Chapter 3

Changes in metabolic demand in the olfactory bulb leads to a decrease in cholinergic- and nitrergic nerve fibers in the anterior cerebral artery of the rat
Introduction

In the previous chapter we hypothesised a coupling between variation in metabolic demand and corresponding flow fluctuations in the afferent arteries leading to adaptation of the nerve fiber densities in these arteries. This was tested by inducing chronic anosmia, through intranasal application of zinc sulfate. We showed that peripherally induced anosmia results in a decreased nerve fiber density in the anterior cerebral artery, which coincides with a decreased metabolic activity in the olfactory bulb. The question remains whether all nerve population(s) is/are affected for the observed decrease in nerve density in the anterior cerebral artery.

It is well established that the cerebrovascular nerves contain a range of neurotransmitter substances as well as peptides and gases and are able to store and release more than one transmitter. For instance, it has been shown that parasympathetic transmitters such as nitric oxide (NO) and acetylcholine (ACh) and NO and vasoactive intestinal polypeptide (VIP) are colocalized in the same perivascular nerves in cerebral blood vessels of the cat (Kimura et al., 1997) and pig (Yu et al., 1998) and have been classified as cholinergic-nitrergic and VIPergic-nitrergic nerves. In the rat, the pterygopalatine ganglion is a major source for these parasympathetic neurons (Suzuki et al., 1989; 1990a) and activation seems to enhance cortical cerebral blood flow (CBF) (Seylaz et al., 1988; Suzuki et al., 1990b) which may be mediated by release of ACh, VIP or NO or a combination of these (Suzuki et al., 1988; Gotoh et al., 1993; Morita-Tsuzuki et al., 1993). The sympathetic nerves originate in the superior cervical ganglion and contain neuropeptide Y (NPY)- and noradrenaline immunoreactive neurons (Edvinsson et al., 1987; Handa et al., 1990). Whereas, the sensory nerves originate in the trigeminal ganglion and contain substance P (SP)- and calcitonin gene-related peptide (CGRP)- immunoreactive neurons (Uddman et al., 1985).

The data accumulated so far support the view that perivascular nerves play a role in the regulation of the cerebral circulation and that their density may alter due to variation in flow and metabolic demand. Therefore, the current study was
undertaken to determine which subpopulation, sympathetic and/or parasympathetic, declines after inducing anosmia. Immunohistochemical techniques were used to localize the general neural marker protein gene product (PGP) 9.5-, the sympathetic nerves containing tyrosine hydroxylase (TH) and the parasympathetic nerves containing vesicular acetylcholine transporter (VACht), VIP and nitric oxide synthase (NOS) in the basal cerebral arteries. In most investigations of quantification of nerve fiber density, superficial nerves and the nerves at the adventitial-medial border were not studied separately. However, it has been shown that the nerves at the adventitial-medial border provide local functional innervation because of their close relationship with smooth muscle cells (Burnstock, 1975). Therefore, also a detailed description of the distribution of PGP 9.5, TH, VACht, VIP and NOS, as measured at the adventitial-medial border, is given for all the segments of the basal cerebral arteries.

Material and Methods

Anosmia procedure

Forty-six male Wistar rats, weighing 300-350 gram, were used in this study. The committee of experiments on laboratory animals approved all experimental procedures. Anosmia was induced as described in detail in chapter 2. Briefly, under anesthesia (Hypnorm 0.01ml/100 gr. body weight s.c., Janssen Pharmaceuticals, Netherlands) a polypropylene tube was inserted 1 cm into each naris followed by administration of 0.05 ml of either 10 % zinc sulfate (Merck, Germany) in 0.9 % saline (n = 23) or 0.9 % saline (n = 23) twice a week during 4 weeks. The anosmic state of the animals was verified by a food location test and the olfactory epithelium was examined by sectioning and hematoxylin/eosin staining at the end of the experiment.
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*Tissue preparation*

Perfusion procedures were performed on all rats with a Watson-Marlow 503S rotation pump (Smith and Nephew, Falmouth, UK). Under deep anesthesia (sodium pentobarbital, 0.1 ml per 100 gram body weight i.p.) a cannula was inserted into the ascending aorta and the rats were perfused with 300 ml 0.9% NaCl containing 500 IE heparin (Leo Pharmaceutical, Weesp, the Netherlands). Then 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, at 4°C) was perfused over 15 minutes, followed by 500 ml 15% sucrose in 0.1 M phosphate buffer (pH 7.4, at 4°C) during 15 minutes. Subsequently, the brains were removed and stored in 15% sucrose in 0.1 M Phosphate Buffered Saline (PBS).

