Roloff, E. V. L., Tomiak-Baquero, A. M., Kasparov, S., & Paton, J. F. R. (2016). Parasympathetic innervation of vertebrobasilar arteries: is this a potential clinical target? *Journal of Physiology*, *594*(22), 6463-6485. https://doi.org/10.1113/JP272450

Parasympathetic innervation of vertebro-basilar arteries: is this a potential clinical target?

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Running title: Vertebro-basilar artery innervation

Size: approximately 10 pages including figures, ~6988 words.

Summary

This review aims to summarise the contemporary evidence for the presence and function of the parasympathetic innervation of the cerebral circulation with emphasis on the vertebral and basilar arteries (the posterior cerebral circulation). We consider whether the parasympathetic innervation of blood vessels could be used as a means to increase cerebral blood flow. This may have clinical implications for pathologies associated with cerebral hypoperfusion such as for stroke, dementia and hypertension. Relative to the anterior cerebral circulation little is known of the origins and neurochemical phenotypes of the parasympathetic innervation of the vertebrobasilar arteries. These vessels normally provide blood flow to the brainstem and cerebellum, but can via the Circle of Willis upon stenosis of the internal carotid arteries supply blood to the anterior cerebral circulation too. We review the multiple types of parasympathetic fibres and their distinct transmitter mechanisms and how these vary with age, disease and species. We highlight the importance of parasympathetic fibres for mediating the vasodilatory response to sympathetic activation. Current trials are investigating the possibility of electrically stimulating the postganglionic parasympathetic ganglia to improve cerebal blood flow to reduce the penumbra following stroke. We conclude that although there are substantial gaps in our understanding of the origins of parasympathetic innervation of the vertebrobasilar arteries, activation of this system under some conditions might bring therapeutic benefits.

List of abbreviations

7-NI	7-nitroindazole
ACA	anterior cerebral artery
AChE	acetyl choline esterase
AcoA	anterior communicating artery
ADMA	asymmetric dimethylarginin
BBB	blood brain barrier
CBF	cerebral blood flow
CGRP	calcitonin gene related peptide
ChAT	choline-acetyl-transferase
CMG	carotid mini-ganglia
DBH	dopamine β-hydroxylase
ECA	external carotid artery
eNOS	endothelial nitric oxide synthase
ICA	internal carotid artery
iNOS	induceable nitric oxide synthase
L-NA	N^G-nitro-L-arginine
NAChR	nicotinic acetylcholine receptor
NAPDH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPR-C	natriuretic peptide clearance receptor
NPY	neuropeptide Y
PACAP	pituitary adenylate cyclase-activating peptide
PCA	posterior cerebral artery
PCoA	posterior communicating artery
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PHI	peptide histidine isoleucine
PPG	pterygopalatine ganglia
RT-PCR	real time polymerase chain reaction
SHR	spontaneously hypertensive rat
SMC	smooth muscle cell
SP	substance P
SPG	sphenopalatine ganglia
SSN	superior salivatory nucleus
TH	tyrosine hydroxylase
VAChT	vesicular acetyl choline transferase
VIP	vasoactive intestinal peptide
VPAC	VIP receptor
WGA-HRP	wheat germ agglutinin-horse radish peroxidase

Introduction

Innervation of the cerebral vasculature was described by Willis as early as 1664, but not until advances in histochemical and immunofluorescent techniques and electrophysiological and pharmacological investigations in the 20th century, did we begin to learn about its origins and function. Innervation of the anterior cerebral circulation, which supply the cerebral hemispheres and deliver the majority of blood to the brain, has been described in detail in several reviews (Purves, 1972); Dahl (1973); (Bevan *et al.*, 1981; Edvinsson, 1987; Bleys & Cowen, 2001; Gulbenkian *et al.*, 2001; Lee *et al.*, 2001; Hamel, 2006; Owman, 2011; Goadsby, 2013). Our aim is to provide an up to date account of the parasympathetic innervation of the posterior vessels (e.g vertebral and basilar), which have received far less attention and whose normal physiological function is still largely unknown.

Clinically, there is a strong interest in cerebral blood flow regulation because of the devastating personal and socio-economic consequences of long term hypo-perfusion and stroke. As the brainstem controls the most essential brain functions for life, the consequences of reduced blood flow in this part of the brain are devastating. Thus, understanding the control of blood flow to the posterior circulation of the brain is critically important and any vasodilating mechanisms highly relevant clinically as it may provide a novel therapeutic target. In the context of ischemic stroke, the parasympathetic innervation to the vertebral and basilar arteries that supply blood flow to the brainstem (and cerebellum) are of great interest and have not been reviewed systematically.

Gross anatomy of cerebral arteries

The brain is one of the most metabolic demanding tissues in the body and it is not surprising therefore that the main cerebral arteries possess several specialisations that make them different from other arteries in the body enabling them to meet the high demand on blood flow. Their organisation is unique such that multiple feed arteries are interconnected with anastomoses forming a ring like structure: the Circle of Willis (Fig 1). The unique blood vessel organisation of the Circle of Willis acts as a distribution centre permitting blood to flow in any direction to meet increased demand and overcome stenosis. However, under normal circumstances blood flow distribution is normally well demarcated with little mixing from the different feeder arteries (McDonald & Potter, 1951). Thus, in the human and rat cerebral blood flow is mainly provided by the internal carotid arteries (ICA) whereas the vertebral and spinal arteries supply the brainstem.

-Insert Fig 1 here-

(human angiogram and schematic of cerebral circulation)

The Circle of Willis is formed from several inter-connecting arteries: including the posterior cerebral artery (PCA), posterior communicating artery (PCoA), anterior cerebral artery (ACA) and anterior communicating artery (AcoA); the latter does not exist in rats as the ACAs fuse to form the azygos anterior cerebral artery (Scremin, 2004). There is considerable variation to this organisation both inter- (Daniel *et al.*, 1953; McFarland *et al.*, 1979) and intra-species (Alpers *et al.*, 1959; Brown, 1966; Frąckowiak & Jakubowski, 2008), as seen in Figure 2. In humans, monkeys and rodents the ICAs supply blood to the Circle of Willis with minimal contribution from the external carotid artery (ECA). In most other mammals including cats, dogs, pigs and sheep, a large proportion of brain blood flow originates from the external

carotid artery via a rete mirabili[1] before joining the Circle of Willis with either no or a small contribution from the internal carotids (Daniel *et al.*, 1953). In humans, primates, rabbits and rats there is no rete.

-Insert Fig 2 here-

(Species variation in cerebral artery organisation)

A unique feature of cerebral arteries is that they are both conduit and resistance vessels and unlike other arteries constrict and dilate (Faraci & Heistad, 1990). Constriction can increase arterial pressure by ~40% in human (Dickinson & Paton, 2012) and rat (Baumbach & Heistad, 1983). This ability protects the brain from sudden rises in BP to prevent stroke (Bill & Linder, 1976; Edvinsson *et al.*, 1976a; Beausang-Linder & Bill, 1981; Sadoshima *et al.*, 1981), whereas vasodilation can protect against ischemia (Santizo *et al.*, 2000; Levi *et al.*, 2012). During constriction maintenance of blood flow to the brain then becomes dependent on compensatory vasodilatation in parenchymal arterioles (Baumbach & Heistad, 1983).

