Short communication

Innervation pattern of the preocular human central retinal artery

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A B S T R A C T
The central retinal artery (CRA) is the main vessel for inner retinal oxygen and nutrition supply. While the intraocular branches lack autonomic innervation, the innervation pattern of the extra-ocular part of this vessel along its course within the optic nerve is poorly investigated. This part however is essential for maintenance of retinal blood supply, in physiological and pathological conditions. Therefore, the aim of this study was the characterization of the autonomic innervation of the preocular CRA in humans with morphological methods. Meeting the Declaration of Helsinki, eyes of body or cornea donors were processed for single or double immunohistochemistry against tyrosine hydroxilase (TH), dopamine-β-hydroxylase (DBH), choline acetyl-transferase (ChAT), vesicular acetylcholine transporter (VACHT), neuronal nitric oxide synthase (nNOS), calcitonin gene-related peptide (CGRP), substance P (SP), vasoactive intestinal polypeptide (VIP), and cytochemistry for NADPH-diaphorase (NADPH-d). For documentation, light-, fluorescence-, and confocal laser-scanning microscopy were used. TH and DBH immunoreactive nerve fibres were detected in the CRA vessel wall, although a distinct perivascular plexus was missing. Further, nerve fibres immunoreactive for ChAT and VACHT were found, while CGRP, SP, and VIP were not detected. NADPH-d staining revealed scattered nerve fibres in the adventitia of the CRA and in close vicinity; however, nNOS-immunostaining could not confirm this finding. The CRA receives adrenergic and cholinergic innervations, indicating sympathetic and parasympathetic components, respectively. Remarkably, a peptidergic primary afferent innervation was missing. Since clinical results suggest an autoregulation of intraretinal vessels, further studies are needed to clarify the impact of CRA innervation for retinal perfusion.

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The central retinal artery (CRA) is the main source of oxygen and nutrition supply for the inner retinal layers. In humans, the CRA is usually the first branch of the ophthalmic artery and one of the main branches of the internal carotid artery (Hayreh, 2006). With a frequency of up to 47% it travels together with the posterior ciliary artery (Erdogmus and Govsa, 2006). The CRA runs medially and caudally of the optic nerve and penetrates it 15.2 to 6.4 mm dorsally of the cribrose lamina (Kocabiyik et al., 2005). It continues from there within the centre of the optic nerve, penetrates the cribrose lamina and eventually forms its terminal branches within the retina. Functionally, the CRA is an end artery, and this implicates that retinal blood flow is essentially influenced by proper CRA function. As early as 1839, the German engineer Gotthilf Heinrich Ludwig Hagen and the French physician Jean Louis Marie Poiseuille independently discovered that the flow through a pipe, or vessel, is directly proportional to the 4th power of its radius (Poiseuille, 1840). For the CRA, this means that small changes in vessel diameter have an enormous impact on retinal blood flow and thus homeostasis.

The diameter of the CRA changes actively via relaxation or contraction of smooth-muscle fibres in the vessel wall. These changes in vessel diameter are controlled by endothelial factors and by the autonomic nervous system (Gugleta et al., 2006; Jabonero, 1956; Koenigsberger et al., 2006). The first histochemical studies on the nature of autonomic innervation of retinal and optic nerve head arteries were performed by Ehinger and Laties (Ehinger, 1966a, b; Laties, 1967; Laties and Jacobowitz, 1966). In experiments with new-world monkeys, these authors showed that an adrenergic innervation exists only dorsally of the cribrose lamina within the intraorbital part of the optic nerve. However,
experiments in monkeys, rabbits, and cats revealed an absence of innervation in vessels rostral of the cribrose lamina (Latties and Jacobowitz, 1966). Regarding blood flow, this led to the assumption that these vessel segments possess an autoregulation (Latties, 1967; Latties and Jacobowitz, 1966). The same is assumed for other intracranial vessels, which are regulated locally mainly via endothelial NO-synthetase (Fodale et al., 2007; Haefliger et al., 1992; Ingyinn et al., 2006). However, the blood flow autoregulation in ocular vessels (here: ophthalmic artery) apparently differs from other brain vessels (here especially: middle cerebral artery): a drop of systemic blood pressure leads to a quick reaction of the middle cerebral artery which compensates these pressure changes before systemic pressure returns to normal, while this happens considerably later in the ophthalmic artery (Kolodjashna et al., 2005).