*Immunohistochemistry*

The basal cerebral arteries were dissected and mounted on sylgard (Dow Corning, USA) with entomology needles. Subsequently, an indirect immunohistochemistry was performed at room temperature.

One series of segments i.e. right parts of the circle of Willis (n = 23) were washed in HEPES buffer containing 0.1 % Triton X-100 for 30 minutes followed by pre-incubation in 5 % normal swine serum (Dako, Denmark) in HEPES buffer for 90 minutes. Subsequently, they were incubated overnight in either Rabbit anti PGP 9.5 (Ultraclone Limited, UK) or Rabbit anti TH (Calbiochem-Novabiochem, USA) both diluted 1 : 400 in HEPES buffer containing 1 % normal swine serum, 0.1 % Triton X-100 and 0.1 % DL-Lysine. After washing in PBS for 30 minutes the segments were incubated in fluorescein isothiocyanate (FITC)- conjugated swine anti rabbit antiserum (Dako, Denmark) diluted 1 : 40 in PBS containing 1 % normal swine serum, 0.1 % Triton X-100 and 0.1 % DL-Lysine for 90 minutes. After washing in PBS the segments were stained for 10 minutes with 0.05 % pontamine sky blue (BDH, UK) to reduce background autofluorescence (Cowen et al., 1985) and washed again in PBS.

A second series of segments i.e. left parts of the circle of Willis (n = 8) were washed in HEPES buffer containing 0.1 % Triton X-100 for 30 minutes followed by pre-incubation in 5 % normal goat serum (Dako, Denmark) in HEPES buffer for 90
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minutes. The segments were incubated overnight in Rabbit anti VAChT (Phoenix Pharmaceuticals, USA) diluted 1 : 1500 in HEPES buffer containing 1 % normal goat serum, 0.1 % Triton X-100, 0.1 % DL-Lysine and 5 % Bovine Serum Albumin (BSA; Sigma, Germany). After washing in PBS for 30 minutes the segments were incubated in 1 : 250 goat anti rabbit biotinylated IgG (Dako, Denmark) for 90 minutes washed again for 30 minutes in PBS and were finally incubated in 1 : 1000 Streptavidin FITC (Dako, Denmark) for 90 minutes. After washing in PBS the segments were stained for 10 minutes with 0.05 % pontamine sky blue.

A third series of segments i.e. left parts of the circle of Willis (n=15) were washed in PBS- 0.2 % Triton X-100 for 30 minutes followed by pre-incubation in either 5 % normal goat serum or 5 % normal swine serum in PBS- 0.2 % Triton X-100 for 90 minutes. The segments were incubated overnight in either guinea pig anti VIP (Eurodiagnostica, Netherlands) diluted 1 : 500 or in 1 : 1000 rabbit anti NOS (Eurodiagnostica, Netherlands) in PBS- 0.2 % Triton X-100 containing 0.1 % DL-Lysine and 1 % BSA. After washing in PBS for 30 minutes the segments were incubated in either 1 : 200 goat anti guinea pig Alexa 594 (Molecular Probes, Netherlands) or in 1 : 40 swine anti rabbit FITC in PBS-0.2 % Triton X-100 containing 0.1 % DL-Lysine for 90 minutes. Only the segments stained for NOS were stained for 10 minutes with 0.05 % pontamine sky blue. All the segments were streched on glass slides and mounted in antifade mountant (Citifluor, London, UK).

Image analysis

PGP 9.5-, TH-, NOS-, VAChT- and VIP- immunoreactivity was quantified using established methods of image analysis (Cowen et al., 1982; Cowen and Thrasivoulou, 1992). The deepest nerve plexus layer, which is functionally important and is situated at the adventitial-medial border (Bleys and Cowen, 2001), was measured by the use of a Zeiss fluorescence microscope (Munchen, Germany) equipped with a 3 CCD color video camera (Sony, Japan) and a Kontron 4.3 image analyzer. Area percentage (percentage of specific fluorescence in the measuring frame) and intercept density per millimeter (ID/mm, number of intersections of the horizontal pixel lines that make up the screen of the image.
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analyzer with the bundles of nerve fibers) were determined. The mean values of the data were determined by analysis of variance (ANOVA) and Fisher PLSD test.

Results

Anosmia

All animals treated with zinc sulfate were unable to locate the chocolate cookie the first day after each irrigation procedure. Their olfactory epithelium was considerably thinner compared to controls (data not shown).

