Innervation of the Superior Tarsal (Müller’s) Muscle in the Cynomolgous Monkey: A Retrograde Tracing Study

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Purpose. The retrograde transport of wheat germ agglutinin–horseradish peroxidase (WGA/HRP) was used to study the localization of the neurons that innervate the superior tarsal muscle in the cynomolgous monkey.

Methods. A 5–10% WGA–HRP solution was applied to the medial part of the superior tarsal muscle. Seventy-two hours later, the animals were killed and perfused with fixative.

Results. The WGA-HRP-labeled neurons were localized in the ipsilateral trigeminal ganglion, the ipsilateral pterygopalatine ganglion, and the ipsilateral superior cervical ganglion. The labeled somata of the sensory neurons were all found in the ophthalmic ganglionic part and the ophthalmic nerve part of the ipsilateral trigeminal ganglion. Most of the labeled somata were distributed somatotopically in the dorsal region of the ophthalmic part of the ganglion. The labeled somata of the sympathetic neurons were all located in the ipsilateral superior cervical ganglion. Most of these labeled somata, which were distributed somatotopically, were found in the cranial part of the ganglion. The labeled somata of the parasympathetic neurons were all located in the ipsilateral pterygopalatine ganglion, particularly in the central ovoid part of the ganglion. No labeled somata were detected in the ipsilateral parasympathetic ciliary ganglion, the ipsilateral nodose ganglion, or the first three ipsilateral sensory spinal ganglia; labeled somata could not be detected in any of the contralateral ganglia examined.

Conclusions. A complete documentation of all projections of the superior tarsal muscle in the monkey has been presented, and convincing data has been given about the parasympathetic innervation of Müller’s muscle. Invest Ophthalmol Vis Sci. 1993; 34:2333–2340.

The superior tarsal (Müller’s) muscle,1–3 the conjunctiva,4–6 the meibomian glands,7 and the blood vessels8 are autonomically innervated structures of the upper and lower eyelids in mammals. The role of the superior tarsal muscle is tonic retraction of the upper eyelid; impulses from the sympathetic nerve fibers cause this muscle to contract.9 Although neuroanatomic studies of the rat,1 mouse,10 rabbit,11 guinea pig,12 cat,7 monkey,12–14 and humans15 have been performed, knowledge about the perikarya of the neurons that take part in the innervation of different components of the eyelid is limited. Acute ganglionectomy of the superior cervical ganglion (SCG) in rats1 produced complete denervation of the superior tarsal muscle. Ganglionectomy of the SCG in the mouse16 caused the disappearance of
adrenergic nerve fibers in the superior tarsal muscle. Acute damage of the adrenergic nerve fibers was found in the superior tarsal muscle 24 hr after superior cervical ganglionectomy in the cynomolgous monkey.15 Denervation of the branches of the ophthalmic part of the trigeminal afferent nerve in the rat16,17 resulted in a decrease in substance-P (SP) immunoreactivity and elimination of the calcitonin gene-related peptide immunoreactivity of sensory nerve fibers in the conjunctiva and the superior tarsal muscle. Partial elimination of the pterygopalatine ganglion (PPG) in the monkey also caused a decrease in the number of nerve fibers in the conjunctiva. Electron-microscopic studies of the superior tarsal muscle in the mouse revealed the existence of Type I nerve endings (adrenergic) and Type II nerve endings (cholinergic). In this study, the mouse, ganglionectomy of the PPG provided support for the hypothesis that cholinergic nerve fibers originate in the PPG. In the developing rat, it was found that excision of the ipsilateral SCG caused a 59% reduction in the density of acetylcholinesterase (AChE)-positive nerves 7 days later, indicating that sympathetic nerves contribute to cholinesterase-positive tarsal muscle innervation. Excision of the PPG concurrent with the SCG caused a virtually complete disappearance of AChE-positive innervation within 7 days, indicating that nonsympathetic cholinesterase-positive fibers derive from the PPG and are presumed to be parasympathetic. Partial diathermic damage of the PPG in the rabbit reduced the number of SP-positive nerve fibers in the upper eyelid.18 These authors did not incorporate the superior tarsal muscle in their study. Intersectioning of the superior levator muscle in the monkey, according to the Fasanella Servat procedure, did not result in total denervation of the upper eyelid.15 By means of histochemical and fluorescent histochemical analysis,11,14,20,21 differences in the number of AChE-positive and adrenergic nerve fibers in the superior tarsal muscles of the guinea pig, rat, monkey, and humans have been demonstrated. Fluorescent retrograde tracing with true blue or diamino yellow of the superior tarsal muscle in the rat showed labeled perikarya in the rostral part of the SCG. AChE in toto and AChE thick-section studies19 of the rhesus monkey and humans revealed the presence of fine-meshed intrinsic nerve plexuses in the inferior and superior tarsal muscles. In these investigations, the origins of the AChE-positive nerve fibers could not be detected.

