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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:

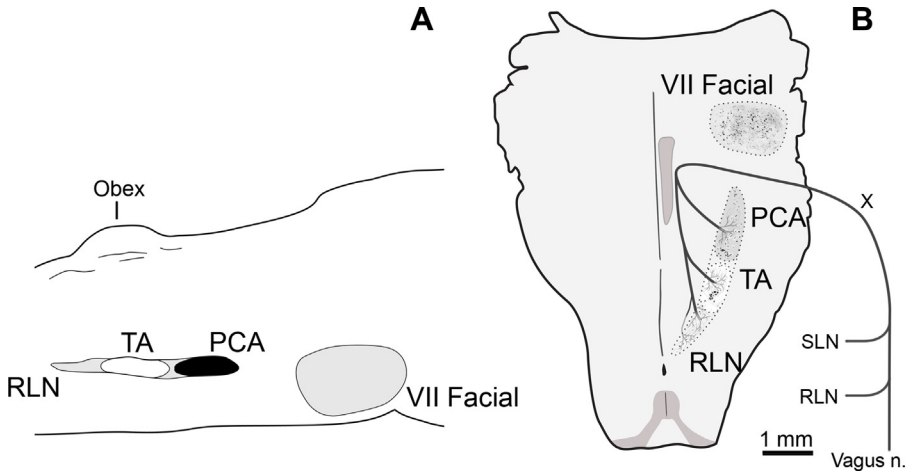

Fig. 1. Location of laryngeal and facial motoneurons within brainstem summarized in a sagittal (A) and a horizontal view (B). DiI retrogradelly transported from Recurrent Laryngeal Nerve and laryngeal muscles showed labelled motoneurons on the ipsilateral nucleus ambiguus. Abductor and adductor motoneurons 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.

1. Wash Buffer: 147 ml of Hibernate A-minusCa (Brainbits, Springfield, IL) plus 3 ml of B27 (Invitrogen), 375 μl of Glutamax (Invitrogen) and 150 μl of DNAse I (Roche Applied Science, Indianapolis, IN).
2. Growth medium: Neurobasal A (98 ml), 2 ml of B27, 250 μl of Glutamine, 46 μl of β-mercaptoethanol, 500 μl of Penicicliline/Streptemicine, and 100 μl of bFGF (all from Invitrogen).
3. Trypsin: 9 ml of Wash buffer plus 1 ml of 10× Trypsin
4. Optiprep Gradient: In each assay we prepared a gradient of Optiprep (Accurate Chemical & Scientific Corp, Westbury, NY). Four solutions with varied concentration were prepared and layered with wash buffer in 15 ml tubes (Milligan and Gifondorwa, 2011) (Table 1).

Table 1
Composition of each layer that composed Optiprep Gradient.

Layer	Optiprep	Wash Buffer	Total
1 (bottom layer)	173 μl	827 μl	1000 μl
2	127 μl	876 μl	1000 μl
3	99 μl	901 μl	1000 μl
4 (top layer)	75 μl	926 μl	1000 μl

2.2. Animals

Sprague Dawley rats were used for the present study. All animals were cared for in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of New York Medical College approved the current protocol.

2.3. Labelling of the cranial motoneurons

Animals at different age were chosen for cell culturing (10, 14, 21, 28, 56 and 84 days after birth). Animals were deeply anesthetized with an intraperitoneal injection of 70 mg/kg of ketamine and 7 mg/kg of xylazine. In the first group (8 animals), the extracranial portion of the left Facial nerve was exposed and cut close to the stylomastoid foramen. A piece of gelfoam soaked with Fast DiI (1,1′-dioctadecyl-3,3,3′,3′-tertamethylindocarbocyanine perchlorate) (Invitrogen) was placed directly on the proximal part of the severed nerve.

In the second group (8 animals), a longitudinal incision was performed on the ventral neck. The strap muscles were split in the midline and retracted laterally to expose the larynx. The right RLN was identified close to the 7th tracheal ring and severed. As explained above, a piece of gelfoam soaked in DiI was placed on the proximal end of the severed RLN.

In the third group (8 animals), the right thyroarytenoid muscle was exposed by excising the superior half of the right ala of the thyroid cartilage. 0,25 μl of Fast DiI was then injected into the muscle using a 10 μl Hamilton syringe attached to stereotaxic micromanipulator. In order to avoid the tracer spilling to surrounding muscles, the wound was rinsed with PBS.

