The Autonomic Facial Nerve Pathway in Birds: A Tracing Study in Chickens

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PURPOSE. In birds, the parasympathetic innervation of the chordoid is via the ciliary (cranial nerve III) and pterygopalatine (cranial nerve VII) ganglia, the latter consisting of a chain of microganglia within the orbit. Because of the scattered nature of these microganglia, lesions of this nerve pathway in birds have not been attempted, making interpretation of the functional contribution of this parasympathetic input to the avian eye uncertain. The purpose of this study was to find an extraorbital approach to the preganglionic part of cranial nerve VII and to reveal its peripheral terminals and its site of origin in the brain stem.

METHODS. The radix autonomica cranial nerve VII was accessed via the tympanic cavity and injected with dextran coupled to Texas red (DTxR). Orbital structures and the brain stem were prepared for tracer detection and immunohistochemistry for neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), galanin (GAL), and somatostatin (SOM). For documentation, fluorescence and confocal laser scanning microscopy were used.

RESULTS. Anterogradely labeled DTxR-positive nerve fibers were detected within the orbital pterygopalatine microganglionic chain, forming boutons closely associated with nNOS-positive neurons. Retrogradely labeled DTxR-positive neurons with cell diameters of approximately 20 μm were found in the brain stem. These were positive for ChAT, but negative for nNOS, VIP, SOM, GAL, and CGRP. They most likely represent the preganglionic neurons of the superior salivatory nucleus. In close proximity, there were larger (40 μm) unlabeled neurons that were positive for ChAT and CGRP, but negative for GAL. These most likely represent motoneurons of the facial nerve.

CONCLUSIONS. This surgical approach offers excellent opportunities for lesioning experiments for the study of the autonomic facial nerve pathway in birds in terms of both its anatomical organization and its function. (Invest Ophthalmol Vis Sci. 2006;47:3225–3233) DOI:10.1167/iovs.05-1279

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superficial veins was stopped by cautery. Under a dissection microscope, a widening of the cut in the direction of the external auditory canal was performed and the external auditory canal was opened from the dorsal aspect with a vertical cut. The resultant flap was brought into an anterior position, resulting in direct access to the tympanic membrane, which was then incised with a 23-gauge needle in the craniocaudal direction just anterior to the insertion of the columella (the bird’s single auditory ossicle). Care was taken to avoid contact with the columella. The underlying bony canal containing the autonomic part of the facial nerve (i.e., the palatine nerve) was thus exposed (Fig. 1). The bony canal was then opened with a 23-gauge needle, and crystals of dextran coupled with Texas red (DTxR; molecular weight [MW] 3000; Invitrogen, Carlsbad, CA) were inserted into the nerve. In another set of experiments, DiI (Invitrogen) was used as a tracer, and applied in the same way. After erythromycin ointment treatment (Fougera, Melville, NY), the external auditory canal and superficial tissue layers were sutured, and erythromycin ointment mixed 1:1 with a 2% lidocaine hydrochloride jelly (Akorn, Buffalo Grove, IL) was put on top of the wound. After a survival time of 4 days, birds were deeply anesthetized with sodium thiopental and transcardially perfused with phosphate-buffered saline (PBS).

**Immunohistochemistry**

After fixation, the brain, the Harderian gland with the adjacent tissue containing the pterygopalatine microganglia and the facial nerve in the area of the injection were removed and prepared for immunohistochemistry. Tissue was rinsed in PBS and transferred into PBS containing 15% sucrose, and then 20-μm-thick sections were cut in a cryostat. Sections were air-dried for 1 hour at room temperature (RT) on poly-L-lysine (Sigma-Aldrich, St. Louis, MO)-coated slides. After a 5-minute rinse in Tris-buffered saline (TBS; Roth, Karlsruhe, Germany) slides were incubated for 1 hour at RT in TBS containing 10% donkey or goat serum (depending on the secondary antibodies used; Sigma-Aldrich), 1% BSA (Sigma-Aldrich), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After a 5-minute rinse, slides were incubated with antibodies for double- and triple-labeling of the markers as listed in Table 1. After a rinse in TBS (four times, 5 minutes each) binding sites of primary antibodies were visualized by corresponding Alexa488, and Cy5/Alexa647-tagged antisera (1:1000; Invitrogen) in TBS, containing 1% BSA and 0.5% Triton X-100 (1 hour at RT) followed by another rinse in TBS (four times, 5 minutes each). Slides were embedded in TBS-glycerol (1:1; pH 8.6).

