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Intrinsic choroidal neurons in the chicken eye: chemical coding and synaptic input

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Abstract

Intrinsic choroidal neurons (ICNs) exist in some primates and bird species. They may act on both vascular and non-vascular smooth muscle cells, potentially influencing choroidal blood flow. Here, we report on the chemical coding of ICNs and eye-related cranial ganglia in the chicken, an important model in myopia research, and further to determine synaptic input onto ICN. Chicken choroid, ciliary, superior cervical, pterygopalatine, and trigeminal ganglia were prepared for double or triple immunohistochemistry of calcitonin gene-related peptide (CGRP), choline acetyltransferase (ChAT), dopamine- β -hydroxylase, galanin (GAL), neuronal nitric oxide synthase (nNOS), somatostatin (SOM), tyrosine hydroxylase (TH), vasoactive intestinal polypeptide (VIP), vesicular monoamine-transporter 2 (VMAT2), and α -smooth muscle actin. For documentation, light, fluorescence, and confocal laser scanning microscopy were used. Chicken ICNs express nNOS/VIP/GAL and do not express ChAT and SOM. ICNs are approached by TH/VMAT2-, CGRP-, and ChAT-positive nerve fibers. About 50% of the pterygopalatine ganglion neurons and

about 9% of the superior cervical ganglion neurons share the same chemical code as ICN. SOM-positive neurons in the ciliary ganglion are GAL/NOS negative. CGRP-positive neurons in the trigeminal ganglion lack GAL/SOM. The neurochemical phenotype and synaptic input of ICNs in chicken resemble that of other bird and primate species. Because ICNs lack cholinergic markers, they cannot be readily incorporated into current concepts of the autonomic nervous system. The data obtained provide the basis for the interpretation of future functional experiments to clarify the role of these cells in achieving ocular homeostasis.

Keywords

Myopia; Blood flow; Immunohistochemistry; Nitric oxide

Introduction

The presence of neurons in the stroma of the choroid has been known since the nineteenth century (Müller 1859). These intrinsic neurons exist in some primates including man and avians, but are absent or scarce in rodents, cats, and rabbits (Flügel-Koch et al. 1994; Flügel et al. 1994; Ramirez et al. 1999). In birds, they express vasoactive intestinal polypeptide (VIP), in combination with nitrergic markers such as neuronal nitric oxide synthase (nNOS) or NADPH-diaphorase (Bergua et al. 1993; Flügel et al. 1994; Miller et al. 1983, for review see Schrödl 2009). In the duck (*Cairina moschata*; Bergua et al. 1996), there are about 1,000/eye, similar to humans, and they exhibit identical chemical coding as humans, with the exception that the duck ICNs express a third neuroactive substance, galanin (GAL), and receive both postganglionic sympathetic and primary afferent input (Schrödl et al. 2000, 2001a; b), indicating that they are integrated in the *extrinsic* neuronal pathways of the eye. In both birds and mammals, these pathways are: (1) primary-afferent (sensory) fibers of trigeminal ganglion origin (Kuwayama and Stone 1987; Shih et al. 1999; Stone and McGlinn 1988) that project to the brainstem; (2) sympathetic fibers that originate in the intermediolateral nucleus, with post-ganglionic fibers from the superior cervical ganglion (Chen et al. 1999; Ehinger 1967; Klooster et al. 1996); (3) parasympathetic fibers that have two origins: one from the superior salivatory nucleus that course via the facial nerve with the post-ganglionic fibers from the pterygopalatine ganglion (Cuthbertson et al. 1997; Nilsson et al. 1985; Ruskell 1970) and the other from the Edinger-Westphal nucleus that course via the oculomotor nerve with the post-ganglionic fibers from the ciliary ganglion (Cuthbertson et al. 1996; Fitzgerald et al. 1990; Marwitt et al. 1971). While in mammals the primary parasympathetic input to the choroid is the pterygopalatine one, in birds there is also input from a population of neurons in the ciliary ganglion called *choroid neurons*, which project exclusively to the choroid (Marwitt et al. 1971) with the rest going to the anterior uvea. To distinguish between these choroid neurons of the ciliary ganglion and the intrinsic neurons *within* the choroid, the term *intrinsic choroidal neurons* (or ICN) was introduced (Schrödl et al. 2000).

To date, there is morphological evidence for one function for these cells: they probably innervate choroidal blood vessel walls and so might influence choroidal blood flow, resulting in alterations in intraocular pressure (IOP). No other functions for them have been

shown; however, it appears that these ICNs make synaptic contact onto the non-vascular smooth muscle (NVSM) cells in the choroid (Schrödl et al. 2001a), leading to speculation that they may play a role in the defocus-induced changes in choroidal thickness in chickens, which position the retina nearer the image plane. As there are also diurnally driven changes in choroid thickness in both birds and man (Brown et al. 2009; Nickla et al. 1998; Stone et al. 2004), these ICNs might play a role in these changes as well.