In the fourth group (8 animals), the larynx was exposed as above. The PCA, located in the dorsal portion of the larynx, was exposed by rotating the larynx laterally. 0,25 μl of the DiI was then injected into the PCA using a stereotaxic micromanipulator.

In another control group (24 animals, 6 rats per intervention), to determine the location of motoneurons in the facial nucleus and nucleus ambiguus, DiI was applied in the facial nerve and RLN and injected into PCA and TA as explained above. The labelled neurons were then identified in serial sections and the topography of their nuclei determined (Fig. 1).

In all animals the strap muscles were re-approximated to the midline and the incision was sutured closed in layers. The animals were returned to the animal facilities with supply of water and food *ad libitum*.

2.4. Dissection and isolation of brainstem

In the control group, five days following the DiI application to either the facial nerve, the RLN, or the laryngeal muscles, rats were euthanized with isoflurane and brainstems were isolated and fixed in 4% Paraformaldehyde in PBS. The brainstems were cryoprotected by immersion overnight in 15% sucrose with PBS, and then in 30% sucrose with PBS until they sank (approximately 2–3 days). 50 μm transverse sections were cut from the brainstem and examined with a fluorescence microscope.

DiI was successfully retrogradelly transported, and the labelling of motoneurons was seen in the appropriate nuclei (Fig. 1). As predicted, motoneurons were labelled in facial nucleus in the first group of animals (Fig. 1A); labelled neurons in nucleus ambiguus following DiI application in the transected RLN (Fig. 1B); motoneurons supplying the PCA labelled with DiI were located in a rostral part within the caudal third of the nucleus ambiguus (Fig. 1C); and motoneurons supplying TA were located in the caudal third of the nucleus ambiguus (Fig. 2D). These studies provided the baseline for culture studies, and showed the ability for DiI to transport successfully to the desired target within 5 days (Popratiloff et al., 2001; Pascual-Font et al., 2011; Weissbrod et al., 2011). Contrary to other tracers like Fluoro Gold, DiI maintains its fluorescence for an extended period of time and does not affect the survival of motoneurons (Yamamoto et al., 1997; Choi et al., 2002; Miyata et al., 2002; Hu et al., 2013).

In experimental groups, the skull was surgically removed and the brainstem dissected out from the beginning of the spinal cord to the pons. The blood vessels and the meninges were removed and the brainstems were placed in 5 ml sterile bottles containing cold wash buffer. Tissue from 6 animals was pooled for isolation of facial nucleus and nucleus ambiguuus motoneurons for culturing purposes.

In the laminar flow fume hood, each brainstem was chopped into 1 mm pieces. The pieces were then transferred into prewarmed trypsin solution 15 ml tubes. The tissue was incubated at 30 °C for 30 min while shaking the tubes at regular intervals in order to homogenize the pieces in the dissociation solutions. The tubes were then centrifuged gently at 500 rpm for 30 s; the trypsin solution was removed and replaced by 2 ml of wash buffer at room temperature.

The pieces were triturated first using a 5 ml pipette (20 times) and then again using the long fire-polished pipette (10–15 times) until homogenized. The mixture was left to settle and the supernatant was transferred to a 50 ml conical tube fitted with a 70 micrometer filter setup. 2 ml of wash buffer was added to the tube and the trituration was repeated with another long fire-polished pipette until all pieces were triturated and passed thorough the 70 micrometer filter fitted on a 50 ml tube. The tubes were centrifuged at 1000 rpm for 3 min, the supernatant was removed and the pellet was resuspended with 5 ml of wash buffer. Following one more centrifugation, the supernatant was discarded and 2 ml of wash buffer was added at room temperature. The cell suspension was added to the Optiprep gradient and was centrifuged at 2200 rpm (800*g*) for 15 min at room temperature. The fraction containing the majority of neurons was collected (for further details see Milligan and Gifondorwa (2011)) and washed with 5 ml of wash buffer x 2. Finally, the supernatant was discarded and the pellet was resuspended in Growth Media (see above).