Therefore, in the monkey, the localization of neurons that innervate the superior tarsal muscle and the possible somatotopic localization of perikarya in various ganglia were investigated by means of the wheat germ agglutinin–horseradish peroxidase (WGA–HRP) retrograde tracing technique.

**MATERIALS AND METHODS**

Five young adult cynomolgous monkeys (Macaca fascicularis) of both sexes, weighing between 2.3–8.1 kg, served as experimental subjects. All experiments in this study were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Each animal was anesthetized with a mixture of ketamine, xylazine, and atropine in the proportion 10:1:0.1 mg/kg, respectively. Two monkeys received an injection of a 10 μl of 5% WGA–HRP solution (Sigma, St. Louis, MO) into the superior tarsal muscle of the right orbit. For this purpose, a 20-gauge needle connected by a short length of polyethylene tubing to a Hamilton syringe was used. In the other three animals, a solidified piece of WGA–HRP (Sigma) in Willospoon (Willpharma, Zwannenburg, The Netherlands), prepared by soaking a small piece of Willospoon in 10% WGA–HRP, was applied to the medial part of the superior tarsal muscle of the right orbit.

All animals were anesthetized 72 hr after surgery with ketamine (0.4 ml/kg) followed by 5000 units/kg of thromboliquine. Next, the animals were killed by an overdose of pentobarbital and perfused directly through the internal carotid artery with 2000 ml of phosphate-buffered saline, pH 7.4, at 37°C, followed by 2–3 l of fixative containing 0.1 mol/l phosphate-buffered 2% paraformaldehyde and 2.5% glutaraldehyde, pH 7.4, and finally, with 0.1 mol/l phosphate buffer containing 10% sucrose at 4°C.

The brainstem, trigeminal ganglia, SCG, PPG, ciliary ganglion, nodose ganglia, and the first three spinal ganglia on both sides were dissected. We cut 50-μm transverse sections of the brainstem with a cryostat microtome from the level of the superior colliculus to the spinal cord. In addition, 20–40-μm serial cryostat sections of the ganglia from both sides were mounted directly on chrome–alum–gelatin-coated slides. The sections on the slides were then tested for the presence of WGA–HRP activity using either the tetramethylbenzidine (TMB) procedure or the gold-substituted silver peroxidase procedure. All samples were examined by bright- and dark-field microscopy for the presence of WGA–HRP-labeled neurons.

Camera lucida drawings of the sections of the ipsilateral trigeminal ganglia and SCG were made to determine the exact localization of the labeled perikarya. The labeled neurons in the ipsilateral trigeminal ganglia, SCG, and PPG were counted to estimate the number of labeled somata in each ganglion. All values are presented as the mean ± the standard error of the mean.

Immediately after perfusion of the animals, the upper eyelid, the lacrimal gland, and the cornea of the right orbit were dissected and processed in the same
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way as the brainstems and ganglia to determine whether the WGA–HRP had leaked into these tissues.