Because the chicken is a useful animal model for emmetropization and its underlying mechanisms, which may include the choroidal thickness changes (Nickla and Wallman 2010; Wallman and Winawer 2004), the aim of this study was (1) to provide a more extensive morphological description of the ICNs in chicken choroid, (2) to ascertain whether the neuropeptide galanin (GAL) is present in chick choroid and eye-related cranial ganglia in order to determine whether this peptide might serve as intrinsic marker, and (3) to determine whether eye-related cranial ganglia have neurons that share the same neurochemical code as ICNs. Addressing these issues is necessary to prepare the morphological basis for upcoming lesion and pharmacological experiments to elucidate function.

Materials and methods

Tissue

Twenty eight heads of chicken (*Gallus domesticus*) were obtained from a local poultry farm. After decapitation, eyes were fixed by intraocular injection with Zamboni's fixative (Brehmer et al. 1998) and kept on ice during transport to the laboratory. All experiments were conducted in accordance with the ARVO statement for the use of animals in ophthalmic and visual research.

Eyes were dissected out of the head, opened along the ora serrata, and the vitreous, retina, and retinal pigment epithelium were carefully removed. Eye cups with choroids attached to the sclera were further fixed by immersion in Zamboni's for 1 h at room temperature (RT) followed by a 1-h rinse in phosphate buffered saline (PBS, pH 7.3) and another rinse in PBS containing 15% sucrose overnight. Eye cups were frozen in nitrogen-cooled methylbutane at -60°C and stored at -20°C until further processing.

Cranial ganglia (i.e. ciliary, pterygopalatine, superior cervical, and trigeminal) were excised under the dissection microscope and fixed through immersion in 4% paraformaldehyde (1 h at RT) followed by a rinse in PBS (1 h) and another rinse in 15% sucrose-PBS overnight. The ganglia were frozen within tissue embedding medium (GSV1, Slee-Technik, Mainz, Germany) at -60°C and stored at -20°C until further processing.

Immunohistochemistry

Choroids were sectioned at $20\text{ }\mu\text{m}$ in a cryostat; all cranial ganglia were sectioned at $18\text{ }\mu\text{m}$. All sections 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% BSA (Roth), and 0.5% Triton X-100 (Merck, Darmstadt,

Germany). After a 5-min rinse, slides were incubated with antibodies for double and triple labeling of the markers listed in Table 1.

Followed by a rinse in TBS (four times 5 min), binding sites of primary antibodies were visualized by corresponding Cy2- or FITC-, Cy3-, and Cy5-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 (four times 5 min). Slides were embedded in TBS-glycerol (1:1 at pH 8.6).

For all antibodies used, negative controls were carried out by replacement of primary antisera by normal serum from those species in which the primary antibodies were raised, or TBS, and resulted in no staining.

NADPH-diaphorase and eosin staining

NADPH-diaphorase (NADPH-d) and eosin staining was done to detect the numbers ganglion cells and the proportion of NADPH-d-positive neurons in the superior cervical ganglion. Slides were incubated in the following solution (1 h at 37°C): 1 mg NADPH (Biomol, Hamburg, Germany) and 0.25 mg nitroblue-tetrazolium chloride (Biomol) per ml PBS, containing 0.5% Triton X-100 (Merck). Incubation was terminated through several rinses in PBS followed by standard eosin-staining protocol.

Documentation

In order to document double and triple 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; $\times 20$ dry or $\times 40$ and $\times 60$ oil immersion objective lenses, with numeric apertures 0.75, 1.30, and 1.4, respectively; Nikon, Düsseldorf, Germany) was applied. Sections were imaged using the appropriate filter settings for Cy3 (568 nm excitation, filter 605DF32; channel 1, coded red), Cy2/FITC (488 nm excitation, filter 522DF32; channel 2, coded green), and Cy5 (647 nm excitation, filter 680DF32; channel 3, coded blue). Co-localization of channels 1 and 2 in the same structure resulted in mixed yellow color. An overlap of channels 1 and 3 resulted in pink, and of channels 2 and 3 in mixed cyan color, respectively. An overlap of all three channels resulted in mixed white color. Extended focus images and z series (z increment of 1–2 μm) were created by electronic superimposition. In order to demonstrate putative synaptic contacts, single sections of z staples of same planes in two different channels were taken and subsequently merged.