An occlusion of the CRA leads to irreversible blindness in the affected eye, but since the pathogenesis for such an event is still poorly understood, the innervation of this vessel might play an important role (Hayreh and Zimmerman, 2005; Pettersen et al., 2005). Also, retinal blood flow has an impact on certain forms of glaucoma (Flammer et al., 2002), and the high prevalence of this disease illustrates the need for further exploration of CRA physiology and pathophysiology (Quigley, 1986; Weih et al., 2001).

While in animal experiments (monkey, rat), a clear peptidergic, adrenergic, nitricergic, and primary afferent (sensory) innervation of the CRA proximal of the cribrose lamina was detectable (Bergua et al., 2003; Toda et al., 1996; Ye et al., 1990), the situation in humans is poorly investigated or controversially discussed. First, a nitricergic and adrenergic innervation could not be found (Latties, 1967; Roufail et al., 1995), while other authors report the presence of cholinergic and aminergic nerves (Komai et al., 1995). Secondly, a cross-over of perivascular fibres of the internal carotid artery to the dura mater of the optic nerve and the adventitia of the ophthalmic artery has been shown (Rusnell, 2003). Furthermore, due to the fact that autoregulation of intracranial/intraocular vessels is well established, autonomic innervation of the CRA was considered dispensable (Delae and Von De Voorde, 2000; Fodale et al., 2007). Therefore, re-investigation of the autonomic innervation of the human CRA along its course within the optic nerve by immunohistochemistry is warranted.

1. Specimens

Meeting the declaration of Helsinki, eyes of body-/cornea-donors (average age 72.2 years; average hrs p.m. 7 h; n = 12) were obtained from either the Cornea Bank of the Department of Ophthalmology or the Institute of Anatomy I, University of Erlangen-Nuremberg.

Eyes with adjacent optic nerves were dissected free, opened along the ora serrata and fixed by immersion in phosphate buffered saline (PBS) containing 4% paraformaldehyde (3 h at room temperature, RT, or 8 h at 4 °C; different fixation protocols did not affect immunohistochemistry). They were rinsed in PBS (24–48 h) and transferred into PBS containing 15% sucrose (24 h at 4 °C). Eyes were embedded in tissue embedding medium (Slee Technik, Mainz, Germany) and frozen at −80 °C by using liquid nitrogen-cooled methylbutane and stored at −20 °C for further processing.

2. Immunohistochemistry

All eye cups with adjacent optic nerve were mounted in a cryostat (HM 500, Microm, Walldorf, Germany) and sagittal serial sections of 12–20 μm were collected and air-dried for 1 hr at RT on poly-L-lysine (Sigma–Aldrich, St. Louis, MO) 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 or goat serum (depending on the secondary antibodies used; Sigma), 1% BSA (Sigma), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After a 5 min rinse, slides were incubated with antibodies for single or double-labeling of the markers as listed in Table 1. After a rinse in TBS (four times 5 min) binding sites of primary antibodies were visualized by corresponding Cy3- or Alexa488-tagged antisera (1:1000; Invitrogen, Karlsruhe, Germany) 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). Negative controls were performed through omission of the primary antibodies and resulted in no staining. All antibodies were successfully used on human tissue in prior studies (Beck et al., 2009; Brehmer et al., 2005, 2006; May et al., 2004; Weidmann et al., 2007).

3. NADPH-d cytochemistry

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 embedding in Kaiser's Glycerol gelatine (Merck, Darmstadt, Germany).