-Insert Figure 3 here-

(Arterial contribution to cerebral blood supply in different species)

[1] A rete mirabili is a complex of arteries and veins in close proximity and built on a counter-current principle, which creates a gradient that enables exchange of heat, ions or gas between the vessels. The function of the rete mirabili varies between species and is controversial in others. Barnett CH & Marsden CD. (1961). Functions of the mammalian carotid rete mirabile. *Nature* **191,** 88-89. It has been suggested it provide means to regulate the temperature of blood flowing to the brain in dogs, cats, sheep and goats. Baker MA. (1982). Brain cooling in endotherms in heat and exercise. *Annual review of physiology* **44,** 85-85. In the dolphin an extensive rete provide means of pressure dampening of BP in the brain. Nagel EL, Morgane PJ, McFarland WL & Galllano RE. (1968). Rete mirabile of dolphin: its pressure-damping effect on cerebral circulation. *Science* **161,** 898-900. A similar function has been attributed to the rete mirabile in the giraffe. Lawrence WE & Rewell RE. (1948). The cerebral blood supply in the giraffidae. *Proceedings of the Zoological Society of London* **118,** 202-212..

Neural innervation of major cerebral vessels

There are two sources of innervation controlling blood flow to cerebral vessels: extrinsic and intrinsic (Hamel, 2006). 'Intrinsic' innervation originating from local neurons within the CNS targeting arterioles to control blood flow in the brain parenchyma. Our focus is on the 'extrinsic' innervation from peripheral ganglia that innervate all major cerebral arteries up to the Virchow-Robin space[2], that is, before they enter the brain parenchyma.

Extrinsic innervation consists of the sensory system and efferent motor-fibres from the autonomic (sympathetic and parasympathetic) nervous system. Numerous proteins have been associated with functionally different subsets of nerve fibres that can be detected using immunocytochemistry. Examples relevant to the cerebral circulation include: acetyl cholinesterase (AChE), vesicular acetyl choline transferase (VAChT) and vasoactive intestinal peptide (VIP) within parasympathetic fibres; tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and neuropeptide Y (NPY) in sympathetic nerves, and calcitonin gene related peptide (CGRP) as a marker of sensory afferents. In basilar arteries from human cadaveric foetuses both noradrenergic and AChE containing fibres first appear around the 12th gestational week whereas NPY and VIP synchronously appeared a month later at 16th gestational week (Kawamura & Takebayashi, 1994). Interestingly, with aging, there was a *decrease* in the expression of vasoconstrictor neurotransmitters (e.g. TH) in cerebrovascular nerves, whereas the expression of vasodilator neurotransmitters (VIP and CGRP) in

[2] Virchow Robin spaces are perivascular spaces or canals surrounding arteries and veins at the point where they leave the subarachnoid on the surface of brain and turn into the parenchyma. See figure in Hamel E. (2006). Perivascular nerves and the regulation of cerebrovascular tone. *Journal of applied physiology* **100,** 1059-1064.

perivascular nerve fibres supplying the rat cerebral arteries *increased* with age (Mione *et al.*, 1988).

Stimulation of sympathetic nerves is generally considered to result in vasoconstriction (D'Alecy & Feigl, 1972; Lee *et al.*, 1976; Baumbach & Heistad, 1983), whereas stimulation of parasympathetic nerves is vasodilatory (D'Alecy & Rose, 1977; Talman *et al.*, 2007). As we shall see later, there are exceptions to this rule. Sensory neurons are a heterogeneous population of either fast transmitting Aδ fibres (being purely afferent) or slowly conducting capsaicin sensitive fibres expressing a range of co-transmitters. Exceptionally, these fibres can also affect cerebrovascular tone due to their additional efferent role; these fibres are referred to as "sensory-motor nerves"(Rubino & Burnstock, 1996) and contain, for example, substance P (SP) and calcitonin gene related peptide (CGRP), a very potent vasodilator.

Extrinsic nerve plexuses and bundles are located on the periphery of the adventitia, mostly run in the longitudinal direction along the vessel and have a smooth ('string like') appearance, whereas single fibres and terminals tend to be located in the deeper layers of the adventitia, running circumferential/perpendicular to the vessel and close to the smooth muscle cells (SMC) (Bleys *et al.*, 1996b; Taktakishvili *et al.*, 2010). The fine terminal nerve fibres are characterised by varicosities and have a 'pearl-on-a string' appearance (Yoshida *et al.*, 1993). These structures contain the highest vesicle numbers and neurotransmitter contents. Autonomic and sensory nerve fibres can be in close proximity to smooth muscle cells (80-100nm) and to each other (Iwayama *et al.*, 1970; Matsuyama *et al.*, 1985). However, on basilar arteries close contacts between varicosities and smooth muscle cells are sparse and distances of 1-2µm are typical (Luff & McLachlan, 1989; Lee *et al.*, 1990). The

functional significance of this is unclear but it may reduce efficacy of transmission in these structures.

The parasympathetic innervation of posterior cerebral vessels

Cerebral arteries are richly innervated with neuronal fibres of parasympathetic origin providing a most powerful vasodilatatory mechanism (Goadsby, 2013) . Parasympathetic fibres follow the sympathetic nerves along cerebral vessels. In comparison to peripheral arteries (femoral and common carotid), where parasympathetic innervation is negligible, it is generally 10-40 times higher in cerebral arteries (Duckles, 1981). The density of innervation is both species and cerebral artery location dependent. Using vasoactive intestinal peptide (VIP) immunopositive fibres as a marker for parasympathetic innervation, relatively low densities have been observed in mouse, rabbit and monkey, with intermediate levels in dog and guinea pig, whereas pig, rat and bat exhibited high levels (Edvinsson *et al.*, 1980; Ando, 1988). In general, fibre densities are greater in the rostral part of the cerebral circulation, than in the caudal (Edvinsson *et al.*, 1980; Kobayashi *et al.*, 1983; Hara & Weir, 1986; Hara & Kobayashi, 1987). The exception is the bat, demonstrating dense VIP positive innervation of the vertebrobasilar arteries (Ando, 1988), In some animals (dogs, rabbits), either no or low parasympathetic innervation of the vertebrobasilar arteries has been reported (Florence & Bevan, 1979; Edvinsson *et al.*, 1980; Saito *et al.*, 1985). However, dogs have NOS positive fibre staining on vertebrobasilar arteries (Yoshida *et al.*, 1993) that was shown not to be sensory, suggesting the presence of some ‘parasympathetic’ fibres.

Types of parasympathetic fibres innervating posterior cerebral vessels

Classically, peripheral parasympathetic nerves are considered to be cholinergic, i.e. using acetylcholine (ACh) as a transmitter. However, it is clear that parasympathetic cells innervating cerebral arteries utilise a host of co-transmitters in addition or in preference to ACh. Some parasympathetic neurones in the cerebral arteries have lost the ability to produce ACh – thus giving rise to the term 'non-cholinergic parasympathetic innervation' (Minami *et al.*, 1994).

Nitric oxide (NO), vasoactive intestinal peptide (VIP), and/or pituitary adenylate cyclase-activating peptide (PACAP) have all been identified as co-transmitters in parasympathetic neurones and all are potent vasodilators (Bevan *et al.*, 1984; McCulloch *et al.*, 1986; Warren *et al.*, 1991; Nozaki *et al.*, 1993; Uddman *et al.*, 1999) producing greater vasodilatation than ACh itself (Lee, 2000). Reviewing the evidence of vasodilation in large cerebral arteries, Lee (2002) concluded that the main effector for vasodilation is NO, with the other neurotransmitters, including ACh, acting as presynaptic modulators of NO release. These findings explained the initial confusion that existed after cholinergic nerves had been identified on cerebral arteries, but were found to be resistant to atropine/physostigmine vasodilation, leading to the misconception that these nerves were non-functional (Lee *et al.*, 1978; Lee, 1980, 1982). Thus, parasympathetic neurons innervating the posterior cerebral arteries consist of a heterogenous population of ACh-VIP-NO-, ACh-NO- and VIP-NO-positive neurons - but this list is not exhaustive (Minami *et al.*, 1994; Kimura *et al.*, 1997; Yu *et al.*, 1998). It is important to note, that NO and PACAP are also co-expressed in sensory neurones on cerebral arteries (Sundler *et al.*, 1996). Therefore, immunocytochemical labelling of parasympathetic neurones is challenging and requires co-labelling.

