A Parasympathetic Ganglion Innervating the Harderian Gland and Lacrimal Gland of the Musk Shrew (Suncus murinus): Fluorescent Tracing and Immunohistochemical Studies

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Abstract: A small ganglion, named the peri-trigeminal ganglion (PTG), was found in the ventromedial border of the rostral half of the trigeminal ganglion (TG) in the musk shrew (Suncus murinus). In frontal sections, the PTG was semicircular or elliptical in shape. Most of the neurons constituting this ganglion were round in shape and much smaller than those of the TG. The retrograde fluorescent tracer fluoro-gold was injected into various regions of the face in order to investigate innervation by the PTG neurons. When the tracer was injected subcutaneously around the external acoustic meatus and around the circumference of the orbit, a number of labeled neurons were seen not only in the TG but also in the PTG. After applying the tracer to the lacrimal gland (LG) and the harderian gland (HG), numerous labeled neurons were detected only in the PTG. A few labeled neurons were found in the PTG after injection into the palatoglossal arch. Immunohistochemically, most of the neurons constituting the PTG were positive for vasoactive intestinal polypeptide (VIP) antisera. And a moderate number of somatostatin (SOM)-immunoreactive neurons and a small number of leucine-enkephalin (L-ENK)-immunoreactive neurons were detected. Numerous substance P-immunoreactive nerve fibers and varicosities were found in the PTG, and fewer L-ENK-, SOM- and VIP-immunoreactive fibers were observed. The present results suggest that the PTG is an autonomic ganglion that resembles in part the pterygopalatine ganglion in other species, and mainly innervates the HG and LG.

Key words: harderian gland, lacrimal gland, pterygopalatine ganglion, suncus murinus, trigeminal ganglion

Introduction

In the musk shrew (Suncus murinus (suncus)), the trigeminal nerve, especially the maxillary nerve, is well developed. In the brain, the nucleus of the spinal trigeminal nerve is wide and occupies a large portion of the medulla oblongata [26]. In the peripheral nervous system, the trigeminal ganglion (TG) of the suncus is

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very large, and contains primary somatic sensory neurons. In our preliminary examination, we found an additional small ganglion in the ventromedial border close to the TG. We named this ganglion the "peri-trigeminal ganglion (PTG)" due to its location. Its cytoarchitecture is obviously different from that of the TG, but whether the PTG is a part of the TG or an independent gathering of neurons remains unclear. The nerve supply of the PTG was therefore examined by neural tracing techniques, and a variety of neuropeptides in the PTG neurons were analyzed by immunohistochemistry.

**Materials and Methods**

The experiments were carried out on 45 adult suncus of both sexes. The males weighed 37–45 g, and the females 50–90 g. These animals (Jic:SUN Strain, originally purchased from Clea Japan Inc.) were bred at the Institute of Laboratory Animals of Hyogo College of Medicine. Four were used for general morphology, 27 for the fluoro-gold (FG) tracing study, and 14 for the immunohistochemical study. All surgical procedures were carried out under deep anesthesia controlled with a sodium pentobarbital solution (40–50 mg/kg, i.p.).

To observe the location and general histology of the PTG, tissue sections were prepared by the following procedures. The animals were anesthetized and perfused transcardially with 50 ml saline, followed by 300 ml of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pb) at pH 7.2. The brain and the trigeminal complexes were removed together, embedded in an agarose-gelatine mixture, immersed in the same fixative overnight, then stored in 20% sucrose solution for 2 days. Serial frontal sections were cut at a thickness of 40 μm with a freezing microtome, mounted on slides, and stained with thionin.