For quantification, we applied two methods: first, ICN or neurons in cranial ganglia were counted using an epifluorescence microscope [Leica Aristoplan, Wetzlar, Germany; filterbloc I3 for Cy2 and N2.1 for Cy3 (both Leica), with $\times 25$ or $\times 40$ objective lenses] or second, plots were created on screen from confocal images out of merged and un-merged pictures, and co-localization rates were defined (see Fig. 5a, b). To avoid double counting of neuronal cell bodies in cranial ganglia, every fifth section was investigated. Only neurons with clearly detectable nuclei were counted, and data are given as means and standard deviations. To document the NADPH-d/eosin staining of the superior cervical ganglion, a digital camera (Spot RT realtime, Visitron Systems, Munich, Germany) attached to a

microscope (Leica Aristoplan) with the SPOT advanced software (version 3.5.6 for Windows, Diagnostic Instruments, Sterling Heights, USA) was used.

Results

Choroid

ICNs—In the NADPH-diaphorase histochemistry, ICNs were found closely associated with choroidal blood vessels (Fig. 1a) where their processes formed a nitrergic peri-vascular fiber network. GAL-immunopositive ICNs were embedded in the α -actin-positive smooth muscle spanning the choroidal stroma and suprachoroid (Fig. 1b), mainly in the temporo-cranial quadrant, with decreasing numbers toward the periphery, and toward the inner choroid. These neurons were round to ovoid, having a maximum cell diameter of 20–40 μ m, in accordance with earlier results in another bird species (Bergua et al. 1996; Schrödl et al. 2000, 2004).

From four eyes, 290 ± 95 ICNs were positive for nNOS. Double-labeling for nNOS and either GAL or VIP was done on one eye for each marker. From a total of 221 neurons, a nNOS/GAL co-localization (Fig. 1c–e) was found in 194 neurons (87.3%); 16 (7.2%) were positive for GAL only and 11 (4.9%) were positive for nNOS only. The co-localization of GAL/VIP (Fig. 1f–h) was found in 390 out of 470 neurons (79.1%). In this eye, 75 neurons were immunoreactive for GAL only (15.9%), while 5 neurons were immunoreactive for VIP only (1.1%). Co-localization of nNOS/VIP (Fig. 1i–k) was found in 385 out of 405 neurons (95.1%); 15 stained for nNOS only (3.7%) and 5 stained for VIP only (1.2%). Finally, TH immunoreactivity was not detected in nNOS- or GAL-positive ICN.

In 54 randomly selected ICNs positive for VIP or GAL, there was no co-localization with either ChAT (Fig. 1l) or SOM (Fig. 1m). Neither did any ICNs show immunoreactivity for CGRP. In summary, the chemical coding of ICNs can be described as nNOS/VIP/GAL positive.

Nerve fibers—GAL- or nNOS (data not shown)-positive nerve fibers co-localizing with VIP were found in perivascular nerve fibers (Fig. 2a), as well as in nerve fibers forming boutons throughout the choroid (Fig. 2b). The density of the peri-vascular plexus was greatest at the entrance sites of the vessels to the choroid. Nerve fibers and boutons positive for either ChAT (Fig. 1l) or SOM (Fig. 1m) were closely apposed to ICNs. ChAT immunoreactivity was also present in perivascular fibers (Fig. 2c) as well as in a dense nerve plexus within the choroidal stroma (Fig. 2d), but was not co-localized with VIP (Fig. 2c, d). Nerve fibers within the choroidal stroma showed a distinct co-localization of ChAT and SOM (Fig. 2e).

Eye-related cranial ganglia

Ciliary ganglion—Numerous cell bodies and nerve fibers within the ciliary ganglion displayed the blue NADPH-d reaction product (Fig. 3a). Here, ciliary neurons (projecting to the ciliary body) and choroid neurons (projecting to the choroid) can be distinguished unequivocally by SOM immunoreactivity, SOM being present in the choroid neurons only (De Stefano et al. 1993). Out of 15 sections, 712 SOM-immuno-positive neurons were

counted, and all of these co-localized for nNOS (Fig. 3b, c). Double-labeling for GAL/SOM revealed no GAL immunoreactivity in the SOM-positive choroid neurons (Fig. 3b). Only very few nerve fibers within the ganglion showed immunoreactivity for GAL as did perivascular nerve fibers in and adjacent to the ganglion.

Trigeminal ganglion—In the trigeminal ganglion, the majority of the neurons were immunoreactive for CGRP. These neurons did not double-label for GAL (Fig. 4a) or VIP, and only very few cells stained solely for GAL. Because SOM is found in trigeminal neurons of mammals (Firth et al. 2002), we tested for this in the chicken. SOM-immunoreactive neurons were absent, and only few SOM-immunoreactive nerve fibers were found throughout the ganglion. Moreover, double immunostaining for CGRP and SOM in the choroid revealed no co-localization of both markers (Fig. 4b).