4. Documentation

Results of single and double label immunohistochemistry were documented using a confocal laserscanning 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 0.75, 1.30, and 1.4, respectively; Nikon, Düsseldorf, Germany). Sections were imaged using the appropriate filter settings for Cy3 (568 nm excitation, filter 605DF32; channel 1, coded red) and Alexa488 (488 nm excitation, filter 522DF32; channel 2, coded green). Colocalization of markers in the same structures in channel 1 and channel 2 resulted in yellow mixed colour. For quantitative assessments, an epi-fluorescence microscope (Aristoplan; Leica, Bensheim, Germany; filterblock N2.1 for viewing Cy 3, filterblock I 3 for Alexa488) with × 25 or × 40 dry objective lenses and equipped with a digital camera (Spot RT, Visitron Systems, Munich, Germany) was used.

Table 1

<table>
<thead>
<tr>
<th>Antibodies against</th>
<th>Raised in</th>
<th>Distributor</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine-β-hydroxylase (DBH)</td>
<td>Rabbit</td>
<td>Biotrend, Cologne, Germany</td>
<td>1:400</td>
</tr>
<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>Sheep</td>
<td>Novus Biologicals, Littleton, USA</td>
<td>1:2000</td>
</tr>
<tr>
<td>Choline acetyl-transferase (ChAT)</td>
<td>Goat</td>
<td>Chemicon, Temecula, USA</td>
<td>1:30</td>
</tr>
<tr>
<td>Vesicular acetylcholine transporter (VACHT)</td>
<td>Rabbit</td>
<td>Phoenix Pharmaceuticals, Belmont, USA</td>
<td>1:1000</td>
</tr>
<tr>
<td>Neuronal nitric oxide synthase (nNOS)</td>
<td>Rabbit</td>
<td>Courtesy of Dr. B. Meyer, Dept. of Pharmacology, Univ. Graz, Austria</td>
<td>1:750</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide (CGRP)</td>
<td>Goat</td>
<td>Peninsula Labs, Belmont, USA</td>
<td>1:250</td>
</tr>
<tr>
<td>Substance P (SP)</td>
<td>Guinea-pig</td>
<td>Novus Biologicals, Littleton, USA</td>
<td>1:1000</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide (VIP)</td>
<td>Guinea-pig</td>
<td>Progen, Heidelberg, Germany</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
5. Tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH)

TH-positive nerve fibres were found in all of the specimens investigated. TH-immuno-positive fibres were detected exclusively in the tunica media and perivascular connective tissue of the CRA (Fig. 1a), but not in the connective tissue septa and nerve fibres of the optic nerve. Likewise, DBH-positive fibres were found in all of the investigated specimens. These fibres were traceable from the perivascular connective tissue almost to the tunica intima. Double labelling of TH and DBH consistently revealed co-localization of both markers, with the DBH-immunoreaction (Fig. 1b) always being weaker than TH-staining.

6. Vesicular acetylcholine transporter (VACHT), choline acetyltransferase (ChAT), and vasoactive intestinal polypeptide (VIP)

VACHT-positive fibres were found in most of the investigated specimens, and were present in the wall of the CRA. ChAT-immunoreactivity revealed the same, and both markers showed almost complete overlap in double labelling experiments (Fig. 1c), with the VACHT-immunoreactivity being more prominent.

While VIP-immunoreactivity was detectable in the perivascular connective tissue of posterior ciliary arteries (Fig. 2a), it was absent in the wall as well as in the perivascular connective tissue of the CRA in all of the specimens investigated (Fig. 2b).

7. Neuronal nitric oxide synthase (nNOS) and NADPH-diaphorase (NADPH-d)

NADPH-d cytochemistry revealed stained nerve fibres in the perivascular connective tissue of the CRA (Fig. 2c), as well as in its media (Fig. 2d). However, nNOS-immunohistochemistry showed...
a complete absence of immunoreactive fibres within the CRA tunica media and adventitia.

8. Calcitonin gene-related peptide (CGRP) and substance P (SP)

Both CGRP and SP were absent in the wall of the CRA (Fig. 3a). However, they were detectable in the choroid of the same donor, showing co-localization (Fig. 3b).

9. Semiquantitative results

A semiquantitative analysis of the obtained data revealed highest density in sympathetic TH/DBH positive nerve fibres (reaching about two thirds of TH/DBH density), followed by nitricergic nerve fibres (low density), and primary afferent CGRP/SP-immunoreactive nerve fibres (absent), and results are discussed below.

