Somatostatin immunoreactivity in quail pterygopalatine ganglion

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Abstract

In the ciliary ganglion of the chicken and quail, somatostatin (SOM) is an exclusive marker for parasympathetic postganglionic neurons innervating the choroid. A second parasympathetic pathway projecting to the choroid originates from the pterygopalatine ganglion. The aim of this study was to investigate SOM immunoreactivity in the pterygopalatine ganglion of the Japanese quail (Coturnix coturnix japonica) and on neurons within the choroid, the intrinsic choroidal neurons (ICN). We did so using immunohistochemistry and subsequent light, electron and confocal laser scanning microscopy. Pterygopalatine neurons were characterized by nNOS-immunohistochemistry or NADPH-diaphorase cytochemistry. SOM immunoreactivity was absent in the perikarya, but neurons were densely surrounded by SOM-positive nerve fibres. Electron microscopy revealed that these fibres formed contacts with and without membrane specializations on pterygopalatine neurons. In the choroid, neuronal nitric-oxide synthase (nNOS)-immunoreactive ICN were likewise closely apposed by SOM-immunoreactive nerve fibres, as revealed by confocal microscopy. There was no detectable co-localization of the markers. In the absence of tracing studies, it is open to speculation whether SOM immunoreactivity originates from preganglionic fibres of the superior salivatory nucleus, postganglionic fibres of the ciliary ganglion or fibres of the brainstem via as yet unknown pathways. SOM may regulate the production of NO in pterygopalatine neurons and ICN, respectively, and is therefore involved in neuronal circuits regulating ocular homeostasis.

Key words autonomic innervation of the eye; blood flow; intrinsic choroidal neurons; NADPH-d; nNOS.

Introduction

In the early 1970s, Gordon Ruskell was the first to describe a parasympathetic pathway to the eye originating in the superior salivatory nucleus, running through the facial nerve to the pterygopalatine ganglion (Ruskell, 1970, 1971); this was in addition to the parasympathetic input to the eye that anatomists already knew, originating from the Edinger–Westphal nucleus and running through the ciliary ganglion. This ganglion represents an important relay in the parasympathetic innervation of the head, including the orbital structures (Ruskell, 2004).

The chemical coding of neurons in the pterygopalatine ganglion is characterized by the presence of nitric oxide and the catalysing enzyme neuronal nitric-oxide synthase (nNOS), vasoactive intestinal polypeptide (Butler et al. 1984; Nilsson, 1996, 2000) and cholinergic markers. Because of its important role in innervating choroidal blood vessels and consequently influencing intraocular pressure (Stone & Kuwayama, 1989), the facial neuronal pathway to the eye has gained strong research interest and has been extensively studied in various laboratory animals (Nilsson et al. 1985; Kuchiwa, 1990).

In birds, which serve as a favourite model in experimental ophthalmology, the same chemical coding is present as in mammals (Zagvazdin et al. 1996; Cuthbertson et al. 1997; Schrödl et al. 2000). Unlike in
mammals, however, the avian pterygopalatine ganglion is not a single solid structure but consists of a chain of interconnected microganglia coursing through the orbit (Giencl & Zaborek, 1984, 1985; Cuthbertson et al. 1997; Schrödl et al. 2000).

A special feature in the avian parasympathetic ciliary ganglion is the existence of two different neuron populations: ciliary neurons project to the ciliary body and iris, whereas choroid neurons project into the choroid (Marwitt et al. 1971). These different neuron populations can be unequivocally identified by the presence of the neuropeptide somatostatin (SOM) in choroid neurons, and the absence in ciliary neurons (Epstein et al. 1988; De Stefano et al. 1993). Because both neuronal pathways (i.e. choroid neurons of the ciliary ganglion and neurons of the pterygopalatine ganglion) project into the choroid, the aim of this study was to determine whether SOM is also present in the pterygopalatine ganglion of quail, which (together with the chicken and pigeon) is one of the most commonly investigated avian species.