Pterygopalatine ganglion—In contrast to mammals, the avian pterygopalatine ganglion is not a single coherent ganglion but a chain of interconnected microganglia located on the medial aspect of the Harderian gland (Gienc and Zaborek 1984). ChAT immunoreactivity in this ganglion was assessed in nine randomly chosen sections from two animals. We found 816 ChAT-immunopositive neurons, all of which co-localized with nNOS (Fig. 4c, d).

Double-labeling for nNOS/GAL (Fig. 4e) in 11 microganglia (20–60 neurons per ganglion) showed that of the total 417 stained neurons, 218 (52.1%) were positive for both nNOS and GAL. Staining for nNOS alone was in 177 neurons (42.3%), whereas 22 neurons were positive for GAL only (5.3%). In double-staining experiments with GAL and VIP (Fig. 4f), ten complete microganglia were investigated, and 222 neurons were counted. 144 neurons showed a co-localization with both markers (64.8%); staining for VIP alone was found in 72 neurons (32.4%), whereas staining for GAL alone was found in 6 neurons (2.7%).

Superior cervical ganglion—Because GAL is present in sympathetic ganglia (Ahren et al. 1990; Kummer 1987; Lindh et al. 1989; Morris et al. 1992), double-labeling experiments with TH, the rate-limiting enzyme in the catecholamine biosynthesis, and GAL were performed (Fig. 5a, b). Out of five sections, 1,575 neurons were counted (~315 neurons per section). TH/GAL was co-localized in 682 neurons (43.3%), whereas 708 neurons stained for TH alone (44.9%), and 184 neurons stained solely for GAL (11.7%). To ascertain whether there is a significant overlapping nitrergic cell population co-localizing GAL and/or VIP (*and therefore matching the chemical code of ICNs*), an indirect approach was used due to antibody incompatibilities (in these sets of experiments TH always served as internal control for identification of the SCG): TH-immunoreactive sections were subsequently treated with NADPH-d staining followed by standard eosin staining (Fig. 5c). In 14 sections, 4,326 neurons were counted (based on eosin staining; ~305 per section), of which 907 (21%) neurons were found to be NADPH-d positive. Based on this, triple immunohistochemistry for TH/VIP/nNOS (Fig. 5d) and TH/VIP/GAL (Fig. 5e) was done (18 sections), and co-localization was found in 76.8% VIP/nNOS (697 out of 907 neurons) and 53.9% VIP/GAL (376 out of 697 neurons), respectively. Therefore, the co-localization of nNOS/GAL-positive neurons (376 out of 4,326 neurons) in the superior cervical ganglion was 8.7%. Perikarya immunoreactive for TH/VIP/nNOS or TH/VIP/GAL were not detected.

Extrinsic contacts on ICN

TH and DBH/VMAT2—Double-labeling for nNOS/TH or GAL/TH was done to reveal putative sympathetic inputs onto ICNs. TH-immunoreactive nerve fibers and boutons were present in the choroidal intervascular stroma, forming dense perivascular plexuses; these fibers were especially numerous in the suprachoroidal layers. TH- and GAL- (Fig. 5f) or nNOS-positive nerve fibers, respectively, ran in common nerve strands, but were not co-localized within the choroidal stroma or perivascular plexus.

We found that TH-immunoreactive nerve fibers formed boutons on nNOS-positive ICNs in 161 out of 263 (61%) nNOS + neurons when screened with fluorescence microscopy. Furthermore, confocal microscopy showed 13 out of 22 (59%) of neurons positive for either nNOS or GAL closely apposed by TH-positive boutons.

To reveal whether the TH-immunoreactive nerve fibers within the choroid indeed originate from the superior cervical ganglion as hypothesized, another marker for sympathetics, DBH, was looked for using immunohistochemistry. Because this antibody did not show positive staining in the chicken choroid (although it did label fibers in prior bird experiments; Schrödl et al. 2001b), VMAT2 was used as an alternative marker for catecholamines. Prior to that, the superior cervical ganglion was screened for VMAT2; VMAT2 was detected in numerous neurons and fibers of the respective ganglion (Fig. 6a). In single optical sections, TH and VMAT2 were found to be colocalized in boutons approaching ICNs (Fig. 6b) as well as in nerve fibers of the choroidal stroma (Fig. 6c).

