Cerebrovascular effects of vasoactive drugs: 
*In vitro, in vivo and clinical investigations*

Yohannes Ayele Mamo

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Department of Pharmacology and Therapeutics
Faculty of Medicine, Dentistry and Health Sciences
University of Melbourne

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Abstract

The brain receives a tightly controlled blood flow that maintains its metabolic needs despite variations in arterial blood pressure. Various physiological factors - neural, humoral and vascular - are involved in the regulation of cerebral blood flow by controlling the tone of cerebral arteries and arterioles. This study characterised the in vitro effects of common vasoactive agents in rat isolated cerebral arteries, as well as the cerebrovascular responses to systemic vasoactive drug administration using in vivo animal models and clinically in patients. Variations were found in effects induced by a number of vasoactive agents in arteries isolated from different regions of the rat cerebral circulation. These were due to heterogeneous distribution of receptors mediating vascular responses. Activation of $\alpha_1$-adrenoceptors that are located on smooth muscle cells of the anterior and middle cerebral arteries, but absent in posterior communicating and basilar arteries, induced vasoconstriction. Activation of $\beta$-adrenoceptors induced vasodilatation in all four arteries. In the middle cerebral artery, $\beta$-adrenoceptors are located on smooth muscle, however they are located on endothelium in the basilar artery. The $\beta$-adrenoceptor-mediated relaxation in basilar artery involved release of nitric oxide (NO) and/or induction of endothelial-derived hyperpolarisation (EDH) depending on which agonist is used. Noradrenaline and isoprenaline favoured release of NO and induction of EDH, respectively, in rat basilar artery. Activation of endothelin receptors induced similar effects in all cerebral arteries tested. The endothelin-1-induced contraction of cerebral arteries involved activation of both voltage-operated calcium channels (VOCC) and non-VOCC. T-type VOCC are activated by low endothelin-1 concentrations, while L-type were activated by high concentrations of endothelin-1. In vivo investigation of cerebral arterial reactivity using the rat cranial window technique showed that the cerebrovascular effects induced by systemic administration of vasoactive agents were predominantly autoregulatory responses to changes in arterial blood pressure induced by the drugs. However, phenylephrine and vasopressin may also have direct constrictor effects on cerebral arteries. The effects of
pre-emptive vasopressor administration on cerebral oxygen saturation during anaesthesia in the ‘beach chair’ position was investigated in patients. Noradrenaline showed a beneficial effect in preventing hypotension and cerebral oxygen desaturation. In contrast, vasopressin did not prevent hypotension and exacerbated cerebral desaturation. This study has shown that regulation of cerebrovascular tone involves a complex integration between the direct effects of vasoactive mediators on cerebral arteries and indirect responses to changes in peripheral blood pressure. Thus, it is a matter of utmost importance to understand the mechanisms mediating effects of vasoactive drugs if they are to be used for neuroprotection during the management of cerebrovascular disorders.
Declaration

This is to certify that:

- The thesis comprises only my original work towards the PhD except where indicated in the statement of contribution of others.
- Due acknowledgement has been made in the text to all other materials used.
- The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Yohannes Ayele Mamo (B.Pharm, M.Sc)

June 2015
Statement of contribution of others

**Chapter 6:** Experiments involving *in vivo* testing of vasoactive drugs in rats were conducted in the Ocular Physiology laboratory, Melbourne Brain Centre. Dr Bang Bui, Dr Zheng He and Mr Shajan Velaedan assisted in surgical preparation, drug infusion and imaging. All the analyses were carried out by Yohannes Mamo.

**Chapter 7:** Experiments involving clinical testing of vasopressor drugs in consented patients were conducted in The Avenue Hospital (Windsor, Melbourne, VIC 3181, Australia) and the data presented in this chapter represents a subset analysis of 24 patients recruited to specifically investigate noradrenaline, vasopressin and the control (saline) group. This is a component of a larger study (ANZCTR No 12610001075077). Associate Professor Paul Soeding, Mr Grey Hoy (Orthopaedic Surgeon) and Mr Martin Spurgeon (Nurse) have been involved in conducting the experiments. Data analyses were carried out by Yohannes Mamo and A/Prof Paul Soeding.
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Abstracts co-authored

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<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABβ</td>
<td>Amyloid-β peptide</td>
</tr>
<tr>
<td>BIS</td>
<td>Bispectral index value</td>
</tr>
<tr>
<td>BK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Large-conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
</tr>
<tr>
<td>COX-1, 2</td>
<td>Cyclooxygenase-1, 2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration of agonist that evokes a half-maximal response</td>
</tr>
<tr>
<td>EDH</td>
<td>Endothelium-derived hyperpolarisation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraamino acetic acid</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum response (effects)</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial NOS</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ET&lt;sub&gt;CO2&lt;/sub&gt;</td>
<td>End tidal carbon dioxide concentration</td>
</tr>
<tr>
<td>ET&lt;sub&gt;SEVO&lt;/sub&gt;</td>
<td>End tidal sevoflurane concentration</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HVA</td>
<td>High voltage-activated</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>IK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Intermediate-conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NOS</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Inositol 1, 4, 5- triphosphate receptor</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>ATP-dependent K&lt;sup&gt;+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>KPSS</td>
<td>PSS containing 124 mm potassium</td>
</tr>
<tr>
<td>K&lt;sub&gt;v&lt;/sub&gt;</td>
<td>Voltage-dependent potassium channels</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N&lt;sup&gt;ω&lt;/sup&gt;-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LVA</td>
<td>Low voltage-activated</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>NIRS</td>
<td>Near-infrared spectroscopy</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal NOS</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Paw</td>
<td>Airway pressure</td>
</tr>
<tr>
<td>pEC$_{50}$</td>
<td>$-\log_{10}EC_{50}$</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC-β</td>
<td>Phospholipase C-β isoform</td>
</tr>
<tr>
<td>PRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>ROCC</td>
<td>Receptor-operated calcium channels</td>
</tr>
<tr>
<td>ScO$_2$</td>
<td>Cerebral oxygen saturation</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SK$_{Ca}$</td>
<td>Small-conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>SOCC</td>
<td>Store-operated calcium channels</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TRPC1</td>
<td>Canonical transient receptor potential type-1</td>
</tr>
<tr>
<td>TXA$_2$</td>
<td>Thromboxane A$_2$</td>
</tr>
<tr>
<td>VOCC</td>
<td>Voltage-operated calcium channels</td>
</tr>
</tbody>
</table>
Chapter 1

General introduction
1.1. Introduction

The brain is one of the most perfused organs receiving about 15-20% of the total cardiac output and 20% of the available oxygen supply since it relies on oxidative metabolism for its high metabolic needs. Glucose is the only substrate for energy metabolism of the brain unlike other tissues (Cipolla, 2009). It maintains a relatively constant blood flow over a wide range of arterial pressure not only to provide optimum supply of oxygen and nutrients but also to protect itself from damage due to overflow as the brain tissue cannot expand due to the skull enclosure. The tone of cerebral arteries is controlled by concerted action of various neural and endothelial mediators, as well as factors circulating in blood. In addition, the water and solute transport into the brain is regulated by the blood-brain barrier (Strange, 1992). Diseases such as stroke dysregulate the normal function of cerebral arteries, such that cerebral blood flow is obstructed consequently leading to cerebral ischaemia. In this chapter, the organization of cerebral circulation, physiological regulation of cerebral blood flow, factors involved in controlling cerebrovascular tone, pharmacological profile of cerebral vasculature and diseases affecting cerebral blood flow and the available treatment options will be reviewed. A detailed discussion of some mediators and pharmacological agents is presented in Chapter 3.
1.2. Anatomy of the cerebral circulation

1.2.1. Organization of the cerebral circulation

The brain receives its arterial blood supply through two pairs of major arteries - the right and left internal carotid and the right and left vertebral arteries, as shown in Figure 1-1 (Day, 1987). The common carotid arteries originate from the aortic arch within the chest and travel to the neck before bifurcating into internal and external carotid arteries at the C3-C4 level of the vertebrae. The external carotid artery then branches into several arteries that supply blood to extracranial tissues as well as the basal and lateral brain surfaces. The internal carotid arteries primarily supply the cerebral hemispheres and the eyes. Each internal carotid artery contributes approximately 40% of the cerebral blood flow (Edvinsson et al., 1993), the remainder being supplied by the two vertebral arteries that distally fuse to form the basilar artery. Branches of the basilar artery provide blood to the brainstem, cerebellum and cerebral cortex.
The brain receives its blood supply through the right and left internal carotid as well as a pair of vertebral arteries. The arteries ultimately join the Circle of Willis, an anatomical anastomosis. Different arteries that branch off the Circle of Willis to distribute blood supply to the two cerebral hemispheres are shown (University of Miami Health System http://surgery.med.miami.edu/images/Circulation_of_brain.gif).

The basilar artery branches into the right and left posterior cerebral arteries that join the two ipsilateral internal carotid arteries via the posterior communicating arteries to form the Circle of Willis, a ring of arteries surrounding the pituitary stalk. Three pairs of main arteries - anterior, middle,
and posterior cerebral arteries - originate from the Circle of Willis and supply the two hemispheres (Figure 1-1 and Figure 1-2). In humans, the proximal portion of the anterior cerebral artery (A1) originates from the internal carotid artery and passes over the optic chiasm connecting with its contralateral artery through the anterior communicating artery that stabilizes blood flow between anterior regions of the brain hemispheres. The two distal segments of the anterior cerebral arteries (A2) enter the longitudinal fissure and run between the hemispheres and pass in front of the corpus callosum. Several arteries branch out of the cerebral artery to supply anterior regions of the brain (Day, 1987). The middle cerebral arteries originate from the internal carotid artery at the medial end of the Sylvian fissure and supply the lateral cerebral hemispheres. Abundant perforating vessels originate along the length of the middle cerebral artery and supply blood to the brain (Day, 1987). Anatomical variations in the Circle of Willis, often lack of anterior and posterior cerebral arteries, are common in humans (Eftekhar et al., 2006; Hashemi et al., 2013). This may potentially contribute to asymmetric flow rates in bilateral arteries and can cause certain clinical consequences including ischaemic stroke (Tanaka et al., 2006; Kardile et al., 2013). The anatomical organizations of the cerebral circulation are generally similar in human and the rat. For this reason, the rat is usually considered a suitable model to study cerebrovascular diseases. Rat isolated cerebral arteries are also widely used to investigate the in vitro effects of pharmacological agents on cerebral arteries. Nevertheless, some differences are noted between the human and rat cerebral circulations in specific arteries as shown in Figure 1-2. For example, the internal carotid artery continues to form the middle cerebral artery in the human, but integrates with the Circle of Willis in the rat cerebral circulation. Further, the anterior communicating artery is present in human but absent in the rat cerebral circulation (Lee, 1995).

Each artery progressively divides into smaller arteries that run on the surface (called pial arteries) until they penetrate into the brain tissue to provide blood to the corresponding regions of the cerebral cortex. The penetrating arteries become parenchymal arterioles and finally form capillaries (Lee, 1995).
Figure 1-2: Comparison of the human and rat cerebral circulations.

The diagram demonstrates the organization of the human (A) and rat (B) cerebral arterial circulation. The major cerebral arteries constituting the Circle of Willis are found both in the human and the rat, but some differences are also shown. In the human, the internal carotid artery continues to form the middle cerebral artery while in the rat, the internal carotid artery integrates with the Circle of Willis. Further, variations in anterior and posterior communicating arteries are noted between the human and rat cerebral circulations (Lee, 1995).

Unlike the arteries, the cerebral venous system is made up of valveless veins and sinuses. The veins draining the cerebral hemispheres are subdivided into outer or superficial cortical veins that are located on the surface of the cortex and the central veins that are located deep inside the brain. The superficial veins drain the cerebral cortex and subcortical white matter, while the deep or central veins, consisting of subependymal veins, internal cerebral veins, basal vein, and the great vein of Galen, drain the brain's interior, including the deep white and grey matter surrounding the lateral and third ventricles. The superficial and cortical veins finally anastomose and empty into the superior sagittal sinus. The superior sagittal sinus is then directed via a confluence of
sinuses toward the sigmoid sinuses and jugular veins (Capra and Kapp, 1987; Schaller, 2004; Kilic and Akakin, 2008) (Figure 1-3).

![Diagram of the human cerebral venous system](http://imueos.wordpress.com/2010/10/10/base-of-the-skull/)

**Figure 1-3: The human cerebral venous system.**

The major superficial and deep lying cerebral veins as well as the common sinuses are shown. The veins ultimately join together and form the sigmoid sinus which descends down to form the jugular vein. The figure also shows various smaller veins and sinuses draining specific regions of the brain (obtained from http://imueos.wordpress.com/2010/10/10/base-of-the-skull/).

1.2.2. **The pial arteries and arterioles**

Intracranial arteries lying on the surface of the brain within the subarachnoid or subpial space are called pial arteries. They are surrounded by cerebrospinal fluid in the pia-arachnoid space (Zhang et al., 1990). The cerebral pial arteries are extensively branched and are characterised by a network of collaterals, branches that interconnect different arteries. When a certain artery is occluded, the collaterals will provide an alternate supply of blood to the tissues.
through another artery as discussed below (Coyle, 1994). The pial arteries receive abundant peripheral innervations (extrinsic innervations) that release different neurotransmitters (discussed in subsequent sections and in Chapter 3). The peripheral innervations are progressively lost as the arteries enter into the brain parenchymal tissue (Figure 1-4) (Hamel, 2006). Remarkably, these arteries contribute to about 50% of the cerebral vascular resistance (Cipolla et al., 2014).

1.2.3. Parenchymal arterioles

The deep penetrating arteries become parenchymal arterioles once they enter the brain tissue and become almost completely surrounded by astrocytes, pericytes and neurons (Figure 1-4). They are usually long unbranched arteries that connect the pial arteries to the capillaries (Cipolla et al., 2014). Parenchymal arteries are structurally distinct from pial arteries and are usually made up of single layer of smooth muscle cells. Parenchymal arterioles are intrinsically innervated by afferent nerves from subcortical nuclei and the local cortical interneurons (Hamel, 2006).

1.2.4. The brain microcirculation

The brain microcirculation consists of extensive capillary networks that are made up of endothelium surrounded by basal lamina and pericytes, but lack smooth muscle cells. The basal lamina is continuously covered by astrocytic end-feet and sparsely by the neurons. The unique tight junctions of endothelial cells, along with pericytes, astrocytes and the basal membrane, play a barrier function (called the Blood-Brain barrier; discussed in section 1.3) that prevents free movement of solutes between blood and brain tissues (Abott et al., 1990; Cipolla, 2009). The tripartite association between neuron, astrocyte and cerebral microvessel is usually referred to as the neurovascular unit (Figure 1-4).
1.2.5. Collateral vessels

The collateral circulation of the brain is a network of arteries and arterioles that maintains stable cerebral blood flow when the primary routes are obstructed or compromised. The Circle of Willis, an anastomotic loop with low resistance, theoretically allows communication between the anterior and posterior circuits. Under normal conditions, the Circle of Willis does not function as a redistributor of cerebral blood flow, however, when the large arteries supplying the brain are occluded, it allows collateral support to the obstructed side depending on the anatomical ‘completeness’ of the anastomosis (Day, 1987). The networks of pial arteries furnish secondary collateral redistribution of blood flow when there is an occlusion of arteries distal to the Circle of Willis. The penetrating arterioles are mostly unbranched and lack collateral connections (Nishimura et al., 2007). Thus, occlusion of these vessels results in infarction of the surrounding tissues.
Figure 1-4: Architecture of the pial and cerebral microcirculation.

The surface pial arteries receive extrinsic innervations: sympathetic nerves originating from superior cervical ganglia (SCG), parasympathetic nerves originating from the sphenopalatine ganglion (SPG) and the otic ganglion (OG), as well as sensory neurons from the trigeminal ganglion (TG). Several neurotransmitters released by the extrinsic and intrinsic nerves are shown. The penetrating arterioles and capillaries are extensively surrounded by intrinsic nerves and astrocytic endfeet. The microcirculation also receives neural projections from subcortical areas such as the locus coeruleus, raphe nucleus, basal forebrain and the thalamus. The inset shows a neurovascular unit (obtained from Hamel (2006)).
1.3. The barriers of the brain

There are three main interfaces that physically isolate the brain from other compartments of the body - the cerebrospinal fluid-brain, blood-brain and cerebrospinal fluid-blood interfaces. These interfaces are the main barriers that provide protective functions to the central nervous system (Abbott et al., 2006). The cerebrospinal fluid is formed by the choroid plexus and its contact with the blood is prevented by the tight junction of ependymal cells lining the plexus. The cerebrospinal fluid-brain barrier is provided by neuroependymal cells interconnected by tight junctions in early life. However, the barrier is gradually lost during adult age due to morphological changes in the ependymal cells (Cipolla, 2009).

The blood-brain barrier is the main barrier that protects the neural tissue from contamination of blood-borne substances. The blood-brain barrier is present in pial arteries and arterioles, veins and capillaries, but is absent in some parts of the brain such as the area postrema, median eminence, neurohypophysis, pineal gland, subfornical organ and lamina terminalis (Ganong, 2000). Structurally, the blood-brain barrier is made by endothelial cells connected paracellularly via extensive tight junctions and adherens junctions (Figure 1-5) (Ballabh et al., 2004). The blood-brain barrier is a selective barrier that allows transcellular passage of small lipid soluble molecules (<400 Da) only. Larger molecules such as glucose, amino acids, nucleotides and vitamins are transported by specific transporters located either on the apical or basolateral membrane of the endothelium. There are also active efflux mechanisms including ATP-binding cassette (ABC) and multidrug resistance (MDR) P-glycoproteins (P-gp) that remove metabolites and drugs from the brain (Cipolla, 2009). The permeability of the tight junctions can be modulated through actin stress fibres in the cytoplasm of the endothelial cells (Wolburg et al., 1994). Certain agonists (e.g. cAMP) decrease permeability of the tight junction by relaxing these fibres, while other substances (e.g. protein kinase C and vascular endothelial growth factors) increase permeability by promoting contraction of the fibres (Abbott et al., 2006; Cipolla, 2009).
1.4. The neurovascular unit

The neurovascular anatomical unit comprises of endothelial cells, basal lamina, pericytes, astrocytes and the neurons. Functional interaction between these components interfaces neuronal activity with blood flow in order to accommodate for the metabolic demands and provide means for removal of the end-products (Merlini et al., 2012). Changes in localized cerebral blood flow in response to increased metabolic need are known as neurovascular coupling or functional hyperaemia. Increased metabolic activities lead to release of neurotransmitters which may directly act on the vascular cells. Since the surface of capillaries is extensively covered by astrocytes, the neurons may activate the astrocytes which in turn alter the vascular tone (Petzold and Murthy, 2011). The astrocytes release factors that regulate the function of endothelial cells. In addition astrocytes provide a cellular link that transmits neural signals to endothelial cells (Figure 1-5).
Figure 1-5: Structural components of the blood brain barrier.

The endothelial cells are encased with basal lamina, pericytes and astrocytic endfeet. The neurons and microglia have sparse direct contact with the capillaries - the astrocytes provide cellular links between the neurons and endothelial cells. a) Example of the transporters expressed by endothelial cells - glucose transporter 1 (GLUT1), L-amino acid transporter 1 (LAT1), excitatory amino acid transporter 1-3 (EAAT1-3) and P-glycoprotein (Pgp); and b) astrocyte-endothelial interactions - astrocytes release various factors that activate specific receptors located on endothelial cells. 5-HT, 5-hydroxytryptamine; ANG1, angiopoetin 1; bFGF, basic fibroblast growth factor; ET1, endothelin; GDNF, glial cell line-derived neurotrophic factor; LIF, leukaemia inhibitory factor; P2Y2, purinergic receptor; TGFβ, transforming growth factor-β; TIE2, endothelium-specific receptor tyrosine kinase 2. The tight junction between individual endothelial cells is shown (obtained from Abbott et al. (2006)).
1.5. Physiological regulation of cerebral blood flow

1.5.1. Cerebral autoregulation

Cerebral autoregulation is defined as an inherent ability of the brain to maintain the blood flow relatively constant over a wide range of peripheral arterial blood pressure (about 50-150 mmHg) (Paulson et al., 1990; van Beek et al., 2008). It allows constant blood flow that matches with the metabolic needs of the brain in the face of fluctuation in perfusion pressure (arterial blood pressure minus intracranial pressure). When the arterial blood pressure falls below the lower limit, cerebral blood flow decreases since the vasodilatation reaches the maximum capacity and the vessels collapse. Equally, as the blood pressure exceeds the upper limit, the arteries are forcefully dilated by the pressure, a condition called ‘breakthrough of autoregulation’, and fail to control the cerebral blood flow (Figure 1-6). Thus, the cerebral blood flow increases/decreases linearly with changes in peripheral arterial blood pressure outside the autoregulatory range (Paulson et al., 1990; Cipolla, 2009). The mechanism underlying cerebral autoregulation involves interaction of multiple components including neurogenic, myogenic and metabolic factors (Busija, 1993).

Cerebral autoregulation can be disrupted by diseases such as chronic hypertension, long-lasting diabetes, cerebrovascular diseases or any factor that causes excessive vasoconstriction or vasodilatation. Hypertension shifts both the lower and upper limits towards higher pressure (called hypertensive adaptation)(Strandgaard et al., 1973). In chronic diabetic patients, the normal autoregulatory plateau is absent so that the cerebral blood flow depends partly on the peripheral arterial blood pressure (Edvinsson et al., 1993). Acute cerebrovascular ischaemia is another common cause of loss of autoregulation, known as vasomotor paralysis. The autoregulatory function of the blood vessels is lost after ischaemic stroke in areas distal to the occlusion (Eames et al., 2002; Immink et al., 2005). A more detailed discussion of cerebral autoregulation is presented in Chapter 6 and Chapter 7.
The vasodilator or vasoconstrictor responses of cerebral arteries are responsible for maintenance of a constant cerebral blood flow (CBF) despite fluctuations in blood pressure within the autoregulatory range (50-150 mmHg). Below the lower autoregulatory limit, impaired dilation of the arteries will lead to cerebral ischaemia, whereas above the autoregulatory limit, forceful dilation of the arteries causes uncontrolled cerebral blood flow that results in vasogenic oedema and brain damage (obtained from Pires et al. (2013)).

