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Short communication

Muscle specific nucleus ambiguus neurons isolation and culturing

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HIGHLIGHTS

- The development of a new protocol for specific brainstem motoneuron isolation from postnatal rats.
- Abductor and adductor motoneurons were isolated in separate plates.
- The utility of neural tracers for a long term neuron culture isolation was addressed.
- The best period for cranial motoneuron isolation and culture in postnatal rats was determined.

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ABSTRACT

Background: Peripheral nerve injury leads to a regenerative state. However, the reinnervation process is highly non-selective. Growing axons are often misrouted and establish aberrant synapsis to abductor or adductor muscles. Determining the complex properties of abductor and adductor motoneurons in a neuron culture, may lay the groundwork for future studies on axon guidance, leading to a clinical treatment for a selective reinnervation.
New method: In the present study we develop a neuron culture protocol to isolate recurrent laryngeal nerve abductor and adductor motoneurons in order to study their unique properties.

Comparison with existing methods the best period to perform the present protocol for postnatal rat cranial motoneurons isolation was determined. In addition, the method allows identification of specific motoneurons from other primary motoneurons and interneurons within brainstem.
Conclusion: The present protocol will allow investigators to perform targeted and novel studies of the mechanisms of peripheral nerve regeneration.

1. Introduction

When peripheral nerves are injured or experimentally transected, they maintain the capabilities of regeneration from the cut end forward. However, reinnervation patterns are not precise and aberrant reinnervation of muscles leads to synkinetic movements. One specific example is the recurrent laryngeal nerve (RLN). Severe injury or transection of this nerve leads to non-specific innervation of the laryngeal muscles, resulting in immobile vocal folds. Immobility in the vocal folds dramatically affects voice production and communication. It can also affect swallowing and breathing (Crumley, 2000; Myssiorek, 2004; Hernandez-Morato et al., 2014a,b). One particularly clinically relevant scenario is after either resection of or unintentional injury to the RLN during removal of the thyroid gland for benign or malignant disease. Permanent vocal fold paralysis occurs in approximately 2.4% of all thyroidectomies and is as high as 8% in patients with carcinoma.

The recurrent laryngeal nerve originates in the ipsilateral nucleus ambiguus that is deeply buried in the brainstem. In rodents, RLN exclusively carries motor axons that innervate the laryngeal muscles. Their cell bodies are located in a rostro-caudal oriented column within brainstem and the nucleus is called nucleus ambiguus. Axons from this nucleus exit with the roots of the vagus to constitute the vagus nerve. The rat RLN ipsilaterally innervates all laryngeal muscles except the cricothyroid muscle, which is innervated by the superior laryngeal nerve. Thus, the RLN contains fibres

Abbreviations: RLN, recurrent laryngeal nerve; PCA, posterior cricoarytenoid; TA, thyroarytenoid.

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transmitting the motor information required for the abduction and the adduction of the vocal folds (Bieger and Hopkins, 1987; Pascual-Font et al., 2011).

The abduction and the adduction of the vocal folds are carried out by two disctinct populations of motoneurons located within the caudal third of the nucleus ambiguus. The length of the rat nucleus ambiguus is approximately 3.5 mm from the rostral spinal cord to the lower pons in the brainstem (Pascual-Font et al., 2011). The entire column can be divided in three major divisions. The compact formation of the nucleus ambiguus constitutes motoneurons that innervate the oesophagus. Below that, the semicompact formation contains motoneurons that innervate the pharynx and the cricothyroid muscle of the larynx innervated by the superior laryngeal nerve. The loose formation of the nucleus ambiguus contains all the motoneurons that innervate the laryngeal muscles except the cricothyroid muscle (Bieger and Hopkins, 1987). Somatotopic organization of the nucleus ambiguus shows laryngeal muscles represented in discrete pools of motoneurons along the nucleus (Fig. 1). 30–40 motoneurons innervating the posterior cricoarytenoid muscle, the unique abductor of the larynx, are located in the rostral third of the nucleus ambiguus. The adductor motoneurons, innervating the thyroarytenoid muscle, are located in caudal third of the nucleus (Bieger and Hopkins, 1987; Pascual-Font et al., 2011; Weissbrod et al., 2011; Hernandez-Morato et al., 2013). Since the RLN carries motor axons that innervate the posterior cricoarytenoid and thyroarytenoid muscles, non-specific reinnervation of these antagonistic muscles occurs and the laryngeal fold remains paralyzed due to this aberrant synkinetic reinnervation (Flint et al., 1991; Crumley, 2000; Pitman et al., 2011). A dramatic change of the somatotopic representation in the nucleus ambiguus after RLN regeneration provides strong evidence of the non-selective motor reinnervation of the larynx (Hernandez-Morato et al., 2013, 2014b). In order for purposeful reinnervation to occur and vocal fold motion to be restored, the axons must be guided back to the correct muscle.