CGRP—The CGRP immunohistochemistry was used to identify putative inputs from primary afferent nerve fibers projecting to the trigeminal ganglion. Of the 135 GAL-positive ICNs that were randomly selected, 130 neurons (96.3%) were approached by CGRP-positive boutons and nerve fibers. Using confocal microscopy, of the 45 randomly selected ICNs positive for either GAL or VIP, 39 (86.6%) were contacted by CGRP-positive boutons (Fig. 6d). CGRP-positive nerve fibers were found in all regions of the choroid, and were not co-localized with GAL or VIP. Few CGRP-positive nerve fibers were present within nerve strands of the ciliary nerve.

Discussion

This study examines the chemical coding of, and synaptic inputs to, the intrinsic choroidal neurons in the chicken, an important animal model of emmetropization. We also report on the histochemical phenotype of nerve fibers within the chick choroid, and of the neurons in all four ocular-related cranial ganglia: the trigeminal, ciliary, superior cervical, and pterygopalatine to elucidate the choroidal innervation patterns of each.

ICN: chemical coding and choroidal innervation

The NADPH-d-positive (presumably nitrergic) ICNs in the chicken choroid have been described only recently (Schrödl et al. 2004). Their phenotype can now be extended to expression of nNOS, VIP, and GAL, in various combinations being found in more than 80% of these neurons, similar to what was found in the duck (Bergua et al. 1996; Schrödl et al. 2000). Furthermore, ICNs did neither express the sympathetic markers TH or VMAT2, nor

do they express CGRP, an indicator (not exclusive marker) of trigeminal origin. These neurons also lack SOM, which along with acetylcholine (Cuthbertson et al. 1996, 1997; Hiramatsu and Ohshima 1999; Pilar et al. 1973) is expressed in the postganglionic fibers of choroid neurons in the ciliary ganglion (De Stefano et al. 1993). Finally, ICNs do not contain ChAT, and therefore, do not fall in the class of “classical” parasympathetic neurons.

We found ChAT-positive nerve fibers coursing within the choroidal stroma as well as forming perivascular plexuses. Most of these fibers are co-localized with SOM, and therefore, presumably are postganglionic fibers from the ciliary ganglion. Another source of ChAT-positive fibers projecting to the bird eye is the pterygopalatine ganglion. Because these fibers colocalize for nNOS and VIP (Cuthbertson et al. 1997), the VIP-positive but ChAT-negative fibers found in the choroid most likely do not derive from the pterygopalatine ganglion, but from the ICNs.

Ciliary ganglion

Unlike the duck (Bergua et al. 1996; Schrödl et al. 2000), the ciliary ganglion of the adult chicken contains numerous NADPH-d-positive neurons and processes, in accordance with data obtained in a study of chicken embryos (Nichol et al. 1995) and pigeons (Cuthbertson et al. 1999). In the choroid neurons of the chick ciliary ganglion, nNOS co-localized with SOM. Because GAL immunoreactivity was absent in these neurons, and GAL-immunoreactive nerve fibers were sparse, a nitroergic–galaninergic input from the ciliary ganglion to the choroid is not supported.

Pterygopalatine ganglion (PPG)

Input from the PPG to the choroid is well established in mammals and birds (Cuthbertson et al. 1997; Ruskell 1970; Stone and Kuwayama 1989); these neurons co-express nNOS, VIP, and ChAT (Cuthbertson et al. 1997, 2003; Hanazawa et al. 1997; Kirch et al. 1995; Schrödl et al. 2000; Stone and Kuwayama 1989; Uddman et al. 1980). In this study we show that about half of the nNOS-positive PPG neurons are immunoreactive for GAL (52%); approximately two-thirds of the neurons show a co-localization for GAL and VIP (64%). Therefore, about 50% of PPG neurons co-express nNOS, VIP, and GAL, thereby having the identical chemical code as the ICNs. If we assume that not all of these nNOS/VIP/GAL-positive PPG neurons project to the eye, and because the colocalization of nNOS/VIP/GAL in the ICNs is greater than 80%, we conclude that the majority of NOS/VIP/GAL-positive nerve fibers in the choroid most likely derive from the ICNs and not the PPG.

Superior cervical ganglion (SCG)

The majority of neurons in the SCG express TH, the rate-limiting enzyme for catecholamine biosynthesis (Purves et al. 2001). We here show that approximately 43% of the TH-positive neurons co-express GAL, similar to many mammals and other birds (Grimes et al. 1994; Kummer 1987; Sakamoto et al. 2000). We propose that these GAL/TH-positive neurons most likely do *not* project to the choroid as no double-labeling for GAL and TH was found in choroidal fibers. We speculate that the neurons positive for TH alone (45%) are those that project to the choroid.