1.5.2. Neural control

The cerebral circulation has been known to receive abundant peripheral innervation, called extrinsic innervation. The sympathetic, parasympathetic and sensory innervations of the cerebral circulation originate mainly from superior cervical, trigeminal and sphenopalatine ganglia, respectively (Lee et al., 1976; Duckles, 1980; Paulson et al., 1990; Lincoln, 1995; Mitsis et al., 2009; ter Laan et al., 2013). In addition, cerebral arteries receive neural projections from various subcortical nuclei (called intrinsic innervations) such as the
thalamus, locus coeruleus, raphe nucleus and basal forebrain (Raichle et al., 1975; Micieli et al., 1994; Hamel, 2006). However, the role of the various neural systems in the regulation of cerebral blood flow has been controversial. For example, several studies have shown that the autonomic nervous system plays a role in the beat-to-beat regulation of cerebral blood flow in humans (Zhang et al., 2002), but others reported contradictory results (Heistad and Marcus, 1978; Tuor, 1992; Roatta et al., 1998; Dunatov et al., 2011). The potential role of perivascular nerves in cerebral autoregulation may be changed during disease conditions such as subarachnoid haemorrhage (Tsukahara et al., 1986a; Tsukahara et al., 1988; Budohoski et al., 2013). In line with this, sympathetic activity has been greatly increased in patients with subarachnoid haemorrhage as manifested by persistent elevation of the noradrenaline concentration in plasma (Naredi et al., 2000). The effects of the nerves releasing common neurotransmitters on cerebral arteries are discussed in Chapter 3.

1.5.3. Myogenic control

An intrinsic property of vascular smooth muscle cells to respond to changes in mechanical stretch or intravascular pressure is known as the myogenic response. Some arteries constrict in response to increased transmural pressure or dilate when the pressure is decreased or removed. The myogenic response is more prominent in small cerebral arteries and arterioles than other vascular beds in several species (Wallis et al., 1996). The myogenic response is important for maintenance of arterial resistance and perfusion of tissues when the arterial blood pressure decreases (Davis, 2012). In addition, it protects smaller arterioles and capillaries from physical damage under conditions of increased perfusion pressure (Paulson et al., 1990). The mechanism by which wall tension or pressure on the vessels elicits the myogenic response is not clearly understood. Multiple ion channels, including the calcium, potassium and chloride channels, may be activated when the smooth muscle cells are stretched (Bulley et al., 2012). Potassium channels – the ATP-sensitive K⁺(KATP), large-conductance Ca2⁺-activated K⁺(BKCa), voltage-activated K⁺(Kv) and inward rectifier K⁺(Kir) channels – are particularly important since the electrochemical
gradient of $K^+$ ion plays critical role in the regulation of membrane potential and vascular tone (Jackson, 2000). Transient receptor potential (TRP) channels, which are non-selective cation channels, also play a significant role in myogenic activation of vascular smooth muscle contraction (Earley and Brayden, 2010). Multiple TRP channels may be involved in the mechanotransduction of myogenic tone in cerebral arteries (Kim et al., 2013). The myogenic response is associated with membrane depolarization that leads to increased calcium influx through the voltage-operated calcium channels (VOCC). It has been indicated that both L- and T-type VOCC may also contribute to the genesis of the myogenic response (Abd El-Rahman et al., 2013).

The myogenic response can be deregulated during cerebral ischaemia and reperfusion (Palomares and Cipolla, 2013). Multiple mechanisms are involved in the ischaemia-induced loss of the myogenic response. First, increased generation of peroxynitrite can lead to endothelial damage which in turn affects release of vasoactive substances such as nitric oxide or endothelin-1 (ET-1). The increased level of vasoactive mediators can directly affect the myogenic tone in cerebral arteries and arterioles. Second, ischaemia may alter the response of vascular smooth muscle cells to an increased pressure by causing certain structural changes such as depolymerisation of the actin cytoskeleton (Cipolla et al., 2001).

### 1.5.4. Endothelial control

In addition to its barrier function of protecting the brain tissues from blood-borne substances, the cerebrovascular endothelium plays a critical role in regulating cerebral blood flow. The vascular endothelium releases several vasoactive mediators that can directly affect the vascular tone. Both vasoconstrictor agents, such as ET-1 and thromboxane $A_2$ (TXA$_2$), and vasodilator agents, such as nitric oxide (NO), prostacyclin (PGI$_2$) and endothelium-derived hyperpolarisation (EDH), are released by the endothelium. More substances with vasodilator properties including carbon monoxide, hydrogen peroxide, hydrogen sulphide, potassium ion ($K^+$) and
methane have been reported to be released by the vascular endothelium (Qi et al., 2011) (Figure 1-7).

**i. Nitric oxide (NO)**

NO is one of the most extensively investigated relaxing factors released by the vascular endothelium. It is constitutively synthesized from L-arginine by enzymatic action of nitric oxide synthase (NOS) (Tousoulis et al., 2012). There are three isoforms of NOS - endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Both eNOS and nNOS are constitutively expressed and well characterised in the cardiovascular and nervous systems. iNOS, however, is activated only during inflammation or after stimulation of the immune system (Arnold et al., 1977; Ignarro et al., 1987; Bryan et al., 2009).

Several vasoactive substances released from nerve terminals, such as acetylcholine, substance P and calcitonin-gene related peptide (CGRP) can induce endothelial production of NO. The NO produced in endothelial cells can freely diffuse into smooth muscle cells where it increases the level of cGMP by activating the soluble guanylyl cyclase enzyme (sGC). cGMP then triggers a cascade of intracellular events that culminate in vascular smooth muscle relaxation. Furthermore, endothelial nitric oxide causes a number of other cellular effects including smooth muscle proliferation, platelet aggregation, and leukocyte recruitment (Zhong et al., 2011).

The basal production of NO contributes to the physiological regulation of cerebral blood flow by inhibiting the resting tone or decreasing cerebral vascular resistance. This maintains the local blood flow to the brain tissues (Faraci, 1994). It has been suggested that NO also contributes to maintenance of the cerebral blood flow by extending the lower limit of cerebral autoregulation. Inhibition of NOS depresses the cerebral blood flow responses to hypotension (vertical autoregulation) in rats (Stephen, 2003), while the autoregulation curve was shifted to the right in eNOS knock-out mice (Atochin and Huang, 2011).
Endothelial impairment or decreased nitric oxide production can result in serious impairment of the cerebral circulation. The production and signalling of NO can be significantly affected by cerebral ischaemia. Occlusion of the middle cerebral artery caused transient increase followed by long-term deficiency of NO leading to neurological damage (Terpolilli et al., 2012). Deficiency of NO due to dysfunctional eNOS has also been implicated in the pathogenesis of cerebral vasospasm following subarachnoid haemorrhage (Pluta, 2006). On the other hand, overproduction of nitric oxide may result in the development or exacerbation of neurological damage in Alzheimer’s disease, multiple sclerosis, head trauma or stroke (Toda et al., 2009).

**ii. Prostacyclin (PGI₂)**

Another vasoactive mediator produced by the vascular endothelium is PGI₂. Activation of phospholipase A₂ leads to liberation of arachidonic acid from membrane-bound lipids. Arachidonic acid is converted to prostaglandin H₂ (PGH₂) by catalytic action of cyclooxygenase-1 (COX-1) and COX-2 enzymes. PGH₂ is further metabolized by prostacyclin synthase enzyme to generate PGI₂ (Marcus et al., 1980). PGI₂ activates IP receptors to elicit relaxation of vascular smooth muscle cells. PGI₂ relaxes cerebral arteries but considerable regional and interspecies variation of the response exist (Brown and Pickles, 1982; Uski et al., 1983). PGI₂ was found to be decreased in primate cerebral arteries after subarachnoid haemorrhage leading to the suggestion that a deficiency in PGI₂ may be implicated in the pathogenesis of cerebral vasospasm (Nosko et al., 1988).

**iii. Endothelium-derived hyperpolarisation (EDH)**

Endothelium-derived hyperpolarisation (EDH) refers to the agonist-induced endothelium-dependent vasodilator mechanism(s) excluding NO and the COX products (Cipolla, 2009). The vascular smooth muscle cell hyperpolarisation may be caused by several factors released from the endothelium as shown in Figure 1-7. EDH is generally characterised by increased potassium conductance with subsequent hyperpolarisation, and thus relaxation, of vascular smooth
muscle cells. Certain agonists like acetylcholine can cause membrane hyperpolarisation by increasing potassium efflux. The major potassium channels involved in the hyperpolarisation are the small- (SK$_{Ca}$) and large-conductance calcium-activated potassium channels (BK$_{Ca}$) expressed in endothelial cells (Marrelli et al., 2003; Ozkor and Quyyumi, 2011). Activation of a receptor on endothelial cells by an agonist causes release of calcium from the endoplasmic reticulum. An increase in intracellular calcium activates the $K_{Ca}$ in the proximity of the myoendothelial junction and triggers a complex process including transmission of hyperpolarisation to the smooth muscle cells via gap junctions. This process ultimately promotes the relaxation of smooth muscle cells. Basal EDH is present in all penetrating cerebral arterioles and is responsible for inhibition of the basal tone. This is typically manifested by vasoconstriction induced in cerebral arteries when the SK$_{Ca}$ and IK$_{Ca}$ channels are inhibited (Ledoux et al., 2006; Cipolla et al., 2009).

Epoxyeicosatrienoic acids (EETs), metabolites of endothelial cytochrome P450 epoxygenase, and H$_2$O$_2$ may also contribute to the EDH function (Campbell et al., 1996; Fleming, 2001; Bellien et al., 2006; Fleming and Busse, 2006). In addition, EDH may work in synergy with NO and PGI$_2$ in regulating the vascular tone. Thus, endothelium-dependent vasodilatation is maintained during states of decreased NO availability such as hypertension, owing to the compensatory increase of EDH activity (Taddei et al., 1999).
Figure 1-7: Relaxation of vascular smooth muscle cells by endothelium-derived factors.

Activation of endothelial cells due to shear stress caused by blood flow or agonist-induced stimulation of specific surface receptors leads to increased intracellular Ca\(^{2+}\) concentration resulting in activation of various enzymes such as endothelial nitric oxide synthase (eNOS), cyclooxygenase (COX) or cytochrome P450 2C (CYP450 2c). Activation of phospholipase A\(_2\) liberates membrane-bound arachidonic acid that is converted to either prostacyclin (PGI\(_2\)) or eicosatrienoic acids (EETs) by the catalytic action of COX or CYP450 2c, respectively. eNOS and COX increase production of nitric oxide (NO) and prostacyclin (PGI\(_2\)) which cause smooth muscle relaxation by elevating the level of cGMP or cAMP in the smooth muscle cells. Elevated cytosolic Ca\(^{2+}\) concentration causes membrane depolarization that is conducted to smooth muscle cells through the myoendothelial gap junction. Depolarization of smooth muscle cells finally causes relaxation of blood vessels (Ozkor and Quyyumi, 2011).
1.5.5. **Metabolic control**

Metabolic demand due to increased neuronal activity of the brain is among the driving factors that increase cerebral blood flow. A number of mediators are proposed to play important roles in coupling neuronal activity to the cerebral blood flow. These include CO$_2$, H$^+$, O$_2$, adenosine and adenosine nucleotides, K$^+$ and Ca$^{2+}$ (Paulson *et al*., 1990). Both hypoxia and hypercapnia seem to play critical roles in cerebral blood flow.

**i. Hypoxia**

It has been well known that hypoxia increases cerebral blood flow by inducing a vasodilator response in cerebral arteries (Poulin and Robbins, 1998). However, the cerebral blood flow does not respond to hypoxia until the tissue PO$_2$ falls below ~50 mmHg. Further decrease in PO$_2$ can significantly increase the cerebral blood flow (up to 400% of resting levels). Hypoxia causes cerebral vasodilation through a number of mechanisms. Decrease in ATP levels due to hypoxia can induce smooth muscle membrane depolarization by activating ATP-dependent K$^+$ channels (K$_{ATP}$). Hypoxia can also increase cerebral blood flow by inducing release of vasodilator mediators. Acute hypoxia promotes upregulation of eNOS thus inducing local release of NO (Min *et al*., 2006). Further, hypoxia induces release of adenosine from endothelial cells (Coney and Marshall, 1998). Prolonged hypoxia can increase cerebral tissue perfusion by increasing capillary density in the brain (Harik *et al*., 1995; Cipolla, 2009).

**ii. Hypercapnia**

Hypercapnia significantly increases cerebral blood flow by dilating the cerebral arteries and arterioles whereas hypocapnia causes the opposite effect (Kety and Schmidt, 1948; Kontos *et al*., 1971). The vasodilatation induced by CO$_2$ may involve different mechanisms including effects of extracellular H$^+$ or through release of NO or the prostanoids (Cipolla, 2009). Increased extracellular H$^+$ concentration, which is an initial step, may lead to membrane hyperpolarisation through direct activation of the K$^+$ channels or indirectly by activating the COX
and NOS enzymes and generating the prostanoids and NO, respectively. These mechanisms ultimately relax the vascular smooth muscle cells by decreasing the level of intracellular calcium (Brian, 1998).
1.6. Pathophysiology of the cerebral circulation

Cerebral blood flow is known to be affected, focally or globally, under several disorders of the brain. Morbidities and mortalities associated with disease states such as ischaemia and haemorrhagic stroke are caused by diminished or complete obstruction of blood flow to certain regions of the brain. Several brain diseases including neurodegenerative disorders and migraine headache are also associated with abnormal cerebral blood flow. Alzheimer’s disease, Huntington’s disease, seizures and migraine are among the pathologic conditions wherein cerebral blood flow is manifestly disrupted. Thus, cerebral vascular disorders need to be emphasised while dealing with these diseases.

1.6.1. Cerebral ischaemia

Cerebral ischaemia is a condition where the blood flow is insufficient to meet its metabolic needs of the brain. It may be caused by obstruction or constriction of the cerebral arteries. The poor oxygen supply can result in brain tissue infarction. Decreases in oxygen and glucose delivery lead to decoupling of oxidative phosphorylation and thus lessen ATP production and disruption of ion transport systems. This causes elevation of intracellular K+ concentration due to the failure of the Na-K pump (Na-K ATPase) and causes neuronal depolarization that promotes Ca2+ influx through voltage-operated calcium channels, subsequently leading to release of excitotoxic amino acids such as glutamate. This condition finally leads to an irreversible neuronal damage or death. In addition, elevated intracellular calcium can decrease cellular integrity and survival by activating a multitude of enzymes that are involved in apoptotic cell death (Turner et al., 2013). Chronic ischaemia can trigger further damage by activating different inflammatory mechanisms. Initially, ischaemia activates the microglia (inflammatory cells in the brain) followed by influx of circulating inflammatory cells including granulocytes, leukocytes, T cells and monocytes from the blood. Excessive release of cytokines and chemokines causes oxidative stress and disruption of the blood-brain barrier further propagating the inflammatory responses (Turner et al., 2013).
There is no clear understanding of the direct effects of acute ischaemia on cerebral autoregulation. It has been suggested that, while ischaemia is caused due to decreased blood flow below a critical level to an area of the brain, it may also induce dysfunction of autoregulation in the surrounding areas. However, there is insufficient evidence showing failure of the autoregulatory capacity of the vessels in the surrounding infarction area (Jordan and Powers, 2012). On the other hand, ischaemia-reperfusion can cause a macro- or micro-vascular dysregulation that may lead to exacerbation of brain injury. Reperfusion after long lasting ischaemia (more than 15-30 minutes) can promote loss of control on cerebral blood flow by impairing the various vascular autoregulatory mechanisms. Decrease in myogenic tone was observed after 6-12 hours of reperfusion. Occlusion of the arteries causes vascular paralysis or dilatation of the arteries downstream by activating release of different vasodilator mechanisms. These include myogenic vasodilation (dilator response due to drop in arterial wall pressure) and build-up of carbon dioxide and lactic acidosis. Ischaemic reperfusion will then induce hyperaemia because the ‘paralysed’ arteries will no more be able to regulate blood flow to the ischaemic area. Exposure of the microcirculation to uncontrolled and excessive pressure results in further damage of the neurons, disruption of the blood-brain barrier and brain oedema. The mechanism underlying diminished tone of the arteries after ischaemia-reperfusion has been suggested to involve oxidative stress (due to generation of reactive oxygen and nitrogen species) and loss of F-actin in vascular smooth muscle cells (Palomares and Cipolla, 2011).

Ischaemia-reperfusion may also alter the vasodilator function of NO. Even though ischaemia induces sustained upregulation of eNOS, postischaemic reperfusion actually diminishes NO-mediated vasodilatation. This could be due to the uncoupling of eNOS that ends up in generation of superoxide anions instead of NO. One reason for uncoupling of eNOS could be decreased availability of substrate (L-arginine) and the cofactor tetrahydrobiopterin (BH₄) which have been depleted during ischaemia and oxidation or inactivation of the cofactor during reperfusion. Ischaemia-reperfusion may also affect dilatation of cerebral arteries by enhancing EDH responses via activation of SKCa and IKCa.
channels in the parenchymal arterioles (Huk et al., 1997; Kidd et al., 2005; Sanchez et al., 2006; Cipolla and Godfrey, 2010).

Furthermore, ischaemia-reperfusion may alter the effects of endothelin-1 in the cerebral arteries. Not only has the concentration of endothelin-1 been increased in plasma, cerebrospinal fluid and brain tissue after stroke, but also antagonism of ET<sub>A</sub> receptors improved microvascular reperfusion after transient middle cerebral artery occlusion (Dawson et al., 1999). Apart from being a potent vasoconstrictor peptide, endothelin-1 induces superoxide production and disrupts the integrity of the blood-brain barrier, thus promoting cerebral oedema.

1.6.2. Cerebral vasospasm

Cerebral vasospasm is a narrowing of arteries usually occurring few days after the rupture of aneurysm. It usually occurs in two phases. There is an acute initial phase starting in 1 to 3 days of subarachnoid haemorrhage. The delayed phase, occurring between 4 to 14 days following subarachnoid haemorrhage, is responsible for most neurological morbidities and mortality (Tani, 2002). Angiographic vasospasm is observed in up to 70% of subarachnoid haemorrhage patients, while 20-30% of them manifest clinical signs and symptoms. Nearly half of the patients with symptomatic vasospasm will develop infarctions that may lead to permanent disability or death (Dumont et al., 2003).

i. Pathogenesis of cerebral vasospasm

The mechanism leading to development of vasospasm is still elusive. It has been proposed that the blood products released when the aneurysm ruptures may directly or indirectly play the main role in the pathogenesis of vasospasm. A number of products released as a result of bleeding have vasoconstrictor activity. Oxyhaemoglobin and fibrin degradation products are among these spasmogens abundantly found in cerebrospinal fluid of subarachnoid haemorrhage patients (Hirano and Hirano, 2010). In addition, endothelin, metabolites of arachidonic acid, catecholamines and 5-hydroxytryptamine (5-
HT) are suggested to contribute to the development of cerebral vasospasm given their potent vasoconstrictor capacity and local production (Edvinsson and Povlsen, 2011). Endothelin is typically increased in cerebrospinal fluid of subarachnoid haemorrhage patients and thus has been at the centre of interest for researchers.

Phenotypic changes of several vasoconstrictor receptors in vascular smooth muscle cells of subarachnoid haemorrhage animal models and human subarachnoid haemorrhage patients have been reported. Edvinsson and Povlsen (2011) have reviewed studies on plasticity of contractile receptors as a mechanism underlying cerebral vasospasm. Generally, receptor proteins and mRNA of endothelin type B receptors (ETB), angiotensin II type 1 receptors (AT1), 5-HT1B, and thromboxane A2 (TP) receptors were found to be up-regulated after experimental subarachnoid haemorrhage.

**ii. Role of inflammation in cerebral vasospasm**

A series of complex inflammatory processes are elicited by presence of blood components in the cerebrospinal fluid following the rupture of an arterial aneurysm. After bleeding, the red blood cells are lysed in the cerebrospinal fluid and release haemoglobin which can trigger an inflammatory cascade. This involves upregulation of cell adhesion molecules on leukocytes and endothelial cells thereby recruiting macrophages and neutrophils to the subarachnoid space which phagocytose the haemoglobin. After some time, the trapped macrophages and neutrophils themselves undergo apoptosis and release their intracellular contents consisting mainly of inflammatory cytokines, reactive oxygen species and vasoconstrictor mediators such as endothelin-1 into the cerebrospinal fluid. This ultimately ends up in propagated inflammation and vasoconstriction (Pradilla et al., 2010).

Overproduction of inflammatory mediators could be among the major mechanisms of underlying delayed cerebral vasospasm. In this regard the expression of tumour necrosis factor-α (TNF-α) in blood and vascular smooth muscle cells of a rat model were shown to be markedly increased suggesting a
role of proinflammatory cytokines in the genesis of the spasm after subarachnoid haemorrhage (Vecchione et al., 2009; Maddahi and Edvinsson, 2010).

Fassbender et al. (2001) evaluated the release of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in patients with subarachnoid haemorrhage. Interestingly, the cerebrospinal fluid level of the cytokines showed significant increments between days 5 and 9, but declined thereafter. There was a parallel decrease in blood flow (demonstrated by increased cerebral blood flow velocity) between days 7 and 11, only slightly lagging behind. In addition, the concentration of IL-6 was found to be significantly elevated in the cerebrospinal fluid of subarachnoid haemorrhage patients with poor clinical outcome.

1.6.3. Migraine

Migraine is a condition characterised by a recurrent moderate or severe headache lasting for up to several days. The pathogenesis of migraine is partly related to dilatation of both intracranial and extracranial arteries (Spierings, 2003; Toda et al., 2009). The vasodilatation may be linked to excessive production and release of NO via stimulation of certain endothelial receptors. For example, activation of 5-HT2A receptors enhances the production of nitric oxide in the trigeminovascular pathway in the rat. In addition to its vasodilator effect, nitric oxide may also sensitize the perivascular as well as central trigeminal nociceptors (Srikiatkhachorn et al., 2002; Barbanti et al., 2014).