The guidance of the regenerating axons is accomplished by several trophic factors expressed differentially in abductor and adductor muscles (Sterne et al., 1997; Simon et al., 2000, 2003; Hernandez-Morato et al., 2014a). It is exceedingly difficult to study selective reinnervation *in vivo* due to the complex environment. Therefore, we seek to gain an insight into trophic factor signalling, by utilizing an *in vitro* setting of isolated motoneurons that innervate abductor and adductor muscles.

Culturing of adult motoneurons in rodents has proven to be quite difficult. This is likely due to the length of the axon, as well as the disruption of cell-cell interactions and many synapses. While a few studies have successfully cultured larger spinal cord motoneurons in mature animals (Milligan and Gifondorwa, 2011), most studies have had greater success culturing motoneurons during development and in early postnatal periods (Kivell et al., 2001; Zuchero, 2014).

In the rat there are approximately 140 motoneurons in the nucleus ambiguus that innervate the ipsilateral laryngeal muscles, PCA and TA (Pascual-Font et al., 2011; Weissbrod et al., 2011). In order to isolate muscle specific neurons for future study of axon guidance, we developed a method to isolate and culture motoneurons from the nucleus ambiguus. Considering the historical difficulty in culturing motoneurons in mature animals, this method was investigated in multiple age groups.

2. Materials and methods

2.1. Materials

8 mm coverslips were used for the present study. They were washed in absolute ethanol, rinsed in double distilled water, dried on a filter paper, and lastly autoclaved.

The coverslips were placed at the bottom of tissue culture plates in a laminar flow hood and were coated with 16 μl of poly-ornithine (0.01%, Sigma, St. Louis, MO) for at least 2 h at room temperature. The poly-ornithine was then siphoned out and the coverslips were coated with laminin (10 μg/ml; Invitrogen, Grand Island, NY) in sterile phosphate buffered saline (PBS). They were later transfer to into the tissue culture incubator (37 °C, 5% CO_2) for 3 h. Before plating we washed the coverslips twice with tissue culturing media (see below).

Several long polished pasteur pippetes were prepared in three different sizes in order to disaggregate the tissue. Under a flame, pipette tips were elongated to narrow the tip and smoothed by the flame. The Pasteur pipettes were rinsed with absolute ethanol, then washed with double distilled water, followed by an autoclave treatment.

All reagents were prepared in a laminar flow hood in order to maintain sterile conditions. The composition of each solution used was:

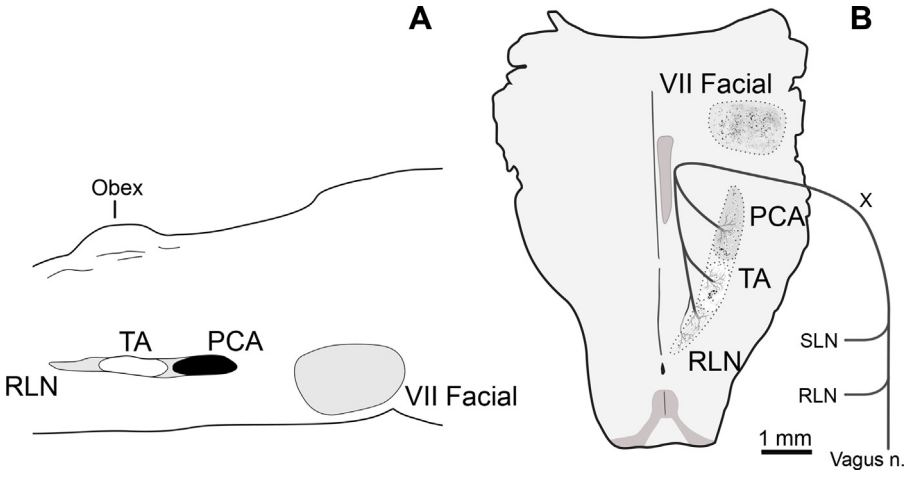

Fig. 1. Location of laryngeal and facial motoneurons within brainstem summarized in a sagittal (A) and a horizontal view (B). DiI retrogradelly transported from Recurrent Laryngeal Nerve and laryngeal muscles showed labelled motoneurons on the ipsilateral nucleus ambiguus. Abductor and adductor motoneurons of the larynx were organized in a somatotopic arrangement along the column of the nucleus ambiguus. X, vagus nerve; VII Facial, Facial nucleus; RLN, Recurrent laryngeal Nerve; SLN, Superior Laryngeal Nerve; PCA, Posterior Cricoarytenoid muscle; TA, Thyroarytenoid muscle.