Interestingly, and of note, we find that in the chicken choroid the TH-immunoreactive fibers do *not* contain for DBH but are positive for the sympathetic marker, VMAT2. VMAT2 has an affinity for serotonin, dopamine, nor-adrenaline, and adrenaline (Peter et al. 1994), and so, cannot be used as an exclusion marker. In fact, early studies of sympathetic innervation of the chicken eye were based on fluorescence-histochemical methods that did not discriminate between the different catecholamines (Ehinger 1967; Guglielmone and Cantino 1982; Kirby et al. 1978); therefore, it is unknown which catecholamines are used by the sympathetics in chicken eyes. Although it remains possible that the absence of DBH labeling in the chick eye could be due to antigen incompatibility, it is possible that the chick system using dopamine or serotonin instead of noradrenaline, in regulation of the homeostasis of the posterior uvea (Ohngemach et al. 1997). In accordance, dopamine has been shown to be involved in the regulation of choroidal blood flow (Reitsamer et al. 2004).

There was a remarkably high number (about 21% of total) of NADPH-d-positive neurons in the SCG, which, to our knowledge, has not previously been found in birds (although it has been shown in some mammals; Grimes et al. 1995; Hisa et al. 1995; Tseng et al. 2000, but see also Dun et al. 1993; Edvinsson et al. 2001; Vanhatalo and Soinila 1994). In human SCG, nNOS-immunoreactive neurons have not been seen (Tajti et al. 1999). Interestingly, in chicks, a subpopulation (about two-thirds) of these nitroergic cells co-localize for VIP, and half of these VIP-positive cells co-localize for GAL. Therefore, these SCG neurons of unknown function share the chemical phenotype of the ICNs.

Trigeminal ganglion

Because very few neurons here were positive for GAL, the trigeminal ganglion presumably does not supply the galaninergic innervation to the choroid. SOM-immunoreactive neurons were also absent, similar to the duck (Schweigert et al. 2003). The presence of SOM in the trigeminal ganglion of mammals (Lazarov 2002) is interpreted as a species difference.

Extrinsic synaptic contacts onto ICNs

It has been shown that in ducks ICNs are approached by sympathetic nerve fibers; this was confirmed at the ultra-structural level (Schrödl et al. 2001b). We postulate that chicken ICNs are also part of the circuit of the sympathetic nervous system as indicated by the presence of TH- and VMAT2-positive boutons in close association.

The ICNs are also contacted by primary afferent CGRP-positive nerve fibers projecting to the trigeminal ganglion, which may represent a pre-central reflex arc within the uvea (Schrödl et al. 2001a). Occasionally, ChAT- or SOM-immunoreactive boutons are also found in close proximity to these cells. These fibers most likely originate from the pterygopalatine or ciliary ganglion as neurons of the superior salivatory nucleus (pre-ganglionic inputs) do not contain SOM (Schrödl et al. 2006), making this input unlikely (although a preganglionic catecholaminergic input from Edinger-Westphal nucleus cannot be excluded). Therefore, it is likely that both sympathetic and parasympathetic systems innervate chicken ICNs as has also been characterized in quail (Schrödl et al. 2005). A summary of the data obtained is given in Fig. 7.

Functional considerations

The choroid of the chicken has recently become the subject of growing interest in the field of eye growth regulation because of the finding more than a decade ago that the choroid changes its thickness in response to retinal defocus, expanding to push the retina forward in response to myopic defocus, and thinning to pull it back in response to hyperopic defocus; these changes occur within hours. The main anatomical correlates of these changes are changes in the size of the large, fluid-filled lacunae in the stroma. Even more interesting is the possibility that these changes in thickness are part of the mechanism that underlies the changes in the rate of elongation of the eye, with a thickening choroid causing ocular growth to be inhibited, and thinning causing an increase in ocular elongation (Nickla and Wallman 2010; Wallman and Winawer 2004). If this conjecture is true, elucidation of the mechanisms underlying these changes in choroid thickness is crucial to understanding the development of ametropias (e.g. myopia). In this context, the existence of such an extensive choroidal nitrergic system is interesting in the light of the fact that nitric oxide is a potent vasodilator and smooth muscle relaxant (Moncada and Higgs 2006). A role for NO in these choroidal thickness changes is supported by the finding that NOS inhibitors inhibit choroidal thickening in response to myopic defocus (Nickla et al. 2009; Nickla and Wildsoet 2004); they also dis-inhibit ocular growth.