In the tracing experiment, 3–5 μl of 1–2% FG (Fluorochrome Inc. U.S.A.) was subcutaneously injected into the dorsum of the nose, the upper lip, the lower lip, the frontal region, the entrance of the external acoustic meatus, and the circumference of the orbit, respectively. In addition, the tracer was also applied to the palatoglossal arch, the hard palate, the orbit, the tongue, the parotid gland and the ocular glands behind the ear. Two or three days after the injections, the animals were killed, and the trigeminal complexes were prepared in the same manner as the histological tissue sections. Serial frontal sections were cut at a thickness of 40 μm with a freezing microtome, mounted on slides and air-dried. The preparations were observed under a fluorescent microscope equipped with an UV filter (Olympus, New Vanox).

For immunohistochemical investigation, the animals were pretreated with an intraperitoneal colchicine injection (3.0 mg/kg, Wako, Japan). After 18–24 hr, the animals were anesthetized and perfused transcardially with 50 ml saline, followed by 300 ml of Zamboni’s solution containing picric acid and paraformaldehyde at pH 7.3, or followed by 300 ml of 4% paraformaldehyde in 0.1 M pb at pH 7.2. The trigeminal complexes were removed and prepared for sectioning as described above. The sections were immunostained by the peroxidase-antiperoxidase (PAP) method [20]. Details of the specific antisera used are shown in Table 1. Each section was incubated with a primary antisera for 2 days at 4°C, and subsequently treated with its respective secondary antibody (goat anti-rat-IgG; E-Y Lab., U.S.A., dilution 1:200, goat anti-mouse-IgG; Cappel, U.S.A., dilution 1:100, or goat anti-rabbit-IgG; Cappel, U.S.A., dilution 1:100). A tertiary antibody (rat-PAP; Rockland, U.S.A., dilution 1:3200, mouse-PAP; Dako, U.S.A., dilution 1:2000).

**Table 1. List of primary antisera used in this study**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Lot No.</th>
<th>Raised</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecystokinin</td>
<td>8843025</td>
<td>Rabbit</td>
<td>Incstar, Stillwater</td>
<td>× 5000</td>
</tr>
<tr>
<td>Leucine enkephalin</td>
<td>080991M1</td>
<td>Mouse</td>
<td>Chemicon, Temecula</td>
<td>× 400</td>
</tr>
<tr>
<td>Neuropeptide tyrosine</td>
<td>71096179</td>
<td>Rabbit</td>
<td>Chemicon, Temecula</td>
<td>× 1000</td>
</tr>
<tr>
<td>Neurectosin</td>
<td>89379050</td>
<td>Rabbit</td>
<td>Incstar, Stillwater</td>
<td>× 8000</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>17110334</td>
<td>Rat</td>
<td>Chemicon, Temecula</td>
<td>× 50</td>
</tr>
<tr>
<td>Substance P</td>
<td>141MCC1</td>
<td>Rat</td>
<td>Chemicon, Temecula</td>
<td>× 400</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide</td>
<td>523237</td>
<td>Rabbit</td>
<td>Incstar, Stillwater</td>
<td>× 5000</td>
</tr>
</tbody>
</table>
Denmark, dilution 1:100, or rabbit-PAP; Dako, Denmark, dilution 1:400) was then added and incubated overnight at 4°C. The immunoreaction was revealed by diaminobenzidine reaction. The specificity of the immunohistochemical reaction was checked as recommended by Sternberger [20], including preabsorption tests with appropriate antigens. Results of all the preabsorption tests were negative in this study.

Results

Location and cytoarchitecture of PTG:

Along the thick bundle of the trigeminal nerve just below the inferior surface of the brain, the TG lay rostrocaudally from the level of the olfactory bulb to the level of the midbrain. Estimated in a series of frontal sections, the rostrocaudal length of the TG was about 5 mm. At the most developed portion of the TG, another small ganglion, tentatively called the PTG, was observed in the ventromedial border of the TG (Figs. 1A and 2). The rostrocaudal length of the PTG was about 1 mm. In frontal sections, the PTG was semicircular or elliptical in shape and much smaller than those constituting the TG (Fig. 1B). A thick bundle of nerve fibers was seen in the center of the PTG (Fig. 1B).