**Documentation**

To document double- and triple-labeled immunohistochemistry, a confocal laser-scanning microscope (MRC 1000; Bio-Rad, Hercules, CA; attached to a Diaphot 300; Nikon, Düsseldorf, Germany; equipped with a krypton-argon laser; ALC, Salt Lake City, UT; and with 20× dry or 40× and 60× oil-immersion objective lenses, with numeric apertures of 0.75, 1.30, and 1.4, respectively; Nikon) was used. Sections were imaged using the appropriate filter settings for GAL, galanin; nNOS, neuronal nitric oxide synthase; VIP, vasoactive intestinal polypeptide; SOM, somatostatin; CGRP, calcitonin gene-related peptide; ChAT, choline acetyl transferase.
DTxR (568 nm excitation, filter 605DF32; channel 1, coded red), Alexa488 (488 nm excitation, filter 522DF32; channel 2, coded green), and Cy5/Alexa647 (647 nm excitation, filter 680DF32; channel 3, coded blue). Colocalization of signals in channels 1 and 2 resulted in a yellow mixed color. An overlap of signals in channels 1 and 3 resulted in pink, and in channels 2 and 3 in turquoise (mixed) colors. For quantitative assessments, a fluorescence microscope (10×/H11003, 20×/H11003, or 40×/H11003 dry objective lenses; model BX50; Olympus, Melville, NY) equipped with a digital camera (model DP70; Olympus) was used.

**Brain Stem Mapping**

Serial sections (18 μm) of traced brain stem areas were photographed in the epifluorescence microscope (Aristoplan; Leica, Bensheim, Germany; ×16 dry objective) with filter settings for DTxR (filter block N2.1) and for background fluorescence (filter block I3). After documentation, sections were stained for light microscopy with a standard Nissl stain and photographed again. These micrographs were digitally mounted (Photoshop 6.0; Adobe Systems, Munich, Germany) and overlaid with the corresponding fluorescence micrographs in such a way that DTxR and Nissl stains of identical neurons were exactly matching (see Fig. 8). Brain stem plots were created from these overlays. Large DTxR-negative neurons (diameter, ≥40 μm) were observed in the immediate vicinity of the smaller DTxR-positive ones. For orientation purposes, nearby brain stem nuclei were identified according to the chicken brain stem atlas of Kuenzel and Masson.24

**RESULTS**

Animals tolerated the surgical procedure very well. Immediately after regaining consciousness, birds started food intake without any signs of diminished capacity. The pupillary light reflex remained intact and no ipsilateral ocular effects were detectable by casual observation. Experiments using DiI showed the same results as those with DTxR, except that DiI was transported in a retrograde direction only. Consequently, only the DTxR results will be documented here.

**Anterograde Tracing to the Orbit**

DTxR-positive nerve fibers were found in the orbit along the course of the radix autonomica cranial nerve VII close to the Harderian gland (Fig. 2A). Immunohistochemistry against nNOS or VIP showed that these postganglionic neurons within the nerve formed a series of microganglia, each of which contained from approximately 20 to 50 neurons; sometimes however, they consisted of a single neuron. nNOS-positive microganglia were present as far proximal as the facial nerve within the tympanic cavity (Fig. 2B). In single confocal optical

![Figure 2](image-url)

**FIGURE 2.** (A) Fluorescence micrograph of a DTxR-labeled nerve fiber (arrowheads) within the autonomic part of the facial nerve within the orbit. (B) Fluorescence micrograph of immunohistochemistry against nNOS: microganglion within the course of the autonomic part of the facial nerve within the tympanic cavity. Note that there were very few nNOS-positive nerve fibers in this nerve.