1.6.4. Alzheimer’s disease

The pathology of Alzheimer’s disease is predominantly caused by neurovascular dysfunction wherein the availability of nitric oxide is decreased (Austin et al., 2013). Chronic cerebral hypoperfusion seems to underlie the cognitive decline associated with Alzheimer’s disease. The structure and function of the endothelium may be altered due to generation of superoxide radicals as a result of interaction with amyloid-β peptide (Aβ). Endothelium-dependent relaxation was found to be profoundly decreased in transgenic mice overexpressing the
amyloid precursor protein (Khalil et al., 2002). The decrease in cerebral blood flow may also be caused by cerebral thrombosis, atherosclerosis and cerebral infarction or decreased cerebral arterial dilatation. Subsequently, there will be reduced oxygen supply to an area in the brain leading to metabolic dysfunction, increased superoxide production as well as Aβ deposition which may cause progressive neurodegeneration and cognitive decline. In some patients the expression of asymmetric dimethyl arginine, an endogenous NOS inhibitor, is found to be increased (Toda et al., 2009; Hunter et al., 2012). Thus, Alzheimer’s disease is thought to be associated with loss of functional vasoregulation due to decreased production of NO.

1.6.5. Huntington’s disease

Dysfunctional cerebral blood flow has been described in Huntington’s disease, a neurodegenerative disorder causing cognitive dysfunction, psychiatric disturbance and movement disorders characterised by involuntary movements as well as altered motor control (Chen et al., 2012). Decreased cerebral blood flow may induce oxidative stress that causes neurodegeneration. The cause of changes in blood flow is not clearly known but may involve overproduction of nitric oxide. Increased nitric oxide production may lead to oxidative stress through conversion into peroxynitrite (Aguilera et al., 2007; Toda et al., 2009).

1.6.6. Seizures

Increased cerebral blood flow and hyperbaric oxygen have been shown to precede the clinical onset of seizures (Moseley et al., 2014). This may be related to an elevated onset of production of nitric oxide that increases cerebral blood flow by dilating cerebral arteries several minutes before onset of the seizure. Endothelial nitric oxide may play a key role in the development of hyperoxic hyperaemia preceding oxygen seizures, while neuronal nitric oxide may mediate toxic effects of hyperbaric oxygen primarily through its reaction with superoxide to generate a stronger oxidant peroxinitrite (De Vasconcelos et al., 2006; Toda et al., 2009). This assertion is supported by a previous finding that
local application of $N^\omega$-nitro-L-arginine, a nitric oxide synthase inhibitor, abolished increases in rat cerebral blood flow due to an induced seizure by topical bicuculline methiodide application via the cranial window (De Vasconcelos et al., 1995).
1.7. **Mechanism of smooth muscle contraction in the cerebral circulation**

1.7.1. **Smooth muscle contraction**

Vascular smooth muscle cells contract in response to mechanical, electrical or chemical stimuli (Bayliss, 1902; Ehrreich and Clopper, 1970). Mechanical stretching of the smooth muscle cells can induce contraction by stimulating calcium channels. Smooth muscle cells also contract when electrically stimulated or due to depolarization of the membrane. Chemical mediators are the most common and physiologically relevant stimuli for smooth muscle contraction. A number of endogenous ligands such as noradrenaline, angiotensin II, vasopressin, endothelin-1 and thromboxane A₂ affect the tone of vascular smooth muscle cells. Binding of an agonist to a G-protein-coupled receptor on smooth muscle cells stimulates the membrane-bound phospholipase C-β isoform phospholipase (PLC-β) enzyme that catalyses conversion of phosphatidylinositol 4,5-bisphosphate (PI₂) into a soluble inositol 1,4,5-triphosphate (IP₃) and a membrane-bound diacylglycerol (DAG) (Alexander et al., 1985). IP₃ stimulates its receptor located on the sarcoplasmic reticulum (SR) and causes initial release of Ca²⁺, which then induces further release of calcium (calcium-induced calcium release). The initial increase in intracellular free calcium concentration [Ca²⁺] may be responsible for the rapid transient phase of smooth muscle contraction (Touyz and Schiffrin, 2000).

The sustained phase of smooth muscle contraction is associated with influx of calcium from the extracellular space via the voltage-, ligand- or store-operated calcium channels. DAG stimulates protein kinase C (PKC), a Ca²⁺- and a phospholipid-dependent kinase that activates specific proteins involved in smooth muscle contraction (Bell et al., 1986; Rasmussen et al., 1987). The free cytosolic Ca²⁺ binds to calmodulin (CAM) to form a Ca²⁺-CAM complex that activates myosin light chain kinase (MLCK). MLCK catalyses phosphorylation of MLC, a 20 kD regulatory subunit of myosin. MLC phosphorylation increases myosin ATPase activity and causes smooth muscle contraction by promoting
cross-bridging of myosin with actin filaments (Khalil, 2010). Furthermore, activation of the Rho kinase pathway promotes sustained contraction of smooth muscle cells by inducing calcium sensitization through inhibition of myosin phosphatase activity (Fukata et al., 2001) (Figure 1-8).
Figure 1-8: Mechanisms of smooth muscle contraction.

Binding of an agonist (A) to a receptor (R) stimulates a membrane bound phospholipase B enzyme (PLCB) which converts phosphatidylinositol 4,5-bisphosphate (PI$_2$) into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ stimulates its receptor on the sarcoplasmic reticulum (SR) and causes release of Ca$^{2+}$. The free cytosolic Ca$^{2+}$ binds to calmodulin (CAM) and thus activates MLC kinase (MLCK) and causes MLC phosphorylation. DAG activates protein kinase C (PKC) which phosphorylates CPI-17, and inhibits MLC phosphatase and thereby increases the sensitivity of myofilament to Ca$^{2+}$. PKC also phosphorylates calponin (Cap) and promotes binding of actin to myosin. PKC may activate a mitogen-activated protein kinase (MAPK) pathway leading to phosphorylation of the actin-binding protein caldesmon (CaD). Activation of the RhoA/Rho-kinase pathway promotes contraction of smooth muscle cells by inhibiting MLC phosphatase. G, heterotrimeric G protein;
1.7.2. Regulation of the intracellular free calcium concentration

Calcium (Ca\(^{2+}\)) is an ubiquitous intracellular messenger that is involved in the regulation of various cellular processes such as muscle contraction, neurotransmission, secretion, gene transcription and cell proliferation (Bootman et al., 2001). Calcium transport across membranes is the basic homeostatic process by which eukaryotic cells maintain these functions. The concentration of calcium in the cytoplasm is normally about four orders of magnitude lower than that of the extracellular space in the resting state; this gradient is maintained by different Ca\(^{2+}\) transporting systems. Intracellular Ca\(^{2+}\) needed for such activities as vascular smooth muscle contraction is provided by the balance between entry from the extracellular space or release from internal stores and extrusion via the plasmalemmal Ca\(^{2+}\)-adenosine triphosphatase (Ca\(^{2+}\)-ATPase) and the Na\(^+\)-Ca\(^{2+}\) exchanger, or uptake into intracellular stores (Alborch et al., 1995; Nikitina et al., 2007).

i. Calcium entry

Cells use several different types of Ca\(^{2+}\) influx channels, which can be grouped on the basis of their activation mechanisms: voltage-operated calcium channels (VOCC), receptor-operated calcium channels (ROCC; also called ligand-gated calcium channels), mechanically-activated calcium channels and store-operated calcium channels (SOCC) (Bootman et al., 2001).

Voltage-operated calcium channels are mostly activated by depolarization of the plasma membrane. They are employed by excitable cells such as muscle and neural cells. ROCC are activated by the binding of agonists (such as acetylcholine, serotonin, ATP or glutamate) to the extracellular domain of the channels, while mechanically-operated channels open in response to cell deformation or stress. SOCC are activated when the calcium in the intracellular
stores is depleted. ROCC include the transient receptor potential channels that are ubiquitously expressed in almost all mammalian tissues. In addition, certain unidentified channels may be activated by intracellular messengers such as DAG or arachidonic acid (Berridge et al., 2000).

**ii. Calcium release from intracellular stores**

Calcium can be released from the endoplasmic or sarcoplasmic reticulum through two classical channels - inositol 1, 4, 5-triphosphate receptors (IP$_3$Rs) and ryanodine receptors (RyRs) (Tykocki and Watts, 2010). IP$_3$ can directly activate IP$_3$R and release Ca$^{2+}$ from SR but DAG inhibits Ca$^{2+}$ release through activation of PKC. RyRs are activated by local increase of calcium in the cytoplasm and furnish additional release of Ca$^{2+}$ from SR amplifying the small Ca$^{2+}$ signal produced by VOCCs or IP$_3$-mediated Ca$^{2+}$ release. RyRs are also involved in the termination of calcium influx through VOCC; i.e., localized release of Ca$^{2+}$ through RyRs, called ‘calcium spark’, activates calcium-sensitive potassium channels, thus causing hyperpolarisation of the plasma membrane which closes the VOCC (Berridge et al., 2000).

1.7.3. **Voltage-operated calcium channels (VOCC)**

**i. Structure of VOCC**

The voltage-operated Ca$^{2+}$ channels comprise a family of structurally-related proteins coupling electrical signals on the cell surface to intracellular events like muscle contraction, secretion, neurotransmission and gene expression (Catterall et al., 2005). Different types of VOCC have been identified based on the properties of the Ca$^{2+}$ current, pharmacological profile and structure of the protein subunits constituting the channels (Catterall, 2000).

The voltage-operated calcium channels described to date are made up or five subunits, namely $\alpha_1$, $\alpha_2$, $\delta$, $\beta$ and $\gamma$ subunits. The $\alpha_1$ subunit has four homologous domains (I-IV) with six transmembrane segments (S1-S6) in each domain as shown in Figure 1-9. The S4 segment in each domain contains the voltage sensor
while the pore loop between S5 and S6 determines ion conductance and selectivity (Catterall et al., 2005). The α₁ subunit can form a functional channel by itself; hence the other subunits are often called auxiliary or ancillary subunits. However they can greatly modulate the electrophysiological and pharmacological properties of the channels (Bergsman et al., 2000). The diversity of VOCC is mostly related to the variation of the α₁ subunits.

**ii. VOCC subtypes**

Based on the degree of depolarization required for their gating, VOCC can be categorized into two major classes - low voltage-activated (LVA) and high voltage-activated (HVA) calcium channels (Bergsman et al., 2000). Alphabetical nomenclature had been used to describe the calcium currents transmitted by these channels. Thus, VOCC were classified as T-, L-, N-, P/Q and R-type. Currently, the α₁ subunits of various VOCCs have been cloned and characterised structurally and their familial relationship revealed. Based on their sequence homology, ten α₁ subunits have been identified and designated a new system of nomenclature. Table 1-1 summarizes the new channel nomenclature along with the alphabetical designation of the respective type of Ca²⁺ currents.
Figure 1-9: Structure of the voltage-operated calcium channel (Ca\textsubscript{v}1).

The different subunits constituting the Ca\textsubscript{v}1 calcium channel (α\textsubscript{1}, α\textsubscript{2}, β, δ and γ) and the different domains of α\textsubscript{1} subunit (I-IV) are shown (from Catterall et al. (2005)).

The L- (Ca\textsubscript{v}1.1-1.4), P/Q- (Ca\textsubscript{v}2.1), N- (Ca\textsubscript{v}2.2), and R-type (Ca\textsubscript{v}2.3) VOCC are activated at less negative voltages and thus are classified as HVA, while T-type (Ca\textsubscript{v}3.1-3.3) calcium channels are categorized as LVA since they are activated at lower depolarization potentials (Nikitina et al., 2007; Kuo et al., 2011).

**iii. Function of VOCC**

Voltage-dependent calcium channels are ubiquitously distributed throughout many different tissues and involved in a variety of functions (Bergsman et al., 2000). They are mainly responsible for such functions as coupling of excitation and contraction, rhythmic activities, excitation-secretion coupling, synaptic nerve transmission, and proliferation as shown in Table 1-1 (Leanne, 2006).
Table 1-1: Classification of the voltage-operated calcium channels, their location, specific inhibitors and physiological functions.

<table>
<thead>
<tr>
<th>Name</th>
<th>Current</th>
<th>Localization</th>
<th>Selective antagonist</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca,1.1</td>
<td>L</td>
<td>Skeletal muscle; transverse tubules</td>
<td>Dihydropyridines, phenylalkylamines and benzothiazepines</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>Ca,1.2</td>
<td>L</td>
<td>Cardiomyocytes; smooth muscles; endocrine cells; neuronal cell bodies; proximal dendrites</td>
<td>Dihydropyridines, phenylalkylamines and benzothiazepines</td>
<td>Excitation-contraction coupling; hormone release; regulation of transcription; synaptic integration</td>
</tr>
<tr>
<td>Ca,1.3</td>
<td>L</td>
<td>Endocrine cells; neuronal cell bodies and dendrites; cardiac atrial myocytes and pacemaker cells; cochlear hair cells</td>
<td>Dihydropyridines, phenylalkylamines and benzothiazepines</td>
<td>Hormone release; regulation of transcription; synaptic regulation; cardiac pacemaking; hearing; neurotransmitter release from sensory cells</td>
</tr>
<tr>
<td>Ca,1.4</td>
<td>L</td>
<td>Retinal rod and bipolar cells, spinal cord, adrenal gland and mast cells</td>
<td>Dihydropyridines, phenylalkylamines and benzothiazepines</td>
<td>Neurotransmitter release, photoreceptors</td>
</tr>
<tr>
<td>Ca,2.1</td>
<td>P/Q</td>
<td>Nerve terminal and dendrites; neuroendocrine cells</td>
<td>ω-Agatoxin IVA</td>
<td>Neurotransmitter release; dendritic Ca(^{2+}) transients; hormone release</td>
</tr>
<tr>
<td>Ca,2.2</td>
<td>N</td>
<td>Nerve terminals and dendrites; neuroendocrine cells</td>
<td>ω-Conotoxin GVIA</td>
<td>Neurotransmitter release; dendritic Ca(^{2+}) transients; hormone release</td>
</tr>
<tr>
<td>Ca,2.3</td>
<td>R</td>
<td>Neuronal cell bodies and dendrites</td>
<td>SNX-482</td>
<td>Repetitive firing; dendritic calcium transients</td>
</tr>
<tr>
<td>Ca,3.1</td>
<td>T</td>
<td>Neuronal cell bodies and dendrites; cardiac and smooth muscle myocytes</td>
<td>Kurotoxin, NNC55-0396 and mibebradil</td>
<td>Pacemaking; repetitive firing</td>
</tr>
<tr>
<td>Ca,3.2</td>
<td>T</td>
<td>Neuronal cell bodies and dendrites; cardiac and smooth muscle myocytes</td>
<td>Kurotoxin, NNC55-0396 and mibebradil</td>
<td>Pacemaking; repetitive firing</td>
</tr>
<tr>
<td>Ca,3.3</td>
<td>T</td>
<td>Neuronal cell bodies and dendrites</td>
<td>Kurotoxin, NNC55-0396 and mibebradil</td>
<td>Pacemaking; repetitive firing</td>
</tr>
</tbody>
</table>

Adapted from Catterall *et al.* (2005).
L-type calcium channels play a central role in excitation-contraction coupling in skeletal muscle, cardiac muscle and other smooth muscles. Other types of VOCC may also play supportive roles during muscle contraction.

The L-type calcium channels of skeletal muscle, localized in the transverse tubules system, are not directly required for contraction although blockade thereof completely inhibits contraction. Rather, they serve as voltage sensors for release of calcium from internal stores (Näbauer et al., 1989). Calcium entry, however, is essential for contraction of cardiac and smooth muscles unlike skeletal muscle (Davis and Hill, 1999). In contrast to striated muscles (i.e., skeletal and cardiac), smooth muscles have a tendency to undergo a biphasic contraction - fast tonic contraction mediated by L-type VOCC followed by a slow phasic response, possibly mediated by T-type VOCCs (Leanne, 2006). There is a growing body of evidence supporting the vasoconstrictor role of T-type channels in renal and cerebral circulations (Navarro-Gonzalez et al., 2009; Kuo et al., 2011). In cardiac cells, T-type channels furnish the major proportion of current required for pacemaker depolarization in the sino-atrial node (Bergsman et al., 2000).

1.7.4. Role of calcium channels in basal tone of cerebral arteries

As in other peripheral blood vessels, calcium influx through VOCCs is the main pathway for electromechanical coupling in cerebral arteries (Nikitina et al., 2007; Kuo et al., 2011). The dynamic range of global cytosolic calcium ([Ca^{2+}]_i) mediating contraction in cerebral arteries is however quite narrow (Wellman, 2006). As a result, cerebral arteries typically display spontaneous submaximal constriction depending on the level of intraluminal pressure or isometric tension. This phenomenon, termed myogenic tone, is an essential mechanism in regulation of local blood flow and tissue perfusion in the cerebral vasculature (Davis and Hill, 1999).
Basal uptake of Ca\(^{2+}\) in the resting state varies in cerebral arteries from different species as measured using a \(^{45}\)Ca\(^{2+}\) isotope. The resting state of Ca\(^{2+}\) influx in dog basilar artery was found to be higher than that in the corresponding mesenteric artery and inhibited by nifedipine (Asano et al., 1993). This shows that the basilar artery may be more depolarized than the mesenteric artery in the resting state. Voltage clamp studies also demonstrated that L-type calcium channels have significant open-state probability around the normal resting potential in canine basilar artery myocytes (Langton and Standen, 1993). On the other hand, verapamil or nifedipine did not affect the basal uptake in bovine isolated middle cerebral arteries, but significantly blocked the \(^{45}\)Ca\(^{2+}\) uptake induced by potassium or serotonin (Wendling and Harakal, 1987). This indicates involvement of non-L-type VOCC in basal tone while L-type VOCCs are mainly responsible for contraction induced by membrane depolarization.

1.7.5. Calcium channels involved in agonist-induced contraction of cerebral arteries

Agonist-induced contraction of smooth muscle cells is dependent on Ca\(^{2+}\) influx from the extracellular space. 5-HT, prostanoids (prostaglandin F\(_{2\alpha}\) and thromboxane A\(_2\)) and endothelins are generally known to increase Ca\(^{2+}\) influx through the VOCCs or ROCC independent of depolarization or release from intracellular stores (Salom et al., 1991; Wendling and Harakal, 1991; Tejerina et al., 1993). However there is variation in the extent of Ca\(^{2+}\) influx induced by the agonists in different species or different arterial beds within the same species.

Despite the acceptance of L-type calcium channels as mediators of agonist-induced cerebral artery contraction, nifedipine did not completely reverse this in basilar arteries from rats, dogs and rabbits (Navarro-Gonzalez et al., 2009). This indicates that other types of calcium channels may be involved in agonist-induced vascular contraction or resting basal tone of cerebral arteries. It may also support the suggestion of the existence of receptor-operated calcium
channels in addition to the conventional VOCCs in the cerebral circulation (Alborch et al., 1995).

The role of T-type VOCCs in contraction of vascular smooth muscle has been controversial mainly because these channels transmit tiny and transient, rather than persistent, Ca$^{2+}$ currents and are more sensitive to depolarization. The window current, defined as the voltage range over which the channel has the capacity to provide persistent calcium influx, for T-type VOCC ranges from -65 to -45 mV (-30 to 0 mV for L-type VOCC) (Kuo et al., 2011). There is strong evidence from electrophysiological and molecular studies supporting the existence of T-type VOCCs in cerebral arteries. To illustrate, T-type VOCCs (along with N-type VOCC) were identified using patch clamp studies in vascular smooth muscle cells isolated from dog basilar arteries (Navarro-Gonzalez et al., 2009).

Expression of T-type VOCCs in renal (Poulsen et al., 2011), coronary (Küng et al., 1995) and mesenteric arteries (Gustafsson et al., 2001) has been previously described. Similarly, Kuo et al. (2010) found expression of mRNA and proteins of T-type (Ca$_{v}$3.1 and Ca$_{v}$3.2) and L-type (Ca$_{v}$1.2) in smooth muscle cells of adult rat basilar and middle cerebral arteries and their branches. They also reported that about 20% of the Ca$^{2+}$ current in smooth muscle cells of major cerebral arteries and about 45% of the current in smooth muscle cells of their branches were constituted by T-channels.

The HVA and LVA calcium channels might be responsible for different functions in cerebral arteries - L-type VOCC playing a major role in vasomotion and essentially responsible for contraction, while T-type VOCC control basal vascular tone (Navarro-Gonzalez et al., 2009). Incubation of basilar arteries with nifedipine abolishes vasomotion without affecting the vascular tone. Application of depolarizing current in the presence of nifedipine, however, caused immediate vasorelaxation. On the other hand, three T-channel antagonists (mibefradil, pimozide and flunarizine), unlike nifedipine, relaxed the basal tone of basilar arteries, further supporting the role of T-channels in
maintaining resting tone of the cerebral arteries. In addition, vasoconstriction induced by 40 mM KCl in the presence of an L-channel blocker was significantly inhibited by mibefradil.

Electrophysiological and molecular studies previously revealed the presence of mRNA and protein for L-(Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3), N- (Ca\textsubscript{v}2.2) and T-type (Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.3) α\textsubscript{1} Ca\textsuperscript{2+} channel subunits (Nikitina \textit{et al.}, 2007) in canine basilar arteries. Nimodipine and mibefradil relaxed intact arteries under isometric tension while ω-agatoxin IVA, ω-conotoxin GVIA or SNX-482 did not relax these arteries. This confirms a functional role of L- and T-type, not N-type, VOCC in canine basilar arteries.
1.8. Therapeutic options for the management of cerebral blood flow disorders

Various therapeutic agents have been tested as treatment modalities to prevent or minimize the neurological morbidities and mortalities associated with the disorders of the cerebral blood flow. Delayed cerebral vasospasm is among the most widely investigated as far as pharmacotherapy of the cerebral circulation disorders is concerned.

Treatment of cerebral vasospasm aims to reduce neurological damages due to ischaemia. The commonly employed treatment modalities include haemodynamic management, endovascular techniques and oral nimodipine pharmacotherapy (Rahimi et al., 2006; Al-Tamimi et al., 2010). Haemodynamic management constitutes the triple H therapy (induced hypertension, haemodilution and hypervolaemia). It was designed to improve cerebral perfusion by increasing blood pressure or cerebral perfusion pressure and volume expansion. The explanation for haemodynamic management is that vasospasm impairs cerebral autoregulation, thus increasing perfusion pressure will passively increase cerebral blood flow according to the Hagen-Poiseuille law. Although triple H therapy improves cerebral perfusion its benefit in decreasing neurological deficits and mortality is not evident (Castanares-Zapatero and Hantson, 2011). Further, triple H therapy is associated with other morbidities such as fluid overload, pulmonary oedema, hyponatremia, congestive heart failure and myocardial infarction (Al-Tamimi et al., 2010).

