Parasympathetic Innervation of the Meibomian Glands in Rats

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Purpose. To determine the location of parasympathetic neurons that innervate the meibomian glands in rats.

Methods. The B subunit of cholera toxin (CTB), fast blue, and a retrograde transneuronal tracer, the Bartha strain of pseudorabies virus (PRV-Ba), were injected into the upper eyelids of adult Sprague-Dawley rats after sectioning the ipsilateral branches of the facial nerve and resecting the superior cervical ganglia. Brains and orbital tissues were processed for the immunohistochemical detection of PRV-Ba and CTB. In selected cases, series of brain sections were double labeled for PRV-Ba and tyrosine hydroxylase to determine the relationship between the A5 noradrenergic cell group and superior salivatory nucleus, or for PRV-Ba and choline acetyltransferase to establish the neurochemical phenotype of parasympathetic preganglionic neurons.

Results. Labeled ganglionic cells were diffusely distributed within the ipsilateral pterygopalatine ganglion (PPG) and along the more proximal portions of the greater petrosal nerve (GPN). Labeled preganglionic neurons were cholinergic and were located immediately dorsolateral to the rostral-most portion of the facial nucleus and caudal superior olive, where they intermingled with A5 noradrenergic cells.

Conclusions. The meibomian glands and other structures within the lid margin are subject to parasympathetic regulation by ganglion cells diffusely distributed within the PPG and along more proximal portions of the GPN. Cholinergic parasympathetic preganglionic neurons that project to meibomian gland–innervating ganglion cells are located immediately lateral, dorsal, and rostral to the facial motor nucleus in the region commonly referred to as the superior salivatory nucleus. (*Invest Ophthalmol Vis Sci.* 2001;42:2434–2441)

Discomfort of the ocular surface, one of the most common problems encountered by ophthalmologists, is usually due to a dry-eye condition characterized by tear film instability and ocular surface abnormalities. Dry-eye conditions can be divided into those with adequate and those with inadequate aqueous tear production.[1] Most dry-eye conditions associated with adequate aqueous tear production are due to meibomian gland dysfunction.[2,3]

The compound, branched acinar meibomian glands are holocrine and produce meibum, an oily substance similar to sebum.[4] The production of meibum appears to be subject to hormonal and neural regulation.[4] Androgen receptors are present in meibomian glands, and androgen deficiency is associated with meibomian gland dysfunction and dry eye.[5–7] The meibomian glands are innervated by sensory, sympathetic, and parasympathetic fibers.[8–13] Nerve fibers are found in close contact with the basal lamina of the meibomian acini.[8–11] However, immunohistochemical and histochemical studies suggest that the majority of nerve fibers found in close contact with the basal lamina of meibomian acini are parasympathetic.[8–11,14]

In the present study, a retrograde transneuronal tracer, the Bartha strain of pseudorabies virus (PRV-Ba), was used to determine the locations of parasympathetic neurons within the pterygopalatine ganglion (PPG) and preganglionic parasympathetic neurons within the central nervous system that innervate the meibomian glands and other structures within the eyelid margin. Double-label immunohistochemistry for PRV-Ba and either tyrosine hydroxylase (TH) or choline acetyltransferase (ChAT) were used to establish the neurochemical phenotype of parasympathetic preganglionic neurons and determine their relationship with the A5 noradrenergic cell group.

Materials and Methods

Animals, Surgery, and Tracer Injections

Experiments were performed on 18 adult Sprague-Dawley rats (250–450 g) in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, and the guidelines of the Institutional Animal Care and Use Committee. Before surgery and the subsequent injection of anatomic tracers, rats were anesthetized with intraperitoneal injections of ketamine (45 mg/kg) and xylazine (5 mg/kg). Because the A5 noradrenergic cell group is premotor to the facial motor nucleus, a second-order afferent of the superior cervical ganglion, and closely related to preganglionic parasympathetic neurons in the superior salivatory nucleus (SSN), all peripheral motor branches of the right facial nerve and both superior cervical ganglia were resected to prevent transport of PRV-Ba into the central nervous system through motor and sympathetic pathways, respectively. Wounds were closed with suture material and tissue adhesive. Because severing the facial nerve paralyzes blinking, Neosporin ophthalmic ointment (GlaxoWellcome, Research Triangle Park, NC) was applied to the right cornea at least twice daily during the postoperative period.

PRV-Ba (1.0 μl, 3.0×10^8 plaque forming units/ml), the B subunit of cholera toxin (CTB; 1.0 μl of a 1% solution; List Biological Laboratories, Campbell, CA), and fast blue (1.0 μl of a 5% solution; Sigma, St. Louis, MO) were used as anatomic tracers. CTB is transported in both the retrograde and anterograde directions and does not move transneuronally. Fast blue is a retrogradely transported fluorescent tracer.