The cells from the media were plated on the coverslips and allowed to attach. The plate was transferred to the incubator at 37 °C with 5% CO_2 for 1.5–2 h. Three ml of growth media was then added in each well. Every 1–2 days, 50% of fresh prewarmed growth

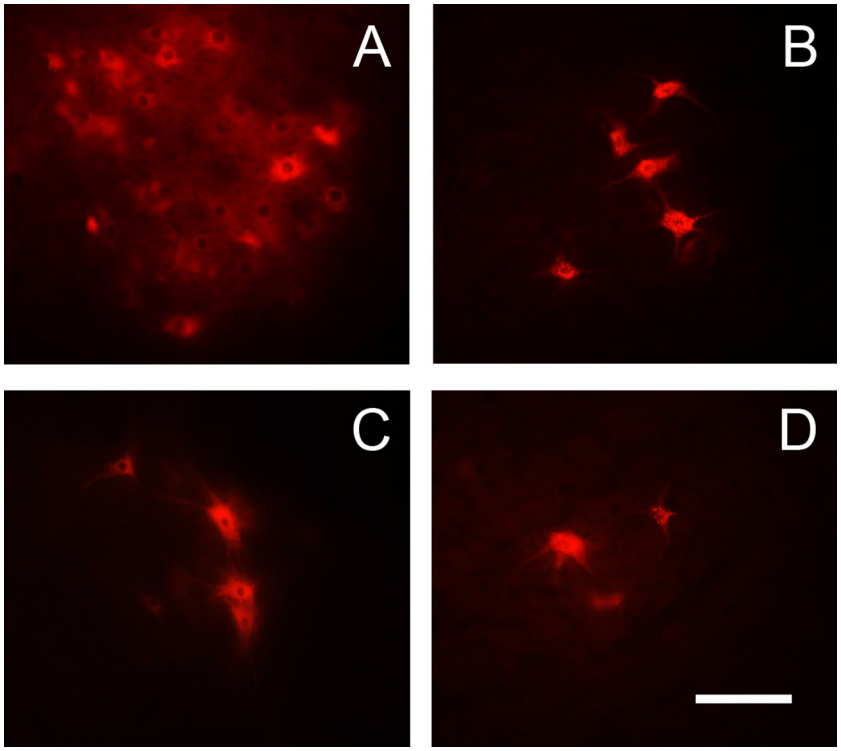

Fig. 2. Neurons located in brainstems with retrogradelly transported DiI (red fluorescence) from severed facial nerve (A), severed recurrent laryngeal nerve (B), injected PCA alone (C), and TA alone (D). The scale bar in D represents 100 μm and is the same for A–C.

Table 2
Number of labelled motoneurons.

Nucleus	Number (Mean ± SD)
Facial motoneurons	2924 ± 690
RLN motoneurons	163 ± 25
PCA motoneurons	40 ± 10
TA motoneurons	30 ± 8

media was replaced in each well. Everyday before the addition of the growth media, coverslips were monitored under the epifluorescence microscope to identify DiI labelled neurons.

As all laryngeal motoneurons are ChAT positive, their identity in the culture was confirmed by staining with ChAT (AB144P, Millipore, Billerica, Massachusetts). (Pascual-Font et al., 2011; Van Daele and Cassell, 2009). To compare the yield between the cultured neurons labelled via RLN application and laryngeal muscle injection, we used Fisher's exact test for statistical analysis.

3. Results and comments

3.1. Motoneurons cell culturing following DiI labelling

Using DiI to retrograde label the motoneurons of the facial nerve, RLN and the PCA and TA of the control group confirmed the location and number of motoneurons as described in previous studies. (Martin et al., 1977; Bieger and Hopkins, 1987; Portillo and Pásaro, 1988; Higashiyama et al., 2005; Pascual-Font et al., 2011; Hernandez-Morato et al., 2013; Pitman et al., 2013). Facial motoneurons were shown located in the ipsilateral facial nucleus in the rostral medulla oblongata (Figs. 1 and 2). The number of labelled motoneurons was 2924 ± 690 (Table 2). RLN motoneurons were located in the caudal end of the nucleus ambiguus. The number of labelled motoneurons was 163 ± 25.