RESULTS

After retrograde tracing with WGA–HRP of the superior tarsal muscle, labeled somata of the neurons only appeared in the ipsilateral trigeminal ganglia, SCG, and PPG (Table 1). The ipsilateral ciliary, nodose, and first three spinal ganglia were devoid of labeled somata. Labeled somata were never observed in the contralateral ganglia in any animal. The brainstem in four of five animals did not contain labeled neurons. In one monkey, 20 labeled motoneurons were located in the ipsilateral facial motor nucleus.

In control sections of the upper eyelid (including the palpebral conjunctiva), lacrimal gland, and whole mount of the cornea, leakage of the tracer could not be detected. The control sections from the monkey with labeled neurons in the ipsilateral facial motor nucleus revealed small amounts of WGA–HRP in the orbicularis oculi muscle of the upper eyelid.

Trigeminal Ganglion

The trigeminal ganglion (Gasser) of the cynomolgous monkey is semilunar in shape. Bundles of nerve fibers originating in the somata of neurons divide the ganglion into three parts, named for the three nerves (ophthalmic, maxillary, and mandibular nerves) that leave the ganglion distally (Fig. 1). Therefore, the terms ophthalmic, maxillary, and mandibular parts of the trigeminal ganglion refer to the topographic distribution of somata of these neurons. The cluster of neurons located proximally in the ophthalmic nerve itself is called the ophthalmic nerve part of the trigeminal ganglion (Fig. 1). An average of 465 labeled somata (Table 1 and 2) was restricted to the ipsilateral ophthalmic part of the ganglion (Fig. 2) and the proximal part of the ophthalmic nerve (Figs. 3, 4). An average of 315 WGA–HRP-positive somata was present in the ophthalmic part of the ganglion. An average of 150 WGA–HRP-positive somata was found in the ophthalmic nerve part of the ganglion; in this part, a somatotopic distribution of the perikarya was detected (Fig. 5). In the transitional zone between the ophthalmic and maxillary parts of the ganglion (Fig. 1) were some labeled perikarya (maximum number, 30). In the ganglionic part, the labeled sensory neurons differed in size; small and large perikarya were positive for the WGA–HRP tracer. In the maxillary part of the ganglion and the principal and mesencephalic nuclei of the brainstem of cranial nerve V, no labeled somata could be detected.

TABLE 1. Presence of Labeled Somata (72 h) after Injection or Application of WGA/HRP into the Superior Tarsal Muscle of a Right Cynomolgous Orbit

<table>
<thead>
<tr>
<th>Ganglia</th>
<th>Ipsilateral</th>
<th>Contralateral</th>
<th>Labeled Somata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trigeminal</td>
<td>+</td>
<td>–</td>
<td>465 ± 62</td>
</tr>
<tr>
<td>Superior cervical</td>
<td>+</td>
<td>–</td>
<td>61 ± 32</td>
</tr>
<tr>
<td>Pterygopalatine</td>
<td>+</td>
<td>–</td>
<td>38 ± 41</td>
</tr>
<tr>
<td>Nodose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ciliary</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spinal 1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spinal 2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spinal 3</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

Table 1. Presence of Labeled Somata (72 h) after Injection or Application of WGA/HRP into the Superior Tarsal Muscle of a Right Cynomolgous Orbit

TABLE 2. Number of Counted Labeled Neurons in Each Ganglion

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>Trigeminal</th>
<th>Superior Cervical</th>
<th>Pterygopalatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 175</td>
<td>396</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>A 519</td>
<td>422</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>Mean (n = 2)</td>
<td>409</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td>710</td>
<td>502</td>
<td>53</td>
<td>48</td>
</tr>
<tr>
<td>Mean (n = 2)</td>
<td>550</td>
<td>112</td>
<td>103</td>
</tr>
<tr>
<td>611</td>
<td>455</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>Mean (n = 3)</td>
<td>502</td>
<td>77</td>
<td>61</td>
</tr>
<tr>
<td>293</td>
<td>465</td>
<td>61</td>
<td>38</td>
</tr>
<tr>
<td>Mean (n = 5)</td>
<td>502</td>
<td>77</td>
<td>61</td>
</tr>
</tbody>
</table>

A = injected animal.