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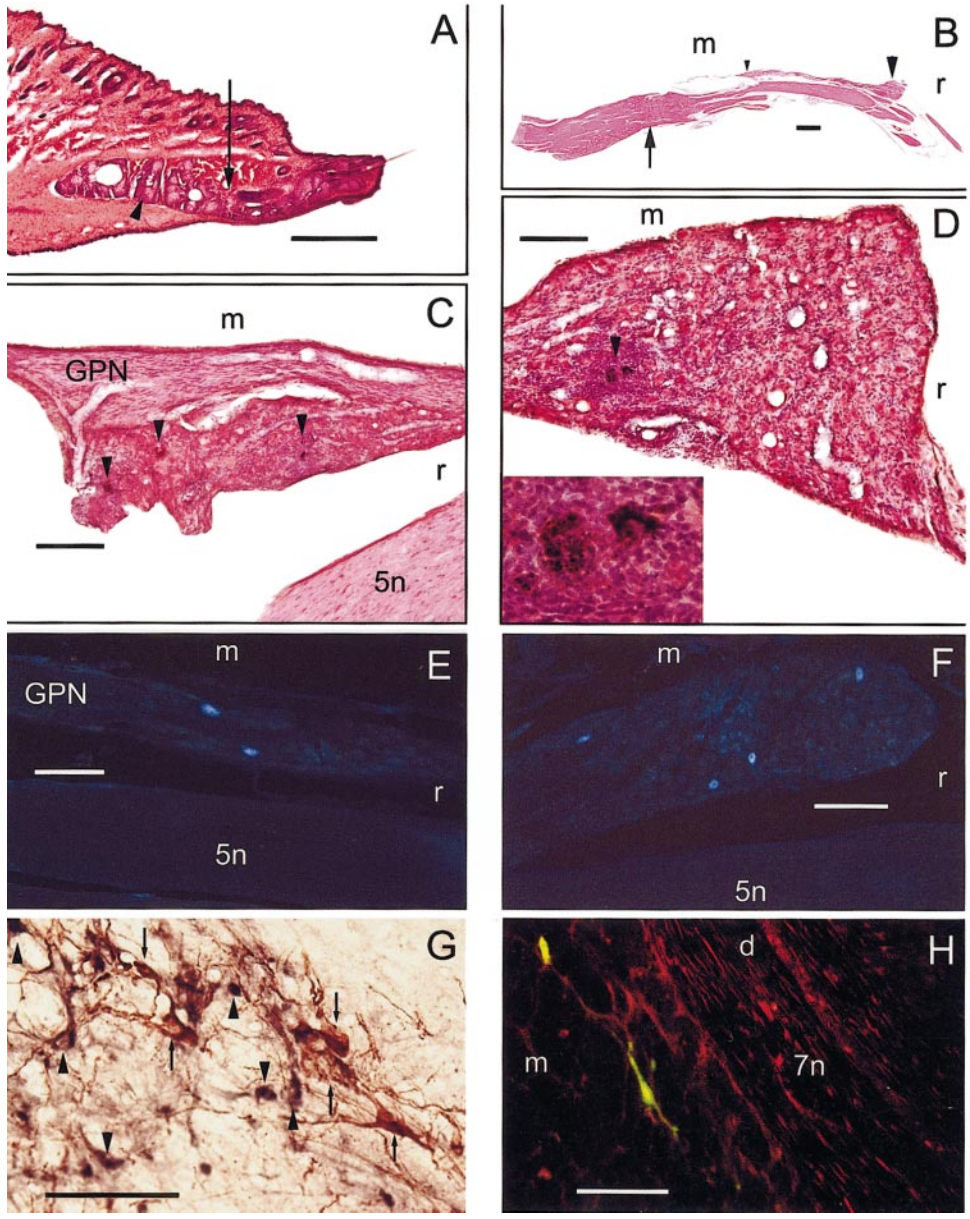

FIGURE 1. (**A**) Meibomian gland (*arrow*) within a parasagittal section through a rat upper eyelid. (**B**) Right-side GPN shown from a caudal collection of ganglion cells (*small arrowhead*) to the PPG (*large arrowhead*). *Arrow*: location of trigeminal ganglion. (**C**) Collection of ganglion cells within the proximal portion of a right-side GPN. *Arrowheads*: locations of three PRV-Ba–labeled ganglion cells. (**D**) A right-side PPG. PRV-Ba–labeled ganglion cells are surrounded by a mononuclear cell infiltrate (*arrowhead*; ×4 enlargement within *inset*). Ganglion cells along (**E**) and within (**F**) a right-side GPN labeled with fast blue. (**G**) Double-label immunohistochemistry for PRV-Ba (*black*) and TH (*brown*) within the SSN. The preganglionic parasympathetic neurons (*black*, *arrowheads*) are closely intermingled with A5 noradrenergic cells (*brown*, *arrows*). (**H**) Double-label fluorescence immunohistochemistry for detection of PRV-Ba (*green*) and ChAT (*red*) within the SSN. PRV-Ba–labeled preganglionic parasympathetic neurons are also ChAT-IR giving their somas a *yellow* color. r, rostral; m, medial; d, dorsal; 5n, trigeminal nerve; 7n, facial nerve. H & E (**A**–**D**). Scale bar, (**A**, **B**) 1 mm; (**C**, **D**) 200 μm; (**E**–**G**) 100 μm.

CTB and fast blue were used to exclude the possibility of monosynaptic parasympathetic innervation of the meibomian glands from the central nervous system and to gauge the specificity of PRV-Ba labeling within the PPG. To avoid contralateral branches of the facial nerves and lacrimal ducts, tracers were injected into the lateral half of either the upper or lower eyelids 1 to 2 mm from the lid margin (Fig. 1A). Injections were performed percutaneously with a syringe (Hamilton, Reno NV) connected to a 30-gauge needle. PRV-Ba injections were made into eyelids on the right side, and CTB and fast blue were injected on the left.

After survival times of 40 to 80 hours, rats were perfused with heparinized saline and then 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4). At time points after 40 hours, PRV-Ba was detected within the SSN. After 72 hours, PRV-Ba began appearing in sites afferent to the SSN. After the perfusion, the orbital contents, along with the trigeminal nerve and greater petrosal nerve (GPN) were removed in toto, up to and including the trigeminal ganglion. Brains and orbital tissues were postfixed in 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4) for 2 hours and then placed in 0.1 M phosphate-buffered 30% sucrose solution (pH 7.4). The complex of neural tissue that includes the trigeminal nerve, trigeminal ganglion, GPN, and PPG were dissected from other structures and sectioned longitudinally in a plane parallel to the GPN (Fig. 1B). Brains were sectioned transversely, and the remaining orbital tissues were sectioned either transversely or parasagittally at 20 to 25 μm on a cryostat and collected in multiple series onto warmed slides. Transverse sections through remaining orbital tissues were perpendicular to the axis defined by the optic nerve. In each case, one series of brain sections was stained with cresyl violet and one series of orbital sections was stained with hematoxylin and eosin (H & E).

Immunohistochemistry

In each case, at least one series of brain sections was processed for detection of PRV-Ba. Additional series were processed for detection of both PRV-Ba and TH or of PRV-Ba and ChAT in the same sections. TH was used as a marker of the A5 noradrenergic cell group. ChAT is a marker of cholinergic neurons. Sections were circled with a hydrophobic slide marking pen (PAP pen; Electron Microscopy Sciences, Fort Washington, PA) and briefly dried on a slide warmer. The steps for detection of PRV-Ba were as follows: (1) Rinse slides in 0.02 M phosphate-buffered saline (PBS) with 0.1% sodium azide (NaN_3; Sigma, St.

Louis, MO) two times over 30 minutes on a rotary shaker; (2) quench endogenous peroxidases with 10% methanol and 3% hydrogen peroxide in PBS for 5 minutes on a rotary shaker; (3) rinse in PBS three times over 30 minutes on a rotary shaker; (4) block with 2% nonfat dry milk and 0.3% Triton X-100 (Sigma) for 1 hour; (5) incubate in primary antibody (goat anti-PRV-Ba generated by two of the authors [PR, MSL]; 1:100,000), 3% rabbit serum, and 0.1% Triton in PBS-NaN_3 overnight; (6) rinse in PBS three times over 30 minutes on a rotary shaker; (7) incubate in secondary antibody (biotinylated rabbit anti-goat (1:500; Vector Laboratories, Burlingame, CA), 2% rabbit serum, and 0.1% Triton in PBS-NaN_3 for 4 hours; (8) rinse in PBS three times over 30 minutes on a rotary shaker; (9) incubate in reagent (Elite ABC; Vector Laboratories) and 0.1% Triton in PBS for 90 minutes at room temperature; (10) rinse in PBS three times over 30 minutes on a rotary shaker; (11) subject to a nickel-intensified diaminobenzidine (black) reaction; and (12) rinse, air dry, dehydrate, clear, and coverslip. In double-staining for PRV-Ba (nickel-intensified diaminobenzidine, black) and tyrosine hydroxylase (diaminobenzidine without nickel intensification, brown) on the same sections, slides were rinsed for 30 minutes after step 11 and then processed starting with step 5, using monoclonal antibody 318 (1:1000; Chemicon International, Temecula, CA).