Labelled motoneurons after DiI injection into PCA were located in the rostral third of the column followed by the pool of labelled TA motoneurons. 40 ± 10 and 30 ± 8 labelled motoneurons were counted from each muscle respectively (Figs. 1–3, Table 2). Labelled neurons from RLN were localized in the ipsilateral part of column of the nucleus ambiguus. On the contralateral nucleus ambiguus, no labelled neurons were observed. As the location and number of RLN motoneurons identified is similar to previous studies using other neuronal tracers such as HRP, Cholera toxin and Dextran Amines (Bieger and Hopkins, 1987; Pascual-Font et al., 2011; Hernandez-Morato et al., 2013; Pitman et al., 2013), it can be assumed cell death or diffusion of the DiI tracer to surrounding cells did not occur.

Location of the labelled neurons in the control group was used to determine the portion of the medulla to be isolated in the first steps of the neuron cell isolation. A somewhat larger portion of the brainstem, located in this area, was removed in each animal to assure the collection of all labelled neurons.

Cultured neurons labelled with DiI were observed at 1, 3, 7 and 14 days in all experimental groups. All labelled neurons were ChAT positive in both the processed brainstem and in cell culturing (Figs. 4 and 5). Neural soma containing granulated DiI and neuritic extension with 1–3 branches were visualized. The DiI granulation in the neurites allowed for easier identification (Figs. 2 and 5). A quantification of the isolated labelled motoneurons is summarized in Table 3. At 28 days and older, hardly any labelled neurons were observed. The tracing step caused an unacceptable death rate in 10 days old rats, whereas 14 day old rats had the largest number of labelled neurons in the cell culturing. As such, 14 day old rats were deemed to be of the best age for this protocol.

More than 40 labelled neurons (approximately 2% of total neurons) were plated at day 1 from Facial nucleus. Only half the number of neurons were alive at day 7 (Table 3). Those alive at 7 days remained robust. At day 14 the identifiable neurons had large varicosities in the cell processes and early signs of degradation. Similarly, 35 labelled RLN neurons were observed at day 1. However, this number was reduced to 16 labelled neurons at 7 days after plating. When labelled from PCA and TA muscle, 9 and 11 DiI posi-