We also studied the possible relationship between SOM and the nitrergic neurons residing within the stroma of the choroid, the intrinsic choroidal neurons (ICN; Schrödl et al. 2000), because they show a similar chemical coding as pterygopalatine neurons and are likewise involved in innervation of choroidal blood vessels (Bergua et al. 1993; Flügel et al. 1994; Schrödl et al. 2003). Both neuron populations, pterygopalatine neurons and ICN, are involved in neuronal control of uveal functions and therefore are crucial for the maintenance of ocular homeostasis.

Materials and methods

Albino and wild-type Japanese quails (Coturnix coturnix japonica), aged between 6 months and 1 year, were housed under 12-h day–night cycles with access to water and food ad libitum. Birds were killed with an intraperitoneal overdose of sodium thiopental (Trapanal, BYK Gulden, Konstanz, Germany), and all orbital structures were immediately removed. Under the dissection microscope, Harderian glands were dissected free, together with the adjacent nerve fibres containing the pterygopalatine microganglia, and fixed by immersion (1 h at room temperature, RT) in a modified Zamboni’s solution (Brehmer et al. 1998). Subsequently, the eyes were opened along the ora serrata, and the vitreous body, retina and retinal pigment epithelium were carefully removed. Eye cups with choroids attached to the sclera were further fixed by immersion in Zamboni’s solution for 1 h at RT, then rinsed for 1 h in phosphate-buffered saline (PBS, pH 7.3), and then overnight in PBS containing 15% sucrose. Eye cups were frozen in nitrogen-cooled methylbutane at −60 °C and stored at −20 °C for further processing.

Immunohistochemistry

Cryostat sections of ganglia and choroids (20 µm) were air-dried for 1 h at RT on poly-l-lysine-(Sigma, Taufkirchen, Germany) coated slides. After a 5-min rinse in Tris-buffered saline (TBS; Roth, Karlsruhe, Germany), slides were incubated for 1 h at RT in TBS containing 10% donkey serum (Dianova, Hamburg, Germany), 1% bovine serum albumin (BSA; Roth), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After a 5-min rinse, slides were incubated with antibodies for SOM (raised in mouse; The Regulatory Peptide Group, MRC, Vancouver, Canada; 1 : 1000) and nNOS (raised in rabbit; courtesy of Dr B. Meyer, University of Graz, Austria; 1 : 500) overnight at RT.

After a rinse in TBS (4 × 5 min) binding sites of primary antibodies were visualized by corresponding Cy3-, and Cy2-tagged antisera (Dianova; 1 : 750) in TBS, containing 1% BSA and 0.5% Triton X-100 (1 h at RT) followed by another rinse in TBS (4 × 5 min). Slides were embedded in TBS-glycerol (1 : 1 at pH 8.6). The specificity of the antibodies has been demonstrated in earlier studies (SOM: Buchan et al. 1985; De Stefano et al. 1993; nNOS: Bergua et al. 1996; Schrödl et al. 2000). Negative controls were performed by omitting the primary antibodies and yielded no immunostaining.

In order to document double immunohistochemistry, a confocal laser scanning microscope (Bio-Rad MRC 1000 attached to a Nikon Diaphot 300 and equipped with a krypton–argon laser, ALC, Salt Lake City, USA; ×20 dry or ×40 and ×60 oil-immersion objective lenses, with numeric apertures of 0.75, 1.30 and 1.4, respectively; Nikon, Düsseldorf, Germany) was used. Sections were imaged using the appropriate filter settings for Cy3 (568-nm excitation, filter 605DF32; channel 1, coded red) and Cy2 (488-nm excitation, filter 522DF32; channel 2, coded green). Co-localization of SOM and nNOS in channels 1 and 2 in the same structure resulted in a mixed yellow colour. Extended focus images and z-series (z-increment of 1–2 µm) were created by electronic superimposition. In order to demonstrate

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putative synaptic contacts, single sections were taken from z-staples of same-focus planes in two different channels, and these channels were merged.

