difference in the opening and closing position of vocal folds during laryngeal muscle spasms [4]. Adductor spasmodic dysphonia is manifested by an abrupt abnormal closing of vocal folds during speech. Abductor spasmodic dysphonia occurs when vocal folds suddenly open involuntarily, while the mixed type contains both conditions. Cannito and colleagues believe that all types should be categorized as “mixed”. The different symptoms should be

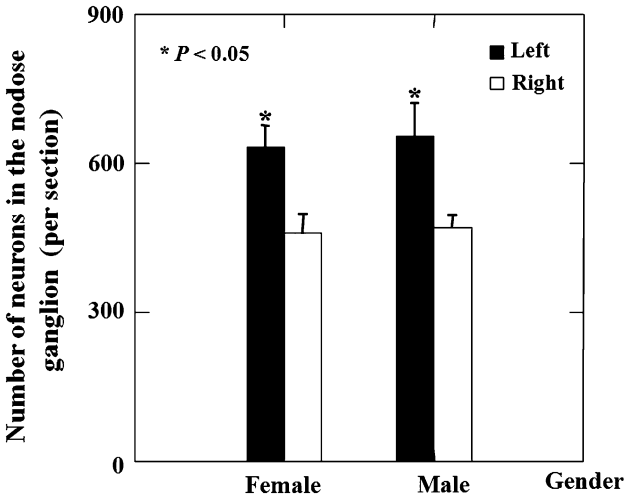

Fig. 1 Comparison of number of neurons in nodose ganglion. In both male and female rats, number of neuron in the nodose ganglion at *left side* is significantly greater than that at *right side* ($p < 0.05$). The total number of neurons in the nodose ganglion at the *same side* is not significantly different between the female and male rats ($p > 0.05$)

attributed to the predominance of either adductor or abductor condition [7].

Spasmodic dysphonia, although not common, is far from rare. In 2006 the prevalence in Iceland was 5.9 cases/100,000 people [3, 10]. Around 50,000 people have spasmodic dysphonia in North America, with an estimated number of 1.1–4.26 cases/100,000 people which predominates in female [27]. Many cases were misdiagnosed as mental illness or hysterical aphasia while the patients did not receive proper medical treatment. Currently the diagnosis of spasmodic dysphonia relies mainly on the perceptual judgments on the unique voice characteristics of such disease [8]. One-third of total patients with spasmodic dysphonia are often accompanied with occurrence of voice tremor. Voice tremor is hardly distinguishable from muscular tension dysphonia, which in hence leads to a high rate of misdiagnosis [15, 23].

It is critical to give these patients an appropriate and effective treatment. Due to the unclear etiology of spasmodic dysphonia, the regular clinical treatment is short-term symptomatic treatment to alleviate the spasticity of vocal folds, while there is no long-term cure. In the early time recurrent laryngeal nerve transection was clinically applied [5]. It was later improved to recurrent laryngeal nerve crush [9]. By removing, crushing or performing the avulsion of left recurrent laryngeal nerves and retaining the right nerves, it significantly reduces the risk of bilateral vocal folds paralysis and alleviates the symptoms. Nevertheless only 36 % of these patients did not recur in 3-year follow-up due to nerve reinnervation, some of whom are accompanied with laryngeal dysfunction [2]. In recent years some scholars have applied local injection of botulinum toxin, which results in better efficacy [3]. The limitation of this method is that it only has short-course effects and the long-term recurrence rate is high. In addition, repeated injections will cause scar formation associated with reduced efficacy. In 2005, the application of bipolar radiofrequency-induced ablation was introduced. It causes minimum damage with the ablation of recurrent laryngeal nerve endings innervating thyroarytenoid muscles, while long-term efficacy remains to be determined [22].