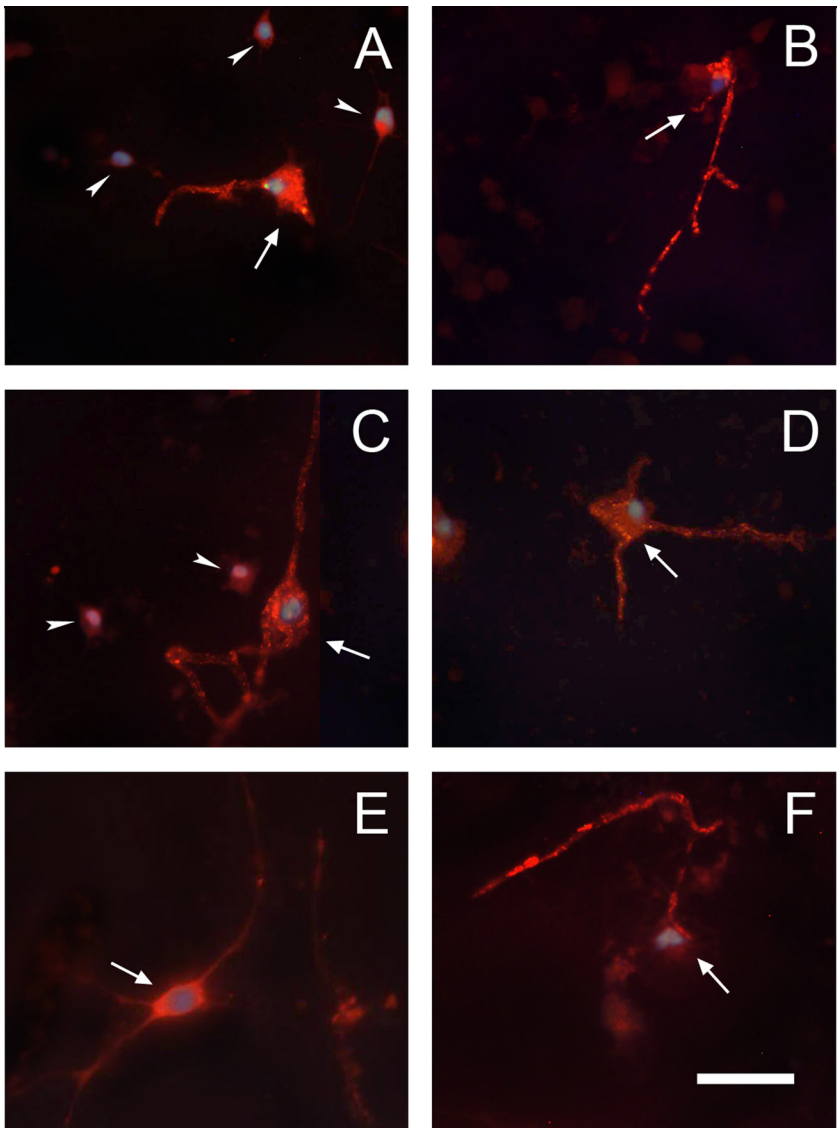

Fig. 3. Cultured motoneurons labelled with DiI (red fluorescence) and nucleus stained with Hoechst (blue fluorescence) from facial nucleus at 3 days (A) and 7 days (B); nucleus ambiguus neurons at 3 and 7 days (C and D); neurons PCA (E) and TA (F) at 7 days. The arrows point to DiI positive motoneurons and the arrowheads point to non-positive cells. The scale bar in the F represents 100 μm. The remaining images are presented at the same magnification.

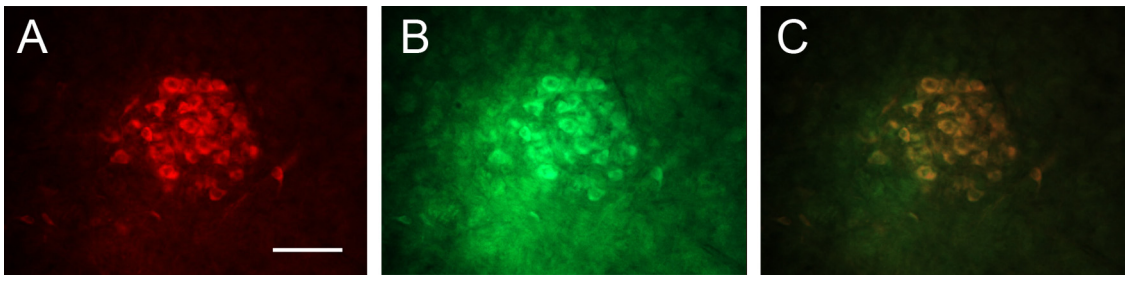

Fig. 4. Neurons located in brainstems from severed facial nerve with retrogradely transported DiI (red fluorescence) (A), immunostained for ChAT as motoneuron marker (green fluorescence) (B), and merge (C). The scale bar in A represents 100 μm and is the same for B and C.

Table 3
Quantification of cell cultured labelled motoneurons at different time points.

Layer	1 day	3 days	5 days	7 days	10 days	14 days
Facial Nerve	46	33	29	21	19	11
Recurrent laryngeal nerve	33	20	17	16	12	9
Posterior Cricoarytenoid muscle	9	8		5		
Thyroarytenoid muscle	11	7		6		

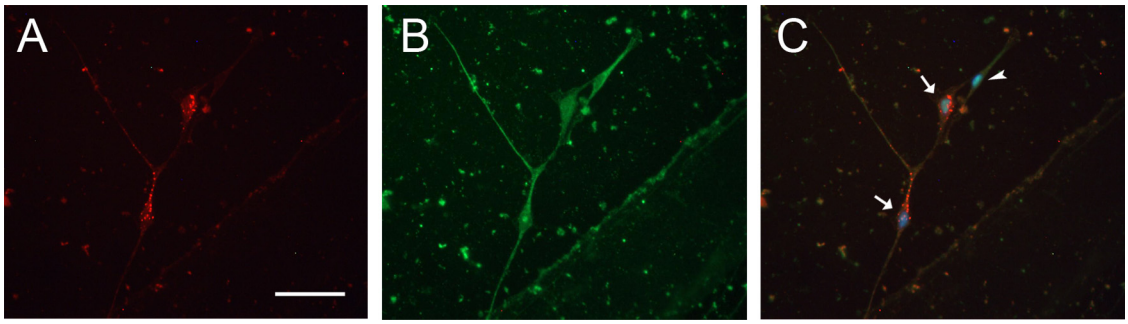

Fig. 5. Cultured motoneurons from RLN at day 1 retrograded traced with DiI (red fluorescence) (A), immunostained for ChAT (green fluorescence) (B), and merge (C). Nucleus is stained with DAPI (blue fluorescence). The arrows point to DiI positive motoneurons and ChAT positive; the arrowheads point to DiI non-positive cells but ChAT positive. The scale bar in A represents 100 μm and is the same for B and C.