Tract tracing studies of parasympathetic input to the cerebral arteries have employed a variety of antibodies including cholinergic markers: choline-acetyl-transferase (ChAT) or the vesicular-acetylcholine-transporter (VAChT), or VIP and the NO producing enzymes: NO synthetase (NOS) or NAPDH-diaphorase. Earlier immunohistochemical studies also used acetyl choline esterase (AChE)[3], though this enzyme is less specific. AChE degrades ACh and was one of the first available cholinergic markers; however, it is not exclusive to cholinergic neurones and has been considered as a pan neuronal marker (Bleys *et al.*, 1996b).

The relative distribution of the different markers (ChAT, VAChT, NOS and VIP) and proportion of neurones expressing them has not been fully elucidated for the vertebrobasilar arteries and reports vary greatly partly due to species differences. ChAT has proven capricious in the periphery and in fine fibres due to low enzyme levels and hence its use underestimates the total cholinergic innervation (Schäfer *et al.*, 1998; Bleys *et al.*, 2001).

Origin of parasympathetic fibres to the posterior cerebral arteries

The origin of the parasympathetic innervation is characterised by a diffuse collection of small extra-cranial ganglia and small cell groups in both the arachnoid and at the base of the skull.

-Insert Figure 4 here-

(Overview of Parasympathetic innervation)

3 Acetylcholine esterase is an enzyme involved in the breakdown of ACh in the synaptic cleft. This was one of the first available cholinergic associated markers. In addition to cholinergic cells it also stains many adrenergic and sensory nerves (Bleys RL, Groen GJ & Hommersom RF. (1996a). Neural connections in and around the cavernous sinus in rat, with special reference to cerebrovascular innervation. *The Journal of comparative neurology* **369,** 277-291..

The anterior circulation is innervated by fibres from the pterygopalatine ganglia (PPG), also referred to as the sphenopalatine ganglia (SPG), the cavernous sinus, the otic and the internal carotid mini-ganglia (CMG) (Chorobski & Penfield, 1932; Kobayashi *et al.*, 1983; Carvalho, 1985; Keller *et al.*, 1985; Hara & Weir, 1986; Walters *et al.*, 1986; Hara & Kobayashi, 1987; Suzuki *et al.*, 1988; Edvinsson *et al.*, 1989; Hara *et al.*, 1989; Hardebo *et al.*, 1991; Suzuki & Hardebo, 1991; Nakai *et al.*, 1993; Suzuki & Hardebo, 1993; Bleys *et al.*, 1996a; Toda *et al.*, 2000a; Toda *et al.*, 2000b; Bleys *et al.*, 2001). The origin of parasympathetic innervation to the vertebrobasilar arteries is less well understood. In monkeys, PPG fibres appeared to be the source of NOS positive fibres to the anterior but not posterior cerebral arteries (Ayajiki *et al.*, 2012) and in rats parasympathetic VIP- (Hara *et al.*, 1989) and NOS- positive fibres (Nozaki *et al.*, 1993) were found on caudal basilar and vertebral arteries following PPG extirpation, suggesting additional sources. Injecting the anterograde tracer wheat germ agglutinin-horse radish peroxidase (WGA-HRP) into the PPG, only stained parasympathetic fibres at the anterior end of the basilar artery (Hara *et al.*, 1993). Similar injections to the otic ganglia did not result in labelling on the vertebrobasilar arteries in rats (Shimizu, 1994). However, using retrograde tract tracing with fluoro-gold applied to the middle portion of the basilar artery resulted in neurone labelling in both the otic ganglia and PPG (Kadota *et al.*, 1996). Labelling in the PPG was also seen following WGA-HRP application to the middle and caudal part of the BAs in cats (Keller *et al.*, 1985). Bleys and Cowen (2001) suggested that PPG, otic and CMG innervation is restricted to the anterior end of the basilar artery with no observable innervation to the caudal basilar and vertebral arteries. Gibbins *et al.* (1984a) lists otic, PPG, submandibular and sublingual ganglia as possible contributions to rostral basilar artery VIP containing fibres. The inconsistencies may be explained by the differences in techniques and species used. Studies

where NOS immunocytochemistry has been performed will not be able to delineate between parasympathetic and sensory nerves. Hence, many of the suggested sources of parasympathetic innervation to the posterior cerebral arteries based on NOS immunochemistry, which included the glossopharygeal and vagal nerves, and upper cervical root ganglia (Suzuki *et al.*, 1988; Hardebo *et al.*, 1991), are all likely to be of sensory origin (Arbab *et al.*, 1986).

Despite evidence for parasympathetic innervation of vertebrobasilar arteries, and the existence of: VAChT (E. Roloff & JFR Paton, personal observation), ChAT and VIP positive fibres in rat (Suzuki *et al.*, 1988; Suzuki *et al.*, 1990c), cat (Gibbins *et al.*, 1984b) and dog (Seki *et al.*, 1995), we have found sparse information regarding the source(s) of these specific parasympathetic inputs. This clearly needs further clarification.

Pre-ganglionic parasympathetic neurones destined for the cerebral vasculature

The location of preganglionic neurones projecting to the PPG of rats has been determined by retrograde transneuronal pseudorabies virus labelling (Spencer *et al.*, 1990). Neuronal labelling was obtained in the ipsilateral superior salivatory nucleus (SSN). Pharmacological stimulation of the greater petrosal nerve cell group within the SSN reduces ipsilateral cerebral vascular resistance. The effect can be countered by PPG section but not with systemic muscarinic antagonism; another confirmation that the vasodilatory mechanism (via the PPG) is mediated by a non-cholinergic mechanism in the cerebral arteries (Nakai *et al.*, 1993). The following nuclei were also labelled and most likely upstream of the SSN: the nucleus tractus solitarii and the dorso medial part of the spinal trigeminal nucleus and giganto cellular reticular nucleus as well as pontine structures of the parabrachial nucleus,

A5 catecholaminergic cells, and sub coeruleus region of the pons. Midbrain areas included the lateral, dorsomedial and paraventricular hypothalamic nuclei, lateral preoptic area. The bed nucleus of the stria terminalis, substantia innominata and the amygdalopiriform were also labelled in the forebrain. Given this extensive convergent connectivity it underpins the importance of the vasodilatory role of the SSN and those regions that may influence it. There is scant information regarding sources of input to the other parasympathetic ganglia innervating cerebral arteries. Gibbins et al. (1984) and Gibbins & Morris (1988) reported the SSN was also a source of input to the otic ganglia and CMG.

Functional role of the parasympathetic transmitters and receptors

The transmitters and receptors are presented in order of importance as vasodilatory mechanisms.