![Figure 3](image-url)

**FIGURE 3.** (A, B) Confocal microscopy (single optical sections) of DTxR-labeled nerve fibers (red) and immunohistochemistry against nNOS (green). Postganglionic nitrergic neurons of the pterygopalatine ganglion were closely associated with anterogradely labeled preganglionic nerve fibers and boutons (yellow mixed color represents sites of closest proximity, arrowheads).
sections, DTxR-positive nerve fibers formed boutonlike endings in close association to the nitrergic neurons of the pterygopalatine microganglia, suggesting that these constitute the preganglionic terminals onto the postganglionic neurons (Fig. 3). There were no anterogradely labeled neurons.

Retrograde Tracing to the Brain Stem

Retrogradely labeled DTxR-positive neurons were detected in the ipsilateral half of the brain stem (Fig. 4A). These labeled neurons were small, with cell diameters of approximately 20 μm, and were ovoid, with a smooth contoured surface (Fig. 4B). They gave rise to several mainly polarly arranged processes. Of the labeled neurons, 77% showed immunoreactivity for ChAT (199/259 DTxR-positive neurons in 18 sections; 76.8%; Fig. 5A). These neurons were immunonegative for nNOS, as well as for VIP, SOM, CGRP, and GAL (Figs. 5B, 6). Fibers with boutonlike endings positive for GAL and SOM were occasionally noted in close association with these DTxR-positive neurons (Figs. 6C, 6D).

Two other types of neurons that were not labeled with DTxR were found in close proximity to the DTxR-positive ones. One type consisted of cells that were of similar size and shape to the DTxR-positive ones, but were immunoreactive for nNOS (Fig. 6B). The second type consisted of large neurons with cell diameters of approximately 40 μm (Figs. 5B, 7A–C). These had a polygonal shape and a smooth contoured surface,

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**FIGURE 4.** (A) Fluorescence micrograph of a brain stem section after DTxR injections into the right autonomic facial nerve showing retrogradely labeled neurons of the superior salivatory nucleus (cross marks the mediosagittal line: D, dorsal; V, ventral; L, left; R, right). (B) Fluorescence micrograph of a retrogradely DTxR-labeled neuron of typical size and shape.

**FIGURE 5.** (A) Confocal microscopy (single optical section) of a retrogradely labeled neuron in the brain stem (DTxR, red), in combination with immunohistochemistry against ChAT (green), showing colocalization of both markers (yellow mixed color). (B) Confocal microscopy (extended focus mode) of retrogradely labeled DTxR-positive neurons in the brain stem (red) followed by immunohistochemistry against GAL (green) and CGRP (blue). DTxR-labeled neurons showed no colocalization with GAL or CGRP. Note a large CGRP-positive neuron in the vicinity, as well as GAL-positive nerve fibers.
and the processes were, when detectable, radially arranged (Fig. 7B). These large neurons showed immunoreactivity for ChAT (Figs. 7A, 7B) and also CGRP (Figs. 5B, 7C), but were negative for GAL (Fig. 5B).

Topography of Labeled Neurons in the Brain Stem

In serial sections (Figs. 8, 9), the area of labeled neurons was observed to extend approximately 1.2 mm in the craniocaudal direction, from the caudal portion of the flocculus to the beginning of the nucleus reticularis gigantocellularis. These labeled neurons were medial to the superior olivary nucleus and lateral to the abducens nerve. In the cranial portion of the nucleus (Figs. 9A–C), they were located between the dorsal and ventral motor nucleus of cranial nerve VII. Perykaria of this nucleus were identified unequivocally based on its cytoarchitecture (Fig. 8). These two facial nuclei merged more caudally (Fig. 9D–L), intermingling with the labeled neurons in such a way that distinct facial and superior salivatory nuclei could not be defined cytoarchitectonically.

DISCUSSION

In this study, we describe a surgical procedure in chickens that accesses the preganglionic autonomic part of the facial nerve via the tympanic cavity and use anterograde and retrograde labeling to trace the pathway to its terminals in the orbit and its origin in the brain stem. We also combine these tracings with immunohistochemistry to determine the chemical coding of the neurons and those in their vicinity.