Peripheral innervation of PTG neurons:

First, to visualize the peripheral innervation of PTG neurons, 3–5 μl of FG was injected into various regions of the face. After applying FG around the external acoustic meatus, labeled neurons were numerous observed in the PTG and also in a wide area of the TG from the lateral border to the caudal pole (Figs. 2A and 3A). After injecting FG into the orbit, many labeled neurons appeared in both the PTG and the medial portion of TG (Figs. 2B and 3B). When FG was injected into the palatoglossal arch, only a few labeled neurons appeared in both the PTG and the caudal portion of TG (Figs. 2C and 3C). When FG was injected into the hard palate, no labeled neurons were found in the PTG, although a few were seen in the ventral border of the middle portion of the TG. After applications of FG to the dorsum of the nose, the upper lip, the lower lip and the frontal region, no labeled neurons were detected in the PTG (Figs. 2D and 3D). In all these cases, although not in the scope of this study, a number of labeled neurons appeared in the TG (Fig. 2D).

In the suncus, the hardierian gland (HG) and the lacrimal gland (LG) stretch rostrocaudally from the orbit to the postauricular region. The results indicated that the peripheral innervation range of PTG neurons coincides with the position of the HG and LG. We therefore cut the skin behind the ear and applied the tracer to the LG and the posterior portion of the HG (pHG). In these cases, a number of labeled neurons were detected only in the PTG (Fig. 3E). For the control, we removed the LG and pHG 2 weeks prior to injection of tracer into the skin behind the ear. In these cases, there were no labeled neurons in the PTG (Fig. 3F).
**Immunohistochemical detection of neuropeptides in PTG:**

Almost all of the neurons constituting the PTG were stained with the antiserum to vasoactive intestinal polypeptide (VIP) (Fig. 4A). Most of them were intensely immunoreactive, but a few immunoreacted weakly. Somatostatin (SOM)-immunoreactive PTG neurons were distributed randomly throughout the ganglion (Fig. 4B). A few neurons immunoreactive to the antiserum to leucine-enkephalin (L-ENK) were observed in the PTG. A great number of substance P (SP)-immunoreactive nerve fibers and varicosities were found throughout the ganglion (Fig. 4C). A small number of SOM- and L-ENK-immunoreactive fibers and varicosities were found randomly in the PTG (Figs. 4B, D). Although VIP-immunoreactive fibers passing through the PTG were often observed, no varicosities were seen in the area of the PTG (Fig. 4A). None of the PTG neurons or fibers were immunoreactive to the antisera to neuropeptide tyrosine (NPY), cholecystokinin (CCK) and neotensin (NT).

In the TG, although not in the scope of this study, a large number of SP-immunoreactive neurons, a moderate number of SOM-immunoreactive neurons, and a small number of L-ENK-immunoreactive neurons were found. None of the TG neurons were immunoreactive to the antisera to VIP, NPY, CCK and NT.

**Discussion**

To investigate the innervation of PTG neurons, the
fluorescent retrograde tracer was injected subcutaneously into various regions of the face. After the injections around the external acoustic meatus and into the orbit, a number of labeled neurons were detected in the PTG. The ocular glands of the suncus are very large and elongated anteroposteriorly from the orbit to behind the ear, unlike the glands of rodents and birds, which never extend outside the orbit [15, 24]. Especially in the postauricular region, well-developed HG and LG are present in the suncus [18]. When the tracer
was applied to this gland directly, numerous labeled neurons were detected exclusively in the PTG. The present study revealed that the PTG appears to innervate primarily the ocular glands of the suncus.