(i) Nitrergic: NO is the main vasodilator in the cerebral arteries (Lee, 2002). It has an estimated diffusion range of up to 300µm and an estimated half-life of 5-15s in the cerebral circulation (Ignarro, 1991; Garthwaite & Boulton, 1995). Nerve fibres with NO production capabilities on cerebral arteries include pure nitrergic, VIPergic and/or cholinergic parasympathetic neurones as well as sensory neurones (Minami *et al.*, 1994; Kimura *et al.*, 1997; Edvinsson *et al.*, 2001). Note that there are multiple additional sources of NO that could produce vasodilation in the vertebrobasilar arteries such as endothelial cells, astrocytes, neurones (Bredt *et al.*, 1990; Faraci & Brian, 1994) and immune cells (Hibbs Jr *et al.*, 1988; Bogdan, 2001). The distribution of NO containing fibres in the cerebral arteries is highly variable in terms of exact location on the cerebral arteries and species. In rats, although present on the basilar artery, the densest NOS/NADPH-diaphorase positive fibre

staining occurred on the anterior part of the cerebral circulation, whereas in humans NOS immuno-reactivity was predominantly on posterior cerebral arteries, especially the basilar artery (Iadecola *et al.*, 1993; Nozaki *et al.*, 1993; Minami *et al.*, 1994; Edvinsson *et al.*, 2001; Taktakishvili *et al.*, 2010).

(ii) Cholinergic: Cholinergic receptors have been located on endothelial cells, smooth muscle cells, and perivascular nerves on cerebral vessels (see Table 1). Electrical or pharmacological stimulation of cholinergic fibres on cerebral arteries can, depending on the species, the site of action, receptor subtype and concentration of ACh released, induce either vasodilatation or vasoconstriction (Edvinsson *et al.*, 1977; Duckles, 1981; Miao & Lee, 1991; Dauphin & Mackenzie, 1995; Toda *et al.*, 1997). In cats and dogs, low concentrations of ACh (10^{-6} to 10^{-5} M) applied to cerebral arteries including the basilar caused vasodilation, whereas higher concentrations result in vasoconstriction (Tsukahara *et al.*, 1986; Dauphin & Hamel, 1992). In humans, however, only vasodilation was observed (Edvinsson *et al.*, 1976b; Tsukahara *et al.*, 1986). ACh can exert its effects via either metabotropic muscarinic (M-) or ionotropic nicotinic (N-type) receptors.

(a). Muscarinic receptors: Four sub types of muscarinic and one nicotinic receptor have been found in on cerebral arteries. Using isolated vessel preparations, Furchgott and Zawadzki (1980) demonstrated that the vasodilatory effect of ACh is endothelium-dependent. ACh acts on muscarinic receptors expressed on endothelial cells that stimulate endothelial NO-synthase activity(eNOS), releasing NO, which then triggers vasodilatation (Figure 6; (Wahl & Schilling, 1993)).

The endogenous origin of ACh for this mechanism is unclear, as it is rapidly broken down by AChE in the blood. Release from perivascular nerves is a possibility, as tritiated ACh applied to the outside of intact vessels readily crossed the media and appeared in the luminal perfusion fluid (Gonzalez *et al.* (1997). An endothelial origin has also been suggested, as endothelial cells contain ChAT and can produce and release ACh (Parnavelas *et al.*, 1985; Kawashima *et al.*, 1990), but what controls this is unclear.

-Insert Table 1 here-

(ACh receptors)

It is likely that muscarinic M_3 receptors located on endothelial cells mediates the NO induced vasodilatation, at least in rabbits (Garcia-Villalon *et al.*, 1991), cats (Dauphin & Hamel, 1990) and human (Dauphin & Hamel, 1992). In the absence of endothelium, ACh (exogenously applied or released by transmural stimulation) induces vasoconstriction via M_1 receptors located on the vascular smooth muscle cells (VSM). M_1 receptors have been identified in VSM of cerebral arteries including basilar arteries of rats (Phillips *et al.*, 1997) and in numerous other species including cat (Dauphin *et al.*, 1991), pig (Garcia-Villalon *et al.*, 1991) and human (Dauphin & Hamel, 1992). Since M_1 receptors have a lower affinity for ACh than M_3 receptors, higher concentrations of ACh are required for their activation and this may provide a mechanism for the differential vasomotor actions of ACh. From a study of pial vessels in mice (Shimizu *et al.*, 1993), there is evidence that the M_1 receptor mechanism is uncoupled/non- functional in the presence of a functional endothelial M_3 receptor, explaining the apparent dominance of M_3 receptor induced vasodilation.

Pre-junctional M_2 receptors are located on nitrergic perivascular neurones and attenuate the vasodilation (or increase vasoconstriction) by inhibiting NO production and release. This

has been found in several species (pig, cat, monkey) and in various locations along the posterior (and anterior) cerebral arteries (van Charldorp & van Zwieten, 1989; Dauphin & Hamel, 1992; Phillips *et al.*, 1997; Toda *et al.*, 1997; Liu & Lee, 1999). In contrast, there is a total absence of M_2 receptors in human cerebral arteries (Dauphin & Hamel, 1992), explaining the absence of ACh induced contraction in man mentioned above.

The M_5 receptor was the last muscarinic receptor to be identified in cerebral arteries and as with M_1 and M_3 receptors it is post-synaptically located. The receptor was found on both smooth muscle cells and endothelium (Phillips *et al.*, 1997; Tayebati *et al.*, 2003). The mRNA content for M_5 in rat basilar artery was found to be equal to that of M_2 and M_3 receptors, and greater than M_1 receptor mRNA (Phillips *et al.*, 1997). Due to the lack of selective M_5 muscarinic receptor ligands, little is known of the functional significance of this receptor on cerebral arteries. To circumvent this problem, Yamada *et al.* (2001b) created a M_5 receptor knock out mouse, in which large cerebral arteries and arterioles did not dilate following topical application of acetylcholine (both in *in vivo* and in organ bath preparation). The effect was specific to the cerebral blood vessels, as peripheral vessels retained the ability to dilate. It has been suggested that some of the effects previously ascribed to the M_3 receptor may be mediated via the M_5 receptor (Elhusseiny & Hamel, 2000).

(b) Nicotinic ACh receptors: There is a dearth of evidence supporting a major role for nicotinic receptors on vertebrobasilar arteries. Though Dauphin and Hamel (1992) did not find nicotinic receptors in cat or human cerebral arteries, Edvinsson *et al.* (1977) found evidence of the α7-NACh receptor on adrenergic nerves of cat middle cerebral arteries. As

its function is relevant to modulation of adrenergic signalling, it will be described in the 'cross talk' section (see below).

(iii) Vasoactive Intestinal Peptide. VIP has been found to co-localise with ChAT in some parasympathetic nerves and ganglia innervating the cerebral vasculature (Gibbins & Morris, 1988; Suzuki *et al.*, 1990a; Hardebo *et al.*, 1992; Uddman *et al.*, 1999) including the basilar artery (Miao & Lee, 1990; Saito et al. 1985). In basilar arteries from cats, only 5% co-localisation between VIP and ChAT positive fibres was found despite these running closely to each other (Miao & Lee, 1990). The density of VIP innervation of cerebral arteries is species dependent with highest densities in the cat, rat and rabbit (Duckles & Said, 1982; Gibbins *et al.*, 1984b; Zhang *et al.*, 1991) and relatively sparse in human (Edvinsson *et al.*, 1987; Edvinsson *et al.*, 1994b). This confers with a study by White (1987) in which 87% of human basilar arteries pre-contracted with prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) displayed a sustained concentration dependent vasodilatation when exposed to VIP. Despite the lack of VIP positive fibre staining on vertebral and basilar arteries in dogs (Edvinsson *et al.*, 1980), the vascular conductance of vertebral arteries was increased by 100% on exposure to VIP (Blitz & Charbon, 1983). These data emphasise the importance of this peptide in mediating vasodilatation of vertebrobasilar arteries but the neural source remains to be ratified.