Because initial experiments that used double-label immunohistochemistry for PRV-Ba and TH with diaminobenzidine substrates demonstrated very close spatial relationships between PRV-Ba- and TH-immunoreactive (IR) neurons (Fig. 1G), fluorescent-tagged secondary antibodies and confocal microscopy were used in subsequent experiments to evaluate the possibility that some parasympathetic preganglionic neurons within the SSN express TH. After incubation with primary antibodies, a cyanine-tagged donkey anti-goat antibody (Cy2; Jackson ImmunoResearch Laboratories, West Grove, PA) and an indocarbocyanine-tagged donkey anti-mouse antibody (Cy5; Jackson ImmunoResearch Laboratories) were used at 1:250 dilutions to detect PRV-Ba and TH, respectively.

For PRV-Ba and ChAT double-labeling, a rabbit polyclonal antibody to ChAT (gift from Miles Epstein, University of Wisconsin, Madison) was used at a 1:500 dilution and the goat anti-PRV-Ba antibody was used at a 1:50,000 dilution. Secondary antibodies, Cy2-tagged donkey anti-goat and Cy5-tagged donkey anti-rabbit, were used at 1:250 dilutions. Tissue was incubated with secondary antibody for 3 hours, rinsed, dehydrated, cleared, and coverslipped with 1,3-diethyl-8-phenylxanthine mounting compound (DPX; Sigma). Fluorescence was visualized with a confocal laser-scanning microscope (Bio-Rad, Hercules, CA).

Orbital tissues, the trigeminal nerve, GPN, and PPG were processed for detection of PRV-Ba, CTB, or fast blue. For detection of CTB, a goat polyclonal antibody was used (1:30,000; List Biological Laboratories) in the sequence of steps described for PRV-Ba. Fast blue was visualized with an epifluorescence microscope equipped with ultraviolet filters (Axioplan; Zeiss, Oberkochen, Germany). In two rats (four eyes), fast blue was the only tracer used. Every section was collected, and labeled cells in the PPG and along the GPN were counted. Care was taken to avoid counting individual fast blue–labeled ganglion cells that appeared on adjacent sections more than once.

Results

Anatomy of the Eyelid Margin

In Sprague-Dawley rats, meibomian glands were present across the entire medial-to-lateral extent of both the upper and lower eyelids. The meibomian glands extended for approximately 3.5 mm from the palpebral margins (Fig. 1A). Modified sebaceous glands were also present around the bases of eyelashes at the palpebral margins. These sebaceous glands had only a few alveoli and were similar to glands of Zeis. There were no eyelid structures that resembled glands of Moll. In addition, there were no accessory lacrimal glands in the eyelids. The superior and inferior tarsal muscles approached the proximal ends of the meibomian glands in the midline of the upper and lower eyelids, respectively.

First-Order Label within the PPG and along the GPN

Ganglion cells were distributed along the GPN for approximately 6 mm proximal to the PPG (Figs. 1B, 1C). A relative concentration of ganglion cells that was approximately 25% the size of the PPG was present caudally within 2 mm of trigeminal ganglion cells (Figs. 1B, 1C). After injection of tracers into the lid margin (Fig. 1A), labeled ganglion cells were widely distributed within the PPG and along the GPN (Figs. 1C–F). There was no clear somatotopic distribution of labeled ganglion cells. CTB (not shown), fast blue (Figs. 1E, 1F), and PRV-Ba (Figs. 1C, 1D) produced similar patterns of ganglionic labeling. There was labeling of cells in the ipsilateral trigeminal ganglion, but no evidence of label in the ciliary ganglion, harderian gland, infraorbital lacrimal gland, or other intraorbital structures with CTB, fast blue, or PRV-Ba. Residual fast blue fluorescence was present at injection sites along the lid margin. Neither CTB nor PRV-Ba could be detected in the lid margin. Both CTB and PRV-Ba must enter neural tissue to prevent clearance by the immune system. There was no labeling of contralateral ganglion cells. The pattern of labeled cells after injection of tracers into the upper lid was similar to that seen after injection into the lower lid.

Figures 1C and 1D show ganglionic labeling with PRV-Ba. In Figure 1C, three ganglion cells along the proximal portion of the GPN are labeled with PRV-Ba. In Figure 1D, two ganglion cells within the proximal portion of the PPG are labeled with PRV-Ba. Figure 1D demonstrates the late effects (78 hours after inoculation) of PRV-Ba infection within the PPG. There was a clear mononuclear cell infiltrate around the ganglion cells infected with PRV-Ba.

Fast blue–labeled ganglion cells were counted in four eyes. In these two cases, fast blue was injected into the upper lid on the right and lower lid on the left. Forty-one ganglion cells were labeled in both cases after upper lid injections. Fifty and 42 ganglion cells were labeled after lower lid injections. The average number of ganglion cells labeled after fast blue injections ($\pm$SD) was 43.5 $\pm$ 4.4. Accurate ganglion cell counts were not possible after CTB and PRV-Ba injections, because only one of three series of slides was processed for detection of the individual tracer.