Surgical Procedure and Anatomy

In birds, the facial nerve enters the internal acoustic meatus together with the eighth cranial nerve (i.e., n. vestibulocochlearis). It divides within the otic bone complex, forming the palatine nerve more rostrally, and the hyomandibular nerve more caudally. Whereas the hyomandibular nerve contains motor and sensory fibers for the lateral neck region, the palatine nerve presumably contains the nerve fibers for the para-
sympathetic innervation of the orbital and nasal structures. This branch enters the orbit as the radix autonomica, where it synapses on the pterygopalatine chain/ethmoidal ganglion. This arrangement is the equivalent of the mammalian greater superficial petrosal nerve26 (or Vidian nerve after merging with sympathetic fibers27). The third branch, the chorda tympani, contains afferent taste fibers, and will not be discussed herein. A posterior approach to the tympanic cavity through the soft tissue of the external auditory canal was chosen because the curved course of the canal makes direct access to the target site impossible. After cutting of the tympanic membrane, the palatine nerve (autonomic part of cranial nerve VII) could be easily detected, entering the tympanic cavity cranially and then descending anterior-medially in its bony canal in the direction of the internal carotid artery. Because the nerve is enclosed within a bony canal, spreading of the DTxR crystals and consequent labeling of structures other than those intended was easily avoided. This surgery did not appear to cause any postoperative complications, and the procedure was well tolerated by the animals. In the long-term survivors, the cut parts of the tympanic membrane reconnected and healed.

Peripheral Targets

Up to this point, our understanding of the course and contents of the radix autonomica has been based on inferences from a variety of dissection approaches.24–27 Our results are the first to show that the facial nerve fibers do indeed course through the tympanic cavity to terminate within the orbital microganglia of the pterygopalatine chain. Furthermore, these microganglia are found as far proximal as the tympanic cavity. Thus, as already mentioned, a surgical approach from within the orbit27 would not be successful. Because these most proximal microganglia (within the proximal facial nerve in the tympanic cavity) are far distal from the eye, it is likely that they innervate nonocular targets.26 Although the existence of connections between the pterygopalatine ganglia and blood vessels supplying the choroid has been demonstrated in pigeons,10 The precise distribution of the pterygopalatine neurons and their exact targets (both ocular and nonocular) remain to be determined.

One important finding of this study is that it has firmly established the superior salivatory nucleus as the site of origin of the preganglionic nerve fibers to the pterygopalatine ganglion. It is not known whether any fibers from the superior salivatory nucleus directly innervate the eye. Although we did find labeled nerve fibers in the autonomic part of the facial nerve, none were detected in the choroid (data not shown). This does not preclude the possibility that these fibers do in fact, innervate the choroid, as we cannot rule out the possibility that the survival time was not sufficient to allow the tracer to travel the distance. It will now be possible to determine the projections from the brain stem to its various targets by the use of tracing techniques.

Central Nuclei

DTxR-Labeled Neurons. The precise connectivity of the superior salivatory nucleus (SSN) and its neurochemical contents has not been established in birds because earlier studies on the facial nerve used conventional horseradish peroxidase (HRP) tracing techniques.30–33 An advantage of the technique used in the current study is that it allows for subsequent immunohistochemistry and therefore reveals the neurochemical coding of a specific target. We believe that the DTxR-labeled neurons observed in the brain stem are indeed the autonomic preganglionic neurons of cranial nerve VII and thus represent the avian equivalent of the superior salivatory nucleus, for the following reasons: First, the small size of the labeled neurons and their ovoid shape are not typical of branchiomatic motoneurons (discussed later). Second, being positive for ChAT is compatible with the neurochemical phenotype of preganglionic parasympathetic neurons.34 Finally, it was a parsimonious conclusion that the anterogradely labeled nerve fibers and the boutonlike endings closely apposed to the nNOS-positive neurons within the autonomic part of the facial nerve have their origin in the retrogradely labeled neurons of the brain stem.

In birds, the exact position of this brain stem nucleus has been uncertain up to this point because of the lack of combined tracing and immunohistochemical studies.24,26 However, studies using retrograde HRP tracing from other nonocular targets have identified a small cell population in the brain stem of the pigeon34 and chicken35 that appears to resemble the cell population presented herein. In mammals, preganglionic neurons of the superior salivatory nucleus express nitrergic markers36–38 as well as cholinergic ones, whereas in our study, nNOS could not be detected in birds. This was also supported by our finding of only a very few nNOS-positive nerve fibers within the radix autonomica of the tympanic cavity. These are presumably either proximal postganglionic

FIGURE 8. Retrogradely labeled DTxR-positive neurons in the brain stem (A, arrows) were matched with their position in the Nissl stain (B, arrows). Note the proximity of large motoneurons to the labeled cells. A brain stem map was created with this technique (see Figure 9). Also see small unlabeled neurons close to the labeled ones.
nerve fibers or nerve fibers of unknown origin. VIP, a ubiquitous marker in the autonomic nervous system\(^3^9\),\(^4^0\) was not found in the DTxR-labeled preganglionic neurons.

