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CHARACTERIZATION OF SOME MORPHOLOGICAL PARAMETERS OF ORBICULARIS OCULI MOTOR NEURONS IN THE MONKEY

D. W. McNEAL, J. GE, J. L. HERRICK, K. S. STILWELL-MORECRAFT AND R. J. MORECRAFT*

Division of Basic Biomedical Sciences, Laboratory of Neurological Sciences, The University of South Dakota, Sanford School of Medicine, Vermillion, SD 57069, USA

Abstract—The primate facial nucleus is a prominent brainstem structure that is composed of cell bodies giving rise to axons forming the facial nerve. It is musculotopically organized, but we know little about the morphological features of its motor neurons. Using the Lucifer Yellow intracellular filling method, we examined 11 morphological parameters of motor neurons innervating the monkey orbicularis oculi (OO) muscle, which plays an important role in eyelid closure and voluntary and emotional facial expressions. All somata were multipolar and remained confined to the intermediate subnucleus, as did the majority of its aspiny dendritic branches. We found a mean maximal cell diameter of 54 μm in the transverse dimension, cell diameter of 60 μm in the rostrocaudal dimension, somal surface area of 17,500 μm^2 and somal volume of 55,643 μm^3. Eight neurons were used in the analysis of dendritic parameters based upon complete filling of the distal segments of the dendritic tree. We found a mean number of 16 dendritic segments, an average dendritic length of 1036 μm, diameter of 7 μm, surface area of 12,757 μm^2 and total volume of 16,923 μm^3. Quantitative analysis of the dendritic branch segments demonstrated that the average number, diameter and volume gradually diminished from proximal to distal segments. A Sholl analysis revealed that the highest number of dendritic intersections occurred 60 μm distal to the somal center with a gradual reduction of intersections occurring distally. These observations advance our understanding of the morphological organization of the primate facial nucleus and provide structural features for comparative studies, interpreting afferent influence on OO function and for designing studies pinpointing structural alterations in OO motor neurons that may accompany disorders affecting facial movement. Published by Elsevier Ltd on behalf of IBRO.

Key words: blinking, facial expression, cranial nerves, pons, non-human primate.

Primate craniofacial muscles, otherwise known as the muscles of facial expression, are unique because they arise from the facial skeleton or subcutaneous aponeurosis, and insert directly into the deep surface of the skin (Huber, 1933; Hollinshead, 1982; Williams et al., 1989). This organization endows these extraordinary muscles with the capability of exerting their biomechanical influence on the skin and facial orifices. Although the muscles of facial expression occupy widespread anatomical regions, they all receive neural innervation from the seventh cranial nerve (CN VII, or the facial nerve) due to their common embryological origin from the second branchial arch.

Motor neurons contributing to the formation of the facial nerve arise from the facial nucleus. The nucleus is located in the lower ventrolateral pontine region of the brainstem and is musculotopically organized (Papez, 1927; Szentagothai, 1948; Crosby and Dejonge, 1963; Friauf and Herbert, 1985; Jenny and Saper, 1987; Satoda et al., 1987; Welt and Abbs, 1990; Morecraft et al., 2001; Sherwood, 2005). In the non-human primate, cell bodies innervating the orbicularis oculi (OO) muscle, which is the focus of this report, are localized in the dorsocentral portion of the nucleus, in an anatomical region designated by most authorities as the intermediate subnucleus (Kuypers, 1958; Jenny and Saper, 1987; Porter et al., 1989; Welt and Abbs, 1990; VanderWerf et al., 1998; Morecraft et al., 2001; Gong et al., 2005).