Location and Characteristics of Preganglionic Parasympathetic Neurons

Second-order labeling after injection of PRV-Ba into the lid margin first became apparent at approximately 40 hours. The intensity of PRV-Ba staining within individual neurons and the number of labeled neurons within the SSN increased up to approximately 72 hours after inoculation. Labeled neurons were not present in the motor trigeminal nucleus, indicating that tracer spread outside the lid was unlikely. TH-IR A5 noradrenergic cells were intermingled with PRV-Ba–labeled preganglionic parasympathetic neurons in the SSN (Fig. 1G). After 72 hours, third-order labeling began to appear in known SSN afferent sites. Second-order labeling (i.e., preganglionic parasympathetic neurons) was concentrated in a region just dorsal and lateral to the superior olive (SO) and medial to the facial nerve root (7n; Figs. 1, 2, and 3). The chartings in Figure 2 indicate that most PRV-Ba-IR neurons were located in the ventral portions of the parvicellular reticular nucleus, pars alpha (PCRtA) and intermediate reticular nucleus (IRt). Caudally (Figs. 2A, 2B), PRV-Ba-IR preganglionic parasympathetic neurons were present dorsal and lateral to the facial motor nucleus, just medial to the spinal trigeminal nucleus. A few

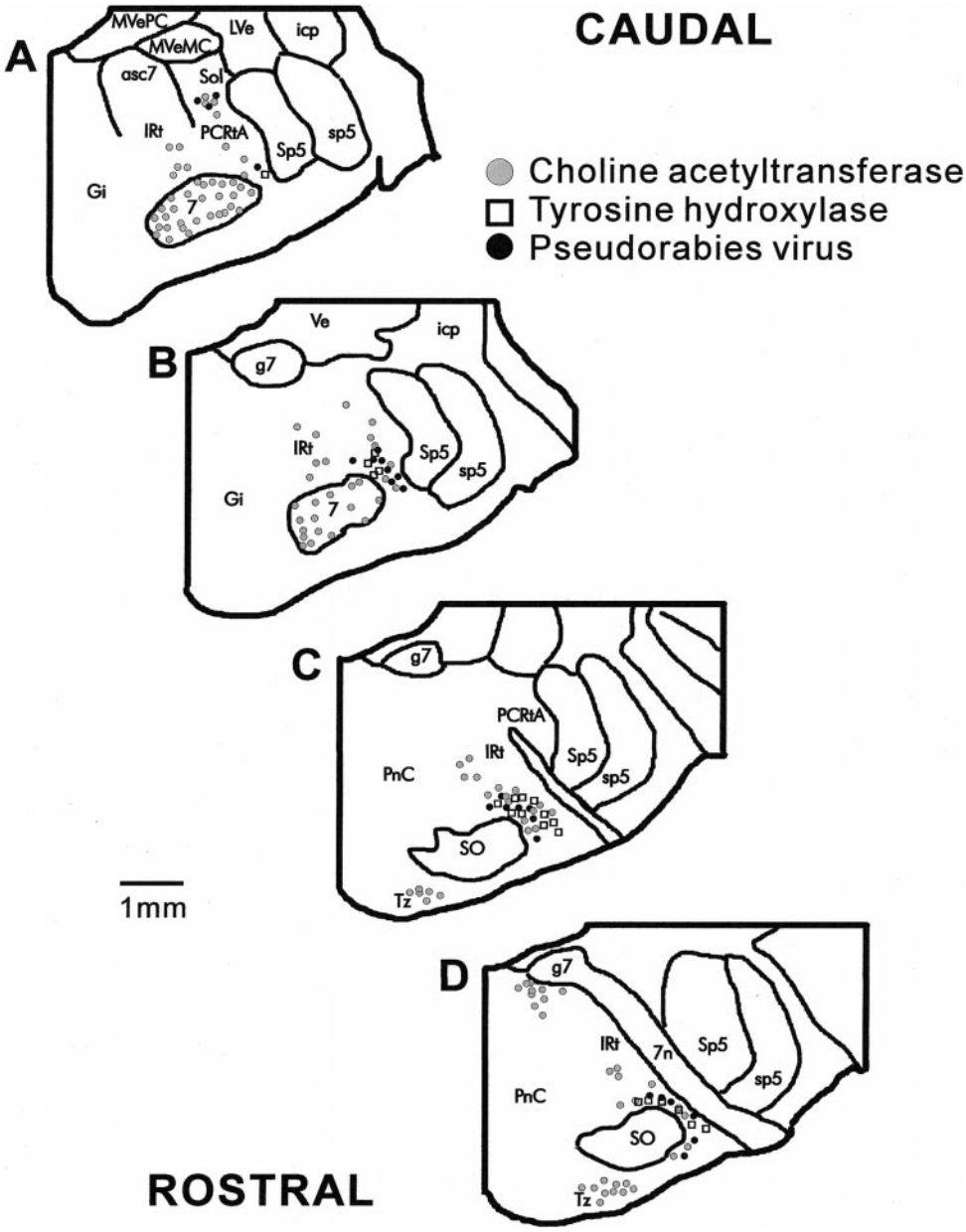

FIGURE 2. The distribution of cell body labeling seen after injection of PRV-Ba into the ipsilateral upper eyelid. Four adjacent series of sections were collected and processed as follows: (**A**) PRV-Ba, (**B**) TH, (**C**) ChAT, and (**D**) cresyl violet. PRV-Ba–labeled cells with were not detected caudal to the section in (**A**) or rostral to the section in (**D**). Each of the four line drawings is a composite of four contiguous sections covering a distance of 100 μm. Note the close relationship between PRV-Ba– and TH–labeled cells. MVePC, medial vestibular nucleus, parvicellular; MVeMC, medial vestibular nucleus, magnocellular; LVe, lateral vestibular nucleus; icp, inferior cerebellar peduncle; asc7, ascending fibers of facial nucleus; IRt, intermediate reticular nucleus; PCRtA, parvicellular reticular nucleus α; Sp5, spinal trigeminal nucleus; sp5, spinal trigeminal tract; Gi, gigantocellular reticular nucleus; 7, facial motor nucleus; Ve, vestibular nuclear complex, g7, genu of facial nerve; PnC, caudal part of pontine reticular nucleus; Tz, trapezoid body; 7n, facial nerve.

neurons were located more dorsally, just ventral to the rostral end of the nucleus of the solitary tract (Sol). Rostrally (Figs. 2C, 2D), PRV-Ba-IR preganglionic parasympathetic neurons surrounded the ventral, lateral, and dorsal margins of the SO. By 65 hours after inoculation, 75 to 100 labeled second-order neurons were present within the SSN. The rostral–caudal, medial–lateral, and dorsal–ventral domains of labeled preganglionic parasympathetic neurons was approximately 500 μm, 800 μm, and 1.5 mm, respectively. Preganglionic parasympathetic neurons labeled with PRV-Ba were limited to the SSN ipsilateral to the side of viral inoculation.

The maximum soma diameter (i.e., long axis) of most labeled neurons within the SSN was 15 to 25 μm. The few neurons located ventral to the rostral tip of the Sol had smaller diameters (8–15 μm). Labeled neurons medial to 7n were oriented such that their long axes were parallel to 7n (Fig. 1H). In a similar fashion, labeled neurons ventral, medial, and dorsal to the SO were oriented so that their long axes were parallel to the surface of the SO and their dendritic arbors respected the boundaries of the SO.