FIGURE 1. Schematic drawing of a cryostat-cut section containing the trigeminal ganglion with its three nerves: ophthalmic nerve, opht; maxillary nerve, max; mandibular nerve, man; ganglionic part, gp; nerve part, np; and transitional zone, arrow.
Most of the labeled somata were present in the cranial part of the spindle-shaped ganglion (Figs. 6, 7). In the upper third of the ganglion, a somatotopic distribution of perikarya was seen (Fig. 8). A few labeled somata (maximum number, ten) were observed in the medial part of the ganglion; none were detected in the caudal part of the ganglion. An average of 61 positive cell bodies was present in this ganglion (Tables 1 and 2).

PPG

WGA-HRP-positive neurons were localized in the ipsilateral PPG. In the three implanted monkeys, labeled somata were detected in the central ovoid part of the ipsilateral PPG (Fig. 9). An average of 61 was present (Table 2). In the two injected animals, a few somata (two and five) were labeled in the ipsilateral PPG (Table 2). The rami orbitalis of this ganglion did not contain labeled somata. In the ipsilateral PPG of the five animals, an average of 38 positively WGA-HRP-labeled cell bodies was counted (Table 1); a somatotopic distribution could not be seen.

DISCUSSION

The results of the current study provide information on the localization and numbers of perikarya innervating the superior tarsal muscle of the cynomolgous monkey. Moreover, the sensory (afferent), sympathetic (effferent), and parasympathetic (effferent) innervation of this muscle has been determined. Afferent innervation was restricted to the ophthalmic part of the trigeminal ganglion; efferent innervation occurs through the SCG and the PPG. There was no
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FIGURE 6. Bright-field light micrograph of WGA–HRP-labeled somata (arrows) in the cranial part of the SCG (TMB procedure). Bar = 50 μm.

evidence for direct projections from the superior tarsal muscle toward the brainstem.

Comparing the injection method versus the Willo- spon implantation method, the best results were obtained with the latter. This is probably the result of the amount of uptake of WGA–HRP into the nerve endings. The WGA–HRP-soaked small piece of Willo- spon localized in the superior tarsal muscle enhanced the uptake of WGA–HRP, thus increasing the amount of WGA–HRP available for transport, which in turn, may increase the neuron labeling.

Controls

Because of selective application of the tracer to the medial part of the superior tarsal muscle, no leakage to the adjacent tissues was detected in four of our five monkeys. The absence of labeled somata in the ciliary ganglia of the five animals investigated further suggested that tracer leakage from the superior tarsal muscle into adjacent tissues of the eyelid was negligible. In one monkey, with some leakage into the adjacent orbicularis oculi muscle, a few labeled motoneurons were found in the ipsilateral facial motor nucleus. This was in full agreement with the results of previous tracer studies on the orbicularis oculi muscle in the cynomolgous monkey. After application of WGA–HRP and HRP to the orbicularis oculi muscle, these authors found labeled motoneurons in the ipsilateral facial motor nucleus with a few neurons in the locus of the contralateral facial motor nucleus.

Trigeminal Ganglion

In the cynomolgous monkey, we chose the same nomenclature for the trigeminal ganglion, the trigeminal nerve, and its branches as is used in humans. In ro-

FIGURE 8. Schematic drawing of serial cryostat-cut sections illustrates the distribution of labeled somata (solid circles) in the SCG (shaded areas). Labeled somata are concentrated mainly in the upper third of the ganglion. The numbers at the bottom of the figures represent the distance in millimeters from the ventral surface of the ganglion.

FIGURE 7. Dark-field micrograph of WGA–HRP-labeled somata with axons (arrows) in the cranial part of the SCG (TMB procedure). Bar = 50 μm.

FIGURE 9. Dark-field micrograph of WGA–HRP-labeled somata (arrows) in the central part of the PPG (TMB procedure). Bar = 50 μm.
The results show that the superior tarsal muscle in the cat was stained positively with the AChE method.