Image analysis

PGP 9.5 immunoreactivity

All animals displayed the same topographical distribution in nerve density, which was greatest in the rostral part of the circle of Willis. The anosmic animals showed a significant decrease in nerve density expressed as area % for the A1-1 segment which is proximal to the origin of the internal ethmoidal artery (IEA) as well as for the A1-2 segment which is distal to the origin of the IEA (11.03 ± 0.1 vs. 13.3 ± 0.1, mean ± S.E.M., \( P < 0.001 \); 11.6 ± 0.2 vs. 14.9 ± 0.4, \( P < 0.001 \), Fig. 1A) compared to controls. For ID/mm the anosmic animals also showed a decreased nerve density for the A1-1 segment as well as for the A1-2 segment (46.6 ± 1.2 vs. 53.5 ± 1.4, \( P < 0.05 \); 49.8 ± 1.9 vs. 58.6 ± 1.7, \( P < 0.05 \), Fig. 1B). However, there was a significant increase in nerve density for the internal carotid artery (ICA) for ID/mm in the anosmic group (48.0 ± 0.8 vs. 42.6 ± 1.8, \( P < 0.05 \), Fig. 1B) compared to controls.

TH immunoreactivity

All the animals displayed the same topographical distribution in nerve density, which was greatest in the IEA followed by both parts of the anterior cerebral artery, the ICA then the middle cerebral artery (MCA), the posterior cerebral artery (PCA), the basilar artery and the vertebral artery in decreasing
order. There was no difference in nerve density between the two groups (Fig. 3A,B,C,D) either expressed as area % (Fig. 2A) or ID/mm (Fig. 2B) in all the segments.

Fig. 1. Mean values and standard error of the mean (A) area percentage and (B) ID/mm of PGP 9.5-immunoreactive nerve fibers in various segments of the basal cerebral arteries from control (open bar; n=16) and anosmic (closed bar; n=15) animals as measured at the adventitial-medial border. * P < 0.05; ** P < 0.01. VERT= vertebral artery; prBAS= proximal part of the basilar artery; distBAS= distal part of the basilar artery; PCA= posterior cerebral artery; ICA= internal carotid artery; MCA = middle cerebral artery; A1-1= first part of the anterior cerebral artery; A1-2= second part of the anterior cerebral artery; IEA= internal ethmoidal artery.

Fig. 2. Mean values and standard error of the mean (A) area percentage of TH-immunoreactive nerve fibers (B) ID/mm of TH-immunoreactive nerve fibers in various segments of the basal cerebral arteries from control (open bar; n=7) and anosmic (closed bar; n=8) animals as measured at the adventitial-medial border. For abbreviations see figure 1 legend.
Fig. 3. Whole mount preparations of perivascular nerves, stained for TH. The deep plexus is transversely orientated and is situated close at the adventitial-medial border (A) A1-1 segment, anosmic (B) A1-1 segment, control (C) A1-2 segment, anosmic (D) A1-2 segment, control. Scale bar = 120 µm

**NOS immunoreactivity**

All the animals displayed the same topographical distribution in nerve density, which was greatest in the second part of the anterior cerebral artery (A1-2) followed by the IEA, the A1-1 segment, the ICA then the MCA, the PCA, the basilar artery and the vertebral artery in decreasing order. The anosmic animals showed a significant decrease in nerve density expressed as area % for the A1-1 segment (Fig. 4A,B) as well as for the A1-2 segment (Fig. 4C,D; 6.6 ± 0.5 vs. 9.4 ± 0.2, p < 0.01; 8.1 ± 0.3 vs. 10.7 ± 0.2, P< 0.01, Fig. 5A) compared to controls. For ID/mm the anosmic animals also showed a significant decrease in nerve density for the A1-1 segment as well as for the A1-2 segment (28.9 ± 2.8 vs. 40.9 ± 1.4, p < 0.01; 36.8 ± 2.6 vs. 47.3 ± 1.2, P < 0.01, Fig. 6A) compared to controls.
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Fig. 4. Whole mount preparations of perivascular nerves, stained for NOS. (A) A1-1 segment, anosmic (B) A1-1 segment, control (C) A1-2 segment, anosmic (D) A1-2 segment, control. Scale bar = 120 µm