We hypothesized that the PTG is functionally equivalent to the pterygopalatine ganglion (PPG) or sphenopalatine ganglion (SPG) of other species. The PPG and SPG are cranial parasympathetic ganglia that receive preganglionic afferent fibers via the greater petrosal branch of the facial nerve. And in turn these ganglia provide postganglionic efferent fibers to orbital structures including the LG, orbital blood vessels and the choroid, as well as mucous glands of nasal, oral and pharyngeal mucosa [1, 4]. In the rat, the PPG is located on the medial surface of the maxillary nerve [14], similar to the position of the PTG in the present study, but the PTG in the suncus is located more caudally than the PPG in the rat, which is situated at the level of the frontal lobe of the brain. Moreover, few labeled neurons were seen in the PTG after injections into the palatal mucosa in the present study. In another study of peripheral nerves of the suncus, we could find a small ganglion, consisting of several neurons, which is located along the pterygopalatine nerve pathway beside the lateral wall of the nasal cavity (Fig. 5) (unpublished data). From its location, we assumed that this ganglion might also correspond to the PPG seen in the rat. Therefore, the suncus may have two parasympathetic ganglia in relation to the maxillary nerve: the large caudal ganglion innervating the ocular glands and a small rostral one. Unfortunately we cannot draw any conclusions from the present study, as we did not examine the targets of the postganglionic fibers from the latter small ganglion. It has been reported that the PPG neurons are constituted of separate groups of neurons in the guinea-pig [6, 19], hen [7], goose [8] and pigeon [3].

In our immunohistochemical study, most of the PTG neurons were VIP-immunoreactive. Motosugi et al.
reported that the most commonly found peptide in the rat PPG neurons was VIP. Based on the results of the present study, neurons containing VIP may innervate the HG and LG. It has been suggested that VIP-positive postganglionic axons are innervated in the PPG innervate the HG in rats [23] and chickens [24] based on immunohistochemical analysis. Because these studies also suggested co-localization of VIP in the cholinergic neurons in the HG, VIP seems to act not only as a neurotransmitter but also as a neuromodulator. In particular, VIP in PPG neurons may mediate vasodilation associated with secretion in the ocular glands, based on the physiological suggestion that VIP promotes secretion from the exocrine glands [10].

In addition to VIP, SOM-immunoreactive response has been demonstrated in some PPG neurons in this study, so that the present results indicate the presence of at least two types of neurons: the VIP and VIP/SOM. SOM has been localized to a number of neurons in the sympathetic ganglia [9, 11], but SOM-immunoreactivity has not been demonstrated in the mammalian parasympathetic ganglion except for the visceral wall of some organs (e.g. intestine, gallbladder, urinary bladder) [2, 13, 22]. A small number of SOM-immunoreactive neurons have been shown to be in the ciliary ganglion of avian species [5]. SOM-immunoreactivity has been identified in the HG of the rat and hamster by radioimmunoassay [16, 17]. And immunohistochemical analysis revealed positive staining by the SOM antibody in some alveolar cells and in the isolated lumina of the rat HG [25]. As far as we know, it seems to be novel that SOM-immunoreactive neurons were found in the PTG. To clarify the role of SOM-containing neurons in the PTG, further investigation of SOM in the suncus HG is necessary.

The somata of PTG neurons appear to be surrounded by a number of synapse-like varicosities of SP-immunoreactive fibers, but we could not determine the origin of these fibers. SP-positive fibers in the rat PPG are known to be derived from primary sensory neurons in the TG [12, 21]. If this occurs in the suncus, a circular neuronal connection may exist, through which a number of postganglionic neurons that innervate the ocular glands may be controlled by the SP-containing axon collaterals of the TG neurons that receive sensory input from the ocular organ, but further examination of this is necessary.

The present study reveals the presence of L-ENK-positive varicose nerve fibers in the PTG. Motosugi et al. [14] showed that L-ENK-immunoreactive fibers formed a synaptic contact on the somatic spine of PPG neurons, and hypothesized that these fibers were derived from preganglionic parasympathetic neurons, but in the present study, few L-ENK-immunoreactive perikarya were seen in either the PTG or the TG. Therefore, in the suncus, the possibility that L-ENK-containing fibers originate in sensory neurons in the TG or inter-neurons in the PTG must be taken into consideration.

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