Nimodipine is the only L-type calcium channel blocker in clinical use for cerebral vasospasm to date offering a modest clinical outcome with negligible effect on the angiographic vasospasm (Pickard et al., 1989). Whether the efficacy of nimodipine in preventing secondary ischaemia is due to its ability to cross the blood brain barrier or its effect on other calcium channels is not clearly known. Nimodipine may protect neuronal death by decreasing calcium influx (Al-Tamimi et al., 2010). Nonetheless, the possibility that nimodipine may dilate the penetrating arteries and arterioles rather than the pial arteries
cannot be ruled out. Despite the improvement in cerebral vasospasm demonstrated by several other drugs tested thus far, most did not modify clinical outcomes. Among the commonly studied agents are neuroprotective agents (such as corticosteroids), statins, magnesium sulphate, nitric oxide donors and endothelin antagonists (Castanares-Zapatero and Hantson, 2011). Recently, clazosentan (selective ET₄ antagonist), which has been in a phase III clinical trial (CONSCIOUS II and III), decreased postaneurysmal subarachnoid haemorrhage vasospasm-related morbidity and mortality, but did not improve outcome (extended Glasgow Outcome Scale) (Macdonald et al., 2012).

Limited progress has been made in the pharmacological treatment of ischaemic stroke as well. The current approach in the management of acute ischaemic stroke mainly employs an anticoagulant or a thrombolytic agent. As such, aspirin and recombinant tissue plasminogen activator are among the few therapeutic agents with demonstrated efficacy in improving clinical outcomes (Ducruet et al., 2009). Statins are also found to be fairly effective in prevention of ischaemic stroke, a benefit independent of their inhibition of cholesterol synthesis. Statins prevent brain tissue damage by maintaining cerebral blood flow through increasing nitric oxide production (Terpolilli et al., 2012). While various agents with thrombolytic activity and neuroprotective benefits are currently being investigated for cerebral vasospasm (Kikuchi et al., 2014; Logallo et al., 2014), the quest for effective therapeutic agents is unanswered. Understanding the responses of cerebral circulation to pharmacological stimulation and the mechanisms involves is vital to for the development of novel drugs that can effectively be used for prevention and treatment of the cerebrovascular disorders. Thus, the current study attempts to analyse the responses of cerebral circulation to activations of different pharmacological targets.
1.9. **Objectives of the study**

The goal of this study is to investigate the responses of cerebral arteries to pharmacological stimulation in isolated arteries, animal models and human subjects. Specifically, the study aims to analyse the:

- Reactivity of cerebral arteries isolated from different regions of the rat cerebral circulation;
- Mechanisms involved in adrenoceptor-mediated responses in rat isolated cerebral arteries;
- Effects of endothelin-1 on rat cerebral arteries and the roles of voltage-operated and non-voltage-operated calcium channels in mediating the responses;
- *In vivo* responses of cerebral arteries to systemically-administered vasoactive agent in the rat; and
- Effects of vasopressor administration on cerebral oxygen saturation during anaesthesia in supine and beach chair positions in patients.
Chapter 2

Methods of *in vitro* small artery reactivity studies
2.1. Introduction

Most of the knowledge regarding effects of vasoactive agents on blood vessels and their targets has been generated *in vitro* in isolated tissue preparations, a technique more than a hundred years old. Various techniques have been employed to characterise the responses of large arteries isolated from various species in an artificially controlled condition without the influence of homeostatic mechanisms such as autonomic nervous system (Moulds, 1983; Spiers and Padmanabhan, 2005). In most cases, the active force induced in the arteries is measured where the vessel diameter is held constant (isometric) or change in diameter is measured under constant pressure (isobaric) or constant tension (isotonic). *In vitro* studies are the most reliable methods to accurately generate agonist concentration-response curves to various vasoactive substances in blood vessels. Although attempts have been made to simulate the *in vivo* parameters (temperature, electrolyte composition, oxygen, pH and transmural pressure), it is impossible to mimic the exact physiological conditions (Yu et al., 2003).

Previous studies have been commonly conducted in spiral strips or ring preparations of large arteries of humans and animals set up in organ baths. Following the understanding of the major contribution of small resistance arteries to total peripheral resistance, however, there has been a growing interest in investigating the contractility of these blood vessels in normal and various disease states. Several investigators attempted to isolate small arteries and characterise the responses using their own custom-made devices. For example, the variation in pharmacological response between different arterial beds was investigated by Bohr *et al.* (1961) in smooth muscle preparations of small resistance arteries isolated from various tissues. Rabbit or dog small resistance arteries (internal diameter 200-300 µm) isolated from mesentery, kidney, lung and brain were sliced into helical strips and mounted on a displacement transducer that was able to record the phasic change in tension. The contractile responses elicited by different vasoactive agents in arteries isolated from different organs were then compared (Figure 2-1).
While this endeavour had advanced technical capability to characterise pharmacological agents in small resistance arteries *in vitro*, there were still concerns about the structural and functional integrity of the vessels. Bevan and Osher (1972) later described an improved method to measure the wall tension in intact cylindrical segments of small arteries. The vessels were dissected and mounted either on a metallic rod and a thin platinum wire (for arteries with internal diameter >200 µm) or on a pair of thin platinum wires (for smaller arteries) that were connected to tension gauge recorder (Figure 2-2). This method allowed continuous measurement of the response in isolated small resistance arteries with accuracy. To date, more advanced myographs with better sensitivity are commercially available.

Myography is an *in vitro* method widely used to characterise functional responses of isolated small resistance arteries of 100-500 µm internal diameter. There are two types of myography techniques – wire and pressure myography. Wire myography is based on measurement of the contraction force under isometric conditions, while pressure myography mostly measures diameter of the pressurized vessels under isobaric conditions. Both wire and pressure myographs are widely used in *in vitro* investigation responses in small vessels, but some arteries showed differences in the pressure-diameter relationship (Lew and Angus, 1992). The response to certain vasoactive mediators may also vary when the arteries are mounted in the wire or pressure myograph. To illustrate, pressurised rat mesenteric arteries were found to be 5-6 fold more sensitive to noradrenaline and phenylephrine than wire-mounted vessels (Buus *et al.*, 1994). Various versions of the wire myograph have been introduced since it was initially developed about four decades ago by Mulvany and Halpern (1976). The four-channel wire myograph (model 620M; Danish Myo Technology; Aarhus, Denmark) is now widely used for *in vitro* characterisation of isolated small resistance arteries because it allows a simultaneous investigation of four arteries in separate baths (Figure 2-3). The complete myography set up consists of a myograph with isometric transducers, a data converter interface (PowerLab) and a computer with software that helps to record the contractile force and analyse data.
Figure 2-1: The first small artery wall tension measurement technique.
A. Preparation of arterial strips and mounting on a displacement transducer and B. a typical recording comparing the contractile responses induced by vasoactive agents in the mesenteric and renal arteries (from Bohr et al. (1961)).
Figure 2-2: An early version of wall tension measurement in intact cylindrical segments of small resistance arteries.

A. An artery (~200 µm i.d.) connected to Statham strain gauge with a stainless steel rod (1.5 cm long; 200 µm i.d.) and a platinum wire (100 µm i.d.); B. A small artery connected to two bridges of supporting plates, one connected to the strain gauge, with a pair of thin platinum wires (25 µm i.d.); and C. A typical trace of spontaneous rhythmic activity of a small mesenteric artery (200 µm i.d.) (slightly modified from Bevan and Osher (1972)).
Figure 2-3: A wire myograph setup

The setup consists of a 4-channel wire myograph (620M; Danish Myo Technology; Aarhus, Denmark) connected to a PowerLab (AD Instruments, Pty. Ltd, Bella Vista, NSW, Australia) and a computer. A segment of a small artery is set up on a pair of jaws with the help of two 40 µm wires. One of the jaws is connected to a micrometer and the other is connected to an isometric force transducer. The tone of the arteries was monitored using LabChart 7 software (AD Instruments, Pty. Ltd, Bella Vista, NSW, Australia).
2.2. Physiological salt solutions

Several physiological salt solutions (PSS) of slightly variable electrolyte composition have been introduced by different researchers (Table 2-1). Despite variation in composition between different PSS types, or sometimes between the same PSS in different labs, they are all isotonic with the blood. The solution is usually made by dissolving analytical grade ingredients in purified water. The critical ions in most PSS are sodium, potassium, bicarbonate and calcium. Most of the osmotic pressure is caused by sodium and chloride ions. Bicarbonate is the principal component of the buffer system controlling pH of the solution. In some cases, synthetic buffers such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) be used instead of bicarbonate. Calcium is needed for smooth muscle contraction. The concentration of potassium can affect the tone of smooth muscle cells - low potassium concentration causes membrane hyperpolarisation while high concentration induces contraction of blood vessels through depolarization. Phosphate and/or sulphate salts are added to increase the buffer capacity and maintain normal anion balance. Some PSS contain EDTA (ethylenediaminetetraamino acetic acid) for chelation of heavy metals that may be present from salt impurities or leaching of metal surfaces. Glucose is commonly included as a metabolic substrate. PSS containing bicarbonate are often aerated continuously with a mixture of 95% O₂ and 5% CO₂ (carbogen). Bicarbonate buffer is most effective in the presence of a high concentration of CO₂. High concentration of O₂ (90-100%) is needed to compensate for the lack of oxygen carriers such as haemoglobin (Moulds, 1983; Radnoti Glass Technology, 2010)
Table 2-1: The composition and application of common physiological salt solutions used in isolated tissue experiments.

<table>
<thead>
<tr>
<th></th>
<th>Frog Ringers</th>
<th>Ringer Locke</th>
<th>Tyrode</th>
<th>Krebs-Henseleit</th>
<th>McEwan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/ml mM</td>
<td>g/ml mM</td>
<td>g/ml mM</td>
<td>g/ml mM</td>
<td>g/ml mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.0-6.5 103-111</td>
<td>9.0 14</td>
<td>83.0 13.7</td>
<td>6.9 118</td>
<td>7.6 130</td>
</tr>
<tr>
<td>KCl</td>
<td>0.075-0.14 1-1.9</td>
<td>0.4 5.4</td>
<td>0.2 2.7</td>
<td>0.35 4.7</td>
<td>0.42 5.6</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1-0.12 0.9-1.1</td>
<td>0.12-0.24 1.1-2.2</td>
<td>0.24 2.2</td>
<td>0.28 2.5</td>
<td>0.24 2.2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.01-0.05 0.1-0.5</td>
<td></td>
<td></td>
<td>0.29 1.2</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td></td>
<td></td>
<td></td>
<td>0.29 1.2</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.01 0.1</td>
<td></td>
<td></td>
<td>0.05 0.04</td>
<td>0.16 1.2</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.1-0.2 1.2-2.4</td>
<td>0.15-0.5 1.8-6.0</td>
<td>1.0 11.9</td>
<td>2.1 25</td>
<td>2.1 25</td>
</tr>
<tr>
<td>Glucose</td>
<td>0-2.0 0-11.1</td>
<td>1.0-2.0 5.5-11.5</td>
<td>1.0 5.5</td>
<td>2.0 11.1</td>
<td>2.0 11.1</td>
</tr>
<tr>
<td>Aeration</td>
<td>Air</td>
<td>Air or O₂</td>
<td>Air or O₂ or 5% CO₂ + 95% O₂</td>
<td>5% CO₂ + 95% O₂</td>
<td>5% CO₂ + 95% O₂</td>
</tr>
<tr>
<td>Uses</td>
<td>Frog and other amphibian tissue</td>
<td>Mammalian perfused heart</td>
<td>Mammalian isolated smooth muscle</td>
<td>Tissue requiring good oxygenation (e.g., nerve, heart)</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Salmon (2014).
2.3. Protocols for *in vitro* vascular studies

2.3.1. Preparation of Krebs’ PSS and dissolution of drugs

The Krebs’ solution was made by dissolving the following ingredients in a water purified by double distillation and ion exchange technique (Milli-Q water) (Angus and Wright, 2000) (Table 2–2). The concentration of Ca$^{2+}$ was decreased for cerebral artery experiments to minimize the occurrence of spontaneous contractions. A 25x concentrated stock solution (Stock A) containing all ingredients except sodium bicarbonate, glucose, CaCl$_2$ and EDTA was initially made and kept in cold storage (4°C) for up to one week at a time. The Stock A was diluted on the day of each experiment; glucose and sodium bicarbonate were added and after vigorously bubbling the solution with a mixture of 5% CO$_2$ and O$_2$ for 5 min, CaCl$_2$ and EDTA were added. The solution was continuously aerated with 95% O$_2$ and 5% CO$_2$ thereafter. In addition, an isotonic potassium salt solution (KPSS) containing 124 mM K$^+$ obtained by equimolar substitution of NaCl with KCl in the Krebs’ PSS was similarly prepared. The KPSS was used to fully depolarise the smooth muscle and contract the arteries. An isotonic solution containing 40 mM K$^+$, used for pre-contraction of the arteries in some protocols, was made by replacing the corresponding amount of Na$^+$ with K$^+$ in Krebs’ PSS. In experiments involving calcium-free PSS, the solution was made without CaCl$_2$ and EDTA was replaced by EGTA (ethylene glycol tetraacetic acid) (1 mM).

All drugs were dissolved in water or other solvents according to the manufacturers’ or suppliers’ instructions and diluted aliquots were made in Milli-Q water just before use. At times, concentrated aliquots were stored in a -20°C freezer and thawed before single use.

2.3.2. Animals

Experiments were approved by The University of Melbourne Animal Ethics Committee in accordance with the *Australian code for the care and use of*
Methods

animals for scientific purposes (8th edition, 2013, National Health and Medical Research Council, Canberra). Rats were obtained from the Biomedical Animal Facility (University of Melbourne) where they were housed in climate-controlled conditions with a 12 h light/dark cycle and free access to normal pellet diet and drinking water.

Table 2-2: The composition of Krebs’ physiological salt solution used in this study

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Composition (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>144</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.9</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.5 (cerebral arteries)</td>
</tr>
<tr>
<td></td>
<td>2.5 (other arteries)</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>128.7</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>25</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>1.18</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>1.17</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.026</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.5</td>
</tr>
</tbody>
</table>

2.3.3. Extraction of the rat brain

Male or female Sprague-Dawley rats (300-350 g) were deeply anaesthetised by spontaneous inhalation of 5% isoflurane (Baxter Healthcare Pty. Ltd.; Toongabbie, NSW, Australia) in 95% O₂ and killed by decapitation; the vertebral column was cut close to the cranial bone to ease opening of the skull. The skin and muscle on the dorsal part of the skull were removed with small scissors. The skull bone was cut along the suture line with a special pair of angled iris surgical scissors inserted from the back and the flap of bone on each side was
removed with rongeurs until the whole brain was clearly visible. The dura overlying the brain was then slightly lifted with fine pair of rat-tooth forceps and cut with small sharp scissors. The olfactory bulb was disconnected by passing a small spatula blade under the brain and gently pushing the bulbs to the side. The brain was then lifted from the floor and the trigeminal nerve was detached near the brain stem using the spatula. The brain was then removed and placed in ice-cold Krebs’ physiological salt solution.

2.3.4. Dissection and setup of cerebral arteries

The brain was placed ventral side up in a petri dish containing ice-cold PSS and continuously bubbled with a mixture of 5% O₂ and 95% CO₂. Fine dissecting forceps and scissors were used to isolate the cerebral arteries. The surrounding meningeal and brain tissues were cleared from the arteries under a dissection microscope. The anterior cerebral artery, middle cerebral artery, posterior communicating artery and basilar artery were carefully dissected and immediately set up in the myograph (Figure 2-4). In some protocols mesenteric arteries were dissected and set up to compare effects in the peripheral circulation; the PSS used for mesenteric artery experiments contained 2.5 mM CaCl₂.

The vessels were cut into 2 mm long segments and mounted on two 40 µm stainless steel wires in a myograph chamber (620M; Danish Myo Technology; Aarhus, Denmark). One of the wires was connected to an isometric force transducer while the other was connected to a screw micrometer used for adjustment of passive force by varying the distance between the wires. Measurement of vascular contractile force was recorded on a computer using LabChart 7 software (AD Instruments, Pty. Ltd, Bella Vista, NSW, Australia). The arteries were allowed to equilibrate for about 30 min in Krebs’ PSS at 37°C (pH 7.4) with continuous aeration by a mixture of 95% O₂ and 5% CO₂.
Figure 2-4: A photographic image of freshly isolated rat brain (ventral surface).

The major cerebral arteries branching out from the Circle of Willis (anterior, middle cerebral and posterior communicating arteries) and the basilar artery are shown.

2.3.5. Normalization

The aim of normalization is to set the initial passive conditions (resting condition) under which the arteries give optimum responses upon pharmacological stimulation or depolarization and to standardise the experimental conditions. After resting for about 30 min, the arteries were normalized while the contractile force was continuous measured. The normalization was carried out by a stepwise distention of the tissue until the tension equivalent to a transmural pressure of 100 mmHg was achieved using the screw micrometer connected to one jaw of the myograph. The normalization window (DMT module plugin) was opened by selecting the DMT menu of the LabChart (Figure 2-5 and Figure 2-6). The vessel length, diameter
of mounting wire, as well as the micrometer reading before (baseline) and after every stretch were entered in the normalization window. The passive tension-
internal circumference curve was automatically generated and the internal circumference of the vessel was estimated by the software. The internal diameter of the artery was calculated from the corresponding internal circumference at 100 mmHg.

\[ D_{100} = \frac{L_{100}}{\pi} \]

Where, \( D_{100} \) is an estimated lumen diameter and \( L_{100} \) represents the internal circumference of the vessel where the point on the exponential curve of effective transmural pressure was 100 mmHg.

The vessels were relaxed by adjusting the micrometer to an estimated reading that corresponds to 90% of the internal diameter that was determined from the curve. All experiments were conducted under the same passive pressure (Mulvany and Halpern, 1977; Angus and Wright, 2000).
Figure 2-5: A representative trace demonstrating normalization of the basilar artery.

The vessel was stretched in a step-by-step fashion by rotating the micrometer connected to one jaw of the myograph, each step lasting for about 1 min, in order to achieve a target pressure of 13.3 KPa (100 mmHg). The artery was then relaxed to a resting tone corresponding to 90% of the internal circumference (IC90).
Figure 2-6: A screenshot display of a typical normalization window (DMT module plugin) in LabChart7 (AD Instruments).

After the wires were brought together to touch each other the force reading was set to zero on the myograph; the length of the vessel, diameter of the wire and the baseline micrometer reading were entered in the space provided. The artery was then stretched step-by-step and the micrometer readings were entered before each next stretch, until the target pressure of 13.3 KPa (100 mmHg) was obtained. The passive wall tension–internal circumference was automatically generated; the internal circumference (IC\textsubscript{100}) and the 90% IC (IC\textsubscript{90}) (micrometer X1) were estimated by the software.

2.3.6. Testing the effects of drugs

The viability of the arteries was initially tested by exposure to an isotonic potassium salt solution (KPSS) containing 124 mM K\textsuperscript{+} obtained by equimolar substitution of NaCl with KCl in the PSS. The KPSS-induced contraction was
repeated after 5 min and the maximum response of the second contraction was used as a reference for quantification of all vascular responses. Only vessels with a KPSS-induced contraction of 2.5 mN or more were used for experimental protocols. The presence of a functional endothelium was tested by the relaxant response to acetylcholine (1-10 µM) in arteries pre-contracted with vasopressin (0.1-1 nM) to 80-90% of the maximum KPSS-induced contraction. The endothelium was considered intact when acetylcholine caused more than 50% relaxation.

The effects of agonists were examined by addition of low-high concentration of the agents with cumulative increments in half-log\(_{10}\) units until no further response was elicited (maximum response). Responses were allowed to plateau before addition of the next higher concentration of the drugs. The relaxation responses were investigated in arteries pre-contracted to 80-100% of the KPSS-induced maximum contraction using vasopressin or with K\(^+\) 40 mM isotonic solution as required. In experiments involving target antagonism or enzyme inhibition, the agents were added to the baths 20 min prior to the agonist unless stated otherwise. The arteries were rinsed three times after every experiment to wash out the drugs completely or once every 15 min between protocols.

### 2.3.7. Removal of endothelium

After normalization, the passive tone of the artery was relaxed (to about 0.5 mN). The bath chamber was temporarily removed from the myograph and a straight and round section of a clean human hair was passed through the lumen of the artery under a light microscope with high magnification. From the inner surface of the vessel, the endothelium was rubbed off by slow back-and-forth movement of the hair, without stretching the artery longitudinally, until all the surface was covered. The chamber was mounted back on the myograph, the PSS solution changed and the tissue was allowed to equilibrate. After 5-10 min, the artery was stretched to its normalization pressure by adjusting the micrometer and allowed to stabilize for 5-10 min. The vessel was then contracted with KPSS to check its viability - tissues contracting less than 50% of
the previous KPSS-induced contraction were not used for experiments. Complete removal of the endothelium was confirmed by absence of relaxation to acetylcholine 1-10 µM (Mulvany, 2004).

2.4. Collection and analysis of data

The absolute contraction force of an individual artery was initially collected on a data pad provided by the LabChart software and expressed as percent of the KPSS-induced contraction. All data were expressed as mean ± SEM. Data analysis and sigmoid curve fitting (non-linear regression) were performed using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). The $pEC_{50}$ (-log$_{10}$ $EC_{50}$) value and the maximum response (Emax) elicited by the drugs were derived from the sigmoid concentration-response curve. $EC_{50}$ is defined as the concentration of agonist that evokes a half-maximal response and $pEC_{50}$ is the negative logarithm of the $EC_{50}$. Statistical comparison between groups of arteries was performed using unpaired t test or one-way ANOVA followed by Bonferroni’s, Tukey’s or Dunnett’s post-test, as appropriate. $P<0.05$ was considered significant in all cases.
Chapter 3

Effects of common vasoactive agents in rat isolated cerebral arteries
3.1. Introduction

Cerebral arteries, like peripheral arteries, are reactive to pharmacological stimulation owing to the presence of various receptors that mediate constriction or dilatation. The sensitivity of cerebral arteries to endogenous vasoconstrictor and vasodilator mediators is critical for maintenance of stable cerebral blood flow in spite of changing peripheral arterial pressure (cerebral pressure autoregulation). It is also important for regulating blood flow according to the metabolic needs of the brain (flow-metabolism coupling) (Peterson et al., 2011). An increased need or decreased supply of oxygen and nutrients to the brain promotes cerebral artery dilatation in order to increase the blood flow and supply more oxygen and nutrients to the brain. Conversely, when there is less need or increased supply, often due to elevated peripheral blood pressure, constriction of cerebral arteries limit the blood flow and thus prevent brain damage (Paulson et al., 1990).