In a recent study in quails, neurons of the pterygopalatine ganglion were shown to be contacted by SOM-positive boutons.\(^4^1\) The origin of these fibers was hypothesized to be the preganglionic neurons of the superior salivatory nucleus. In our study, however, we found that preganglionic neurons do not contain SOM. It is likely that other sources, such as the ciliary ganglion\(^4^2\),\(^4^3\) or fibers from other areas of the brain stem,\(^4^5\) are the sources of the terminals described in the quail study. Although this discrepancy could be attributable to species differences, the fact that chickens and quails belong to the same family weakens this supposition. The origin of the nNOS- and SOM-positive boutons observed in close association with the retrogradely labeled DTxR-positive neurons shown herein is not known. Verification of synaptic contact awaits confirmation by electron microscopy.

Unlabeled Neurons. We found two populations of unlabeled neurons in the vicinity of the DTxR-labeled ones. The first were nitrergic, were of similar size and shape to the DTxR-positive ones, and have an as yet unknown function. A second population of large neurons displayed the typical size and shape of cranial nerve motoneurons\(^3^1\),\(^4^6\),\(^4^7\) in birds. These are positive for ChAT and CGRP, which is similar to the chemical coding shown for some mammalian cranial motoneurons.\(^4^8\),\(^4^9\),\(^5^0\) However, these putative cranial motoneurons were negative for GAL, unlike the GAL-positive ones found in rats\(^5^1\); presumably, this is a species difference. Based on our cytoarchitectural studies, these large neurons most likely represent facial motoneurons. However, confirmation requires double-tracing experiments, because in birds the facial and trigeminal...
nuclei are in close proximity to one another and in fact, intermingling.\textsuperscript{52}

**Functional Considerations**

The potential functions of the autonomic facial nerve pathway in birds are unknown. In pigeons, a substantial number of cholinergic nerve fibers in the inferior-nasal quadrant of the choroidal survival removal of the ciliary ganglion. It has been proposed that this innervation field reflects the innervation territory of the facial nerve\textsuperscript{6} (apparently this “split- innervation pattern” also exists in mammals\textsuperscript{55}). Further, it is known but has not been demonstrated that fibers of the pterygopalatine ganglion in birds innervate choroidal blood vessels\textsuperscript{15} and therefore may influence intraocular pressure.\textsuperscript{54} Moreover, the intrinsic choroidal neurons\textsuperscript{1,13,55} and possibly the pterygopalatine ganglion comprise a source of innervation of nonvascular smooth muscle cells\textsuperscript{1,13,56} that span the choroid. It has been hypothesized that a change in tonus of these nonvascular smooth muscle cells could lead to the changes in choroidal thickness that constitute part of the compensatory response of the eye to retinal defocus\textsuperscript{57} in chicks. Specifically, the choroid rapidly (within hours) changes its thickness in response to myopic and hyperopic defocus\textsuperscript{58} by moving the retina toward the image plane. In addition, the thickness of the choroid shows a diurnal rhythm, becoming thicker at night and thinner during the day.\textsuperscript{58,59} Evidence supporting a role for the pterygopalatine ganglion and possibly the intrinsic choroidal neurons on these choroidal responses is that intravitreal injections of the nitric oxide synthase inhibitor L-NAME (N\textsuperscript{G}-nitro-arginine methyl ester) inhibits the choroidal expansion in response to myopic defocus.\textsuperscript{60} Because both the pterygopalatine ganglion and intrinsic choroidal neurons are nitrergic,\textsuperscript{13,61,62} it is plausible that L-NAME acts at these sites. The elucidation of the mechanisms underlying this function, as well as other possible ocular functions that might be mediated through the autonomic pathway of the seventh cranial nerve are now feasible with the approach demonstrated herein.

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