10. Sympathetic innervations

The postganglionic sympathetic innervation originates in the superior cervical ganglion, which is the uppermost ganglion of the sympathetic ganglion chain situated at the height of the 2nd/3rd cranial vertebra. Neurons within this ganglion predominantly (>75%) contain noradrenaline and neuropeptide Y, while a minority (<5%) also contain VIP (Baffi et al., 1992; Cavallotti et al., 2001; Tajti et al., 1999). Noradrenaline probably represents the most important sympathetic transmitter for the eye (Sears, 1975), and here, for the detection of sympathetic innervation, antibodies against TH, the rate-limiting enzyme in catecholamine synthesis, and DBH were used. All of our immunohistochemical experiments revealed TH and DBH positive nerve fibres in the vessel wall of the CRA and adjacent to it, thus a sympathetic innervation can be assumed. Most likely, this sympathetic CRA innervation in humans originates from the perivascular plexus of the ophthalmic artery.

Still, it is unknown what a stimulation of sympathetic receptors on the wall of the CRA provokes: generally, in peripheral vessels of resistance vasoconstriction is mediated via β1- and β2-adrenergic receptors (Muszkat et al., 2004), while β2-adrenergic receptors mediate vasodilation (Schutzer et al., 2006). Noradrenaline preferentially has an effect on α-adrenergic receptors, while adrenaline also stimulates β-adrenergic receptors; however, the distribution of receptors in the CRA is still unknown. Pharmacological studies using brimonidine, an α2-receptor agonist approved in glaucoma therapy, revealed a heterogeneous effect on retinal vessel diameter: topical application dilates grade 1 CRA arterioles, while it constricts grade 2 arterioles (Rosa et al., 2006). If α2-receptors are expressed there at all, one would expect a marked decrease in blood flow caused by the adrenergic vasoconstriction. However, an increase in blood noradrenaline concentration higher than 10 times above plasma level reveals no significant change in retinal blood flow (Jandrasisits et al., 2002), and this has been attributed to retinal autoregulation (Delaeys and Van De Voorde, 2000) as already postulated by Laties (1967) while topical application of timolol, a non-selective β-blocker, revealed no significant change in retinal blood flow (Arend et al., 1998). This heterogeneity in blood flow dynamics might not be caused due to different calibres in the CRA (Wang et al., 2007) but due to complex intrinsic retinal mediators (Laties, 1967), such as nitric oxide or endothelins (MacCumber et al., 1991; Rakowski et al., 2003).

11. Parasympathetic innervations

In primates including man, parasympathetic innervation of the eye courses via the ciliary ganglion, but also via the pterygopalatine ganglion (Agassandian et al., 2002; Cuthbertson et al., 1997; Neuhuber and Schrödl, 2010; Ruskell, 1970a, b), and both pathways have also been demonstrated in experimental animals (qual: Schrödl et al., 2005; rat: Yasuhara et al., 2004). Further, there are hints that the otic ganglion is also involved in innervation of orbital blood vessels (Cheng et al., 2001). Here, we were able to show that ChAT and VACHT were co-localized in nerve fibres supplying the CRA, indicating a parasympathetic innervation within the optic nerve. Although ChAT and VACHT have been demonstrated in human cranial vessels, this was not the case for the CRA. In animal experiments with rats (Bergua et al., 2003), rabbits (Kumagai et al., 1988), and rhesus monkeys (Ye et al., 1990), further parasympathetic substances have been demonstrated using antibodies against VIP, nNOS, and cytochemistry for NADPH-d. VIP/nNOS were absent in our experiments, and this might be interpreted as a species difference.