Electron microscopy

For electron microscopy, pre-embedding protocols were used to demonstrate NADPH-d activity followed by SOM immunohistochemistry and the diaminobenzidine (DAB) reaction. Pieces of Harderian gland with adjacent nerve fibres were fixed by immersion in PBS containing 4% paraformaldehyde and 0.01% glutaraldehyde. After several rinses in PBS, pterygopalatine microganglia were identified within these whole-mounts using the NADPH-diaphorase (NADPH-d) technique (1.5 h at 37 °C): 1 mg NADPH (Biomol, Hamburg, Germany) and 0.25 mg nitroblue-tetrazolium chloride (Biomol) per ml PBS, containing 0.1% Triton X-100 (Merck), followed by a rinse in PBS (1 day at 4 °C). Under the dissection microscope, the blue NADPH-d reaction product enabled us to visualize the microganglia, and these were trimmed for further processing.

After endogenous peroxidase blocking (10% methanol containing 3% hydrogen peroxide; 10 min at RT), the tissue was pre-incubated for 1 h at RT in TBS containing 10% donkey serum (Dako), 1% BSA, 0.05% thimerosal and 0.1% Triton X-100, followed by a 10-min rinse in TBS. Tissue was incubated with SOM antibody (diluted in TBS, 1 : 1000, containing 1% BSA, 0.1% Triton X-100 and 0.05% thimerosal; 72 h at 4 °C) followed by a rinse with 0.02% NaN₃ in TBS (72 h at 4 °C). Binding sites were visualized using biotinylated sheep anti-mouse immunoglobulin (1 : 100, Amersham, Freiburg, Germany) in TBS containing 1% BSA, 0.1% Triton X-100 and 0.05% thimerosal (48 h at 4 °C), followed by streptavidin-peroxidase (Dako; 1 : 1000; 24 h at 4 °C) and the DAB reaction. Whole-mounts were postfixed in 1% osmium tetroxide with 1.5% potassium hexacyanoferrat (Merck), dehydrated through graded alcohols, embedded in Epon and mounted on Epon blocks. Semi-thin sections were stained with methylene blue. Silver-grey serial ultrathin sections were lightly contrasted with lead citrate, examined in a Zeiss EM 906 transmission electron microscope and photographed for documentation. Micrographs were digitized (Agfa SnapScan e50, Agfa, Köln, Germany) and slightly adapted in contrast and brightness (Adobe Photoshop 6.0, Adobe, San Jose, USA).

Results

In the quail, nerve fibres containing the pterygopalatine ganglionic chain were found on the medial aspect of the harderior gland, as described in earlier studies (Gienc & Zaborek, 1984, 1985; Cuthbertson et al. 1997; Schrödl et al. 2000).

Neurons were visualized using nNOS immunohistochemistry or NADPH-d cytochemistry. The number of neurons per microganglion generally varied between 3 and 5, up to about 50 (Figs 1 and 2a). Single cells within pterygopalatine nerve strands were also visible. Long axes of perikarya ranged from 10 to 20 µm, but a few cell diameters of up to 40 µm were also found (Figs 1 and 2a). Pterygopalatine ganglionic cell bodies mainly showed an eccentric nucleus; in shape they were round to ovoid, with a smooth contoured surface. The number of processes varied; sometimes up to four per neuron were detected, but in this study no attempt was made to characterize these processes further.

Within the pterygopalatine nerve strand, numerous varicose nerve fibres immunoreactive for SOM were present. These nerve fibres showed no co-localization with nNOS (Fig. 1).

No SOM-immunoreactive neuronal cell bodies were detectable. On their course within the pterygopalatine nerve strands, these fibres were seen to be forming boutons, and SOM-immunoreactive boutons were found in close proximity to the pterygopalatine neuronal perikarya, as demonstrated by light microscopy (Fig. 2a) or in single confocal optical sections (Fig. 1).