Neurochemical Characteristics of Preganglionic Parasympathetic Neurons

Before 72 hours had passed since inoculation, all PRV-Ba–labeled central nervous system neurons were cholinergic, as indicated by colocalization with ChAT (Fig. 1H). Some PRV-Ba–labeled neurons in the region of the SSN were TH-IR after 72 hours (Figs. 3E–G). These TH- and PRV-Ba–IR neurons were probably A5 noradrenergic cells afferent to PRV-Ba–labeled preganglionic parasympathetic neurons in the SSN.

Figures 1G, 2, and 3 clearly show that the SSN and A5 noradrenergic cell group are not anatomically separate structures. Double-label immunohistochemistry allowed for differentiation of SSN and A5 noradrenergic neurons. A5 noradrenergic cells had both larger somas and dendritic arbors than preganglionic parasympathetic neurons. TH-IR fibers encircled the somas of some PRV-Ba–labeled preganglionic parasympathetic neurons. Although electron microscopy was not performed, TH-IR fibers appeared to make synaptic contact with

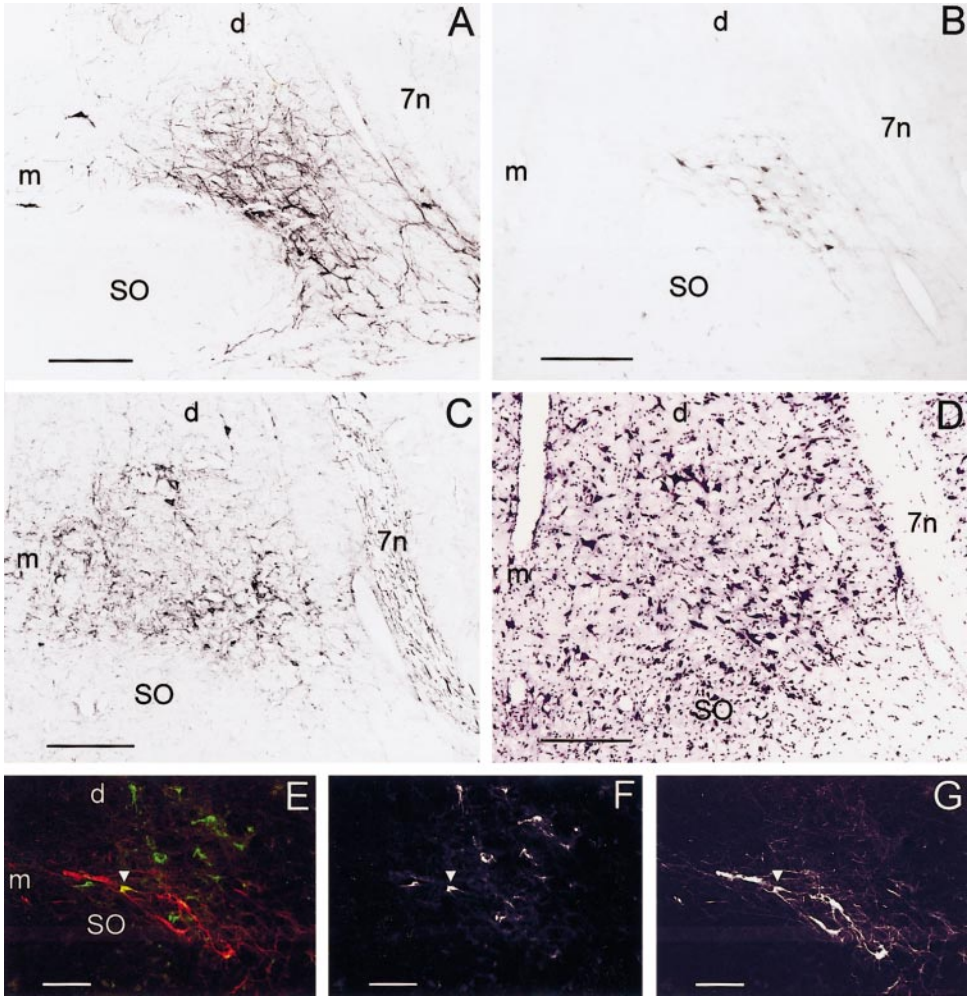

FIGURE 3. Localization of neurochemical markers within the ventral portion of the SSN. (**A–D**) Serial sections through the ventral SSN processed for (**A**) TH, (**B**) PRV-Ba, (**C**) ChAT, and (**D**) cresyl violet. (**E**, **F**) Double-label fluorescence immunohistochemistry for detection of PRV-Ba (*green*) and TH (*red*) within the ventral SSN 72 hours after injection of virus into the eyelid margin. (**E**) A neuron double labeled for PRV-Ba and TH appears *yellow* (*white arrowhead*). (**F**) Gray-scale image of PRV-Ba–IR corresponding to (**E**). (**G**) Gray-scale image of TH-IR corresponding to (**E**). Abbreviations are defined in Figures 1 and 2. Scale bar, (**A–D**) 200 μm; (**E–G**) 100 μm.

PRV-Ba–labeled neurons, based on their light microscopic appearance.

DISCUSSION

Peripheral Findings

The consistent presence of labeled ganglion cells in the PPG after injection of different neuroanatomic tracers (PRV-Ba, CTB, and fast blue) into the eyelid margins of rats, along with previous studies that demonstrated parasympathetic axons and nerve terminals in close contact with the basal lamina of meibomian gland acini, strongly suggests that the parasympathetic nervous system modulates meibomian gland function. Other structures are contained in the eyelid margin, however. In primates, van der Werf et al.[13] injected fast blue 2 mm from the marginal part of the palpebral conjunctiva to study conjunctival innervation and found labeled ganglion cells randomly distributed in the ipsilateral PPG. In their article, they raised the possibility that spread of tracer to the adjacent meibomian glands might explain the presence of labeled cells in the PPG. Along these lines, Seifert and Spitznas[11] systematically examined the parasympathetic innervation of human eyelid structures using both electron microscopy and a marker of parasympathetic nerve fibers, vasoactive intestinal peptide (VIP). They were unable to detect nerve fibers innervating conjunctival goblet cells, although VIP-IR nerve fibers were found in association with conjunctival blood vessels. Other structures in the human eyelid margin that may receive parasympathetic innervation include the glands of Zeis, glands of Moll, sweat glands, and accessory lacrimal glands of Wolfring.[11] Therefore, it is possible that the parasympathetic nervous system acts on several glandular and vascular structures of the eyelid simultaneously to promote secretion of meibum and other substances onto the cornea and palpebral margin.