Both chick and primate choroids have vascular and non-vascular smooth muscle cells, both of which are innervated by nitrergic fibers (Bergua et al. 1996; Cuthbertson et al. 1997; Fischer and Stell 1999; Flügel-Koch et al. 1994; Poukens et al. 1998; Schrödl et al. 2000, 2001a, 2003, 2004), indicating inputs from any of the following: ICNs, the ciliary ganglion, or the pterygopalatine ganglia. The functions of the non-vascular smooth muscle that spans the stroma and suprachoroid, and the ICNs, are as yet mysterious. It is possible that changes in the tonus of the non-vascular smooth muscle mediated by either division of the parasympathetic system or the ICNs might result in changes in choroidal thickness by exerting forces on the fluid-filled lacunae. Furthermore, the diurnal changes in choroidal thickness (Brown et al. 2009; Nickla et al. 1998) might be mediated by the same circuit.

It is quite reasonable that NO results in relaxation of non-vascular smooth muscle, and we propose that acetylcholine may result in contraction. The action of acetylcholine (ACh) on vascular smooth muscle is relaxation, mediated by NO (formerly endothelium-relaxing factor). However, ACh may result in contraction of non-vascular smooth muscle, as supported by the study by Meriney and Pilar (1987) in which stimulation of the chick ciliary ganglion resulted in a contraction of the choroid, as a whole. While the role for NO in choroidal thickness changes is supported by evidence, the system(s) and/or cells that are involved are as yet unknown. Single lesions of neither the ciliary nor the PPG pathways result in significant changes in the ability of the choroid to respond to defocus. However, intriguingly, lesions of both pathways together result in a diminished response of the choroid to myopic defocus (Nickla and Schrödl 2008). The definitive role for the various possible players in this response awaits the ability to selectively lesion the ICNs.

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Fig. 1.

a NADPH-diaphorase cytochemistry (light microscopy): ICN are closely associated with choroidal blood vessels (*asterisks*). **b** Immunohistochemistry for α -smooth muscle actin (*green*) and galanin (*red*): ICN-forming processes with boutons are enmeshed within the smooth-muscle framework of the choroid (confocal microscopy, extended focus mode). **c–e** Double immunohistochemistry for nNOS (*red*, **c**) and GAL (*green*, **d**) reveals a colocalization of both markers in ICN (**e**, *mixed yellow color*). **f–h** Double immunohistochemistry for GAL (*red*, **f**) and VIP (*green*, **g**) reveals a colocalization of both markers in ICN (**h**, *mixed yellow color*). **i–k** Double immunohistochemistry for VIP (*red*, **i**) and nNOS (*green*, **j**) reveals a colocalization of both markers in ICN (**k**, *mixed yellow*

color). **l** Double immunohistochemistry for ChAT (*red*) and VIP (*green*) reveals no co-localization in ICN; CHAT-immunoreactive nerve fibers forming boutons closely approached ICN (*arrowhead, mixed yellow color*). **m** Double immunohistochemistry for GAL (*green*) and SOM (*red*) reveals no co-localization in ICN, but ICN show close association with boutons immunoreactive for SOM (*arrowheads*). **b–m** Confocal microscopy, **b** extended focus mode, **c–m** single optical sections

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Fig. 2.

a, b Double immunohistochemistry for GAL (*red*) and VIP (*green*) reveals a co-localization of both markers in the perivascular plexus (**a**, *asterisks*) as well as in nerve fibers in the choroidal stroma (**b**) as indicated by *mixed yellow color*. **c, d** Double immunohistochemistry for ChAT(*red*) and VIP (*green*) reveals no co-localization of both markers within the perivascular plexus (**c**, *asterisks*) as well as none in nerve fibers within the choroidal stroma (**d**) as indicated by the absence of *mixed yellow color*. **e** Double immunohistochemistry for ChAT (*red*) and SOM (*green*) reveals a co-localization of both markers in nerve fibers within the choroidal stroma as indicated by *mixed yellow color*. All images: confocal images; **a, b** extended focus mode; **c–e** single optical sections

Fig. 3.

Ciliary ganglion. **a** NADPH-d cytochemistry (light microscopy): numerous structures and processes display the *blue* NADPH-d reaction product. **b, c** Double immunohistochemistry for nNOS (**b**) and SOM (**c**): numerous nNOS-positive neurons with cap-like endings (*arrowheads*, representing ciliary neurons) and bouton-like endings (*arrows*, representing choroid neurons) are visible. While all choroid neurons show co-localization for SOM (**c**, *arrows*), this is not the case for the ciliary neurons (**c**, *arrowheads*; confocal image, single optical section). **d** Double immunohistochemistry for GAL (*green*) and SOM (*red*): GAL is not present in SOM-positive choroid neurons (confocal image, extended focus mode)

Fig. 4.