There are two VIP receptors with similar affinity for VIP: VIP_1 ($VPAC_1$) and VIP_2 ($VPAC_2$). In addition to these, VIP can also act via the natriuretic peptide clearance receptor (NPR-C) (Akiho *et al.*, 1995; Murthy *et al.*, 2000; Grant *et al.*, 2005). Immunohistochemical labelling for $VPAC_1$ in rat cerebral arteries found it on the surface of smooth muscle cells in the medial layer only (Fahrenkrug *et al.*, 2000). This was confirmed using *in situ* hybridization

mRNA, where $VPAC_1$ and $VPAC_2$ located into smooth muscles cells in posterior (and anterior) cerebral vessels (Baun *et al.*, 2011). Similar results have been obtained in humans, using RT-PCR against the two VPAC receptors (Knutsson & Edvinsson, 2002). In pig basilar arteries, mRNA is present for $VPAC_1$, $VPAC_2$ and NPR-C. Using immunocytochemistry, $VPAC_1$ was found in the endothelium, $VPAC_2$ on the outer layers of SMCs and endothelium and NPR-C throughout the artery including the perivascular nerves themselves (Grant *et al.*, 2005).

The vasodilatory effect of VIP may or may not involve a NO dependent mechanism. Denuded cerebral arteries from sheep and cow showed blunted VIP induced vasodilation, indicating the presence of a mechanism involving NO of endothelial origin (Gaw *et al.*, 1991; Gonzalez *et al.*, 1997). VIP stimulation of isolated rat basilar arteries results in vasodilation, which is inhibited by the nNOS selective antagonist: 7-nitroindazole (7-NI), indicating involvement NO from perivascular nitrergic nerves (Seebeck et al, 2002). VIP receptors present on SMC, however, induce vasodilation directly, via an NO independent mechanism, probably through the cAMP pathway (Grant *et al.*, 2005).

(iv) Pituitary adenylate cyclase-activating peptide (PACAP) belongs to the same family of glucagon/secretin neuropeptides as VIP and shares 68% amino acid sequence homology. The two co-transmitters are expressed in different subpopulations of parasympathetic neurones. In addition, PACAP is also expressed by both trigeminal sensory neurones and sympathetic neurons making its association with the parasympathetic nervous system equivocal (Sundler *et al.*, 1996). PACAP mRNA is expressed in most otic and PPG cells in the rat (Sundler *et al.*, 1996). PACAP acts through 3 different G protein coupled receptors: the VIP receptors: $VPAC_1$ & $VPAC_2$, and PAC_1, all increase adenylate cyclase activity. There is

evidence of vasodilatory effects of PACAP in rat basilar arteries mediated by nitregic nerves (Seebeck *et al.*, 2002). Although PACAP is up to 100x more potent as a vasodilator than VIP (Kashimoto *et al.*, 1996), PACAP fibre innervation of cerebral arteries is less dense than that of VIP and NO (Warren *et al.*, 1991; Edvinsson *et al.*, 2001). In pre-constricted rat basilar arteries a stronger vasodilatory capacity of VIP than PACAP was found, whereas VIP and PACAP acted similarly in the anterior vessel segments, which may indicate a differential receptor distribution between these arteries (Baun *et al.*, 2011).

(v) Peptide histidine isoleucine. VIP-like neuropeptides such as peptide histidine isoleucine (PHI) in rat and the human equivalent peptide histidine methionine (Linder *et al.*, 1987) are also coexpressed in the VIP population of parasympathetic fibres on basilar arteries and have weaker vasodilatory properties relative to VIP (Suzuki *et al.*, 1984; Uddman *et al.*, 1993).

Cross talk between parasympathetic and sympathetic nerves

Parasympathetic and sympathetic nerves are often intertwined on cerebral arteries (Owman *et al.*, 1974) or run in parallel within the same perineural sheath (Iwayama *et al.*, 1970; Edvinsson *et al.*, 1972a). Axo-axonal contact distances between sympathetic and parasympathetic fibres can be as little as 25 nm, compared to the 100 nm seen for neuro-muscular contacts in the same vessels (Edvinsson *et al.*, 1972a). This close apposition suggests cross talk between parasympathetic and sympathetic nerve fibres.

-Insert Figure 5 here-

(Close apposition of NA and Ach nerves)

Support for interactions between the parasympathetic and sympathetic innervations to cerebral arteries is that they develop simultaneously in both mice and rats (Kobayashi *et al.*, 1981; Tsai *et al.*, 1992) and human: (Kawamura & Takebayashi, 1994). In pre- and postnatal rats, parasympathetic and sympathetic fibres (visualised with VIP and NPY markers, respectively) show similar patterns of development in their distribution, density and pattern (Tsai *et al.*, 1992). This close association in development was not observed for sensory fibres (visualised using CGRP), their development occurs after sympathetic innervation (Tsai *et al.*, 1989). There is not the same degree of close apposition between sensory fibres with either sympathetic or parasympathetic nerves (Tsai *et al.*, 1989; Edvinsson *et al.*, 1994a). Functional studies in basilar arteries from rat and pig have found that NA and ACh have limited vasoreactivity, yet adrenergic nerves express nicotinic α-7 NACh receptors, which upon stimulation, cause increases in intracellular calcium leading to noradrenaline release from their varicosities (Si & Lee, 2001; Si & Lee, 2002). This triggers vasodilatation via activation of β_2-adrenergic receptors; vasoconstriction does not occur as there is paucity of α-adrenoceptors. β_2-adrenoceptors are located on the intertwined acetylcholinergic-nitrergic fibres leading to NOS activation and NO production and stimulation of guanylyl cyclase (Fig 7). Removal of the endothelium does not affect the arteries ability to dilate, indicating the source of NO must be neuronal (Lee, 1980; Toda, 1982). Indeed, NO is stored and released with ACh. ACh acts on nitrergic neurones stimulating prejunctional M_2 receptors and inhibits NO synthesis, thus modulating NO release (Liu *et al.*, 2002). Following unilateral excision of the superior cervical ganglion in rats, NO mediated vasodilation in cerebral arteries was reduced, indicating a dependence on an intact sympathetic innervation (Smith *et al.*, 2002).

-Insert Figure 6 here-

(Mechanism of crosstalk)

Cross talk may have a role in the development and maintenance of autonomic fibres and their phenotype. For example, after sympathectomy: (i) parasympathetic fibres proliferate and make more direct contacts with vascular smooth muscle cells, whereas prior to sympathectomy they mainly contact sympathetic fibres (Smith *et al.*, 2002); (ii) parasympathetic cells can display increased dopamine-β-hydroxylase (DBH) immunoreactivity (though this is functionally irrelevant), and this is matched by a similar decrease in the number of PPG neurones expressing parasympathetic markers (Mione *et al.*, 1991; Fan & Smith, 1993); (iii) both NOS and VIP activity in the PPG is decreased, resulting in a reduction in NOS dependent vasodilation in various target areas (Fan & Smith, 1993; Smith *et al.*, 2002).

In face of increased sympathetic drive as occurring during “fight or flight” situations vertebrobasilar arteries may thus vasodilate (instead of constrict) via the mechanisms described above to ensure the brainstem and or cortex remains well perfused at times when peripheral organs experience vasoconstriction and reduced vascular conductance (Lee *et al.*, 2011).

Other proposed functions of parasympathetic innervation to cerebral arteries *in vivo*

With such a dense parasympathetic innervation of the cerebral arteries it is a natural question to ask: What are the physiological circumstances in which these nerves are activated?? Beside the suggested function in ensuring adequate CBF during periods of high sympathetic activity, what else does it do? The question has not been answered equivocally,

though various clues to its function are appearing: As mentioned earlier activation of the parasympathetic innervation causes vasodilation. Thus it is likely to be involved in any process requiring centrally regulated increases in CBF that are independent of the endothelial NO system. Examples include, increased brain metabolism, thermoregulation (it is essential to keep the brain thermoregulated) and hypercapnia.