VACHT immunoreactivity

All the animals displayed the same topographical distribution in nerve density, which was greatest in the IEA followed by both parts of the anterior cerebral artery, then the ICA, the MCA, the PCA, the basilar artery and the vertebral artery in decreasing order. The anosmic animals showed a significant decrease in vesicle content expressed as area % for the A1-1 segment (Fig. 7A,B) as well as for the A1-2 segment (Fig. 7C,D; 1.4 ± 0.1 vs. 2.4 ± 0.3, P < 0.05; 1.2 ± 0.1 vs. 2.1 ± 0.3, P < 0.05, Fig. 5B) compared to controls. For ID/mm there was also a significant decrease in vesicle content in the anosmic animals for the A1-1 segment as well as for the A1-2 segment (13.1 ± 1.1 vs. 21.1 ± 1.6, p < 0.01; 11.6 ± 1.0 vs. 20.2 ± 2.0, P < 0.01, Fig. 6B) compared to controls.
Fig. 5. mean values and standard error of the mean of area % (A) NOS- (B) VAChT- (C) VIP- immunoreactive nerve fibers from control (open bar, n=8) and anosmic (closed bar, n=7) animals. * $P < 0.05$ ** $P < 0.01$. For abbreviations see figure 1 legend.

Fig. 6. mean values and standard error of the mean of ID/mm (A) NOS- (B) VAChT- (C) VIP- immunoreactive nerve fibers from control (open bar, n=8) and anosmic (closed bar, n=7) animals. ** $P < 0.01$. For abbreviations see figure 1 legend.
VIP immunoreactivity

All the animals displayed the same topographical distribution in nerve density, which was greatest in the IEA followed by both parts of the anterior cerebral artery, then the ICA, the MCA, the PCA, the basilar artery and the vertebral artery in decreasing order. There was no difference in nerve density between the two groups (Fig. 8A,B,C,D) either expressed as area % (Fig. 5C) or ID/mm (Fig. 6C).

Fig. 7. Whole mount preparations of perivascular nerves, stained for VACHT (A) A1-1 segment, anosmic (B) A1-1 segment, control (C) A1-2 segment, anosmic (D) A1-2 segment, control. Scale bar = 120 µm.
Discussion

Topographical distribution

Until now, quantitative techniques for determining nerve densities in the basal cerebral arteries in rats did not distinguish between the functional deep plexus, at the adventitial-medial border, and nerves of passage, except for a pilot study by Bleys and Cowen (2001). In our view, it is important to make this distinction since a regionally specific distribution of terminal fibers may indicate functional differences. This becomes especially important when one investigates the parasympathetic innervation of the rostral cerebral circulation in rat. Parasympathetic nerves, and sensory as well, gain access to the cerebral arterial tree at the level of the IEA, and as a consequence high nerve densities in rostral arteries are expected when terminal fibers as well as nerves of passage are included. In this study, the density of the nerve fibers in controls containing NOS, VACH T, VIP and TH were higher in the rostral part compared to the caudal part of the circle of Willis. Moreover, the
rostral part of the circle of Willis receives the highest density in NOS-immunoreactive nerve fibers compared to VIP- and TH-immunoreactive nerve fibers. The topographical distribution of VACHT-immunoreactivity appears to be similar to that of NOS. Nevertheless, several studies were conducted in which they used semiquantitative techniques to measure the nerve densities of acetylcholine esterase (AchE)- (Hara et al., 1985), TH- (Jacobowitz et al., 1987), VIP- (Hara et al., 1985; Kobayashi et al., 1981) and nicotinamide adenine dinucleotide phosphate (NADPH)- containing nerve fibers (Nozaki et al., 1993; Suzuki et al., 1994). They demonstrated that the distribution of NADPH revealed a similar pattern to that of VIP and the distribution of AChE– containing nerves appeared to be similar to VIP. However, AchE is not a specific marker for cholinergic nerves, since it has also been found in adrenergic and sensory nerves (Chubb et al., 1980). Moreover, quantitative measurements as done in this study, will give a more accurate overview of the density of the perivascular nerves in cerebral arteries.

Within the pterygopalatine ganglion colocalization has been found for NOS and VIP, NOS and ChAT and for VIP and ChAT in different species (Nozaki et al., 1993; González et al., 1997; Kimura et al., 1997; Yu et al., 1998). Colocalization of these transmitters has also been found in the perivascular nerves except for ChAT and VIP. Yu and colleagues (1998) suggested that ChAT and VIP, which are localized within the same cell body, could distribute differently and independently at the terminal level. However, it has been shown that ChAT immunoreactivity is present in cholinergic superficial nerve fibers and in cell bodies, while VACHT, a functional acetylcholine transporter, is especially present in nerve terminals (Weihe et al., 1996; Schäfer et al., 1998). This makes VACHT a more reliable marker to study colocalization with VIP in the perivascular nerves and to determine whether there is a different distribution at the terminal level between VIP and acetylcholine.