Endogenous mediators may be involved in the pathogenesis of different cerebral blood flow disorders such as migraine or cerebral vasospasm (Crowley et al., 2008). Most of the drugs employed in the treatment of these disorders or new therapeutic agents under investigation target the tone of cerebral arteries (Castanares-Zapatero and Hantson, 2011). In the same way, exogenous vasoactive substances used for other conditions may affect blood flow to the brain. Nevertheless, presence of functional blood-brain barrier is thought to limit the direct effect of most systemically administered pharmacological agents on the cerebrovascular smooth muscle (Ballabh et al., 2004). Vasoactive agents may also indirectly affect the tone of cerebral arteries by inducing systemic hypertension or hypotension, consequently activating the autoregulatory vasoconstrictor or vasodilator response.

Several endogenously released factors affect the tone of vascular smooth muscle cells (Table 3-1). The response of cerebral arteries to common vasoactive drugs is generally similar to peripheral arteries, but some qualitative and quantitative differences exist. Further, arteries located in different regions
of the cerebral circulation may exhibit differential reactivity to the vasoactive mediators. The aim of this chapter is to investigate the responses of arteries isolated from different regions of the rat cerebral circulation to common vasoactive mediators. In addition, previous studies on the effects of these agents on cerebral arteries have been reviewed and compared with the results of this study.

3.1.1. Effects of endothelial factors on cerebrovascular tone

The role of the endothelium in the regulation of cerebral circulation is discussed in section 1.5.4. Briefly, vascular endothelium releases both constrictor and dilator mediators. The most widely characterised constrictor agents produced by the endothelium are endothelin (further discussion on endothelin is presented in Chapter 5) and arachidonic acid metabolites such as thromboxane A₂, which is also produced by platelets and brain tissues. Thromboxane A₂ is a potent vasoconstrictor in cerebral as well as peripheral arteries (Ellis et al., 1976; Ellis et al., 1977). Enhanced expression of thromboxane A₂ receptors (TP) is associated with a decrease in cerebral blood flow following subarachnoid haemorrhage (Saema et al., 2010).

Nitric oxide, prostacyclin (PGI₂) and endothelium-derived hyperpolarisation (EDH) are the major dilators produced and released by the vascular endothelial cells. Peptide dilators (e.g., bradykinin, substance P, calcitonin gene-related peptide (CGRP) and arginine vasopressin) and certain neurotransmitters (e.g., acetylcholine and noradrenaline) induce vasodilatation by stimulating release of endothelium-derived relaxing factors (Toda and Okamura, 1998). Deficiency of endothelial vasodilator agents and/or increased production of vasoconstrictor mediators have been implicated in the pathogenesis of cerebral vasospasm and ischaemia (Suhardja, 2004).

3.1.2. The sympathetic nervous system

Cerebral arteries receive abundant noradrenaline-containing neurons from the peripheral sympathetic nervous system. The majority of these neurons
innervating the cerebral circulation originate from the superior cervical ganglion. Some adrenergic projections originating from subcortical nuclei such as the locus coeruleus also synapse on cerebral arteries (Raichle et al., 1975; Micieli et al., 1994; Hamel, 2006). In terms of number of nerve axons per mass of smooth muscle, cerebral arterial beds are probably more densely innervated than other vascular beds. The nerve terminals contain similar amounts of noradrenaline as those of well-innervated peripheral arteries and the release and reuptake processes are not different (Edvinsson and Owman, 1974; Edvinsson et al., 1993). Noradrenaline, a potent vasoconstrictor in peripheral arteries, induces a weak constrictor effect in arteries isolated from certain regions of the cerebral circulation. On the other hand, noradrenaline induces a variable degree of relaxation in different cerebral arteries actively contracted with an agonist. Consistent with these findings, \textit{in vivo} sympathetic stimulation barely affected cerebral blood flow under normal conditions. Heterogeneous distribution of the constrictor and dilator adrenoceptors in smooth muscle and endothelium of cerebral arteries may be responsible for the diversity of responses to pharmacological stimulation. A further discussion of cerebrovascular adrenoceptor stimulation is presented in Chapter 4.

3.1.3. The parasympathetic nervous system

It is well established that cerebral arteries are innervated by the extracerebral parasympathetic nervous system originating from sphenopalatine, otic and internal carotid ganglia. Biochemical investigations have shown the presence of nerves containing acetylcholine in cerebral arteries. The distribution of cholinergic nerves is similar to adrenergic nerves in cerebral arteries of most species (Edvinsson and MacKenzie, 1976). \textit{In vitro} or \textit{in situ} application of acetylcholine or cholinomimetic drugs can elicit a potent relaxation in cerebral arteries (Edvinsson et al., 1977). However, the sensitivity of cerebral arteries to exogenous acetylcholine varies among different species (Tsukahara et al., 1986b). Acetylcholine causes relaxation of vascular smooth muscle cells by releasing nitric oxide via activation of endothelial muscarinic (M$_3$) receptors (Dauphin and Hamel, 1990; Toda and Okamura, 1990; Parsons et al., 1991).
Acetylcholine may also cause vasoconstriction of cerebral arteries by stimulating $M_1$ receptors located on smooth muscle cells (Dauphin et al., 1991). The peripheral parasympathetic innervation is limited to the pial arteries, but cholinergic neurons originating from the basal forebrain affect the cortical microcirculation (Hamel, 2004).

### 3.1.4. 5-Hydroxytryptamine (5-HT)

Earlier histochemical and autoradiographic studies provided evidence that nerves containing 5-HT (serotonin) are found in cerebral arteries. It was subsequently demonstrated that major cerebral arteries of various species are innervated by serotonergic nerves originating from the superior cervical ganglia along with adrenergic neurons (Cowen et al., 1986; Lincoln, 1995). Intrinsic serotonergic neurons projecting from raphe nucleus also innervate the cerebral microcirculation (Cohen et al., 1996) indicating that 5-HT may be involved in coupling cerebral blood flow to the metabolic needs of the brain (Nobler et al., 1999). Although in vitro application of 5-HT induces a potent vasoconstrictor effect mediated by 5-HT$_{2A}$ receptors in peripheral arteries, it has weak effects in cerebral arteries. The 5-HT-induced constriction of cerebral arteries involves activation of both 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors (Kovacs et al., 2012) but 5-HT$_{1B}$ receptors appear to be more important in cerebral arteries and are found to be upregulated in subarachnoid haemorrhage (Ansar et al., 2007).

### 3.1.5. Neuropeptides

The cerebral arteries are also innervated by sensory nerve fibres, originating largely from the trigeminal and pterygopalatine ganglion and containing calcitonin gene-related peptide (CGRP), substance P, neurokinin A, pituitary adenylate-cyclase activating peptide (PACAP) and vasoactive intestinal peptide (VIP) (Ruskell and Simons, 1987; Tsai et al., 1988; Ando et al., 1990; Ando et al., 1994; Baeres and Moller, 2004). The neuropeptides mostly induce in vitro relaxation responses in cerebral arteries obtained from different species (Edvinsson et al., 1985; Toda and Okamura, 1998). CGRP may be involved in the
pathogenesis of migraine headache by inducing dilatation of cerebral and dural blood vessels. Further, the antimigraine effects of triptans are related to inhibition of CGRP release through activation of 5-HT1B/1D receptors in trigeminovascular neurons (Tepper et al., 2002).

3.1.6. Bradykinin

Bradykinin belongs to a family of endogenous peptides called kinins which are produced by the enzymatic action of kallikreins on kininogen precursors (Regoli and Barabe, 1980). It has two types of G-protein coupled receptors - B1 and B2 receptors. Bradykinin relaxes blood vessels by activating B2 receptors predominantly located on endothelial cells and inducing the release of nitric oxide, cyclooxygenase products and EDH (Mombouli and Vanhoutte, 1995; Golias et al., 2007; Rahman et al., 2014). It is a potent vasodilator peptide in peripheral as well as cerebral arteries (Whalley and Wahl, 1983). Bradykinin may also contract blood vessels by inducing release of endothelium-derived contracting factors such as thromboxane A2 and prostaglandin H2 (Miyamoto et al., 1999; Ihara et al., 2000). Bradykinin causes a variable degree of dilatation in cerebral arteries isolated from different species (Görlach and Wahl, 1996). Additionally, bradykinin increases the permeability of the blood-brain barrier by opening the endothelial tight junctions (Wahl et al., 1999).

3.1.7. Vasopressin

Vasopressin is known to induce both constrictor and dilator effects in vascular smooth muscle cells. The vasoconstrictor activity is caused by stimulation of the vasopressin V1 receptors located on smooth muscle cells (Fernandez et al., 2001). The dilator effect involves release of the endothelial nitric oxide via activation of endothelial V1 receptors (Suzuki et al., 1992; Suzuki et al., 1994). Vasopressin exerts a variable degree of constriction in cerebral arteries isolated from different regions. This may be related to heterogeneous distribution of the constrictor and dilator receptors in different arterial beds (Garcia-Villalon et al., 1996). For instance, removal of endothelium enhanced the
Common vasoactive agents

vasoconstrictor effect of vasopressin in rabbit basilar artery (Garcia-Villalon et al., 1996), but not in human cerebral arteries (Martin de Aguilera et al., 1990). Vasopressin may be involved in the development of cerebral vasospasm following subarachnoid haemorrhage (Trandafir et al., 2004).

3.1.8. Angiotensin

It is widely accepted that angiotensin II causes constriction of peripheral as well as cerebral arteries either by directly stimulating AT$_1$ receptors located on smooth muscle cells or eliciting release of endothelial prostaglandins in different species (Wei et al., 1978; Edvinsson et al., 1979; Kanaide et al., 2003). It can also induce endothelium-dependent relaxation by activating AT$_1$ receptors located on vascular endothelium (Toda, 1984). Recent studies have shown that AT$_2$ receptors may also play role in the vasodilator effect of angiotensin II (Carey et al., 2000). The vasoconstrictor effect of exogenous angiotensin II in cerebral arteries can be attenuated due to simultaneous activation of dilator receptors (Näveri et al., 1994; Vincent et al., 2005). The AT$_1$-mediated contraction of cerebral arteries has been found to be upregulated in focal cerebral ischaemia (Stenman and Edvinsson, 2004; Ansar et al., 2007).

3.1.9. Soluble guanylyl cyclase (sGC) stimulation/activation

Soluble guanylyl cyclase (sGC) is the primary target of the gaseous ligands NO and CO (Evgenov et al., 2006; Bryan et al., 2009). Binding of NO to the haem moiety activates sGC which then catalyses the conversion of GTP to cyclic GMP (cGMP). cGMP induces vascular smooth muscle relaxation by a number of mechanisms. These include inhibition of inositol-1,4,5-triphosphate generation, enhancing efflux of cytosolic calcium, dephosphorylation of myosin light chain kinase (MLCK), inhibition of calcium influx and potassium channel opening (Priviero and Webb, 2010). Increased cGMP levels may also promote other cellular functions such as smooth muscle proliferation, platelet aggregation, and leukocyte recruitment (Zhong et al., 2011). Impaired NO/cGMP signalling is involved in the aetiology of several cardiovascular disorders such as systemic
arterial hypertension, pulmonary hypertension, coronary artery diseases and peripheral vascular diseases including erectile dysfunction and atherosclerosis (Stasch et al., 2011). A novel class of drugs directly acting on sGC has been emerging during the last two decades. They can be subclassified into two categories depending on the mode of action. The first category, called haem-dependent sGC stimulators, constitutes drugs that activate sGC with intact haem (e.g., YC-1, BAY 41-2272, BAY 41-8543, BAY 63-2521 and A-350619). They sensitize sGC to physiological concentrations of NO depending on the presence of reduced (ferrous) iron in the haem bound to sGC. The second group, known as sGC activators, bind to and activate the enzyme without the need for haem and NO binding (NO- and haem-independent) (Evgenov et al., 2006; Stasch et al., 2011). The currently available sGC activators include cinaciguat (BAY 58-2667), ataciguat (HMR 1766) and BAY 60-2770. Some of these drugs have demonstrated promising potential for the treatment of cardiovascular diseases (Schäfer et al., 2006; Weissmann et al., 2009; Andreas et al., 2010; Tamargo et al., 2010).
Table 3-1: Summary of the common mediators released by extrinsic and intrinsic perivascular nerves, their respective receptors and the responses elicited in cerebral arteries.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Origin/source</th>
<th>Receptor - location</th>
<th>Vascular response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peripheral (extrinsic)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>Superior cervical ganglion</td>
<td>α₁ - SMC</td>
<td>Constriction</td>
<td>(Edvinsson, 1975; Hamel, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α₂ - Endothelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>β₁, β₂ - Endothelium</td>
<td>Dilatation</td>
<td></td>
</tr>
<tr>
<td>ACH</td>
<td>Pterygopalatine ganglion</td>
<td>M₅ - Endothelium</td>
<td>Dilatation</td>
<td>(Edvinsson, 1975); Hamner et al. (2012)</td>
</tr>
<tr>
<td>NO</td>
<td>Pterygopalatine ganglion</td>
<td>-</td>
<td>Dilatation</td>
<td>(Nozaki et al., 1993; Westcott and Segal, 2013)</td>
</tr>
<tr>
<td>CGRP</td>
<td>Trigeminal ganglion</td>
<td>NK₁R - Endothelium</td>
<td>Dilatation</td>
<td>(Tsai et al., 1988; Edvinsson et al., 2001)</td>
</tr>
<tr>
<td>SP</td>
<td>Multiple</td>
<td>VAPC₁ - Endothelium</td>
<td>Dilatation</td>
<td>(Shimizu et al., 1999)</td>
</tr>
<tr>
<td>VIP</td>
<td>Pterygopalatine ganglia</td>
<td></td>
<td></td>
<td>(Edvinsson et al., 2001)</td>
</tr>
<tr>
<td>NPY</td>
<td>Superior cervical ganglion</td>
<td>NPY₁ - Endothelium</td>
<td>Constriction</td>
<td>(Nilsson et al., 1996; Edvinsson et al., 2001)</td>
</tr>
<tr>
<td><strong>Central (intrinsic)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>Locus coeruleus</td>
<td>α₁ - SMC</td>
<td>Dilatation</td>
<td>Cohen et al. (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α₂ - Endothelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>β₁, β₂ - Endothelium/SMC</td>
<td>Constriction</td>
<td></td>
</tr>
<tr>
<td>ACH</td>
<td>Nucleus basalis</td>
<td>M₅ - Endothelium</td>
<td>Dilatation</td>
<td>Sato et al. (2001)</td>
</tr>
<tr>
<td>5-HT</td>
<td>Raphe nucleus</td>
<td>5-HT₁B - SMC</td>
<td>Constriction</td>
<td>(Lincoln, 1995)</td>
</tr>
<tr>
<td>GABA</td>
<td>Unidentified</td>
<td>GABA₆ - SMC</td>
<td>Dilatation</td>
<td>Imai et al. (1991)</td>
</tr>
</tbody>
</table>

ACH, acetylcholine; CGRP, calcitonin gene-related peptide; GABA, gamma aminobutyric acid; 5-HT, 5-hydroxytryptamine; NA, noradrenaline; NO, nitric oxide; NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal peptide; and VSM, vascular smooth muscle.
3.2. Materials and methods

3.2.1. Isolation of arteries

The major arteries were isolated from anterior (anterior and middle cerebral arteries) and posterior (posterior communicating and basilar arteries) regions of the rat cerebral circulation and the vasomotor responses to common vasoactive agents were characterised using the wire myography technique as previously described in Chapter 2. The concentration-contraction or concentration-relaxation curves were constructed for each drug and the pEC$_{50}$ values were derived whenever possible.

3.2.2. Experimental protocols

The viability of the arteries was initially assessed by contraction with KPSS, the maximum value of which has been used as a reference to compare the vascular responses induced by vasoactive drugs. Drugs were added to the baths in cumulative concentrations where sufficient time was allowed for the responses to plateau before adding the next higher concentration of the drug. Relaxant effects of the drugs were similarly assessed in arteries pre-contracted with vasopressin. All responses were expressed as the % of the KPSS-induced maximum contraction.

3.2.3. Drugs and chemicals

The drugs used in this study were obtained from the following suppliers: Acetylcholine, noradrenaline and 5-hydroxytryptamine (Sigma Aldrich, St Louis, Missouri, USA); endothelin-1 (GenScript, Piscataway, NJ, USA); bradykinin and vasopressin (AusPep, Parkville, Victoria, Australia); sodium nitroprusside (Hospira Inc., Lake Forest, USA) and BAY 41-8543 (gift from Dr. Johannes-Peter Stasch, Bayer Pharma AG, Berlin, Germany). All drugs were dissolved and/or diluted in water except endothelin-1 and BAY 41-8543 which were initially dissolved in 10% dimethylformamide and then diluted in water.
3.3. Results

3.3.1. Effects of noradrenaline in cerebral arteries

Noradrenaline induced weak concentration-dependent contractile effects in rat isolated anterior and middle cerebral arteries. The maximum contraction (Emax) induced by noradrenaline was less than 20% of the KPSS-induced maximum contraction in both arteries. The contraction pEC$_{50}$ values for noradrenaline were 5.7±0.1 (n=8) in the anterior cerebral artery and 5.6±0.1 (n=7) in the middle cerebral artery (Figure 3-1). The basilar and posterior communicating arteries were not contracted by noradrenaline. In contrast, noradrenaline induced relaxation responses in all four arteries when pre-contracted with vasopressin (Figure 3-2). The relaxation pEC$_{50}$ values of noradrenaline were 6.2±0.1 (n=7), 6.5±0.2 (n=8), 6.8±0.3 (n=7) and 6.2±0.2 (n=6) in anterior cerebral, middle cerebral, posterior communicating and basilar arteries, respectively.
Figure 3-1: Contractile effects of noradrenaline in rat cerebral arteries.

Contractile effects of noradrenaline in rat isolated anterior cerebral, middle cerebral, posterior communicating and basilar arteries. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.
Figure 3-2: Relaxant effects of noradrenaline in rat cerebral arteries.
Rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries were pre-contraction with vasopressin (AVP) and when the plateau was obtained, cumulative concentrations of noradrenaline were added. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC₅₀±SEM. n, number of arteries from separate rats.
3.3.2. **Contractile effects of endothelin-1 in rat cerebral arteries**

Endothelin-1 induced potent constrictor effects in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries. The middle cerebral and posterior communicating arteries tended to be more sensitive to endothelin-1 than the anterior cerebral artery or basilar artery. The $pEC_{50}$ values of endothelin-1 were 8.4±0.06, 8.7±0.11, 8.8±0.14 and 8.3±0.05 in anterior cerebral, middle cerebral, posterior communicating and basilar arteries, respectively. The maximum effects caused by endothelin-1 in the cerebral arteries ranged from 108±2% ($n=11$) (in basilar artery) to 122±2% ($n=10$) (in anterior cerebral artery) of the KPSS-induced maximum contraction.
Figure 3-3: Contractile effects of endothelin-1 in rat cerebral arteries.
Contractile effects of endothelin-1 in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n; number of arteries from separate rats.

3.3.3. Contractile effects of 5-hydroxytryptamine (5-HT) in rat cerebral arteries

The contractile effects of 5-HT were characterised in the rat middle cerebral and basilar arteries. The contractile potency of 5-HT appeared to be similar in the two arteries - the pEC$_{50}$ values of 5-HT were 6.6±0.4 (n=4) in the middle
cerebral and 6.8±0.03 in the basilar artery. However, the maximum contraction induced by 5-HT was much greater in the rat middle cerebral artery (Emax 90±6%) than in the rat basilar artery which responded poorly (Emax 22±5% of the KPSS-induced maximum contraction; p<0.05, unpaired t test) (Figure 3-4).

![Graph of contractile effects of 5-hydroxytryptamine (5-HT) in rat middle cerebral and basilar arteries.](image)

**Figure 3-4:** Contractile effects of 5-hydroxytryptamine (5-HT) in rat middle cerebral and basilar arteries.

Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC\textsubscript{50}±SEM. n, number of arteries from separate rats.

### 3.3.4. Contractile effects of vasopressin in rat cerebral arteries

Vasopressin induced a strong contraction in both rat middle cerebral and basilar arteries (Figure 3-5). The maximum response induced by vasopressin was similar in the two arteries - 110±8% in the middle cerebral and 100±2% of the KPSS-induced maximum contraction in the basilar artery (p>0.05, unpaired t test). However, vasopressin was more potent in the middle cerebral artery, pEC\textsubscript{50} 10.0±0.2 (n=4), than in the basilar artery, pEC\textsubscript{50} 9.1±0.1 (p<0.05, unpaired t test; n=6). It was observed that some basilar arteries were resistant to
vasopressin contraction or rapidly desensitized upon exposure to vasopressin and their data were excluded from the analysis.

Figure 3-5: Contractile effects of vasopressin in rat middle cerebral and basilar arteries.
Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.

3.3.5. Effects of acetylcholine in rat cerebral arteries

Acetylcholine caused a concentration-dependent relaxation of rat isolated middle cerebral and basilar arteries pre-contracted with vasopressin (Figure 3-6). The relaxation was more pronounced in basilar artery than in the middle cerebral artery which was only weakly relaxed (EC$_{50}$ could not be determined). Acetylcholine induced a maximum relaxation of 83±8% of the pre-contraction tone in basilar arteries with a pEC$_{50}$ value of 6.2±0.2 (n=7). High concentrations of acetylcholine (≥30 µM) induced a weak contraction in some arteries.
Figure 3-6: Relaxant effects of acetylcholine in rat middle cerebral and basilar arteries.

The arteries were pre-contracted with vasopressin (AVP) and when the plateau was obtained, cumulative concentrations of acetylcholine were added. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. $n$, number of arteries from separate rats.