Electrical stimulation of the PPG in rats increases cerebral blood flow by 40-50% on the ipsilateral side as measured by Doppler flow over the parietal cortex and tissue PO2 (Seylaz *et al.*, 1988; Talman *et al.*, 2007). In dogs, stimulation of the PPG and the superficial petrosal evoked vasodilatation in the middle cerebral artery, posterior communicating arteries and basilar artery (D'Alecy & Rose, 1977; Toda *et al.*, 2009). Anti-cholinergic drugs fails to attenuate the response, emphasising that the vasodilatory response is mediated via a non-cholinergic mechanism (Nakai *et al.*, 1993).

What evidence is there for vasodilatory tone? Following removal of the PPG in the awake rat Suzuki *et al.* (1990b) reported no change in blood flow, suggesting an absence of tone. However, these data were not confirmed by Boysen *et al.* (2009), who saw ablation of the PPG evoking a 30% decrease in blood flow increasing cerebrovascular resistance (CVR) in rats. Given that the muscarinic antagonist, atropine, does not affect cerebral blood flow, non-cholinergic transmission must underlie this tone (Toda *et al.*, 1993; Yoshida *et al.*, 1993; Toda *et al.*, 2000a). Further evidence of tonic parasympathetic tone in the form of reduced blood flow upon NO inhibition has also been observed in monkeys (Toda *et al.*, 2000b) and dogs (Toda *et al.*, 1993; Yoshida *et al.*, 1993; Toda *et al.*, 2000a), where the vasodilatory responses to nerve stimulation was found to be attenuated by the NO inhibitor N^G-nitro-L-arginine (L-NA). Indeed, NO of neuronal origin has been associated with vasodilation and

decreased cerebral vascular resistance (Toda & Okamura, 2003). In nNOS deficient mice a reduced vasodilator response following stimulation of both efferent and afferent nerves was observed, an effect not seen in eNOS or iNOS deficient mice (Toda *et al.*, 2009). Thus, there appears to be an apparent loss of ACh dependency as primary transmitter in the parasympathetic innervation and transfer to NO mediated vasodilatory processes, which is distinct to the innervation of peripheral arteries.

Parasympathetic innervation from the PPG acting directly or indirectly on nociceptive sensory fibres have also been suggested to participate in regulation of blood brain barrier (BBB) permeability (Delépine & Aubineau, 1997; Yarnitsky *et al.*, 2004a; Yarnitsky *et al.*, 2004b) and development of cluster headaches/migraine (Yarnitsky *et al.*, 2003). Additionally, it is involved in the dilation of cerebral arteries following "breakthrough of autoregulation" in acute hypertension (Agassandian *et al.*, 2003).

Parasympathetic targets with clinical relevance for the cerebral vasculature

The critical importance of the brainstem for life means that its perfusion needs to be tightly regulated. At rest, autonomic tone of the cerebral vessels is small (Heistad & Marcus, 1978; Heistad *et al.*, 1978). However, it is reasonable to assume that any imbalance in the activity of autonomic nerves innervating the posterior cerebral circulation could have pathological implications for blood flow control and be a potential clinical target for various conditions in which cerebral blood flow is impeded, such as stroke or hypertension. The importance of the brainstem is highlighted by the finding that during extreme acute hypotension, cerebral blood flow is prioritised to the brainstem (Mueller *et al.*, 1977). In this regard, we consider how disease is affected by the parasympathetic regulation of the cerebral vasculature and

ways in which modulation of parasympathetic input to the cerebral arteries may be used to alleviate pathology.

During pathological increases of blood pressure above the upper limit of autoregulation, cerebral vessels dilate. Denervation of parasympathetic fibres originating from the PPG dampens this effect suggesting a baroreceptor mediated cerebral vasodilatation in rats (Talman & Dragon, 2000). It was later found that neuronal NOS (nNOS) drove the observed dilatory response; using a specific nNOS inhibitor the breakthrough dilatation that occurs above the autoregulatory threshold was attenuated (Talman *et al.*, 2007). One possibility is that the shift in the autoregulatory curve to the right in hypertension (e.g. as in the SHR) may reflect reduced parasympathetic nitrergic input.

The importance of NO in cerebral vascular regulation is highlighted by the association of the endogenous NOS inhibitor - asymmetric dimethylarginin (ADMA) which is elevated in conditions of hypoperfusion such as stroke and hypertension (Yoo & Lee, 2001; Napoli *et al.*, 2004; Kielstein & Zoccali, 2005). By targeting ADMA availability (Fan *et al.*, 2013) and reducing plasma levels (Fan *et al.*, 2013; Tsai *et al.*, 2014) hypertension is prevented in young SHRs. Furthermore, ADMA infusion at levels that do not affect systemic blood pressure decreased CBF and induces vascular stiffness in healthy volunteers (Kielstein *et al.*, 2006). In rats topical application of ADMA results in basilar vasoconstriction and an inhibition of ACh mediated vasodilatation and in rabbits it has the same effect in cortical vessels (Faraci *et al.*, 1995). Thus, NO in conjunction with parasympathetic innervation plays a major role in maintaining blood flow to the brain in health and disease states. The role of ADMA in ischemic stroke and its potential as a predictive disease marker has been discussed and reviewed elsewhere (Chen *et al.*, 2012).

The endogenous NOS inhibitor monomethylarginine (L-NMMA), has been reported to be present at lower concentrations than ADMA in the circulation (Vallance *et al.*, 1992). Like ADMA, L-NMMA was shown to reduce cortical blood flow when injected into the internal carotid artery without affecting systemic blood pressure (Thompson *et al.*, 1996). When challenged with hypercapnia, the chemoregulatory response normally resulting in increased cerebral blood flow in monkeys, was prevented by prior intracarotid injection of L-NMMA. The same was not true for cerebral autoregulation due to changes in blood pressure (Thompson *et al.*, 1996). This result provides further evidence for the role of NO in control of cerebral blood flow.

Nitric Oxide is synthesised by NOS from L-arginine. The active site of NOS is subject to inhibition by the assymetric methylarginines, ADMA and L-NMMA. These inhibitors could be a good clinical target in conditions where the synthesis or bioavailability of NO is compromised (Leiper & Nandi, 2011). However, it is not specific to nNOS but also work on eNOS.

The finding of markers associated with cardiovascular risk, and possibly autonomic imbalance, such as ADMA (Faraci *et al.*, 1995), could provide other therapeutic pathways with targeted prophylactic medicine. In humans, sulfhydryl ACE inhibitor to reduce blood pressure also restored the NO/ADMA balance in hypertension (Napoli *et al.*, 2004). Similarly, in SHRs 10 week treatment with an ACE-inhibitor not only decreased blood pressure but improved the vasodilatatory response of the basilar artery and blood flow during hypotension as well as the NO-mediated dilatation during topical application of ACh; these data all suggest an improvement in endothelial function of the basilar artery (Toyoda *et al.*,

1998). Furthermore, NO or NO donor administration in stroke models has been shown to reduce lesion size and increase cerebral perfusion (Willmot *et al.*, 2005).