The uncertain cause of spasmodic dysphonia has been the main obstacle for further research. It is not clear whether one or more regions, located in cerebral cortex, brain stem, nucleus of solitary tract, nodose ganglion, and recurrent laryngeal nerves are essential for the occurrence and progression of spasmodic dysphonia. In addition, neither basic nor clinical research has been done to elucidate the underlying mechanism of female predominance. Our study used the rat nodose ganglion as our model to determine whether there is a significant anatomical difference

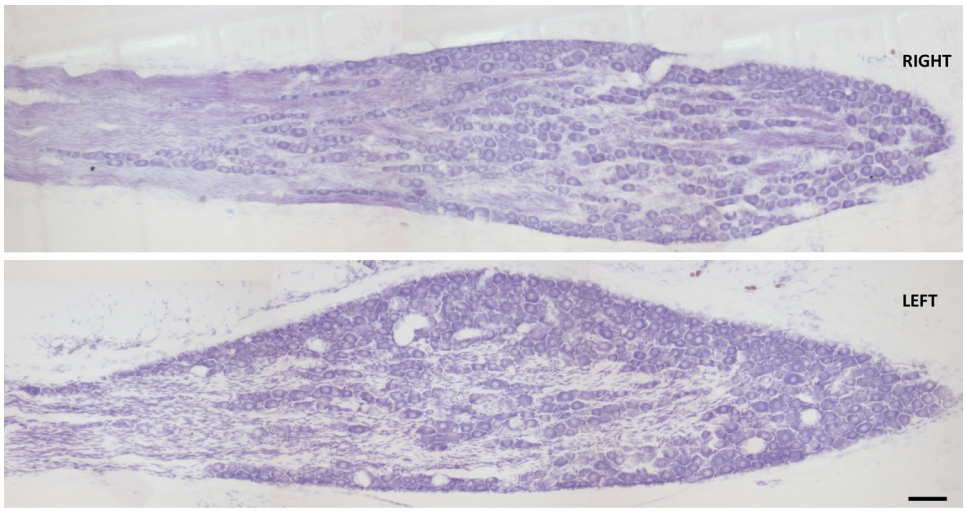

Fig. 2 Cresyl-violet staining of nodose ganglion neurons. It showed that the amount of neurons in the *left* nodose ganglion (*lower*) was more than that in the *right* nodose ganglion (*upper*) (*Scale bar* 200 μm)

between male and female rat by counting and comparing their total nodose ganglion neuron number, which may potentially shed some light on a new research field of gender difference mechanism existing in this disease.

In this study, total neuron number of rat nodose ganglions was compared in combination of two variables (gender and body side). The results showed that total neuron number of nodose ganglion in the female rat did not differ significantly from that in the male at the same side. However, we found an unanticipated result in this study that the total neuron number of left nodose ganglion is significantly greater than that of right nodose ganglion in the same gender. There is no similar report up to date while no related research on nodose ganglion of human or animal subjects have been performed. Rat visceral sensory neurons are located in the rostral, middle or caudal part of nodose ganglion. And these neurons form synapses with visceral structures extensively located at neck, chest, abdomen, etc. They mainly collect impulses from the thoracic and abdominal organs, plus general visceral sensory impulses from the pharynx and larynx. There is no clear projection map of neurons in response to different visceral inputs but cells corresponding to the same organ tend to aggregate [12, 14, 21, 25]. In general, neurons in rostral part of nodose ganglion are receiving impulses from upper esophagus and aortic baroreceptor, and neurons in middle and caudal parts are directly related to gastrointestinal and pancreatic sensory transmission [11, 16, 19, 20]. These nerve impulses are projected via ascending nerves of nodose ganglion to NST [17]. One study in dogs revealed that neurons located at rostral nodose ganglion send their contacting process to superior laryngeal neuron fibers, while neurons contacting recurrent laryngeal nerve fibers are widely distributed throughout nodose ganglions [28]. The shortcoming of this study is that no retrograde tracing on laryngeal afferent neurons had been performed. This in hence makes us unable to determine how many neurons out of the total population are receiving laryngeal afferents. We will examine the proportion and number of these neurons in nodose ganglion with retrograde tracing method subsequently, to further explore the gender and body side difference of this particular neuron population in the animal model. However, the same results may not be expected between the animal model and human due to the species specificity. Similar studies in human tissues will be more valuable to illuminate the gender differences in laryngeal neuroanatomy and explore the mechanisms underlying the laryngeal disorders due to these differences in human.

Our study reports, for the first time, that left nodose ganglions in rats contain significantly more neurons than right nodose ganglions. This difference may be related to the fact that left and right side of nodose ganglion are receiving different visceral sensory impulses associated with specific physiological functions respectively in the rats. Further work needs to be done to elucidate which internal organ(s) are directly related, and the outcome will contribute significantly to this side differences. Studies using human laryngeal tissues are expected to provide more direct and valuable information for exploring neurological mechanisms underlying the spasmodic dysphonia and its gender predominance.

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