Simons and Smith[12] transconjunctivally injected the retrograde tracer, Fluoro-Ruby (Molecular Probes, Eugene, OR), into the everted upper eyelids of six rats. They used four to six injection sites and a total volume of 8 to 12 μl of tracer (10% solution) to encompass the superior tarsal muscle and meibomian glands of the upper eyelid. Their injections labeled only 31 ± 6 cells in the PPG. Their methodology did not distinguish tarsal muscle– from meibomian gland–innervating PPG ganglion cells. In our study, using a much smaller volume of tracer (1 μl) injected transcutaneously (Fig. 1A), an average of 43.5 ganglion cells per eyelid were labeled with fast blue. Apparently, fast blue is a more effective tracer than Fluoro-Ruby in the rat parasympathetic nervous system. When compared with Fluoro-Ruby, fast blue also labels a significantly larger number of cat motoneurons.[15]

A study in cynomolgus monkeys demonstrating the presence of labeled ganglion cells in the PPG after injection of tracer into the superior tarsal muscle,[16] a lesion study indicating that nonsympathetic cholinesterase-positive fibers within the superior tarsal muscle derive from the pterygopalatine ganglion,[17] and the demonstration of VIP-immunoreactivity within the superior tarsal muscle in rats[12] support the notion

that the parasympathetic nervous system innervates the superior tarsal muscle through the PPG. The possibility that some labeling seen in the PPG in our experiments was due to spread of tracer to the superior tarsal muscle cannot be entirely excluded. However, we used small injection volumes (1.0 μl), with injection sites located at least 2 mm away from the superior tarsal muscle. In addition, careful review of eyelid sections did not demonstrate fast blue, CTB, or PRV-Ba within the superior tarsal muscle.

According to Motosugi et al.,[18] the PPG in Sprague-Dawley rats contains 4932 ± 291 ganglion cells, whereas Simons and Smith[12] reported a much higher number of cells (8238 ± 1610). The explanation for these substantial numerical differences is not obvious. Because each injection in our study covered approximately one half of an eyelid and labeled an average of 43.5 ganglion cells, it can be reasonably assumed that a minimum of 170 (43.5 labeled ganglion cells per half-lid × four half-lids) or 3% (170 labeled ganglion cells per eye/total of 5000 ganglion cells) of all PPG ganglion cells innervate rat meibomian glands. This is a minimum number, because it is unlikely that fast blue labeled every ganglion cell in the population covered by the injection site. The other 97% of PPG ganglion cells presumably innervate other cranial structures such as the lacrimal gland, nasal and palatal mucosa, cerebrovasculature, and choroidal blood vessels.[16,19–22]

As shown in Figure 1, labeled ganglion cells were randomly distributed throughout the PPG and along the GPN after tracer injection into the dorsal eyelid margin. Similar to previous results seen with conjunctival injections of tracer,[12,13] there was no clear somatotopy to the pattern of labeled cells. In contrast, after injection of tracer into the extraorbital lacrimal gland in rats, labeled neurons are limited to the rostral portion of the PPG, and after injection of tracer into the anterior chamber of the eye, labeled neurons are limited to the proximal ganglionic collections along the PPG.[22] Perhaps, the widespread distribution of PPG ganglion cells that innervate the meibomian glands and conjunctiva suggests that these eyelid structures increase their secretions in response to a wider variety of external triggers than the lacrimal glands.

The presence of certain neuropeptides and neurotransmitter markers in both the PPG and terminal projection fields in the eyelids supports the idea that the parasympathetic nervous system innervates the meibomian glands. Most meibomian gland input appears to be parasympathetic, with smaller contributions from sympathetic and sensory nerves. A dense accumulation of cholinergic nerve fibers forms a network around the acinar and ductal tissue of the meibomian glands.[14] Nerve fibers near the acini of meibomian glands are also IR for other parasympathetic markers including VIP and nitric oxide synthase (NOS).[8,9,11] Kirch et al.[9] colocalized neuropeptide Y (NPY) with both DBH and VIP, but found that VIP-NPY–IR terminals were much more numerous than DBH-NPY–IR terminals. In addition, DBH-NPY–IR terminals were preferentially localized to perivascular nerves, whereas VIP-NPY–IR terminals were concentrated at the basement membranes of meibomian gland acini. In rat, nearly all PPG ganglion cells are VIP-IR, and the majority are NPY-IR[18,23,24] and ChAT-IR.[23] Ganglion cells in the PPG also use nitric oxide as a neurotransmitter. Using a combination of (reduced nicotinamide adenine dinucleotide phosphate [NADPH]) diaphorase histochemistry and NOS immunohistochemistry, Yamamoto et al.[25] showed that most PPG ganglion cells were NOS-IR and stained with NADPH. Nearly all NOS-IR PPG ganglion cells were also VIP-IR.

Central Findings

Viral transneuronal tracing is a potentially powerful tool for understanding the neural regulation of ocular functions such as aqueous tear[26] and meibum production, choroidal blood flow,[20] and blinking. Viruses are able to replicate in recipient neurons, thereby amplifying signal in higher-order afferents. Potential problems with using viruses as neuronal tracers include virus-induced neuronal degeneration with longer postinoculation survival times, failure of certain subsets of neurons to label with virus, and variability between cases of transneuronal labeling. Use of the smallest doses of virus to produce transneuronal labeling, use of several animals at each of multiple different postinoculation survival times, and comparison with conventional tracers limits the pitfalls of viral transneuronal tracing experiments and maximizes the amount of valuable information obtained. In this study, PRV-Ba was used to show that the preganglionic parasympathetic neurons that project to meibomian gland-innervating PPG ganglion cells are concentrated in the ventral portion of the SSN immediately rostral, lateral, and dorsal to the rostral-most portion of the facial motor nucleus.

The SSN comprises a collection of preganglionic parasympathetic neurons that project to the PPG through the GPN and to the submandibular ganglion through the chorda tympani.[26–31] The PPG sends projections to the lacrimal gland, nasal and palatal mucosa, cerebral vasculature, conjunctiva, and choroidal blood vessels. The submandibular ganglion innervates the submandibular and sublingual salivary glands. Using a variety of neuroanatomic methods, it has been shown that the SSN neurons projecting to the PPG are located ventral to those projecting to the submandibular ganglion, although the borders between the two neuronal populations are not strictly defined. In addition, after tracer injections into the PPG or GPN, a small number of neurons have been localized to an area immediately ventral to the rostral pole of Sol. Comparison of our study with Tóth et al.[26] suggests that within the ventral portion of the SSN, the neuronal population responsible for lacrimal innervation may be centered slightly caudal to that responsible for meibomian gland innervation. Future studies using two different transneuronal tracers will be needed to determine whether the ventral SSN is divided into spatially distinct subpopulations of neurons innervating the lacrimal gland, nasal and palatal mucosa, cerebral vasculature, conjunctiva, choroidal blood vessels, and meibomian glands.