The direct effects of parasympathetic innervation can be seen during a hypotensive challenge in SHRs in which forebrain blood flow was reduced after chronic parasympathetic denervation compared to sham controls (Koketsu *et al.*, 1992). These findings were not corroborated in normotensive Sprague Dawley rats (Branston *et al.*, 1995). The parasympathetic system is involved in vasodilatation at the lower end of autoregulation (Branston *et al.*, 1995). To what extent this is true for the posterior circulation of the brainstem remains to be established. In stroke, parasympathetic denervation exacerbates infarct size following middle cerebral artery occlusion (Kano *et al.*, 1991; Koketsu *et al.*, 1992). This evidence supports a role for parasympathetic nerves in protecting against brain ischemia. In this context, stimulation of the PPG provided protection by increasing CBF and reducing the infarct size following cerebral artery occlusion (Henninger & Fisher, 2007). A reduction in ischemic area as well as protection of blood-brain barrier integrity were found in a model of photothrombotic stroke with PPG stimulation (Levi *et al.*, 2012).

It is worth considering the potential clinical importance of cross talk between sympathetic and parasympathetic branches mentioned above, as it can result in alterations in autonomic regulation in pathological circumstances. For instance stimulation of sympathetic nerves in normotensive rats results in basilar artery vasodilatation and increased blood flow, the same is not true for hypertensive animals or animals with chronic superior cervical ganglion denervation (Chang *et al.*, 2012). The attenuated response is likely to be, at least in part, due to a reduction in the sympathetic to parasympathetic terminal density at the level of

the basilar artery in the hypertensive animals (Lee & Saito, 1986) or a lack of sympathetic input (Chang *et al.*, 2012). Conversely, an overactive sympathetic and underactive parasympathetic nervous system limits basilar artery dilatation in face of a hypotensive challenge (Barry *et al.*, 1982). This may cause ischemic damage especially in conditions where cerebrovascular resistance is high (e.g. SHRs), (Paton *et al.*, 2009; Cates *et al.*, 2011; Cates *et al.*, 2012). Furthermore the limited vasodilatation of the basilar artery (Toyoda *et al.*, 1998) contributes to the shift of the autoregulatory curve to higher BP levels in hypertension (Barry *et al.*, 1982) further increasing the susceptibility of hypoperfusion.

In case of ischemic stroke, stimulation of the parasympathetic nervous system to increase cerebral blood flow and restrict ischemia has clinical potential particularly considering the limiting treatments currently available and the restrictions of their use, as is the case with thrombolysis therapy (Cheyuo *et al.*, 2011). Stroke in the vertebrobasilar circulation is a clinical emergency and accounts for about 20% of all ischemic strokes (Schoen *et al.*, 2011) and has a high morbidity and mortality rate. Evidence shows that parasympathetic input provides some protection from ischemic damage (Kano *et al.*, 1991; Koketsu *et al.*, 1992; Henninger & Fisher, 2007; Cheyuo *et al.*, 2011; Levi *et al.*, 2012). Importantly this can be effective even after 24 hours of stroke onset (Solberg *et al.*, 2008; Levi *et al.*, 2012). The mechanisms of action require further investigation, as does the relevance of this treatment to the vertebrobasilar circulation. A comprehensive review on the potential of harnessing the parasympathetic system as a therapy in stroke has been published recently (Cheyuo *et al.*, 2011). Whether this can also be targeted to assist with vertebrobasilar artery hypoperfusion in neurogenic hypertension (Cates *et al.*, 2012; Marina *et al.*, 2015), for example, remains unknown but holds therapeutic potential. Indeed, the Brainsgate Ischemic

Stroke System comprises an stimulator implanted adjacent to the PPG via the greater palatine canal under local anaesthesia (Khurana *et al.*, 2009). This device is currently undergoing phase 3 trials (Clinical Trials.gov: NCT00826059 & NCT01874093). Another device directed to stimulation of the otic ganglion has also been described (Shalev & Gross, 2010). By extrapolation, once the source(s) of input to the posterior cerebral circulation has been clarified, these nerves/ganglia could be targeted in a similar fashion.

Systemically, activation of the parasympathetic efferent vagus nerve has been shown to inhibit pro-inflammatory cytokine release and decrease the inflammatory response. This function is coined “the cholinergic anti-inflammatory pathway” (Borovikova *et al.*, 2000; Pavlov *et al.*, 2003; Tracey, 2007). Evidence suggest it works via nicotinic α7-acetyl cholinergic receptor stimulation (Wang *et al.*, 2003) and has been reported to have beneficial effects in a plethora of diseases from irritable bowel syndrome to depression (Wang *et al.*, 2004; Rush *et al.*, 2005; van Westerloo *et al.*, 2006; Ghia *et al.*, 2007; van Maanen *et al.*, 2009) as well as improving kidney transplant outcomes (Hoeger *et al.*, 2010). We propose that activation of the parasympathetic pathways innervating the cerebral arteries could also yield a similar anti-inflammatory response. In the anterior circulation this may be relevant for Alzheimer’s disease (de la Torre, 2002; Reale *et al.*, 2004) and for posterior cerebral arteries: hypertension, which has been linked to inflammatory activity in brainstem walls (Waki *et al.*, 2007; Waki *et al.*, 2010; Zubcevic *et al.*, 2011; Waki *et al.*, 2013).

In this regard, both VIP and PACAP have been reported to exert similar anti-inflammatory effects through the VPAC1 receptor, which could be a relevant mechanism for the

parasympathetic innervation of cerebral arteries (Leceta *et al.*, 2000; Martinez *et al.*, 2002; Grimm *et al.*, 2003).

Yarnitsky *et al.* (2005) have shown that electrical stimulation of the PPG can prevent cerebral vascular spasm after subarachnoid haemorrhage and improve blood flow. Acetylcholine (given via intracisternal injection) can counter effects of cerebral vasospasm in basilar arteries after subarachnoid haemorrhage by supressing the production of cytokines (Song *et al.*, 2014). However, it is worth noting that PPG stimulation has also been reported to disrupt the BBB and induce neurogenic inflammation via a reflex involving nociceptive sensory fibres (Delépine & Aubineau, 1997). A similar mechanism has also been ascribed to pain in migraine: Yarnitsky *et al.* (2003) reported that PPG activation during migraine is involved in sensitization of intracranial nociceptors by direct or indirect activation of sensory pain fibres by the release of ACh, VIP and NO. This effect of PPG stimulation has been used clinically to deliver macromolecules through the BBB (Yarnitsky *et al.*, 2004a). Hence the (over-) stimulation of the parasympathetic innervation to the cerebral arteries may not only yield positive effects. Clinical application will require careful adaptation depending on the effect sought.

Finally, and hypothetically, peptide targeting of the cerebral vasculature may also be possible. Using phage display proteins were identified that targeted the endothelial cells of the aorta and heart (Greig *et al.*, 2010). Using such a technique may allow selective targeting of the cerebral arteries and may even differentiate between posterior versus the anterior circulation. Such targeting might allow enhancement of efficacy of vasodilating transmitter substances, for example.

Concluding remarks

Control of blood flow to the brain is highly specialised and regionally specific. Uniquely, resistance is controlled in part by the main supply vessels as well as the microcirculation. We have discussed how the Circle of Willis and the vertebrobasilar circulations have different patterns of nervous innervation from parasympathetic, sympathetic and sensory systems. Because of similar phenotypes, we stress caution as to how to separate parasympathetic from sensory innervations immunocytochemically. Whilst the parasympathetic innervation of the anterior cerebral circulation is well described, this review highlights the need for clarification of the sources of parasympathetic innervation to the posterior cerebral circulation. It is clear that there are multiple sources of parasympathetic innervation and these change with age and in disease states and between species. The distinct sources of origin provides an opportunity for targeted regulation affecting sub-components of the cerebral circulation, which may be relevant to the treatment of stroke. The review highlights the number of transmitters systems used by the parasympathetic nerves innervating the cerebral vessels (e.g. ACh, NO, VIP, PACAP & PHI); this may provide fail safe mechanisms, state-dependent control, and regulation of the magnitude of the vasoactive response, for example. The evidence is unequivocal that there is a strong interdependence between the sympathetic and parasympathetic innervation both developmentally and functionally as they modulate each other. An example is the basilar artery vasodilatation induced by sympathetic nervous stimulation triggered by NO release from parasympathetic terminals. Malfunction of one of these systems will functionally alter the performance of the other. Thus, therapy targeting the parasympathetic nervous system to increase brain blood flow should not only be assured of a functional parasympathetic but also sensory and sympathetic innervation.