### 3.3.6. Relaxant effects of bradykinin in rat cerebral arteries

Bradykinin caused a potent relaxation in rat middle cerebral and basilar arteries pre-contracted with vasopressin (Figure 3-7). The relaxation $p$EC$_{50}$ value of bradykinin was greater in the middle cerebral artery (8.1±0.3, n=5) than in the basilar artery (7.2±0.2, n=4; $p<0.05$, unpaired t test). However, the maximum relaxation induced by bradykinin was similar in the two arteries: 65±13% in middle cerebral artery and 82±12% of the pre-contraction tone in the basilar artery ($p>0.05$, unpaired t test). It appeared that the tone of middle cerebral arteries did not relax further, or in some cases, started to constrict slightly after a high concentration (10 µM) of bradykinin was added.
Figure 3-7: Relaxant effects of bradykinin in rat middle cerebral and basilar arteries.

The arteries were pre-contracted with vasopressin (AVP) and when the plateau was obtained, cumulative concentrations of bradykinin were added. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.

3.3.7. Relaxant effect of sodium nitroprusside in rat cerebral arteries

Sodium nitroprusside induced a marked concentration-dependent relaxation in middle cerebral and basilar arteries pre-contracted with vasopressin (Figure 3-8). While the basilar artery was completely relaxed by cumulative additions of sodium nitroprusside, some contractile tone was left in the middle cerebral artery. The relaxation potency of sodium nitroprusside was similar in middle cerebral and basilar arteries with pEC$_{50}$ values of 5.6±0.3 (n=5) and 6.2±0.3 (n=3), respectively (p>0.05, unpaired t test).
Figure 3-8: Relaxant effects of sodium nitroprusside in rat middle cerebral and basilar arteries.

The arteries were pre-contracted with vasopressin (AVP) and when the plateau was obtained, cumulative concentrations of sodium nitroprusside were added. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC\textsubscript{50}±SEM. n, number of arteries from separate rats.

3.3.8. The effects of BAY 41-8543 in rat cerebral arteries

The relaxant effects of BAY 41-8543, a soluble guanylyl cyclase stimulator, were characterised in rat cerebral arteries. BAY 41-8543 caused concentration-dependent relaxation of rat cerebral arteries isolated from different regions (Figure 3-9). The pEC\textsubscript{50} values were 8.3±0.1 (n=4), 7.6±0.2 (n=4), 7.7±0.2 (n=4) and 8.2±0.2 (n=4) in anterior cerebral, middle cerebral, posterior cerebral and basilar arteries, respectively. The potency of BAY 41-8543 was not significantly different in the four arteries (p>0.05, one way ANOVA).
Figure 3-9: Relaxant effects of BAY 41-8543 in rat cerebral arteries.
The anterior cerebral, middle cerebral, posterior communicating and basilar arteries were pre-contracted with vasopressin (AVP) and when the plateau was obtained, cumulative concentrations of BAY 41-8543 were added. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.
3.4. Discussion

The rat cerebral arteries were sensitive to most vasoactive drugs tested, similar to other resistance arteries in the peripheral circulation. However, some qualitative and quantitative differences in the response to vasoactive agents existed between the cerebral and other arteries, as well as within the cerebral arteries owing to the heterogeneous distribution of targets or because of morphological differences.

Noradrenaline induced both constriction and relaxation responses that varied according to the location of the artery. The constriction was induced only in the anterior and middle cerebral arteries. This suggests that the distribution of α₁-adrenoceptors that mediate the constrictor response is limited to the anterior or rostral region of the rat cerebral circulation. The magnitude of the constrictor response, which was weak compared to peripheral vessels, may reveal that the sympathetic nervous system has limited effects on cerebral arteries. This is consistent with previous reports that the contraction induced by noradrenaline was significantly less in cerebral arteries than peripheral arteries (Hogestatt and Andersson, 1984). Vasodilator responses were induced by noradrenaline in all arteries taken from different regions of the rat cerebral circulation, demonstrating that the receptors mediating vasodilatation are distributed throughout the major cerebral arteries. The mechanisms involved in noradrenaline-induced responses in rat isolated cerebral arteries are analysed in Chapter 4.

Endothelin-1 was shown to induce a potent vasoconstrictor response with no significant regional variation in sensitivity between the four arteries. This indicates that ETₐ receptors mediating contraction are present throughout the rat cerebral cerebral circulation and suggests that the peptide may be involved in pathogenesis of cerebral vasospasm. Further analysis of the mechanism involved in endothelin-1-induced contraction of rat cerebral arteries is presented in Chapter 5.
5-Hydroxytryptamine induced potent constriction in rat cerebral arteries. The response obtained in the middle cerebral artery (90%) was remarkably greater than in the basilar artery (22%). The variation in response between arteries of different regions of the cerebral circulation demonstrates heterogeneous distribution of constrictor 5-HT receptors. Previous studies have shown disparity in the magnitude of 5-HT-induced constriction of cerebral arteries obtained from different species (Toda and Okamura, 1993). The constriction induced by 5-HT in cerebral arteries is mediated by 5-HT$_{2B}$ receptors (Deckert and Angus, 1992; Kaumann and Levy, 2006). Thus rat basilar arteries may have a sparse population of the constrictor 5-HT$_{2B}$ receptors unlike the middle cerebral arteries.

Vasopressin induced a strong constrictor response (100-110%) in rat cerebral arteries, but with greater potency in the middle cerebral than in basilar arteries. This reveals that the rat anterior cerebral circulation may have greater sensitivity to vasopressin compared to the posterior circulation, as represented by the basilar artery. Previous studies found that vasopressin caused potent constriction in human and goat cerebral arteries (Lluch et al., 1984; Martin de Aguilera et al., 1990), but endothelium-dependent relaxation was also reported in canine basilar arteries which was converted to a weak constriction when the endothelium was removed (Katusic et al., 1984; Suzuki et al., 1993). There may be a similar dilator effect that attenuates the constrictor effect of vasopressin in the rat basilar artery. However, this requires further investigation.

Acetylcholine relaxed cerebral arteries - greater relaxation was caused in the basilar artery than in middle cerebral artery. This is an indication that stimulation of the parasympathetic nervous system may increase cerebral blood flow by dilating arteries in the rat. Acetylcholine has been reported to dilate rabbit (Parsons et al., 1991), human (Tsukahara et al., 1986b) and dog basilar arteries (Toda, 1979). However, it induced both dilator and constrictor responses in dog (Tsukahara et al., 1986b) and pig cerebral arteries (Lee et al., 1982). The variation in the acetylcholine-induced response between cerebral arteries of different species, or sometimes between different vascular beds of
the same species, may be due to heterogeneous distribution of muscarinic receptors in endothelium mediating dilator responses and constrictor receptors on the smooth muscle cells (Lee, 1982). Both M1 and M2 receptors may be involved in mediating the constrictor and dilator responses depending on their location on smooth muscle or endothelium (Garcia-Villalon et al., 1991; Dauphin et al., 1994).

Bradykinin induced a dilator effect in the rat cerebral circulation with significantly greater potency in the middle cerebral artery than in the basilar artery. The current result is consistent with previously reported in vitro dilator effects of bradykinin in cerebral arteries of different species (Wahl et al., 1983; Whalley et al., 1983; Kamitani et al., 1985). It can be concluded that bradykinin may be involved in the regulation of the basal tone of cerebral arteries by promoting release of endothelial vasodilator mediators such as NO and production of EDH.

The sensitivity of cerebral arteries to exogenous nitric oxide was also assessed. Sodium nitroprusside, an inorganic nitric oxide-releasing drug, induced a marked and sustained relaxation with similar potency in rat middle cerebral and basilar arteries. This demonstrates the sensitivity of the healthy cerebral arterial tissue to the relaxation effects of nitric oxide, a major regulator of vascular tone and cerebral blood flow. Similar results have been previously reported in cerebral arteries of various species (Kimura et al., 1998; Salom et al., 1998; Salom et al., 1999; Schubert et al., 2004).

Similarly, stimulation of the sGC enzyme with BAY 41-8543 induced a potent dilatation of the cerebral arteries. It can be inferred that basally-released nitric oxide can relax contracted arteries if sGC is stimulated. This highlights the critical role played by the NO/cGMP pathway in the regulation of basal tone of cerebral arteries, and therefore, in the maintenance of cerebral blood flow. Direct stimulation of sGC may be a new therapeutic option for the management of cerebral ischaemia or any condition associated with compromised cerebral
blood flow. However, sGC stimulators need to be further investigated in cerebral arteries since only limited studies are available in this regard.

Overall, this study showed that cerebral arteries exhibit remarkable regional variation in their responses to common vasoactive substances. Often, the arteries were very sensitive to these agents, but in some cases they responded weakly or not at all. Further, some mediators such as noradrenaline can induce opposing effects depending on the type or location of the receptors stimulated. Drugs which decrease blood flow to some regions such as the frontal cortex may increase the flow or have no effect in other regions such as the brain stem. Thus, results obtained from studies limited to only some parts of the cerebral vascular bed should be examined carefully before conclusions are drawn for generalised effects of drugs on cerebral blood flow.
Chapter 4

Mechanisms involved in adrenoceptor-mediated responses in rat cerebral arteries
4.1. Introduction

The cerebral circulation receives abundant sympathetic innervation, mainly originating from the superior cervical ganglion (Lee et al., 1976; Duckles, 1980; Paulson et al., 1990; Lincoln, 1995; Mitsis et al., 2009; ter Laan et al., 2013). Cerebral arteries also receive adrenergic projections from certain nuclei of the central nervous system such as the locus coeruleus (Raichle et al., 1975; Micieli et al., 1994; Hamel, 2006).

Electrical stimulation experiments and studies involving systemic administration of sympathomimetic agents indicated that the sympathetic nervous system may be involved in regulation of cerebral blood flow (Hamner et al., 2010; ter Laan et al., 2013). However, the precise role of the sympathetic nervous system in cerebral autoregulation is not clearly known. Activation of the sympathetic nervous system with a cold pressor test did not alter cerebral blood flow despite increasing arterial blood pressure. This finding then led to an assertion that sympathetic activation may induce constriction of cerebral arteries and thus prevent increases in cerebral blood flow (Roatta et al., 1998). Other studies have shown that the sympathetic nervous system plays a protective role by preventing increases in cerebral blood flow during acute elevation of blood pressure (Heistad and Marcus, 1978; Tuor, 1992; Dunatov et al., 2011) or maintaining it during acute hypotension (Ogoh et al., 2008). These observations indicate that the sympathetic nervous system plays a dual role – inducing vasoconstriction under conditions of elevated blood pressure and vasodilatation of cerebral arteries under hypotensive states. The constrictor and dilator responses induced by sympathetic nerve activation may be mediated by different adrenoceptor subtypes located in cerebral arteries. Therefore, the location of specific adrenoceptors and their particular roles in mediating these effects should be rigorously investigated in cerebral arteries.
Previous studies have reported variable effects of noradrenaline in cerebral arteries obtained from different species, along with limited mechanistic studies. Noradrenaline generally induced variable degrees of contraction in human, monkey, sheep, dog, cat, rabbit and rat middle cerebral arteries (Edvinsson and Owman, 1974; Edvinsson et al., 1993). Relaxant effects of noradrenaline have been observed in basilar arteries obtained from various species including the rat (Winquist and Bohr, 1982; Edvinsson et al., 1993). However, Tanishima (1983) reported that noradrenaline contracted the human basilar artery. Both contractile and relaxant effects were induced by catecholamines in the feline middle cerebral artery (Edvinsson and Owman, 1974). Variation in responses induced by noradrenaline in arteries obtained from different regions of the cerebral circulation is probably related to the distribution of adrenoceptors mediating vasoconstriction or vasodilatation. This was demonstrated by differences in regional effects of noradrenaline in bovine cerebral arteries where β-adrenoceptors mediated relaxation of the caudal arteries and α-adrenoceptors mediated contraction of the rostral arteries. Blockade of α-adrenoceptors converted the contraction to relaxation, while inhibition of β-adrenoceptors attenuated the relaxant effect of noradrenaline (Ayajiki and Toda, 1992).

While β-adrenoceptors are generally accepted to mediate relaxation of actively contracted cerebral arteries (Edvinsson et al., 1993), the precise mechanisms leading to vasodilatation are not clearly known. A study in rat isolated cerebral arteries found that the relaxation induced by noradrenaline involves release of nitric oxide through activation of the β-adrenoceptors located on the endothelial cells (Hempelmann and Ziegler, 1993). Other studies, however, indicated that activation of presynaptic β2-adrenoceptors located on nitrergergic neurons (previously known as non-adrenergic non-cholinergic neurons) releases nitric oxide (Zhang et al., 1998; Lee et al., 2000; Chang et al., 2012). The involvement of other factors such as prostacyclin or endothelium-derived hyperpolarisation in mediating relaxation of cerebral arteries induced by β-adrenoceptor activation and their possible connection with specific β-adrenoceptor subtype is unknown. The relationship between changes in
sympathetic activity and aetiology of cerebrovascular diseases such as the vasospasm that often occurs following subarachnoid haemorrhage is poorly understood. Sympathetic activity has reportedly been elevated in patients with subarachnoid haemorrhage (Naredi et al., 2000; Lambert et al., 2002), but the link between sympathetic activation and development of cerebral vasospasm has not been established (Moussouttas et al., 2012). Damage to the vascular endothelium or uncoupling of nitric oxide synthase during subarachnoid haemorrhage (Sabri et al., 2011) may increase sensitivity of cerebral arteries to vasoconstrictor agents including noradrenaline. Thus, a thorough investigation of the mechanisms involved in adrenoceptor-mediated responses in cerebral arteries is important to understand the process leading to the development of cerebrovascular disorders.

The aim of this study was to analyse the regional variation in responses induced by adrenoceptor activation, the adrenoceptor subtypes involved, as well as the role of endothelium-derived relaxing factors in mediating the relaxant responses in arterial segments isolated from different regions of the rat cerebral circulation.
4.2. Materials and methods

4.2.1. Methods

The responses of cerebral arteries obtained from different regions of the rat cerebral circulation were analysed using wire myography as described in Chapter 2.

4.2.2. Experimental protocols

Experiments were conducted as described in Chapter 2, unless specified otherwise. Briefly, the viability of the arteries was initially assessed by contraction with KPSS, the maximum value of which has been used as a reference to compare the vascular responses induced by vasoactive drugs. Drugs were added to the baths in cumulative concentrations where sufficient time was allowed for the responses to plateau before adding the next higher concentration of the drug, except where single concentrations of the drugs were tested. In protocols involving assessment of relaxant effects, arteries were pre-contracted to about 80-100% of the KPSS-induced maximum contraction using vasopressin followed by addition of the test drugs. The inhibitors or antagonists were added 20-25 min before testing an agonist. All responses were expressed as the % of the KPSS-induced maximum contraction.

4.2.3. Drugs and chemicals

The drugs used in this study were obtained from the following suppliers: (-)-Adrenaline, atenolol, formoterol, (-)-isoprenaline, (-)-noradrenaline, methoxamine, \( N^{\omega} \)-nitro-L-arginine methyl ester (L-NAME) and prazosin (Sigma Aldrich, St Louis, Missouri, USA); ICI 118,551 (Tocris Bioscience, Bristol, UK); propranolol (Imperial Chemical Industries Ltd, Macclesfield, UK); and apamin, charybdotoxin and vasopressin (AusPep, Parkville, Victoria, Australia).
4.3.  Results

4.3.1.  Contractile effects of $\alpha_1$-adrenoceptor stimulation in cerebral arteries

The effects of $\alpha_1$-adrenoceptor stimulation in arteries taken from different region of the rat cerebral circulation were investigated using the $\alpha_1$-adrenoceptor selective agonist methoxamine. Methoxamine caused concentration-dependent contraction of the rat anterior (Figure 4-1A) and middle cerebral (Figure 4-1B) arteries. The potency of methoxamine was similar in the anterior cerebral ($pEC_{50} 5.2\pm0.08, n=4$) and middle cerebral ($pEC_{50} 5.3\pm0.1, n=6$) arteries. Similarly, the maximum contractile effects elicited by methoxamine were not significantly different in the anterior (79±5%) and middle cerebral (58±8% of the KPSS-induced maximum contraction, $P>0.05$) arteries. However, methoxamine failed to contract either the posterior communicating (Figure 4-1C) or the basilar (Figure 4-1D) artery isolated from the rat.

4.3.2.  Relaxant effects of $\beta$-adrenoceptor stimulation in cerebral arteries

Stimulation of $\beta$-adrenoceptors using isoprenaline (nonselective $\beta_1$- and $\beta_2$-adrenoceptor agonist) caused concentration-dependent relaxation of rat cerebral arteries actively constricted with vasopressin (1-3 nM for basilar, or 0.1-1 nM for other arteries). Isoprenaline (1 nM-1 µM) induced similar degrees of relaxation in the rat anterior cerebral (Figure 4-2A), middle cerebral (Figure 4-2B), posterior communicating (Figure 4-2C) and basilar arteries (Figure 4-2D) pre-contracted with vasopressin. There was no significant difference between the relaxation potencies of isoprenaline in the four arteries ($P>0.05$) - the $pEC_{50}$ values were 7.4±0.3 (n=5), 7.4±0.1 (n=7), 7.1±0.4 (n=5) and 6.8±0.2 (n=7) in anterior cerebral, middle cerebral, posterior communicating and basilar arteries, respectively.
Figure 4-1: Effects of α₁-adrenoceptor stimulation in rat cerebral arteries.

Effects of α₁-adrenoceptor activation with methoxamine in A) anterior cerebral; B) middle cerebral; C) posterior communicating; and D) basilar arteries isolated from the rat. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC₅₀±SEM. n, number of arteries from separate rats.
Figure 4-2: Relaxant effects of β-adrenoceptor stimulation in cerebral arteries

Relaxant effect of isoprenaline in rat A) anterior cerebral; B) middle cerebral; C) posterior communicating; and D) basilar arteries pre-contracted with vasopressin (AVP) 0.1·3 nM. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. $n$, number of arteries from separate rats.
4.3.3. Effects of endothelium removal and inhibition of nitric oxide production on isoprenaline-induced relaxation of cerebral arteries

The roles of endothelium and nitric oxide in mediating the relaxation induced by β-adrenoceptor stimulation with isoprenaline were investigated in arteries isolated from anterior (middle cerebral artery) and posterior regions (basilar artery) of the rat cerebral circulation. Physical removal of the endothelium did not affect the relaxant effect of isoprenaline (1 nM-1 μM) in the middle cerebral artery (Figure 4-3A). However, removal of endothelium inhibited the isoprenaline-induced relaxation in rat basilar artery (Figure 4-3B). The relaxation pEC$_{50}$ value of isoprenaline in the endothelium-denuded middle cerebral artery was not significantly different from corresponding arteries with intact endothelium ($P>0.05$). Inhibition of nitric oxide production with L-NAME 100 μM did not significantly affect the isoprenaline-induced relaxation either in middle cerebral (Figure 4-4A) or basilar arteries (Figure 4-4B) isolated from the rat. The relaxation potency of isoprenaline in the presence of L-NAME was similar in the middle cerebral ($pEC_{50}$ 6.9±0.3, n=7) and basilar arteries ($pEC_{50}$ 6.8±0.2, n=7; $P>0.05$).
Figure 4-3: Effects of endothelium removal on isoprenaline-induced relaxation of cerebral arteries.

Relaxant effect of isoprenaline in rat A) middle cerebral; and B) basilar arteries with or without endothelium. The arteries were pre-contracted with vasopressin (AVP) 0.1-3 nM and subsequently relaxed with cumulative addition of isoprenaline. The endothelium was removed from the arteries by rubbing the lumen with a human hair. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. $n$, number of arteries from separate rats.
Figure 4-4: Effects of inhibition of nitric oxide production on isoprenaline-induced relaxation of cerebral arteries.

The effects of nitric oxide production inhibition with Nω-nitro-L-arginine methyl ester (L-NAME) on isoprenaline-induced relaxation of rat A) middle cerebral; and B) basilar arteries. The arteries were pre-contracted with vasopressin (AVP) 0.1-3 nM and subsequently relaxed with cumulative addition of isoprenaline. L-NAME was added 20 min prior to AVP contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC_{50±SEM}. n, number of arteries from separate rats.

4.3.4. Contractile effects of noradrenaline and the role of endothelium and nitric oxide

The contractile effects of noradrenaline, an endogenous neurotransmitter released by sympathetic nerves, were characterised in rat cerebral arteries. As with methoxamine, noradrenaline induced contraction only in anterior and middle cerebral arteries. However, the contractile responses induced by noradrenaline were relatively weak – E_{max} ~17% of the KPSS-induced maximum contraction in anterior (Figure 4-5A) and middle cerebral arteries (Figure 4-5B). There was no significant difference in pEC_{50} values of noradrenaline in anterior
cerebral (5.6±0.06, n=7) and middle cerebral arteries (5.7±0.12, n=7; P>0.05). However, noradrenaline did not contract rat isolated posterior communicating (Figure 4-5C) and basilar arteries (Figure 4-5D). Removal of endothelium from the middle cerebral artery did not affect the contraction induced by noradrenaline (Figure 4-6). However, nonselective inhibition of the nitric oxide synthase enzyme with L-NAME 100 µM significantly increased the contraction induced by noradrenaline in both anterior (E_max 69±10%) (Figure 4-7A) and middle cerebral arteries (E_max 69±5%) (Figure 4-7B) compared to the weak response obtained in the absence of L-NAME (P>0.05). No contractile effects of noradrenaline were observed in posterior communicating or basilar arteries pre-treated with L-NAME.
Figure 4-5: Contractile effects of noradrenaline in rat cerebral arteries

The effects of noradrenaline in rat isolated A) anterior cerebral; B) middle cerebral; C) posterior communicating; and D) basilar arteries. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC\(_{50}\)±SEM. \(n\), number of arteries from separate rats.
Figure 4-6: Effect of endothelium removal on the noradrenaline-induced contraction of middle cerebral artery.

Contractile effects of noradrenaline in the rat middle cerebral artery with or without endothelium. The endothelium was removed from the arteries by rubbing the lumen with a human hair. Data are shown as percent KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol). n, number of arteries from separate rats.
Figure 4-7: Effects of inhibition of nitric oxide production on noradrenaline-induced contraction of cerebral arteries.