Fig. 1 Pterygopalatine ganglion. Double immunohistochemistry for nNOS (red) and SOM (green). Neurons are densely surrounded by SOM-immunoreactive nerve fibres forming bouton-like contacts. nNOS and SOM are not co-localized (confocal image, single optical section; scale bar 25 µm).
Using electron microscopy, pterygopalatine neurons were visualized with the NADPH-d technique and could therefore be identified under the light microscope before trimming (Fig. 2a). The electron-dense NADPH-d reaction product was found in the perikarya and processes of the pterygopalatine ganglionic chain, and was clearly distinguishable from the DAB reaction product, indicating SOM immunoreactivity (Figs 2b and 3a). The DAB reaction product was found in nerve fibres within the ganglion as well as in profiles directly contacting perikarya or processes of pterygopalatine ganglion neurons. Both contacts with (Figs 2c and 3b) and without membrane specializations were observed. Owing to the use of the DAB method and the density of the reaction product, we were not able to discern any vesicles within the profiles contacting pterygopalatine ganglion neurons.

In the choroid, intrinsic choroidal neurons were detected by means of nNOS immunohistochemistry; their morphology and the quantities found matched our expectations from our previous studies (Schrödl et al. 2000, 2004). In double staining experiments for nNOS and SOM, intrinsic choroidal neurons were consistently found to be closely apposed by SOM-immunoreactive nerve fibres (Figs 4 and 5). In single confocal optical sections, these nerve fibres were forming boutons on both soma (Fig. 4) and dendrites (Fig. 5) of intrinsic neurons. Co-localization of both markers within the same structure was not detectable.

![Fig. 2 Pterygopalatine ganglion. (a) Microganglion, histochemistry for NADPH-d (blue) followed by immunohistochemistry for SOM (brown): note the concentration of SOM-positive nerve fibres (arrows). The neuron in the white box is the same as represented in (b) (N; scale bar 25 µm). (b) Electron micrograph of boxed area in (a): the neuron (N) shows the electron-dense reaction product of the NADPH-diaphorase and is closely approached by nerve fibres containing DAB reaction product indicating SOM immunoreactivity (box; scale bar 2 µm). (c) Electron micrograph of the boxed area in (b): SOM-immunoreactive profile (asterisk) closely approaching the neuron, forming a thin membrane specialization (arrowheads; scale bar 1 µm).](image)

![Fig. 3 Pterygopalatine ganglion. (a) Electron micrograph of a neuron positive for NADPH-diaphorase (N), contacted by an SOM-immunoreactive bouton (boxed area; scale bar 5 µm). (b) Magnification of the boxed area shown in (a): an SOM-immunoreactive profile (asterisk) is in contact with the neuron (N), forming a thin membrane specialization (arrowheads; scale bar 0.5 µm).](image)
Discussion

This study demonstrates SOM-immunoreactive nerve fibres in the pterygopalatine ganglion of the quail. Using electron microscopy, these nerve fibres were seen to form contacts with and without membrane specialization on the pterygopalatine neurons. Although SOM has been described in the autonomic ganglia of various species (McLachlan, 1995), data regarding the presence of SOM immunoreactivity in the pterygopalatine ganglion have been lacking until now. Furthermore, as with pterygopalatine ganglion neurons, ICN were closely approached by SOM-immunoreactive fibres.

Over the last few decades, there have been several morphological investigations of the avian pterygopalatine ganglion (Gienc & Zaborek, 1984, 1985; Walcott et al. 1989; Cuthbertson et al. 1997, 1999; Hiramatsu & Ohshima, 1999; Kuder et al. 1999; Schrödl et al. 2000). However, it has not been possible until now to make a morphological distinction of the functionally different pterygopalatine neurons as described in the cat (Kuchiwa et al. 2000). Studies in quail pterygopalatine ganglion at the electron microscopic level revealed synaptic contacts with boutons containing granular and agranular vesicles (Kuder et al. 1999), and it is tempting to speculate that these granular vesicles contained SOM, as described in the present study. Because DAB was used in this study to demonstrate the presence of SOM, we were unable to characterize the type of vesicles contacting the pterygopalatine neurons.