Moreover, in the rat, the trigeminal ganglion has a common ophthalmic–maxillary ganglion. A somatotopic distribution was present in the ophthalmic nerve part, specifically in the dorsal region. This finding is in accordance with the results of studies in which transsected branches of the ophthalmic nerve of the cat were labeled. Immunohistochemical studies of the eyelids of the rat and clinic showed calcitonin gene-related peptide- and SP-positive nerve fibers in the superior tarsal muscle. In that experiment, the calcitonin gene-related peptide-positive nerve fibers disappeared after denervation of the ophthalmic and maxillary nerves; the SP-positive nerve fibers were reduced in number but did not disappear. These results are in agreement with the findings of the current investigation.

SCG

The results show that the superior tarsal muscle in the clinical study is sympathetically innervated by moderate numbers of neurons located in the ipsilateral SCG. Several other studies confirm this observation. A fluorescent tracer applied to the superior tarsal muscle and the nictitating membrane in rats resulted in the appearance of labeled somata in the ipsilateral rostral part of the SCG. In the double-labeling experiments, double labeling of the somata of the SCG could not be detected. It can be concluded that collateral innervation does not exist in the SCG of the rat. In our investigation, a cranial somatotopic distribution of labeled somata in the SCG was seen in the cynomolgous monkey. This sympathetic connection between SCG and superior tarsal muscle has also been observed in the mouse and in one cynomolgous monkey after ganglionectomy of the SCG. Histochemical studies (using the Falck and Hillarp and the AChE methods) showed both adrenergic and cholinergic innervation in the superior tarsal muscle; destruction of the PPG in the rabbit led to a decrease in the number of SP-positive nerve fibers in the upper eyelid, particularly in the conjunctiva and not in the superior tarsal muscle.

Experiments combined with immunohistochemical studies revealed that, in the rat, neither the number nor the distribution of SP-immunoreactive nerve fibers in the superior tarsal muscle and the palpebral conjunctiva were affected by ganglionectomy of the SCG or denervation of the ophthalmic and maxillary nerve parts of the trigeminal ganglion. It is well known that innervation of the (palpebral) conjunctiva is parasym pathetic. Some nerve fibers originating in the PPG pass through the superior tarsal muscle and are probably destined for the conjunctiva. In the cynomolgous monkey, electron microscopy revealed parasympathetic nerve fibers and endings in the palpebral conjunctiva. A dense meshwork of sensory and autonomic nerve fibers was found in the palpebral conjunctiva of both humans and the rhesus monkey. In the same studies, dense intrinsic nerve plexuses were identified in the superior and inferior tarsal muscles. The two plexuses are connected with one another. These AChE studies did not give a definite answer about the origin of autonomic parasympathetic nerves, such as the sympathetic and sensory nerves. Definite demonstration of the origin of parasympathetic innervation in the PPG has not been obtained with this retrograde tracing study of the cynomolgous monkey. So, in this study, it has been proved that, in the primates, the tarsal muscles have parasympathetic (PPG) and sympathetic (SCG) innervation. These findings suggest a possible mechanism of interactions between sympathetic and parasympathetic nerves in this smooth muscle, as was postulated in the rat. Sympathetic nerves can modulate parasympathetic nerves indirectly by influencing production and/or release of neurotrophic substances from smooth muscle cells. Neuroanatomic studies of the eyelids of humans have shown that pathways of sympathetic nerves accompany the branches of the ophthalmic nerves toward the tarsal muscles. However, these authors neglected the parasympathetic compo-
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nent. Dissection of the palpebral levator muscle resulted in partial denervation of the upper eyelid, which could be explained by the fact that the autonomic nerve pathways run alongside the blood vessels and branches of the ophthalmic nerve. In conclusion, the parasympathetic nerve fibers located in the superior tarsal muscle in the primate originate in the ipsilateral PPG. To our knowledge, this is the first observation of parasympathetic innervation of Müller's muscle in the monkey.

Key Words
WGA–HRP, innervation, superior tarsal muscle, trigeminal ganglion, superior cervical ganglion, pterygopalatine ganglion, cynomolgous monkey

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