Historically, the paired OO muscles have received significant attention due to their prominent role in emotional expression and social communication, as well as their critical contribution to ocular reflexes (Duchenne, 1862; Darwin, 1872; Yerkes and Yerkes, 1929; Huber, 1931; Ekman and Friesen, 1982; Manning and Evinger, 1986; Evinger et al., 1989; Ekman et al., 1990; Ekman, 2003). In addition, the OO muscle and its affiliated motor neurons have long been integral components of experimental models designed to understand classical conditioning, the functional organization of brainstem sensorimotor networks, and voluntary and emotional facial expression. However, recent efforts have focused on examining the role of the OO in neurological movement disorders affecting eyelid closure. Indeed, OO abnormalities occur in a host of neurological disorders including blepharospasm, hemifacial spasm, Meige syndrome, Parkinson's disease and Tourette's syndrome (Basso and Evinger, 1996; Hallett and Daroff, 1996; Smith et al., 1996; Schicatano et al., 1997; Jankovic and Tolosa, 1998; Peshori et al., 2001; Evinger et al., 2002; Hallett, 2002; Holstege, 2002). In many of these diseases there is a cumulative breakdown in the structural organization and functional regulation of the neural circuitry mediating OO function. In response to the growing interest in the roles of the OO, we examined the normal morphological characteristics of OO motor neurons in the facial nucleus of the non-human primate by capitalizing on the application of the postmortem Lucifer Yellow (LY) intracellular filling technique.

*Corresponding author. Fax: +1-605-677-6381.
E-mail address: rmorecra@usd.edu (R. J. Morecraft).
Abbreviations: FB, Fast Blue; HRP, horseradish peroxidase; LY, Lucifer Yellow; M1, primary motor cortex; OO, orbicularis oculi; PB, phosphate buffer; TBS, tris-buffered saline; 1°, primary; 2°, secondary; 3°, tertiary; 4°, quaternary.

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Table 1. Experimental details

Animal	Sex	Species	Weight (kg)	Side	Tracer	Muscle location	Injection sites	Total amount (μl)
Case 1	M	Rhesus	7	R,L	FB	Upper and lower orbital, upper pretarsal	18	36
Case 2	M	Rhesus	8	R,L	FB	Upper and lower orbital, upper pretarsal	8	20
Case 3	F	Fascicularis	4.5	R,L	FB	Upper and lateral orbital	8	20
Case 4	M	Rhesus	3.2	R,L	FB	Upper orbital	4	25

EXPERIMENTAL PROCEDURES

The morphological characteristics of lower motor neurons innervating the orbicularis oculi (OO) muscle were examined in four healthy adult monkeys (three male and one female) (Table 1). All protocols were approved by the Institutional Animal Care and Use Committee of the University of South Dakota and were conducted in accordance with the United States Department of Agriculture, National Institutes of Health and the Society for Neuroscience guidelines for the ethical treatment of experimental animals. Every effort was made to minimize the number of animals used and their suffering.

To localize neurons within the facial nucleus innervating the OO muscles, we injected the retrograde neural tracer, Fast Blue (FB) (Dr. Illing Plastics, Breuberg, Germany) directly into the OO muscles of anesthetized monkeys (Fig. 1). To minimize involvement of the surrounding muscles, which interdigitate with OO fibers, we placed our injections into the orbital portion of the OO muscle located directly over the bony orbital margin of the facial skeleton. In cases 1 and 2, injections were also made directly into the pretarsal (palpebral) portion within the upper eyelid (Table 1).

Following a survival period of 26–32 days to enable neuronal transport of the tracer to the facial nucleus, the monkeys were reanesthetized, perfused transcardially with fixative and the CNS and OO muscles were removed. We then filled FB-labeled neurons in the facial nucleus with LY in fixed, 250–300 μm thick tissue sections. Infusing each neuron with LY enabled us to visualize the somata and dendritic extensions of OO motor neurons, and to quantify these morphological elements with confocal microscopy. Following confocal microscopy and photographic documentation, the 250–300 μm thick tissue sections were sliced using a cryostat at a thickness of 10 μm and these sections were processed for immunohistochemical localization of LY and histochemical localization of Nissl substance for facial nucleus cytoarchitecture. The FB injection sites in the OO muscles were also evaluated using histochemical and fluorescent methods for injectate localization.