Since nNOS and NADPH-d show extensive co-localization (Mayer et al., 1991), the discrepancy in our nNOS-NADPH-d results might also be caused by unspecific NADPH-d reaction, unable to discriminate between the different isoforms of NOS: while histologically, e-NOS could be unequivocally distinguished due to its endothelial localisation, we cannot rule out an iNOS expression (Koziel et al., 2000), leading to a false positive NADPH-d reaction (although eyes of the body/cornea donors had no clinical signs of inflammation and no indications of inflammation were evident in the clinical records). On the other hand, it is also known that a positive NADPH-d reaction without nNOS-immunoreactivity is possible due to independent expression of both enzymes (Spessert et al., 1994).

Clinically, flicker light stimulation leads to an increase in retinal blood flow, and i.v.-application of l-NMMA, an unspecific NOS inhibitor, leads to a significant reduction of retinal vessel diameters (Garhofer et al., 2003; Toda et al., 1996). In pigs, stimulation of NOSynthase leads to a retinal vasodilation (Hein et al., 2006; Yuan et al., 2008). That means that although morphologically (our results) a nitricergic innervation of the CRA within the optic nerve is doubtful, clinically at least a modulating nitricergic effect is plausible.
12. Primary afferent innervations

Primary afferent nerve fibres travel to their neurons residing in the trigeminal ganglion. These trigeminal axons contain several neuropeptides, such as neurokinin A, SP, CGRP or cholecystokinin (Goadsby and Hoskin, 1997), with vasodilatory properties (Chou et al., 1977; lwase et al., 2003). In humans, about half of the primary afferent neurones contain CGRP, and 25% of these neurones co-localize with SP (Quartu et al., 1992). While in rats, rabbits, and rhesus monkeys primary afferent nerve fibres reach the CRA (Bergua et al., 2003; Kumagai et al., 1988; Ye et al., 1990), this was not the case in human CRA in our series of experiments. The absence of these peptides does not necessarily indicate an absence of primary afferent innervation, since autolytic processes in human post-mortem tissue can lead to false negative results. However, since both tested peptides, SP and CGRP, were not detectable here, but in choroid of the same donor, it is likely that a primary afferent innervation is indeed absent in this part of the human CRA. Nevertheless, a primary afferent innervation of the CRA feeding vessel, the internal carotid artery, has been described (Suzuki and Hardebo, 1991).

13. Perspectives and clinical consequences

The CRA holds an exceptional position between retinal autor-regulation on one side and cerebral vessels on the other. However, maintenance of constant retinal blood flow is essential for the function of retinal neurones, and therefore the upstream CRA plays an important role in various oculo-pathological mechanisms. In that context, retinal blood supply has to compensate for large changes in pressure: this holds true for variability in systemic pressure, such as hypotension, but also for local intraocular pressure. Here, IOP can be several times above normal, as it is the case in glaucoma (Dayan et al., 2008), or almost zero, as in open trauma (Lima-Gomez and Cornejo-Mendoza, 2004). Uncompensated pressure changes lead to visual impairment, or, in the worst case, to a total loss of retinal function with consecutive amaurosis (Li et al., 2005). Knowledge of CRA innervation and the associated competence of dilatation of this vessel might result in new therapeutic approaches in such thromboembolic events.

In summary, this study has settled some uncertainties concerning CRA innervation. While sympathetic and parasympathetic components were confirmed in the post-cibriiform CRA, the lack of nNOS and CGRP/SP immunoreactive nerve fibres as well as the lack of innervation anterior to the cribose lamina probably indicates differences between human and non-human primates and other mammals. The clinical consequence of the observed density in innervation pattern remains to be elucidated. Nevertheless, the pre-ocular CRA innervation as demonstrated in this study may be relevant for understanding the physiology and pathology of blood supply of retina and optic nerve.

Disclosure

All authors disclose any actual or potential conflict of interest; all authors have approved the final article.

References


Contribution of the authors

All authors have approved the final version of the article.

Antonio Bergua: involved in conduction of experiments, manuscript preparation, study design.

Markus Kapseiret: involved in the conduction of the experiments, manuscript preparation, study design.

Winfried L. Neuhuber: involved in the study design, manuscript preparation.

Herbert A. Reitsamer: involved in the study design, manuscript preparation, fund raising.

Falk Schrödl: involved in the conduction of the experiments, manuscript preparation, fund raising, study design.

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