Innovative methods are being used to selectively stimulate specific nerves and ganglia innervating components of the cerebral circulation to increase blood flow. Whether specific endothelial markers on aspects of the cerebral circulation can be identified and target to modulate parasympathetic mediated vasodilatation remains a project for future scientific enquiry.

Additional information

Competing interests

The authors have no competing interests to report.

Author contributions

Eva v.L. Roloff: Research, 1st draft of article, contribution to figures and revision; Ana Tomiak-Baquero: Figures, research and contribution to article (sections in 1st draft), revision; Sergey Kasparov: Contributions to concept, grantholder & revision, Julian F.R. Paton: Developer of concept, contribution to main article and major revision, and primary grantholder. All authors approve the final version of the manuscript.

Funding

This article was written with the support of The British Heart Foundation (BHF) RG/12/6/29670

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Figure Legends

Figure 1. Human angiogram and schematic of cerebral circulation.
(A) CT scan showing the anatomy of the cerebral arterial supply in humans. The image was kindly provided by Dr Nathan Manghat from University Hospital Bristol Trust. (B) Labelled schematic representation of (A) including origins of the circulation form the aortic arch, Figure modified from Schuenke *et al.* (2010). Abbreviations: A, anterior; R, right; P, posterior; L, left.

Figure 2. Species variation in cerebral artery architecture.

Schematic representation of the cerebral arterial tree and difference in contribution to anterior and posterior circulation by the ECA and ICA in (A) rats, (B) goat/sheep and (C) rabbit. Notice the presence of the carotid rete in (B) goat/sheep (red arrow), the lack of ICA and the existence of a V-Oa in the same species. CoW and VA labelled for orientation purposes. Abbreviations: APAr, ascending pharangeal artery residual; BA, basilar artery; CCA, common carotid artery; CoW, Circle of Willis; SA, spinal artery; VA, vertebral artery; V-0a, vertebral-occipital anastomoses. Figure adapted from Daniel *et al.* (1953), Andersson and Jewell (1956) and Baldwin and Bell (1963).

Figure 3. Approximate spatial contribution to cerebral blood supply by carotid and vertebral arteries in different species.

Schematic representation of the cerebral blood supply contribution by the carotid and vertebral arteries in (A) man, (B) rat, (C) goat/sheep, (D) rabbit, (E) dog, (F) cat and (G) calf. Image based and adapted from Baldwin and Bell (1963) and Bralet *et al.* (1977).

Figure 4. **Parasympathetic innervation to cerebral vasculature.**

(A) Schematic overview of the anatomic position of various parasympathetic ganglia which are sources of parasympathetic input to the cerebral arteries. The level of parasympathetic innervation to the cerebral arteries varies as shown by (B) VIP innervations in the posterior communicating artery (dense innervation) and basilar artery (moderate innervation); image from Hara *et al.* (1985); x172 in original article, reproduced with permission. (C) Schematic representation of parasympathetic innervations to the rostral and posterior cerebral vasculature. The gradient represents reported density of innervation, checkered filling indicates unreported innervation. Note that species variation regarding innervation is not depicted in the figure. CmG, Carotid miniGanglia; CS, Cavernous Sinus; OG, Otic Ganglia; PTG; Pterygopalatine Ganglia; VIP, vasoactive intestinal peptide. Original figure collated from data from Gibbins *et al.* (1984a); Hara *et al.* (1985); Keller *et al.* (1985); Shimizu (1994); Kadota *et al.* (1996); Bleys *et al.* (2001); Ayajiki *et al.* (2012).

Figure 5. Electron microscopy image showing the close proximity of sympathetic and parasympathetic terminals on a cerebral artery.

Image of the anterior cerebral artery of cat showing the close apposition of a parasympathetic (Ch, cholinergic) and sympathetic (A, adrenergic) neurons. Schwann cell is visible at the bottom. Image from Edvinsson *et al.* (1972b). x60000 in original reference, with permission.

Figure 6. Mechanisms of endothelial dependent cross-talk leading to NO induced vasodilatation.

There are multiple sources of nitric oxide production including the endothelium. (A) ACh exerts endothelium dependent vasodilatation acting on muscarinic (M) type 3 and/or 5 receptors on endothelial cells inducing an increase in intracellular calcium which leads to NO production via eNOS activity. ACh still evokes a dilatation in denuded vessels suggesting a non-endothelial source of nitric oxide (NO). (B) Norepinephrine (NE) can stimulate the production of NO from cholinergic/nitrergic neurons via β_2 receptors leading to increases in Ca2+ and calcium channel stimulation. The NO diffuses to nearby smooth muscle cells causing relaxation. Modulation of NO release in this system is achieved by ACh mediated NO inhibition via pre-junctional muscarinic (M) type 2 receptors on nitrergic neurons. Abbreviations: ACh, acetylcholine; cGMP, cyclic guanosine monophosphate; GC, guanylate cyclase; GTP, guanosine triphosphate; NO, nitric oxide; NOS, nitric oxide synthase. Figure based and adapted from Wahl and Schilling (1993), Zhang *et al.* (1998b), Liu *et al.* (2000) and Si and Lee (2002).

Tables

Receptor subtype	Cellular location	Vessel location	Effect	Proportion of total receptors	Species	Reference
M_1-AChR	SMC	MCA	Vasoconstriction	15-27%	Cat	(Dauphin *et al.*, 1991)
				26-54%	Human	(Dauphin & Hamel, 1992)
M_2- AChR	Perivascular nerves	Absent!! BA	Auto regulatory	35% 0%	Cat & human	(Dauphin & Hamel, 1992)
	Nitrergic nerves		NO-modulatory-inhibition of NO - vasoconstriction	-	Pig	(van Charldorp & van Zwieten, 1989; Liu & Lee, 1999)
	Pre-junctionally NO-nerves	MCA[4]	Attenuate vasodilation by inhibiting NO-release from nitrergic nerves		Monkey	(Toda *et al.*, 1997)
M_3- AChR	Endothlial	MCA	Vasodilation	10-18%	Cat & human	(Dauphin & Hamel, 1990; Dauphin *et al.*, 1991)
M_5- AChR	?	BA	?	?	Rat	(Phillips *et al.*, 1997; Tayebati *et al.*, 2003)
	Endothelium & SMC (less) media	CoWs	Vasodilation		GM-mouse	(Yamada *et al.*, 2001a)
α7-N AChR				none	Cat & human	(Dauphin & Hamel, 1992)
		BA	Modulation of sympathetic		Pig	(Zhang *et al.*, 1998a; Si & Lee, 2001)
		BA	perivascular neurones		Rat	(Chang *et al.*, 2012)
α3β2- N AChR			Modulation of sympathetic perivascular neurones		Rat	(Lee *et al.*, 2011)

Table 1. Cholinergic receptors identified on cerebral arteries.

[4]Endothelial denuded vessel prep

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