tive neurons respectively observed in culture at day 1. This number decreased to 5 and 6 neurons at day 7 (Table 3). Using Fisher's exact test, the difference in the number of motor neurons identified after applying the tracer directly to the cut RLN compared to injecting it into the PCA and TA was statistically significant ($P = 0.026$). This is not an unexpected result as it was found previously in study of the facial nerve (Popratiloff et al., 2001).

The findings of this study suggest that muscle specific neurons can be isolated and cultured by labelling motoneurons via DiI muscle injection and then isolating and culturing the motoneurons as described. This is useful not only for the nucleus ambiguus but also for isolating neurons from small nuclei of the brainstem innervating other specific muscles, such as the hypoglossal nuclei (Ternaux and Portalier, 1993a,b).

Using this particular approach we believe will be useful for isolating neurons innervating specific muscles within small nuclei of the brainstem like hypoglossal nuclei (Ternaux and Portalier 1993a,b). Using DiI as a tracer for the nucleus ambiguus, the diffusion of the tracer did not occur in the surrounding cells as confirmed by the total number of labelled neurons as shown in different studies using other type of neuronal tracers as HRP, Cholera toxin and Dextran Amines (Bieger and Hopkins, 1987; Pascual-Font et al., 2011; Hernandez-Morato et al., 2013).

3.2. Technical considerations

Our study presents technical innovations regarding identifiable neural cell culturing of cranial motoneurons. Neuronal isolation from adult rat brainstem presented several key difficulties which prevented us from harvesting a large enough number of cells for further essays (Milligan and Gifondorwa, 2011; Halum et al., 2012; McRae et al., 2012).

In order to obtain enough number of isolated neurons, one should use a minimum of 8 animals per group: 2 animals to check the neuron labelling in brainstem slices compared to controls, and 6 for cell culturing purposes. Considering the number of motoneurons that supply the laryngeal motoneurons we determined that the number of animals per group is a critical factor (Hernandez-Morato et al., 2014a).

Age of the animal is also a critical factor when culturing motoneurons. Initial experiments were performed on 1, 2 and 3 months old animals. Because the number of viable neurons isolated was very low, further experiments were performed on rats 10, 14 and 21 days old.

At 10 days postnatal, rats were so young that 30% died during the surgery, at the time of tracer application. Additionally, more animals died within 2–3 days after the surgery (40–50%). We assume that the need for precise anesthesia at very small volumes, as well as surgical stress, was the cause of death. In addition, due to anmal size, surgical exposure of the PCA was extremely difficult. Due to these considerations, isolation performed 5 days after the surgery obtained poor results.

In 14 day postnatal animals, the first surgery was performed successfully in the majority of the cases. By taking care to use accurate amounts of anesthetic and performing the surgery slowly to minimize animal intraoperative stress, no animals died from the surgery at the day of motoneuron isolation. The number of isolated neurons was the largest in all groups studied at this time point.

At 21 days, animals can feed independently and the tracing of Facial Nerve, RLN, and TA was relatively easy. Unfortunately, the number of labelled neurons isolated was lower than in the 14 days postnatal animals.

These considerations and results suggest that, 14 day old rats are the most suitable for cranial motoneuron isolation and cell culturing.

3.3. Further studies

The isolation of abductor and adductor motoneurons in a cell culture could aid in further studies by defining the role of different trophic factors during nerve regeneration. It is known that some trophic factors act on neuromuscular formation and maintenance (Ip et al., 2001; Hernandez-Morato et al., 2014a). However, it is still not clear what effect trophic factor expression has on abductor and adductor muscles reinnervation (Sterne et al., 1997; Simon et al., 2000, 2003; Hernandez-Morato et al., 2014a). Further cell culture isolation experiments could provide additional insight the specific motoneuron reinnervation following a nerve injury.