The effects of nitric oxide production inhibition with Nω-nitro-L-arginine methyl ester (L-NAME 100 μM) on noradrenaline-induced contraction of rat A) anterior; and B) middle cerebral arteries. L-NAME was added 20 min prior to the noradrenaline contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC50±SEM. n, number of arteries from separate rats.

4.3.5. Relaxant effects of noradrenaline in rat cerebral arteries and the role of endothelium and nitric oxide

Noradrenaline elicited a concentration-dependent relaxation of rat cerebral arteries actively contracted with vasopressin. The maximum relaxation response to noradrenaline appeared to be lesser in anterior (~60%) (Figure 4-8A) and middle cerebral arteries (~52%) (Figure 4-8B) than in posterior communicating arteries (~69%) (Figure 4-8C) and basilar arteries (~76% of the pre-contraction tone) (Figure 4-7D). The relaxation pEC50 values of noradrenaline were 6.5±0.2 (n=7), 6.2±0.1 (n=7), 6.8±0.3 (n=8) and 6.2±0.2 (n=6) in anterior cerebral, middle cerebral, posterior communicating and
basilar arteries, respectively. No significant difference was found in relaxant potency of noradrenaline between the four arteries \((P>0.05)\). Removal of the endothelium inhibited the relaxant effects of noradrenaline in the basilar artery as shown in Figure 4-9. The absence of spontaneous relaxation in the control arteries left contracted during experimental period further confirmed that the relaxation responses were induced by noradrenaline. Pre-treating the arteries with L-NAME 100 µM inhibited the relaxant effects of noradrenaline in rat cerebral arteries (Figure 4-10A-D). Noradrenaline slightly increased the pre-contraction tone in anterior and middle cerebral arteries pre-treated with L-NAME as shown in Figure 4-10A and B.
Figure 4-8: Relaxant effects of noradrenaline in rat cerebral arteries.

Relaxant effects of noradrenaline in rat isolated A) anterior cerebral; B) middle cerebral; C) posterior communicating; and D) basilar arteries pre-contracted with vasopressin (AVP) 0.1-3 nM. Arteries in the treatment group were relaxed by cumulative addition of noradrenaline, while those in the time control group were left contracted during this time. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC50±SEM. n, number of arteries from separate rats.
Figure 4-9: Effects of endothelium removal on noradrenaline-induced relaxation of rat basilar artery.

Relaxant effect of noradrenaline in rat basilar artery pre-contracted with vasopressin (AVP) 0.1-3 nM. The endothelium was removed from the arteries by rubbing the lumen with a human hair. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. $n$, number of arteries from separate rats.
Figure 4-10: Effects of inhibition of nitric oxide production on the noradrenaline-induced contraction of cerebral arteries.

The effect of inhibition of nitric oxide production with N\textsuperscript{\textmu}-nitro-L-arginine methyl ester (L-NAME) on noradrenaline-induced relaxation of rat A) anterior cerebral; B) middle cerebral; C) posterior communicating; and D) basilar arteries pre-contracted with vasopressin (AVP) 0.1-3 nM. L-NAME was added 20 min prior to the noradrenaline contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC\textsubscript{50}±SEM. \( n \), number of arteries from separate rats.
4.3.6. Roles of α- and β-adrenoceptors in the responses induced by noradrenaline in rat cerebral arteries

The roles of α- and β-adrenoceptors in the responses induced by noradrenaline were further analysed in rat isolated cerebral arteries. The contractile responses were analysed in middle cerebral artery, while the relaxant responses were analysed in all four arteries in the presence of the adrenoceptor antagonists prazosin, propranolol, atenolol or ICI 118551. Inhibition of α₁-adrenoceptors with prazosin 0.1 µM completely inhibited the contraction induced by noradrenaline (10 nM-10 µM) in the rat middle cerebral artery (Figure 4-11A). Pre-treatment of the arteries with propranolol 0.1 µM, a nonselective β-adrenoceptor antagonist, increased the contractile response to noradrenaline of the middle cerebral artery to about 52% of the KPSS-induced maximum contraction (Figure 4-11B). Inhibition of β₁-adrenoceptors with atenolol 1 µM increased the contractile effect of noradrenaline in the middle cerebral artery (Figure 4-11C). Similarly, pre-treating the arteries with the β₂-adrenoceptor antagonist ICI 118551 (1 µM) increased the contractile response to noradrenaline (Figure 4-11D). Both atenolol and ICI 118551 caused similar increases in the maximum contractile effects of noradrenaline (Émax 41-43% of KPSS-induced contraction) in the rat middle cerebral artery. The pEC₅₀ of noradrenaline was not significantly changed by the antagonists (vehicle, 5.7±0.12; propranolol, 6.2±0.09; atenolol, 6.2±0.13; and ICI 118551, 6.0±0.13; P>0.05). Stimulation of α₂-adrenoceptors with clonidine (10 nM-1 µM) did not induce any contraction in the rat middle cerebral artery (n=2; data not shown).

Non-selective inhibition of the β-adrenoceptors with propranolol 1 µM caused a rightward shift of the noradrenaline concentration-relaxation curve in rat cerebral arteries (Figure 4-12). Noradrenaline initially caused a small increase of the pre-contraction tone in some anterior and middle cerebral arteries pre-treated with propranolol (Figure 4-12A and B). However, propranolol diminished the maximum relaxant effect of noradrenaline in these arteries. Specific inhibition of β₁-adrenoceptors using atenolol 1 µM also caused a rightward shift of the concentration-relaxation curve without affecting the maximum response.
in the basilar artery (Figure 4-13A). Similarly, ICI 118551 0.1 µM, a β2-adrenoceptor specific antagonist, shifted the noradrenaline concentration-relaxation curve to the right as shown in Figure 4-13B. In another set of experiments, the basilar artery was relaxed with selective agonists to characterise the presence of β1- and β2-adrenoceptors. Adrenaline, a selective β2-adrenoceptor agonist, caused a concentration-dependent relaxation of the basilar artery with a pEC50 of 6.4±0.08 (n=4) as shown in Figure 4-14A. However, another β2-selective agonist formoterol (0.1 nM-1 µM) caused poor relaxant effects in the basilar artery (Figure 4-14B). Likewise, prenalterol (10 nM-10 µM), a β1-adrenoceptor partial agonist, did not relax basilar arteries pre-contracted with vasopressin (n=2; data not shown). Presence of α2-adrenoceptors was tested using a selective agonist clonidine (10 nM-1 µM). Clonidine did not cause any relaxant effect in rat middle cerebral or basilar arteries pre-contracted with vasopressin (Figure 4-15).
Figure 4-11: Effects of adrenoceptor antagonism on the noradrenaline-induced contraction in middle cerebral artery.

The effects of A) prazosin 0.1 µM; B) propranolol 0.1 µM; C) atenolol 1 µM; and D) ICI 118551 1 µM pre-treatment on the noradrenaline-induced contraction of rat middle cerebral arteries. The antagonists were added 20 min prior to noradrenaline contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats. *P<0.05 compared to the vehicle group.
Figure 4-12: Effects of β-adrenoceptor antagonism on the noradrenaline-induced relaxation of cerebral arteries.

The effects of nonselective inhibition of β-adrenoceptors with propranolol 1 µM on the noradrenaline-induced relaxation of rat A) anterior cerebral; B) middle cerebral; C) posterior communicating; and D) basilar arteries pre-contracted with vasopressin (AVP) 0.1-3 nM. Propranolol was added 20 min prior to AVP contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC_{50±SEM}. n, number of arteries from separate rats.
Figure 4-13: The roles of $\beta_1$- and $\beta_2$-adrenoceptors in noradrenaline-induced relaxation of basilar arteries.

The effects of A) atenolol 1 $\mu$M; and B) ICI 118551 0.1 $\mu$M pre-treatment on the noradrenaline-induced relaxation of the rat basilar artery pre-contracted with vasopressin (AVP). The antagonists were added 20 min prior to AVP contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. $n$, number of arteries from separate rats.
Figure 4-14: Relaxant effects of adrenaline and formoterol in rat basilar arteries.

Relaxant effects of A) adrenaline; and B) formoterol in the rat basilar artery pre-contracted with vasopressin (AVP). Arteries were relaxed by cumulative concentrations of adrenaline or formoterol. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC_{50}±SEM. n, number of arteries from separate rats.
Figure 4-15: Relaxant effects of clonidine in rat cerebral arteries.
The effects of clonidine in rat A) middle cerebral; and B) basilar arteries precontracted with vasopressin (AVP). There arteries were pre-contracted with vasopressin (0.1-3 nM) and when the plateau was attained, clonidine was added in cumulative concentrations. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol). n, number of arteries from separate rats.

4.3.7. Involvement of the endothelium-derived hyperpolarisation (EDH) in $\beta$-adrenoceptor-mediated relaxation of cerebral arteries

Cerebral arteries were pre-contracted with a depolarizing $K^+$ solution (equimolar Krebs’ solution containing 40 mM $K^+$) and subsequently relaxed by cumulative addition of the nonselective $\beta$-adrenoceptor agonist isoprenaline (Figure 4-16). Isoprenaline induced a slight relaxation in both middle cerebral and basilar arteries. Addition of sodium nitroprusside 1-10 $\mu$M (an NO donor drug) after isoprenaline caused a complete relaxation of the arteries. Inhibition of EDH production in basilar arteries with a combination of apamin 50 nM and charybdotoxin 50 nM slightly shifted the concentration-relaxation curve of
noradrenaline, but the $pEC_{50}$ was not significantly changed (Figure 4-17A). The isoprenaline-induced relaxation of the basilar artery was, however, markedly inhibited by the combination (the EC$_{50}$ value could not be derived after the inhibition) (Figure 4-17B). Combination of apamin 50 nM and charybdotoxin 50 nM in the presence of L-NAME 100 µM and indomethacin 3 µM only slightly inhibited the isoprenaline-induced relaxation in the middle cerebral artery (Figure 4-18A), but completely inhibited the relaxation in the rat basilar artery (Figure 4-18B). A moderate to strong constriction was initially observed in some middle cerebral arteries after addition of apamin and charybdotoxin, but subsided in most arteries within 10 min.
Figure 4-16: Relaxant effects of isoprenaline in cerebral arteries pre-contracted with K⁺

Relaxant effects of isoprenaline in rat A) middle cerebral; and B) basilar arteries pre-contracted with Krebs’ buffer solution containing K⁺ 40 mM (K40). Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol). n, number of arteries from separate rats.
Figure 4-17: Effects of apamin and charybdotoxin combination on the relaxation induced by noradrenaline and isoprenaline in rat basilar arteries.

Effects of apamin 50 nM and charybdotoxin (ChTx) 50 nM pre-treatment on A) noradrenaline-induced; and B) isoprenaline-induced relaxation of rat basilar artery pre-contracted with vasopressin (AVP). The antagonists (inhibitors) were added 20 min prior to AVP contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.
Figure 4-18: Effects of endothelial-derived EDH, NO and PGI$_2$ inhibition on the isoprenaline-induced relaxation of cerebral arteries.

Effects of endothelium-derived hyperpolarisation (EDH), nitric oxide (NO) and prostacyclin (PGI$_2$) inhibition on isoprenaline-induced relaxation in the rat A) middle cerebral; and B) basilar arteries pre-contracted with vasopressin (AVP). EDH production was inhibited by using a combination of apamin (Apam) 50 nM and charybdotoxin (ChTx) 50 nM. L-NAME 100 µM and indomethacin (Indo) 3 µM were added to inhibit NO and PGI$_2$ production. The antagonists (inhibitors) were added 20 min prior to AVP contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.
4.4. Discussion

This chapter analysed the effects of adrenoceptor activation, the location and subtypes of receptors involved and the role of endothelial factors in different arteries of the rat cerebral circulation. Activation of adrenoceptors in rat cerebral arteries caused heterogeneous responses depending on the regional location of the arteries. Both contractile and relaxant effects were induced in anterior and middle cerebral arteries, while only relaxant effects were induced in posterior communicating and basilar arteries. The relaxation response in the middle cerebral artery is endothelium-independent, but involved release of endothelial NO and EDH in the basilar artery. $\alpha_1$-Adrenoceptors mediate the contractile responses in rat anterior and middle cerebral arteries, while $\beta_1$- and $\beta_2$-adrenoceptors mediate the relaxant effects in rat cerebral arteries.

Selective stimulation of $\alpha_1$-adrenoceptors with methoxamine contracted arteries isolated from the anterior region of the rat cerebral circulation – anterior and middle cerebral arteries. However, no contractile response was induced by methoxamine in arteries obtained from the caudal region of the rat cerebral circulation, namely the posterior communicating and basilar arteries. Noradrenaline elicited similar effects as methoxamine in cerebral arteries, but the constrictor responses were relatively weak. The contractile responses were inhibited by prazosin, a selective $\alpha_1$-adrenoceptor antagonist. These findings indicate that the distribution of $\alpha_1$-adrenoceptors may be limited to arteries in the anterior parts of the rat cerebral circulation. Stimulation of $\alpha_2$-adrenoceptors with clonidine did not cause contraction in rat cerebral arteries. Similar findings were previously reported by Hogestatt and Andersson (1984) that noradrenaline induced an $\alpha_1$-adrenoceptor-mediated contraction in rat middle cerebral artery, but not in the basilar artery. Similarly, noradrenaline contracted bovine artery strips taken from anterior cerebral, middle cerebral and internal carotid arteries through $\alpha_1$-adrenoceptor stimulation (Ayajiki and Toda, 1990).
The relaxant effects of adrenoceptor stimulation were also investigated in rat cerebral arteries pre-contracted with vasopressin. Nonspecific activation of the β-adrenoceptors with isoprenaline caused strong relaxation in cerebral arteries pre-contracted with vasopressin. Similarly, noradrenaline induced relaxant effects that varied between arteries taken from different regions – greater relaxation was obtained in caudal (posterior communicating and basilar) than rostral (anterior and middle cerebral) arteries. No relaxation was induced by α-adrenoceptor stimulation in cerebral arteries. This indicates that β-adrenoceptors mediate the relaxation responses in rat cerebral arteries. The relaxation effects in anterior and middle cerebral arteries counteract the contraction induced by noradrenaline. As a result, noradrenaline induced weak contractile responses that were attenuated by simultaneous stimulation of β-adrenoceptors, unlike methoxamine which caused a relatively strong contraction of anterior and middle cerebral arteries. The assertion was supported by increases in the contractile responses to noradrenaline when β-adrenoceptors were inhibited with propranolol; or selective β1-(atenolol) and β2-adrenoceptor (ICI 118551) antagonists. These findings indicate that both β1- and β2-adrenoceptors are involved in the relaxation induced by noradrenaline. Similar findings were previously reported in rat middle cerebral arteries where pre-treatment with propranolol enhanced the maximum contraction induced by noradrenaline (Hogestatt and Andersson, 1984). Additionally, the presence of β-adrenoceptors was confirmed by inhibition of the noradrenaline-induced relaxation caused by adrenoceptor antagonists in rat cerebral arteries pre-contracted with vasopressin. Propranolol 1 µM significantly attenuated the relaxation, while atenolol 1 µM and ICI 118551 1 µM shifted the concentration-relaxation curve of noradrenaline to the right. This shows involvement of both β1- and β2-adrenoceptors in the relaxation induced by noradrenaline. Relaxation induced by adrenaline, a drug with preferential affinity for β2-adrenoceptors (pKi 6.2), reveals the presence of these receptors in the rat basilar artery. However, the lack of response to formoterol, a selective β2-adrenoceptor agonist (Baker, 2010), is intriguing and these observations need further investigation.
In the middle cerebral artery, removal of the endothelium neither inhibited the relaxation induced by isoprenaline nor enhanced the noradrenaline-induced contraction. The findings suggest an absence of relaxant β-adrenoceptors on endothelial cells, but these may be located on the smooth muscle cells of the rat middle cerebral artery. In contrast, removal of the endothelium inhibited the relaxation induced by noradrenaline or isoprenaline in the basilar artery. This indicates that β-adrenoceptors are located on the endothelium of the rat basilar artery. Previous studies have shown the presence of both endothelial and smooth muscle β-adrenoceptors in various peripheral arteries isolated from different species (see Guimarães and Moura (2001) for a detailed review). It appears that the presence of endothelial adrenoceptors in cerebral arteries is now widely accepted (Winquist and Bohr, 1982; Hempelmann and Ziegler, 1993). However, most studies were conducted in basilar artery preparations and did not compare the endothelial/smooth muscle distribution of β-adrenoceptors in other cerebral arteries. Thus, this study has shown heterogeneous regional distribution of endothelial and smooth muscle β-adrenoceptors in rat cerebral arteries.

Inhibition of NO production with L-NAME inhibited the relaxation responses to noradrenaline (up to a maximum concentration of 30 µM) in cerebral arteries. Furthermore, L-NAME increased the contraction induced by noradrenaline in anterior and middle cerebral arteries in contrast to the finding that the contractile effect of noradrenaline was not enhanced by removing the endothelium in the middle cerebral artery. However, L-NAME alone or in combination with indomethacin did not inhibit the relaxation induced by isoprenaline in rat middle cerebral and basilar arteries. This disparity observed in the inhibitory effects of L-NAME on the responses induced by noradrenaline and isoprenaline may possibly be related to a potentiation of vasopressin-induced pre-contractile tone that has resisted the relaxant effects of noradrenaline, a relatively weaker relaxant than isoprenaline. The increases in the contractile responses to noradrenaline in anterior and middle cerebral arteries pre-treated with L-NAME also illustrates a potentiation effect. L-NAME can potentiate the effects of vasoconstrictor agonist by inhibiting the basal
production of nitric oxide which is responsible for regulating tone of cerebral arteries (Faraci, 1994).

The difference in efficacy between noradrenaline and isoprenaline with regard to the quantity of cAMP generated has been previously noted. For example, isoprenaline produced three times more cAMP than noradrenaline at 10-fold less concentration in human lymphocytes (MacGregor et al., 1996). Whether these variations are related to the differences in relative affinities for the β-adrenoceptor subtypes or efficacy of the individual agonist is unknown. The variations are unlikely to be due to differences in selectivity of the agonists for β-adrenoceptor subtypes since both drugs have slightly greater affinities for β₁ (pKᵢ: noradrenaline 6.0 and isoprenaline 6.6-7.0) than β₂-adrenoceptors (pKᵢ: noradrenaline 5.4 and isoprenaline 6.4) (IUPHAR/BPS http://www.guidetopharmacology.org/). The difference in efficacies of noradrenaline and isoprenaline in inducing relaxation of cerebral arteries following activation of either β₁ or β₂-adrenoceptor subtypes is unknown and needs to be investigated.

The possible role of EDH in adrenoceptor-mediated relaxation of rat cerebral arteries was also analysed using a combination of apamin 50 nM and charybdotoxin 50 nM, a combination that has been shown to block EDH by selectively inhibiting potassium channels in the endothelium without affecting the channels in smooth muscle cells (Doughty et al., 1999). The chemical identity of EDH is not clearly identified, but it is generally accepted as an agonist-induced endothelium-dependent relaxation of smooth muscle cells due to an increased K⁺ conductance. It may involve efflux of ions or secondary mediators from the endothelium into the myoendothelial space and/or transfer into smooth muscle cells via the gap junctions (Ozkor and Quyyumi, 2011). This study found that apamin 50 nM and charybdotoxin 50 nM only slightly inhibited the relaxation induced by noradrenaline, but significantly inhibited the isoprenaline-induced relaxation in the basilar artery. This indicates the presence of EDH in the rat basilar artery that is favourably released by isoprenaline. Further, a combination of apamin 50 nM and charybdotoxin 50 nM,
L-NAME 100 μM and indomethacin 3 μM, inhibited the relaxation induced by isoprenaline in the basilar artery, but not in the middle cerebral artery. This shows an involvement of EDH and NO in the relaxation of the basilar artery induced by β-adrenoceptor stimulation. In addition, these results reiterate the fact that relaxation of the rat middle cerebral artery induced by β-adrenoceptor activation is primarily an endothelium-independent phenomenon. In another attempt to attenuate the production of EDH, arteries were pre-contracted by using a depolarizing concentration of K+ (40 mM), as previously indicated (Ozkor and Quyyumi, 2011). However, only weak relaxations were induced by isoprenaline in both middle cerebral and basilar arteries pre-contracted with 40 mM K+ demonstrating that high K+ may simultaneously inhibit both NO and EDH-mediated relaxations.

Stimulation of vascular β-adrenoceptors has been known to induce hyperpolarisation associated with smooth muscle relaxation (Goto et al., 2001). Stimulation of β-adrenoceptors on endothelial cells causes increases in intracellular calcium ([Ca^{2+}]_i) that subsequently lead to synthesis of NO or generation of EDH. It appears that NO-mediated relaxations require lower concentrations of endothelial [Ca^{2+}]_i than EDH-mediated relaxation. This was demonstrated in uridine triphosphate-induced relaxation of rat cerebral arteries where a lower threshold concentration of endothelial [Ca^{2+}]_i (~220 nM) was required for production of NO than for generation of EDH (~340 nM) (Marrelli, 2001). Thus, stimulation of β-adrenoceptors with isoprenaline may cause high [Ca^{2+}]_i that induces generation of EDH in the rat basilar artery.

Taken together, this study found that adrenoceptor activation induced variable responses in the rat cerebral circulation due to heterogeneous distribution of the constrictor and dilator adrenoceptors as summarised in Figure 4-19. Constrictor α1-adrenoceptors are found in arteries taken from medial and anterior locations, but β-adrenoceptors mediating relaxation are distributed in both anterior and posterior regions of rat cerebral arterial circulation. The β-adrenoceptors are located on smooth muscle in middle cerebral arteries, unlike in the basilar artery where they are located only on the endothelium. Activation
of endothelial β-adrenoceptors in the basilar artery induces release of variable proportions of EDH and NO depending on the agonist used. Isoprenaline favours release of EDH, while noradrenaline induces preferential release of NO in the rat basilar artery.

Figure 4-19: Summary of the location and effects mediated by β-adrenoceptors and effects of noradrenaline and isoprenaline in rat cerebral arteries.