**Functional considerations**

In this study we demonstrated that peripheral induced anosmia leads to a decreased density in PGP 9.5- (16% decline), NOS- (27% decline) and VACHT- (39% decline) containing nerves in the anterior cerebral artery, whereas there was
no difference in nerve density between controls and anosmic animals for VIP- and TH- containing nerve fibers. The question is, how in our model the presumed changes in flow following metabolic changes lead to a decreased nerve density. We propose a role for neurotrophic factors. The neurotrophic factors responsible for parasympathetic neuronal survival and outgrowth are not well defined. Nevertheless, neurturin (NRTN) a member of the glial cell-derived neurotrophic factor (GDNF), signaling via the GDNF family receptor α2 (GFRα2), has been identified as a target derived factor for parasympathetic neurons (Rossi et al., 2000; Laurikainen et al., 2000a; Laurikainen et al., 2000b). It has been shown that mice lacking GFRα2 have a significant decrease in NOS containing nerves in the dorsal penile and cavernous nerves (Laurikainen et al., 2000a). However, in the duodenum of GFRα2 deficient mice no difference in NADPH- diaphorase staining was observed (Rossi et al., 1999). Whereas, GFRα2 deficient mice as well as mice lacking NRTN have a reduced density of AChE-positive fibers in the myenteric plexus of the duodenum but the VIP positive fibers appeared not to be affected (Rossi et al., 1999; Heuckeroth et al., 1999). Therefore, the decrease in NOS- and VACHT containing nerves in the present study could be due to a decreased availability of NRTN/GFRα2. Since, the density of VIP-immunoreactive nerves were equal in both groups, this could be due to the fact that VIP does not need NRTN/GFRα2 for survival. Moreover, it has been shown that a majority of neurotrophin-3 (NT3) immunoreactive neurons contained VIP in the ganglion cells of the rat small intestine (De Giorgio et al., 2000). Whether some of the VIP containing neurons in the pterygopalatine ganglion also contain NT3 has to be elucidated. However, it has been shown that nerve growth factor (a member of the neurotrophin family), is expressed within a majority of pterygopalatine neurons (Hasan and Smith, 2000). Therefore, it seems likely that the parasympathetic neurons expressing NOS, VIP and or VACHT are sustained by different neurotrophic factors i.e. NOS and VACHT requires NRTN/GFRα2 for signaling and VIP requires NT3 for signaling.
On a more functional level, the long-term decrease in density of parasympathetic fibers in cerebral arteries following a decrease in metabolic demand of a brain region supplied by these arteries, suggests a role for these fibers in coupling metabolism and CBF. This coupling has thus far been ascribed to chemical and metabolic regulation mechanisms at the level of small arteries and arterioles (Gotoh and Tanaka, 1988). Based on the present results, we speculate that involvement of large arteries is a prerequisite for the occurrence of flow changes in the small vessels. The importance of this involvement of large arteries may be to prevent vascular steal in which another brain area supplied by the same artery suffers from increases of flow to the active brain region (Faraci and Heistad, 1990). For this phenomenon we ascribe a role to the dilatory parasympathetic system, which may explain why the density of TH-containing nerves remained unaltered in the present experiment. It has been shown that NO and not ACh or VIP mediates the major component of the neurogenic vasodilatation. Support for this comes from studies in which inhibition of NOS affected cerebral blood flow in the same way as did denervation (Nozaki et al., 1993; Minami et al., 1994; Talman and Dragon, 1995; Talman and Dragon, 2000). Furthermore, based on pharmacological studies in which they showed that transmural nerve stimulation elicited neurogenic vasodilatation, which is not blocked by atropine but is blocked by inhibitors of NO synthesis indicating that NO mediates the predominant neurogenic vasodilator response in cerebral arteries (Lee, 1980; Lee and Sarwinski, 1991).

In conclusion, our results show that the cerebral arteries are supplied with numerous parasympathetic and sympathetic nerve fibers and that the cholinergic- and nitrergic nerve fibers are subjected to changes whenever there is a chronic change in metabolic demand. Whether these changes in nerve density are due to changes in blood flow and changes in availability in growth factors needs to be elucidated in further experiments.