Surgery

For surgical preparation, each monkey was injected with atropine sulfate (0.05 mg/kg), immobilized with ketamine hydrochloride (10 mg/kg) and either anesthetized with an i.p. injection of sodium pentobarbital or intubated, placed on a mechanical respirator and anesthetized with isoflurane gas (1–1.5%) and a surgical grade oxygen/air mixture. Several injections of a 3–4% solution of FB were placed into the OO muscle (Table 1). This was accomplished by inserting a sterile, 1 inch, 20 gauge hypodermic needle through the skin and into the orbital portion of the OO muscle, and then passing a 10 μl Hamilton microsyringe (Fisher Scientific, Pittsburg, PA, USA) filled with FB through the bore hole of the needle. The use of the hypodermic needle allowed for precise penetration into the OO muscle and prevented potential tissue blockage of the Hamilton microsyringe. Animals were then removed from anesthesia, placed into their home cage and carefully monitored throughout the recovery process.

Tissue processing

After a survival period of 26–32 days, the animals were injected with atropine sulfate (0.05 mg/kg) and immobilized with ketamine hydrochloride (10 mg/kg). They then were deeply anesthetized with an overdose of pentobarbital (50 mg/kg or more) and perfused transcardially with 0.9% saline, followed by 2 l of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Sucrose was not used during the perfusion to limit the possibility of tissue shrinkage in the brainstem for our cell filling procedure. The brain and brainstem were removed, blocked and photographed for neuroanatomical reconstruction. Subsequently, the cortex was placed into a 30% sucrose solution in 0.1 M PB and the brainstem placed into cold 0.1 M PB buffer (without sucrose) for preparation of vibratome slicing.

The OO muscles and surrounding tissue were removed from the facial skeleton and processed for fluorescent and brightfield microscopy to localize the placement of the neural tracers, and to determine if the tracer had spread into adjacent muscle fibers (Fig. 1). To accomplish this, the muscles were placed in a solution of 20% glycerol and 2% dimethyl sulfoxide in 0.1 M PB adjusted to a pH of 7.4. After thorough infiltration of the cryoprotectant, the tissue was flattened and frozen sectioned at 50–75 μm on a sliding microtome (American Optical model AO 860, Buffalo, NY, USA). Even-numbered serial sections were placed on subbed slides, stained with Eosin Y, for identification of cytoplasm and muscle striations, and then coverslipped with Permount (Fisher Chemicals) for microscopic analysis of muscular organization in relation to the location of the injected tract tracer (Fig. 1A). Odd-numbered serial sections were mounted on subbed slides, and coverslipped using D.P.X. neutral mounting medium (Aldrich Chemical Co. Inc., Milwaukee, WI, USA) for fluorescent microscopic analysis (Fig. 1B).

The entire rostral to caudal extent of the pons was blocked from the brainstem and transferred to an oscillating vibratome (Electron Microscopy Sciences, Model OTS-3000-03, Washington, PA, USA). The pons was then cut transversely into 250 or 300 μm thick serial sections, which were transferred to well plates filled with cold 0.1 M PB at a pH of 7.4.

The locations of FB-labeled neurons within the facial nucleus were then recorded. This was accomplished by placing each thick section into a bath of 0.1 M PB solution supported on a microscope slide. The brainstem outline and locations of labeled neurons were then plotted using epifluorescent illumination on an Olympus microscope (BX-51 or BX-60, Leeds Precision Inc., Minneapolis, MN, USA). The microscope was equipped with a computer-controlled MAC 5000 motorized microscope stage (Ludl Electronic Products, Hawthorne, NY, USA) which was electronically coupled to a Neurolucida neuroanatomical data collection and analysis system (Microbrightfield Inc., Colchester, VT, USA) loaded in a Dell 4400 desktop computer (Dell Computer Corp., Round Rock, TX, USA).

Intracellular filling method

To intracellularly fill the FB-labeled neurons, borosilicate glass micropipettes (10 cm length, 0.2 mm O.D., 0.69 mm I.D.; Sutter