a Trigeminal ganglion: double immunohistochemistry for CGRP (*green*) and GAL (*red*). Neurons of the trigeminal ganglion show no co-localization of GAL (confocal image, extended focus mode). **b** Choroid: double immunohistochemistry for CGRP (*green*) and SOM (*red*): primary afferent nerve fibers in the choroid do not co-localize for SOM as indicated by absence of *mixed yellow color* (confocal image, single optical section). **c, d** Pterygopalatine ganglion: double immunohistochemistry for ChAT (**c**) and nNOS (**d**) revealed a complete colocalization of both markers (confocal images, single optical sections). **e, f** Pterygopalatine ganglion: double immunohistochemistry for GAL (*red*) and nNOS (*green*, **e**), and VIP (*red*) and GAL (*green*, **f**), revealed a co-localization of the

aforementioned markers as indicated by *mixed yellow color* (*arrowheads*; confocal images, single optical sections)

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Fig. 5.

a, b Double immunohistochemistry of the superior cervical ganglion for GAL (*green*) and TH (*red*): out of confocal images (**a**), plots were created (**b**) to define co-localization rates (*red* TH only, *green* GAL only, *yellow* co-localization of TH and GAL). **c** In the superior cervical ganglion, 21% of all neurons are nitrergic as revealed by NADPH-diaphorase cytochemistry combined with eosin counterstaining. **d** Triple immunohistochemistry for nNOS (*green*), VIP (*blue*), and TH (*red*) in the superior cervical ganglion: co-localization of nNOS and VIP was amounted to 76.9% as indicated by *mixed cyan color*. **e** Triple immunohistochemistry for TH (*red*), VIP (*blue*), and GAL (*green*) in the superior cervical ganglion: colocalization of VIP and GAL was amounted to 54.4% as indicated by *mixed*

cyan color. **f** Double immunohistochemistry for GAL (*green*) and TH (*red*): nerve fibers in the choroid show no co-localization (single optical section)

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Fig. 6.

a Immunohistochemistry for VMAT2 in the superior cervical ganglion reveals immunoreactivity in neurons as well as nerve fibers (single optical section). **b** Triple immunohistochemistry for VMAT2 (*red*), TH (*blue*), and VIP (*green*): nerve fibers co-localizing VMAT2/TH (*mixed pink color*) forming bouton-like appositions (*cyan* and *white mixed colors*, *arrowheads*) onto VIP-positive ICN (single optical section). **c** Double immunohistochemistry for VMAT2 (*green*) and TH (*red*): nerve fibers in the choroid show a co-localization as depicted by *mixed yellow color* (single optical section). **d** Double immunohistochemistry for CGRP (*green*) and VIP (*red*): primary afferent nerve fibers forming bouton-like appositions onto VIP-positive ICN (single optical section)

Fig. 7. Colocalization rates of ICN and subsets of neurons in ganglia supplying the choroid (in percentages; *SCG* superior cervical ganglion, *PPG* pterygopalatine ganglion, *CIL* ciliary ganglion, *TRIGEM* trigeminal ganglion). Possible interaction of corresponding ganglia onto ICNs is indicated by *arrows*

Table 1

Antibodies used in this study

Antibody against	Raised in	Distributor	Dilution
CGRP	Rabbit	Peninsula, Belmont, USA	1:200
	Sheep	Biotrend, Köln, Germany	1:300
ChAT	Goat	Chemicon, Hofheim, Germany	1:30
DBH	Rabbit	Affiniti, Nottingham, UK	1:400
GAL	Guinea pig	Peninsula, Belmont, USA	1:750
	Rabbit	Affiniti, Nottingham, UK	1:600
nNOS	Rabbit	Courtesy of Dr. B. Mayer, Department of Pharmacology, University of Graz, Austria	1:500
SOM	Mouse	Courtesy of Dr. ME. De Stefano, Department of Cell Biology, University of Rome "La Sapienza", Italy	1:1,000
TH	Mouse	Chemicon, Hofheim, Germany	1:400
VIP	Guinea pig	Peninsula, Belmont, USA	1:750
VMAT2	Rabbit	Phoenix Pharmaceuticals, Karlsruhe, Germany	1:1,000
α -SMA	Mouse	Sigma-Aldrich, Taufkirchen, Germany	1:400

CGRP calcitonin gene-related peptide, *ChAT* choline acetyltransferase, *DBH* dopamine- β -hydroxylase, *GAL* galanin, *nNOS* neuronal nitric oxide synthase, *SOM* somatostatin, *TH* tyrosine hydroxylase, *VIP* vasoactive intestinal polypeptide, *VMAT2* vesicular monoamine-transporter 2, *α -SMA* α -smooth muscle actin