The origin of these SOM-immunoreactive fibres is still unclear. On the one hand, they may represent preganglionic fibres from the superior salivary nucleus. In birds, no data regarding the chemical coding of this nucleus are available. In humans, cats and rats, preganglionic fibres from the superior salivary nucleus contain nitricergic markers, as do postganglionic neurons (Gai & Blessing, 1996; Zhu et al. 1997; Cuthbertson et al. 2003). Our study revealed no co-localization between SOM and nNOS/NADPH-d, and given that there are no species differences, it seems improbable that SOM-positive fibres originate from the superior salivatory nucleus. It may be that connections from regions of the brainstem (as yet unknown) will prove to be a more likely source (Shiosaka et al. 1981; Takatsuki et al. 1981). However, there are no tracing data available in the literature, and therefore future studies will be needed to test this hypothesis. A third possibility is that SOM-immunoreactive postganglionic fibres of the ciliary ganglion may connect to the radix autonomica of the facial nerve within the orbit and course further on to the pterygopalatine microganglia. Again, this possibility will need to be properly tested in tracing or transection studies. Indications that such a pathway may exist stem from tracing studies in rat, where tracer injections into the pterygopalatine ganglion led to labelled nerve fibres in the ciliary ganglion (ten Tusscher et al. 1990; Beckers et al. 1993), and therefore it seems reasonable to believe that there may be such a pathway in the opposite direction.

Of interest is the observation of SOM-immunoreactive nerve fibres closely approaching ICN. These profiles may represent synaptic contacts, but until now it has not been possible to confirm this using electron microscopy. However, this theory is supported by the fact that earlier studies, using the same confocal method as

Figs 4 and 5  Choroid. ICN immunoreactive for nNOS (red), their soma (Fig. 4) and processes (Fig. 5) closely approached by SOM-immunoreactive nerve fibres (green, arrowheads; confocal image, single optical section; scale bar 20 µm).
in this study (Schrödl et al. 2001a,b), revealed true synaptic contacts on ICN, and therefore it is likely to be the case here as well. As already mentioned with regard to pterygopalatine neurons, these SOM approaches may represent preganglionic ‘facial’ input from the superior salivatory nucleus. Second, they may also derive from perikarya of different brainstem regions via yet unknown paths. In both cases, SOM in the avian choroid would no longer represent an exclusive marker of ciliary ganglion origin. A third possibility, these fibres may indeed originate from postganglionic neurons of the ciliary ganglion, which would implicate a parasympathetic influence onto ICN. In humans, parasympathetic boutons on intrinsic neurons have been described morphologically, using an antiserum against the vesicular acetylcholine transporter (May et al. 2004).

We can only speculate about the functional aspect of SOM input: given that SOM of avian choroid neurons in the ciliary ganglion modulates the release of acetylcholine from the same nerve terminals within the choroid (Pilar et al. 1996), this might be also the case with pterygopalatine neurons. In non-human primates, it is speculated that SOM may act as a subtype of primary afferent fibres (Firth et al. 2002), but in duck trigeminal ganglion no SOM-immunoreactive perikarya were found (Schweigert et al. 2003). Because both cell populations – pterygopalatine neurons and ICN – are nitrergic in nature, it seems most likely that NO production is regulated by SOM, as has been shown in vitro for nNOS (Arena et al. 2004). Together with the already known contacts from sympathetic and primary afferent nerve fibres (Schrödl et al. 2001a,b), SOM modulation on ICN would highlight their role as an integrator within the choroid. This means that besides well-established neuronal pathways to the eye (ten Tusscher et al. 1994), an intrinsic source would be able to regulate choroidal needs locally. Because the targets of ICN are non-vascular smooth muscle cells of the choroidal stroma and choroidal blood vessels (Bergua et al. 1993; Flügel et al. 1994; Schrödl et al. 2001a,b, 2003) virtually every function of the choroid can be controlled by ICN, e.g. blood flow and choroidal thickness, and could also be in part responsible for diurnal changes within the eye (Nickla et al. 1998, 2001).

Taken together, further investigations are required to clarify the origin of SOM-immunoreactive fibres on either pterygopalatine neurons or ICN. This will be helpful for a better understanding of neuronal regulation regarding choroidal blood flow or changes in choroidal thickness.

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