4. Conclusions

This study describes a method for culturing postnatal neurons from rat brainstems. The method allows investigators to identify neurons innervating abductor and adductor muscles by labelling them with DiI before plating. Facial nerve, RLN, and laryngeal motoneurons can be optimally cultured 14 days after birth and are easily identifiable from the rest other neurons secondary to the presence of the tracer in the cell body and their neurites. This method allows for future research into the unique properties of abductor and adductor cranial motoneurons that supply the rat larynx. Determination these properties could allow for development of clinical treatments for selective reinnervation following peripheral nerve injury.

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References

Bieger, D., Hopkins, D.A., 1987. Viscerotopic representation of the upper alimentary tract in the medulla oblongata in the rat: the nucleus ambiguus. J. Comp. Neurol. 262 (August (4)), 546–562.

Choi, D., Li, D., Raisman, G., 2002. Fluorescent retrograde neuronal tracers that label the rat facial nucleus: a comparison of Fast Blue, Fluoro-ruby, Fluoro-emerald, Fluoro-Gold and DiI. J. Neurosci. Methods 117 (June (2)), 167–172.

Crumley, R.L., 2000. Laryngeal synkinesis revisited. Ann. Otol. Rhinol. Laryngol. 109 (April (4)), 365–371.

Flint, P.W., Downs, D.H., Coltrera, M.D., 1991. Laryngeal synkinesis following reinnervation in the rat. Neuroanatomic and physiologic study using retrograde fluorescent tracers and electromyography. Ann. Otol. Rhinol. Laryngol. 100 (10), 797–806.

Halum, S.L., McRae, B., Bijangi-Vishehsaraei, K., Hiatt, K., 2012. Neurotrophic factor-secreting autologous muscle stem cell therapy for the treatment of laryngeal denervation injury. Laryngoscope 122 (September (11)), 2482–2496.

Hernandez-Morato, I., Pascual-Font, A., Ramírez, C., Matarranz-Echeverría, J., McHanwell, S., Vazquez, T., et al., 2013. Somatotopy of the neurons innervating the cricothyroid, posterior cricoarytenoid, and thyroarytenoid muscles of the rat's larynx. Anat. Rec. 296 (February (3)), 470–479.

Hernandez-Morato, I., Isseroff, T.F., Sharma, S., Pitman, M.J., 2014a. Differential expression of glial-derived neurotrophic factor in rat laryngeal muscles during reinnervation. Laryngoscope 124 (September (12)), 2750–2756.

Hernandez-Morato, I., Berdugo-Vega, G., Sanudo, J.R., McHanwell, S., Vazquez, T., Valderrama-Canales, F.J., Pascual-Font, A., 2014b. Somatotopic changes in the nucleus ambiguus after section and regeneration of the recurrent laryngeal nerve of the rat. Anat. Rec. 297 (January (5)), 955–963.

Higashiyama, F., Fukushima, N., Yokouchi, K., Kawagishi, K., Moriizumi, T., 2005. The minimum number of facial motor neurons essential for minimal whisker movement in neonatally nerve-transected young adult rats. J. Neurosurg. 102 (May (4)), 390–395.

Hu, W., Liu, D., Zhang, Y., Shen, Z., Gu, T., Gu, X., et al., 2013. Neurological function following intra-neural injection of fluorescent neuronal tracers in rats. Neural Regen. Res. 8 (May (14)), 1253–1261.

Ip, F., Cheung, J., Ip, N., 2001. The expression profiles of neurotrophins and their receptors in rat and chicken tissues during development. Neurosci. Lett. 301 (July), 107–110.

Kivell, B.M., McDonald, F.J., Miller, J.H., 2001. Method for serum-free culture of late fetal and early postnatal rat brainstem neurons. Brain Res. Brain Res. Protoc. 6 (February (3)), 91–99, Available from: http://ac.els-cdn.com/S1385299X00000374/1-s2.0-S1385299X00000374-main.pdf?_tid=89da8f74-2498-11e3-99cc-00000aab0f6b&acdnat=1379972511_fb740384177b644698922562719f7aa2.

Martin, M.R., Caddy, K.W., Biscoe, T.J., 1977. Numbers and diameters of motoneurons and myelinated axons in the facial nucleus and nerve of the albino rat. J. Anat. 123 (Pt. 3), 579–587.