The close relationship between the A5 noradrenergic cell group and SSN reported in our study is consistent with previous descriptions of this region.[26,32] Noradrenergic TH-IR neurons were intermingled with PRV-Ba–IR neurons in the ventral–lateral pons. Using electron microscopy, Nemoto et al.[32] showed TH-IR synapses on dendrites of SSN neurons. Therefore, studies of A5 or SSN afferents using local injection of neuroanatomic tracer are not possible.

Projections to the SSN have been analyzed with anterograde and retrograde transneuronal tracers. These studies were not specific to lacrimal or meibomian preganglionic parasympathetic neurons. Spencer et al.[31] injected PRV-Ba into the PPG and mapped the distribution of first- and presumptive second-order retrograde labeling. They did not inject ganglion cells along proximal portions of the GPN with tracer. In addition, because they did not section the superior cervical ganglia, some of the label that they detected in the "SSN" could have been A5 noradrenergic cells retrogradely labeled from the intermediolateral cell column, and some presumptive PPG second-order afferents may have actually been afferents of the A5 noradrenergic cell group. In any case, they reported transneuronally labeled SSN afferent neurons in nearly 20 sites in the brain stem, hypothalamus, and telencephalon. Of these, only the specificity of projections from the lateral hypothalamic area and hypothalamic paraventricular nucleus to SSN rather than the A5 noradrenergic neurons has been demonstrated by use of anterograde tracers.[33,34]

In all probability, many of the projections to the ventral SSN described by Spencer et al.[31] synapse on meibomian and lacrimal parasympathetic preganglionic neurons. However, no specific information is available regarding the role of these nuclei in the control of either meibomian or lacrimal gland function. Insights into the central nervous system regulation of aqueous tear formation provided by clinical-pathologic correlation in patients with neurologic diseases and the physiological characteristics of other preganglionic parasympathetic cell groups may apply, in part, to meibum production. Both acute and chronic lesions of the parasympathetic pathways to the eyes are typically associated with clinically inconsequential decreases in aqueous tear production and absence of dry-eye symptomatology.[35] Neural control may be critical when production above basal levels is required, as in reflex and emotional tearing. Projections from Sp5 to the SSN may mediate tearing in response to ocular or nasal irritation, and pathways that include Sol may mediate tearing in response to strong gustatory stimuli. Pathways from limbic areas (e.g., amygdalopyriform transition zone and bed nucleus of the stria terminalis) to the SSN may be disinhibited in patients with degenerative diseases and pseudobulbar palsy. In these patients, tearing and outward expressions of grief may occur without an inward emotional counterpart. The projections from the hypothalamus to the SSN may be divided into those primarily related to general autonomic functions (paraventricular nucleus, dorsomedial hypothalamus, posterior hypothalamus) and those associated with the limbic system (lateral preoptic area, lateral hypothalamus), with the understanding that there is significant functional overlap between the two.[36–38]

The general autonomic projections may help to regulate tearing within the context of ambient temperature, serum osmolarity, hormonal levels, and sleep. The hypothalamic limbic projections may be part of a pathway for emotional tearing. Noradrenergic and serotonergic projections to the SSN from noradrenergic cell groups (A1 and A5) and the raphe nuclei, respectively, are similar to the innervation of motor[39] and other brain stem preganglionic parasympathetic[40] cell groups. These noradrenergic and serotonergic projections may modulate the excitability of preganglionic parasympathetic neurons according to mood, acute and chronic stressors, and sleep.[41–43]

In summary, we have shown that rat meibomian glands are innervated by ganglion cells in the PPG. Parasympathetic preganglionics that project to meibomian gland-innervating PPG ganglion cells are cholinergic and are concentrated in the ventral half of the SSN around the lateral and dorsal surfaces of the SO and immediately rostral, dorsal, and lateral to the rostralmost portion of the facial motor nucleus. These parasympathetic preganglionic neurons are intermingled with A5 noradrenergic neurons, so that the ventral SSN is not spatially distinct from the A5 noradrenergic cell group. The neural regulation of meibomian gland function provides a potentially rich therapeutic target for treatment of those dry-eye conditions associated with adequate aqueous tear production.

References

1. Pflugfelder SC. Differential diagnosis of dry eye conditions. *Adv Dent Res.* 1996;10:9–12.
2. Pflugfelder SC, Tseng SCG, Sanabria O, et al. Evaluation of subjective assessments and objective diagnostic tests for diagnosing tear-film disorders known to cause ocular irritation. *Cornea.* 1998;17:38–56.
3. Yokoi N, Mossa F, Tiffany JM, Bron AJ. Assessment of meibomian gland function in dry eye using meibometry. *Arch Ophthalmol.* 1999;117:723–729.
4. Bron AJ, Tiffany JM. The meibomian glands and tear film lipids: structure, function, and control. In: Sullivan DA, Dartt DA, Meneray MA, eds. *Lacrimal Gland, Tear Film, and Dry Eye Syndromes 2*. New York: Plenum Press; 1998:281–295.
5. Krenzer KL, Reza Dana M, Ullman MD, et al. Effect of androgen deficiency on the human meibomian gland and ocular surface. *J Clin Endocrinol Metab.* 2000;85:4874–4882.
6. Sullivan DA, Sullivan BD, Ullman MD, et al. Androgen influence on the meibomian gland. *Invest Ophthalmol Vis Sci.* 2000;41:3732–3742.
7. Wickham LA, Gao J, Toda I, Rocha EM, Ono M, Sullivan DA. Identification of androgen, estrogen and progesterone receptor mRNAs in the eye. *Acta Ophthalmol Scand.* 2000;78:146–153.
8. Chung CW, Tigges M, Stone RA. Peptidergic innervation of the primate meibomian gland. *Invest Ophthalmol Vis Sci.* 1996;37:238–245.
9. Kirch W, Horneber M, Tamm ER. Characterization of meibomian gland innervation in the cynomolgus monkey (*Macaca fascicularis*). *Anat Embryol.* 1996;193:365–375.
10. Seifert P, Spitznas M. Immunocytochemical and ultrastructural evaluation of the distribution of nervous tissue and neuropeptides in the meibomian gland. *Graefes Arch Clin Exp Ophthalmol.* 1996;234:648–656.
11. Seifert P, Spitznas M. Vasoactive intestinal polypeptide (VIP) innervation of the human eyelid glands. *Exp Eye Res.* 1999;68:685–692.
12. Simons E, Smith PG. Sensory and autonomic innervation of the rat eyelid: neuronal origins and peptide phenotypes. *J Chem Neuroanat.* 1994;7:35–47.
13. van der Werf F, Baljet B, Prins M, Ruskell GL, Otto JA. Innervation of the palpebral conjunctiva and the superior tarsal muscle in the cynomolgus monkey: a retrograde fluorescent tracing study. *J Anat.* 1996;189:285–292.
14. Perra MT, Serra A, Sirigu P, Turno F. Histochemical demonstration of acetylcholinesterase activity in human meibomian glands. *Eur J Histochem.* 1996;40:39–44.
15. Richmond FJR, Gladdy R, Creasy JL, Kitamura S, Smits E, Thomson DB. Efficacy of seven retrograde tracers, compared in multiple-labelling studies in feline motoneurones. *J Neurosci Methods.* 1994;53:35–46.
16. van der Werf F, Baljet B, Prins M, Timmerman A, Otto JA. Innervation of the superior tarsal (Müller's muscle) in the cynomolgus monkey: a retrograde tracing study. *Invest Ophthalmol Vis Sci.* 1993;34:2333–2340.
17. Sharp CE, Smith PG. Developmental regulation of parasympathetic nerve density by sympathetic innervation in the tarsal smooth muscle of the rat. *Neuroscience.* 1992;49:229–236.
18. Motosugi H, Chiba T, Konno A, Kaneko T. Distribution of neuropeptides in rat pterygopalatine ganglion: light and electron microscopic immunohistochemical studies. *Arch Histol Cytol.* 1992;55:513–524.
19. Nakai M, Tamaki K, Ogata J, Matsui Y, Maeda M. Parasympathetic cerebrovasodilator center of the facial nerve. *Circ Res.* 1993;72:470–475.
20. Cuthbertson S, LeDoux M, Ryan P, Reiner A. The retrograde transneuronal tracer pseudorabies virus used to define central neural circuits that regulate choroidal blood flow in rat [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 2000;41(4):**S**549. Abstract nr 2907.
21. van der Werf F, Baljet B, Prins M, Otto JA. Innervation of the lacrimal gland in the cynomolgus monkey: a retrograde tracing study. *J Anat.* 1996;188:591–601.
22. Ten Tusscher MPM, Klooster J, Baljet B, van der Werf F, Vrensen GFJM. Pre- and post-ganglionic nerve fibers of the pterygopalatine ganglion and their allocation to the eyeball of rats. *Brain Res.* 1990;517:315–323.
23. Leblanc GG, Trimmer BA, Landis SC. Neuropeptide Y-like immunoreactivity in rat cranial parasympathetic neurons: coexistence with vasoactive intestinal peptide and choline acetyltransferase. *Proc Natl Acad Sci USA.* 1987;84:3511–3515.
24. Kuwayama Y, Emson PC, Stone RA. Pterygopalatine ganglion cells contain neuropeptide Y. *Brain Res.* 1988;446:219–224.
25. Yamamoto R, Bredt DS, Snyder SH, Stone RA. The localization of nitric oxide synthase in the rat eye and related cranial ganglia. *Neuroscience.* 1993;54:189–200.

26. Tóth IE, Boldogkõi Z, Medveczky I, Palkovits M. Lacrimal preganglionic neurons form a subdivision of the superior salivatory nucleus of rat: transneuronal labelling by pseudorabies virus. *J Auton Nerv Syst.* 1999;77:45–54.
27. Contreras RJ, Gomez MM, Norgren R. Central origins of cranial nerve parasympathetic neurons in the rat. *J Comp Neurol.* 1980; 190:373–394.
28. Jansen ASP, Ter Horst GJ, Mettenleiter TC, Loewy AD. CNS cell groups projecting to the submandibular parasympathetic preganglionic neurons in the rat: a retrograde transneuronal viral cell body labeling study. *Brain Res.* 1992;572:253–260.
29. Nicholson JE, Severin CM. The superior and inferior salivatory nuclei in the rat. *Neurosci Lett.* 1981;149–154.
30. Ng YK, Wong WC, Ling EA. A light and electron microscopical localisation of the superior salivatory nucleus of the rat. *J Brain Res.* 1994;35:39–48.
31. Spencer SE, Sawyer WB, Wada H, Platt KB, Loewy AD. CNS projections to the pterygopalatine parasympathetic preganglionic neurons in the rat: a retrograde transneuronal viral cell body labeling study. *Brain Res.* 1990;534:149–169.
32. Nemoto T, Konno A, Chiba T. Synaptic contact of neuropeptide- and amine-containing axons on parasympathetic preganglionic neurons in the superior salivatory nucleus of the rat. *Brain Res.* 1995;685:33–45.
33. Hosoya Y, Matsushita M, Sugiura Y. A direct hypothalamic projection to the superior salivatory nucleus neurons in the rat: a study using anterograde autoradiographic and retrograde HRP methods. *Brain Res.* 1983;266:329–333.
34. Hosoya Y, Sugiura Y, Ito R, Kohno K. Descending projections from the hypothalamic paraventricular nucleus to the A5 area, including the superior salivatory nucleus, in the rat. *Exp Brain Res.* 1990; 82:513–518.
35. Pulec JL, House WF. Facial nerve involvement and testing in acoustic neuromas. *Arch Otolaryngol.* 1964;80:685–692.
36. Bernardis LL, Bellinger LL. The dorsomedial hypothalamic nucleus revisited: update. *Proc Soc Exp Biol Med.* 1998:218:284–306.
37. Bushnik T, Bielajew C, Konkle AT. The substrate for brain-stimulation in the lateral preoptic area. I: anatomical mapping of its boundaries. *Brain Res.* 2000;27:103–111.
38. Chiba T, Kayahara T, Nakano K. Efferent projections of infralimbic and prelimbic areas of the medial prefrontal cortex in the Japanese monkey *Macaca fuscata. Brain Res.* 2001;888:83–101.
39. Bayliss DA, Vianna F, Talley EM, Berger AJ. Neuromodulation of hypoglossal motoneurons: cellular and developmental mechanisms. *Respir Physiol.* 1997;110:139–150.
40. Haxhiu MA, Jansen AS, Cherniack NS, Loewy AD. CNS innervation of airway-related parasympathetic preganglionic neurons: a transneuronal labeling study using pseudorabies virus. *Brain Res.* 1993; 30:115–134.
41. Boutrel B, Franc B, Hen R, Hamon M, Adrien J. Key role of 5-HT1B receptors in the regulation of paradoxical sleep as evidenced in 5-HT1B knock-out mice. *J Neurosci.* 1999;15:3204–3215.
42. Shih JC, Chen K, Ridd MJ. Monoamine oxidase: from genes to behavior. *Annu Rev Neurosci.* 1999;22:197–217.
43. Stark KL, Hen R. Knockout corner. *Int J Neuropsychopharmacol.* 1999;2:145–150.