McRae, B.R., Shew, M., Aaron, G.P., Bijangi-Vishehsaraei, K., Halum, S.L., 2012. A rapid, novel model of culturing cranial nerve X-derived motoneurons for screening trophic factor outgrowth response. Neurol. Res. 34 (July (6)), 564–575.

Milligan, C., Gifondorwa, D., 2011. Isolation and Culture of Postnatal Spinal Motoneurons. Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 77–85.

Miyata, T., Kawaguchi, A., Saito, K., Kuramochi, H., Ogawa, M., 2002. Visualization of cell cycling by an improvement in slice culture methods. J. Neurosci. Res. 69 (August (6)), 861–868.

Myssiorek, D., 2004. Recurrent laryngeal nerve paralysis: anatomy and etiology. Otolaryngol. Clin. North Am. 37 (1), 25–44.

Pascual-Font, A., Hernandez-Morato, I., McHanwell, S., Vazquez, T., Maranillo, E., Sañudo, J., 2011. The central projections of the laryngeal nerves in the rat. J. Anat. 219 (May (2)), 217–228.

Pitman, M.J., Weissbrod, P., Roark, R., Sharma, S., Schaefer, Steven D., 2011. Electromyographic and histologic evolution of the recurrent laryngeal nerve from transection and anastomosis to mature reinnervation. Laryngoscope 121 (2), 325–331.

Pitman, M.J., Berzofsky, C., Alli, O., Sharma, S., 2013. Recurrent laryngeal nerve transection and anastomosis: rat laryngeal motoneuron survival and effect of the anastomosis site. Ann. Otol. Rhinol. Laryngol. 122 (4), 283–287.

Popratiloff, A.S., Neiss, W.F., Skouras, E., Streppel, M., Guntinas-Lichius, O., Angelov, D.N., 2001. Evaluation of muscle re-innervation employing pre- and post-axotomy injections of fluorescent retrograde tracers. Brain Res. Bull., 115–123.

Portillo, F., Pásaro, R., 1988. Location of bulbospinal neurons and of laryngeal motoneurons within the nucleus ambiguus of the rat and cat by means of retrograde fluorescent labelling. J. Anat. 159 (August), 11–8.

Simon, M., Terenghi, G., Green, C.J., Coulton, G.R., 2000. Differential effects of NT-3 on reinnervation of the fast extensor digitorum longus (EDL) and the slow soleus muscle of rat. Eur. J. Neurosci. 12 (March (3)), 863–871.

Simon, M., Porter, R., Brown, R., Coulton, G.R., Terenghi, G., 2003. Effect of NT-4 and BDNF delivery to damaged sciatic nerves on phenotypic recovery of fast and slow muscles fibres. Eur. J. Neurosci. 18 (November (9)), 2460–2466.

Sterne, G.D., Coulton, G.R., Brown, R.A., Green, C.J., Terenghi, G., 1997. Neurotrophin-3-enhanced nerve regeneration selectively improves recovery of muscle fibers expressing myosin heavy chains 2b. J. Cell Biol. 139 (November (3)), 709–715.

Ternaux, J.-P., Portalier, P., 1993a. Influence of tongue myoblats on rat dissociated hypoglossal motoneurons in culture. Int. J. Dev. Biol. 11 (1), 33–48.

Ternaux, J.-P., Portalier, P., 1993b. Culture of hypoglossal cells, dissociated from foetal and new-born rats. J. Neurosci. Methods 49, 33–47.

Van Daele, D.J., Cassell, M.D., 2009. Multiple forebrain systems converge on motor neurons innervating the thyroarytenoid muscle. Neuroscience 162 (August (2)), 501–524.

Weissbrod, P., Pitman, M.J., Sharma, S., Bender, A., Schaefer, S.D., 2011. Quantity and three-dimensional position of the recurrent and superior laryngeal nerve lower motor neurons in a rat model. Ann. Otol. Rhinol. Laryngol. 120 (November (11)), 761–768.

Yamamoto, N., Higashi, S., Toyama, K., 1997. Stop and branch behaviors of geniculocortical axons: a time-lapse study in organotypic cocultures. J. Neurosci. 17 (May (10)), 3653–3663.

Zuchero, J.B., 2014. Purification of dorsal root ganglion neurons from rat by immunopanning. Cold Spring Harb. Protoc. 2014 (August (8)), 826–838.