A) β-adrenoceptors mediating relaxation are located on smooth muscle and endothelium of the rat middle cerebral and basilar artery, respectively, while α₁-adrenoceptors are located only on the smooth muscle of the middle cerebral artery and mediate a relaxation response. B) Noradrenaline activated β₁/β₂-adrenoceptors predominantly inducing release of nitric oxide (NO), but isoprenaline favours the release of endothelium-derived hyperpolarisation (EDH) in the rat basilar artery.
Limitations and future directions

Due to time limitations on the part of the candidate to submit this thesis, experiments pertaining to further characterisation of β-adrenoceptors in rat cerebral arteries have not been completed. Thus, the following points have been identified for future investigation based on the findings of this study:

- To examine the relationship between specific activation of $\beta_1/\beta_2$-adrenoceptor subtypes and the release of NO and EDH and the relevance of relaxations induced by noradrenaline and isoprenaline in the rat basilar artery.
- To assess the link between the efficacy of β-adrenoceptor agonists (ligands) and the release of NO and EDH in the basilar artery and beyond.
Chapter 5

Effects of endothelin in the rat cerebral circulation and the role of voltage-operated and non-voltage-operated calcium channels
5.1. Introduction

Endothelins are vasoactive peptides mainly produced and released by endothelial cells. They were initially isolated from endothelial cells and subsequently characterised by researchers in Japan (Yanagisawa et al., 1988; Barton and Yanagisawa, 2008). The family includes endothelin-1, endothelin-2 and endothelin-3; endothelin-1 (a 21-amino acid peptide) being the most powerful vasoconstrictor agent. Endothelins are synthesised as 212-amino acid precursor peptides, called preproendothelins that are subsequently converted to the respective big-endothelin intermediates (or proendothelins) by furin-like endopeptidases. The big-endothelins are then converted to endothelins primarily by the membrane-bound endothelin converting enzymes as shown in Figure 5-1 (Barton and Yanagisawa, 2008). Apart from their vascular effects, endothelins also have potent effects in the heart and kidney, as well as in the central nervous, respiratory, endocrine, urogenital, and gastrointestinal systems (Neylon, 1999).

Endothelins have two distinct G-protein-coupled receptors - ET\textsubscript{A} and ET\textsubscript{B} (Davenport, 2002). ET\textsubscript{A} receptors have high affinities for endothelin-1 and endothelin-2 but about 100-fold lower affinity for endothelin-3. The affinities of ET\textsubscript{B} receptors are similar for all three endothelins (Kedzierski and Yanagisawa, 2001). ET\textsubscript{A} receptors located on vascular smooth muscle cells mediate the vasoconstrictor and growth promoting effects of endothelins, while endothelial ET\textsubscript{B} receptors mediate vasodilatation, growth inhibition and clearance of ET\textsubscript{A} receptors (Kawanabe and Nauli, 2005). However, endothelin ET\textsubscript{B} receptors may also be located on vascular smooth muscle cells and mediate vasoconstriction (Haynes et al., 1995).

The production of endothelin is mostly regulated at the level of mRNA transcription since most cells producing endothelins lack storage vesicles. Endothelin-1 mRNA transcription is upregulated by tumour necrosis-alpha, growth factor-beta interleukins, noradrenaline, angiotensin II, thrombin, insulin and hypocapnia. The expression of endothelin-1 is downregulated by
nitric oxide, prostacyclin and atrial natriuretic peptide and hypoxia, while initially increased then decreased by shear stress or stretch (Kedzierski and Yanagisawa, 2001).

Preclinical and clinical studies have shown endothelin-1 is involved in the pathogenesis of various diseases including pulmonary and arterial hypertension, heart failure, renal diseases, atherosclerosis, diabetes and cancer (Kedzierski and Yanagisawa, 2001). Endothelin-1 has also been identified as a potential cause of ischaemic stroke and cerebral vasospasm that often occurs after subarachnoid haemorrhage. Studies have reported increased concentrations of endothelin-1 in cerebrospinal fluid of subarachnoid haemorrhage patients during cerebral vasospasm (Kobayashi et al., 1995; Kästner et al., 2005). In addition, increased sensitivity of cerebral vessels to endogenous vasoconstrictor agents may also contribute to the pathogenesis of the vasospasm due to upregulation of receptors (Edvinsson and Povlsen, 2011). This indicates that even relatively low concentrations of vasoconstrictor mediators may cause significant vasospasm.
Figure 5-1: The endothelin pathway.

Endothelins are initially expressed as preproendothelin precursor peptides (preproET-1, preproET-2 and preproET-3) that are subsequently cleaved by furin-like proteases to form big-endothelins (proendothelins). Big-endothelins are then converted to the respective endothelins by endothelin converting enzymes (ECE). The endothelin peptides activate the G-protein-coupled endothelin ET<sub>A</sub> and ET<sub>B</sub> receptors eliciting different physiological responses (From Kedzierski and Yanagisawa (2001)).

The vasoconstrictor action of endothelin-1 depends predominantly on influx of calcium from the extracellular space. It can also cause release of calcium from the sarcoplasmic/endoplasmic reticulum that normally constitutes a small proportion of the calcium source (QingHua et al., 2009). Despite initial reports that endothelin-1 activates voltage-operated calcium channels (VOCC) in porcine coronary artery smooth muscle cells (Goto et al., 1989), it appears that these channels constitute only a small part in the vasoconstrictor response since nifedipine, a specific inhibitor of L-type VOCC, did not inhibit calcium influx in this tissue (Kawanabe et al., 2002; Kawanabe and Nauli, 2005). Endothelin-1
has also been reported to enhance calcium entry into cardiac smooth muscle cells via T-type VOCC in rat ventricular myocytes (Furukawa et al., 1992). Non-voltage-operated calcium channels, namely receptor-operated calcium channels and store-operated calcium channels, are known to play a principal role in the sustained increase of intracellular calcium that maintains the contractile effect of endothelin-1 (Enoki et al., 1995; Iwamuro et al., 1998; Iwamuro et al., 1999; Zhang et al., 1999; Zhang et al., 2000; Kawanabe et al., 2002). On the other hand, the dynamic range of global cytosolic calcium mediating contraction in cerebral arteries is quite narrow (Wellman, 2006) and, as a result, cerebral arteries are typically sensitive to constriction induced by an agonist or increased intraluminal pressure. Understanding the functional relationship between endothelin-1 and calcium channels in the cerebral circulation may help to unravel the mechanism of endothelin-1-induced vasospasm. The aim of this study was to characterise vasoconstrictor effects of endothelin-1 and the roles of L- and T-type VOCC and non-VOCC in arterial segments isolated from different regions of the rat cerebral circulation using wire myography.
5.2. Materials and methods

5.2.1. Methods

Arteries isolated from different regions of the rat cerebral circulation (anterior cerebral, middle cerebral, posterior communicating and basilar arteries) were characterised in vitro using wire myography as described in section 2.3. Rat small mesenteric arteries were also isolated and characterised in a similar manner.

5.2.2. Experimental protocols

The effects of endothelin-1 (0.01–100 nM) were examined by addition of cumulative concentrations in half-log_{10} increments. Responses were allowed to plateau before addition of the next higher concentration. The functional presence of endothelin ET\textsubscript{B} receptors in cerebral arteries was assessed using sarafotoxin S6c, a selective endothelin ET\textsubscript{B} receptor agonist. The vessels were pre-contracted to 80–90% of the maximum KPSS-induced contraction using vasopressin (0.1–1 nM), and relaxed with cumulative concentrations of sarafotoxin S6c (0.01–3 nM). Nifedipine (1 μM), NNC 55-0396 (1 μM) or SK&F 96365 (10 or 30 μM) were used to assess the roles of L-type VOCC, T-type VOCC or SOCC and ROCC, respectively. Combinations of 1 μM nifedipine with 10 or 30 μM SK&F 96365 were also used to investigate effects of combined blockade of L-type VOCC and non-voltage-operated calcium channels. All calcium channel inhibitors were added 20–25 min before addition of endothelin-1.

In a second series, the ability of these calcium channel antagonists to relax endothelin-1-induced contraction was investigated. The vessels were contracted with endothelin-1 (10 nM) followed by addition of cumulative concentrations of nifedipine, NNC 55-0396 or SK&F 96365. In a third series, the middle cerebral artery was pre-contracted with endothelin-1 (10 nM) before adding a single concentration of nifedipine (0.01 or 1 μM), SK&F 96365 (1 or 10 μM) or their combinations. Some vessels acted as time controls after the endothelin-1 pre-contraction.
5.2.3. Drugs and chemicals

The drugs used in this study were obtained from the following suppliers: Endothelin-1 (GenScript, Piscataway, NJ, USA), vasopressin and sarafotoxin S6c (AusPep, Parkville, Victoria, Australia), bosentan (gift from Actelion Pharmaceutical Ltd., Allschwil, Switzerland), nifedipine (Sigma Aldrich, St Louis, Missouri, USA), NNC 55-0396 and SK&F 96365 (Tocris Bioscience, Bristol, UK). Endothelin-1 was dissolved in 10% dimethylformamide (DMF). Nifedipine was dissolved in dimethylsulfoxide (DMSO) and diluted in water. Vasopressin, bosentan, sarafotoxin S6c, NNC 55-0396 and SK&F 96365 were dissolved in water.
5.3. Results

5.3.1. Effects of endothelin receptor stimulation in rat cerebral arteries

The effects of endothelin receptor activation were investigated in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries. Endothelin-1 induced potent constrictor effects in cerebral and mesenteric arteries. The contraction induced by endothelin-1 was characterised by vasomotion (a wave-like oscillation of tone), typically in the middle cerebral artery, which was abolished by the calcium channel inhibitors (Figure 5-2). The middle cerebral and posterior communicating arteries tended to be more sensitive to endothelin-1 than anterior cerebral artery or basilar artery (Figure 5-3 and Table 5-1). The maximum effects caused by endothelin-1 in the cerebral arteries ranged from 108±2% (n=11) (in basilar artery) to 122±2% (n=10) (in anterior cerebral artery) of the KPSS-induced maximum contraction; similar effects were obtained in mesenteric arteries as shown in Table 5-1. Sarafotoxin S6c (0.01-1 nM) caused significant relaxation of vasopressin pre-contracted arteries with similar potency in each vessel (pEC50 10.2±0.2-10.4±0.2). The maximum relaxation was, however, variable between arteries taken from different regions of the rat cerebral circulation - anterior cerebral artery and middle cerebral artery (77%), posterior communicating artery (88%) and basilar artery (100%) of vasopressin pre-contraction (Figure 5-3). Sarafotoxin S6c did not induce any contraction in cerebral arteries, with or without endothelium.
Figure 5-2: Representative trace of the endothelin-induced contraction of cerebral arteries.

Individual experimental traces of endothelin-1-induced vascular contraction in middle cerebral arteries showing (left panel) the oscillations in tone and (right panel) the effect of pre-treatment with the L-type calcium channel inhibitor nifedipine 1 μM.
Figure 5-3: Contractile effects of endothelin-1 in rat cerebral arteries.
Contractile effects of endothelin-1 in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC_{50}±SEM. n, number of arteries from separate rats.
Figure 5-4: Relaxant effects of sarafotoxin S6C in rat cerebral arteries.

Relaxant effects of sarafotoxin S6C in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries pre-contracted with vasopressin (AVP) 0.1-1 nM. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.

5.3.2. Antagonism of endothelin receptors in rat cerebral arteries

Specific inhibition of the ET$_{A}$ receptors with bosentan caused a rightward shift of the endothelin-1 concentration-response curve in rat cerebral and mesenteric arteries (Figure 5-5 and Table 5-1). At 0.1 µM, bosentan caused a significant inhibition of endothelin-1-induced contraction only in the anterior
cerebral artery (1.5 fold shift) and basilar artery (2.5 fold shift). However bosentan 1 µM, significantly antagonised endothelin-1 in all arteries with fold rightward shifts in EC$_{50}$ of 4, 5, 8, 4 and 5 in the anterior cerebral, middle cerebral, posterior communicating, basilar and mesenteric arteries, respectively ($P<0.05$, compared with respective vehicle group) (Table 5-1).
Figure 5-5: Effects of bosentan on the endothelin-induced contraction of cerebral arteries.

Effects of bosentan (Bos) 0.1 or 1 µM on the contraction induced by endothelin-1 in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries. Bosentan was added 20 min before endothelin contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. n, number of arteries from separate rats.
Table 5-1: Comparison of the $pEC_{50}$ and $E_{\text{max}}$ values of ET-1 and the fold shift caused by bosentan pre-treatment in rat cerebral and mesenteric arteries

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>ACA</th>
<th>MCA</th>
<th>Pcom</th>
<th>BA</th>
<th>Mes</th>
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</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>$E_{\text{max}}$</td>
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<td>119±2</td>
<td>117±4</td>
<td>108±2</td>
<td>109±3</td>
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<tr>
<td>$pEC_{50}$</td>
<td>8.4±0.06</td>
<td>8.7±0.11</td>
<td>8.8±0.14</td>
<td>8.3±0.05</td>
<td>8.4±0.12</td>
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<td><strong>Bosentan 0.1 µM</strong></td>
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<tr>
<td>n</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>$E_{\text{max}}$</td>
<td>122±3</td>
<td>112±4</td>
<td>123±2</td>
<td>109±3</td>
<td>116±2</td>
</tr>
<tr>
<td>$pEC_{50}$</td>
<td>8.2±0.04*</td>
<td>8.3±0.14</td>
<td>8.5±0.16</td>
<td>7.9±0.08*</td>
<td>8.2±0.08</td>
</tr>
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<td>EC$_{50}$ Fold shift$^a$</td>
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<td>-</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Bosentan 1 µM</strong></td>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>5</td>
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<td>5</td>
</tr>
<tr>
<td>$E_{\text{max}}$</td>
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<td>113±5</td>
<td>123±4</td>
<td>110±4</td>
<td>117±1</td>
</tr>
<tr>
<td>$pEC_{50}$</td>
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<td>8.0±0.07*</td>
<td>7.9±0.11*</td>
<td>7.7±0.04*</td>
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</tr>
<tr>
<td>EC$_{50}$ Fold shift$^a$</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

ET-1, endothelin-1; ACA, anterior cerebral artery; MCA, middle cerebral artery; Pcom, posterior communicating artery; BA, basilar artery; Mes, mesenteric artery. $E_{\text{max}}$, the maximum contractile response produced by ET-1 expressed as % of KPSS-induced maximum contraction; $E_{\text{max}}$, the maximum contractile response produced by ET-1 expressed as % of KPSS-induced maximum contraction; $EC_{50}$, the concentration of ET-1 that caused 50% of the maximum response; $pEC_{50}$, the negative logarithm of $EC_{50}$; n, the number of arteries (each from a separate rat). *P<0.05, compared to respective vehicle, one-way ANOVA with Dunnett’s post-test for multiple comparisons. $^a$Rightward shift caused by bosentan.
5.3.3. Effects of inhibiting nitric oxide production and endothelium removal on the endothelin-1-induced contraction of cerebral arteries

Inhibition of endothelial nitric oxide production with L-NAME 100 µM caused a significant leftward shift of the endothelin-1 concentration-contraction curve in rat anterior cerebral artery (p<0.05 compared to vehicle, unpaired t test). L-NAME did not, however, affect the endothelin-1 response in middle cerebral, posterior communicating and basilar arteries (Figure 5-6). Physical removal of the endothelium increased the potency of endothelin-1 in both anterior cerebral and basilar arteries, but it did not significantly change the $pEC_{50}$ of endothelin-1 in the rat middle cerebral and posterior communicating arteries (Figure 5-7). The maximum contraction elicited by endothelin-1 in the rat cerebral arteries was not affected by L-NAME treatment or removal of the endothelium.
Figure 5-6: Effects of inhibiting nitric oxide production on the endothelin-1-induced contraction of cerebral arteries.

Contractile effects of endothelin-1 in rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries pre-treated with Nω-nitro-L-arginine methyl ester (L-NAME). L-NAME was added 20 min before endothelin-1 contraction. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC50±SEM. n, number of arteries from separate rats. *P<0.05 compared to vehicle, unpaired t test.
Figure 5-7: Effects of endothelium removal on endothelin-1-induced contraction of cerebral arteries.

Contractile effects of endothelin-1 in endothelium-denuded rat anterior cerebral, middle cerebral, posterior communicating and basilar arteries. The endothelium was removed from the arteries by rubbing the lumen with a human hair. Data are shown as percent of KPSS-induced maximum contraction. Vertical bars represent ±SEM (those not shown are contained within the symbol); horizontal bars indicate EC_{50±SEM}. n, number of arteries from separate rats. *P<0.05 compared to vehicle, unpaired t test.

5.3.4. Contractile effects of endothelin in calcium-free physiological salt solution
Calcium-free physiological salt solution was used to analyse the endothelin-1-induced release of calcium from intracellular stores. Only a small contraction was obtained with addition of endothelin-1 in the absence of extracellular calcium (Figure 5-8). The estimated $pEC_{50}$ value of endothelin-1 in calcium-free solution, however, was not significantly different from the respective vehicle (normal PSS) in all arteries ($P>0.05$) (Table 5-2). The maximum contraction induced by endothelin-1 ranged from about 13% (anterior cerebral artery) to 24% (basilar artery) of the maximum KPSS-induced contraction (Table 5-3).
Figure 5-8: The contractile effects of endothelin-1 in calcium-free medium.

The contractile effects of endothelin-1 in rat cerebral arteries: A. in physiological salt solution (PSS) containing 1.5 mM calcium; and B. in PSS without calcium. Data are shown as percentage of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC$_{50}$±SEM. $n$, number of arteries from separate rats. ET-1, endothelin-1; ACA, anterior cerebral artery; MCA, middle cerebral artery; Pcom, posterior communicating artery and BA, basilar artery.

5.3.5. Effects of nifedipine pre-treatment on endothelin-1-induced contraction

Inhibition of the L-type VOCC with 1 µM nifedipine did not affect the pEC$_{50}$ values of endothelin-1 in the cerebral arteries ($P>0.05$) (Table 5-2). Similarly, the maximum contraction induced by endothelin-1 was not significantly decreased except in the middle cerebral artery ($P<0.05$) where a maximum effect was decreased by about 19% compared with the (Table 5-3 and Figure 5-9A). Nifedipine did not affect the baseline tone of the arteries during the pre-treatment period.
5.3.6. **Effects of NNC 55-0396 pre-treatment on endothelin-1-induced contraction**

Pre-treatment of the different cerebral arteries with NNC 55-0396 1 μM, a putative selective T-type voltage-operated calcium channel blocker did not significantly change the pEC$_{50}$ of endothelin-1 (Table 5-2). The maximum contractile effect of endothelin-1 was, however, significantly decreased in the cerebral arteries (Table 5-3; shown for middle cerebral artery in Figure 5-9B). NNC 55-0396 1 μM did not affect the baseline arterial tone, but moderate to strong contractions were observed in most vessels after addition of 3 or 10 μM. Thus, a higher concentration of NNC 55-0396 was not considered in this study.

5.3.7. **Effects of SK&F 96365 pre-treatment on endothelin-1-induced contraction**

Pre-treatment of the cerebral arteries with SK&F 96365 10 μM was without effect, while 30 μM caused a concentration-dependent decrease in the maximum endothelin-1 contractile response without significantly altering the pEC$_{50}$ values (Table 5-2 and Table 5-3). As shown in Figure 5-9C for the middle cerebral artery, the maximum contractile effect of endothelin-1 was not significantly affected by 10 μM of SK&F 96365. At 30 μM, however, SK&F 96365 significantly decreased the maximum contractile effects of endothelin-1 ($P<0.05$) in the cerebral arteries, with changes in E$_{max}$ of 23% in basilar arteries to 41% in middle cerebral arteries compared with respective vehicle pre-treatment values (Table 5-3 and Figure 5-9C). SK&F 96365 100 μM completely abolished the contraction induced by endothelin-1 in cerebral arteries. SK&F 96365 10 or 30 μM did not affect the baseline arterial tone, while a slight decrease was observed in some arteries after addition of 100 μM.
5.3.8. Combined effects of nifedipine and SK&F 96365 on endothelin-1-induced contraction

Rat cerebral arteries were pre-treated with a combination of nifedipine 1 µM with SK&F 96365 10 µM or 30 µM to assess the effects of simultaneous blockade of L-type VOCC and non-VOCC on the contractile effects of endothelin-1. Combinations of nifedipine and SK&F 96365 did not significantly change the pEC\textsubscript{50} of endothelin-1 (Table 5-2); however, maximum contractile effects of endothelin-1 were significantly attenuated (P<0.05; Table 5-3). Thus, the response to endothelin-1 10 nM (alone) was 110±4% of the KPSS-induced contraction and the combination of nifedipine 1 µM with SK&F 96365 10 µM decreased the response to 44±7% of KPSS-induced contraction (Figure 5-9D). Similarly, combination of nifedipine 1 µM with SK&F 96365 30 µM caused a further decrease in the maximum effect of endothelin-1 (36% in middle cerebral artery, Figure 5-9D; and 52% in basilar artery, Table 5-3; P<0.05). In all arteries, the resting baseline tone was not affected by either combination of antagonists.

Further examination of the responses to endothelin-1 at 3 nM (taken from the concentration-response curves when neither nifedipine 1 µM nor SK&F 96365 10 µM affected the endothelin-1 contraction), showed that the combined treatment attenuated the response to just 17±5% of KPSS-induced contraction in the middle cerebral artery (Figure 5-10). The combination of nifedipine 1 µM and NNC 55-0396 1 µM did not show this enhanced effect.
Figure 5-9: Effect of calcium channel inhibition on endothelin-induced contraction of middle cerebral arteries.

Effects of endothelin-1 in rat isolated middle cerebral arteries pre-treated with: A. nifedipine; B. NNC 55-0396; C. SK&F 96365; or D. Combination of nifedipine and SK&F 96365. ET-1, endothelin-1; SK&F, SK&F 96365; NNC, NNC 55-0396; Nifed, nifedipine. Data are shown as percentage of KPSS-induced maximum contraction. Vertical bars represent ± SEM (those not shown are contained within the symbol); horizontal bars indicate EC50±SEM. n, number of arteries from separate rats. The calcium channel antagonists were added 20 min prior to endothelin-1 contraction.
Figure 5-10: Comparison of the effects of calcium channel antagonists on endothelin-1-induced contraction of cerebral arteries.

The effects of pre-incubation with calcium channel antagonists, alone or in combination, on the contraction induced by endothelin-1 at 3 nM in rat middle cerebral arteries (data are derived from the ET-1 concentration-response curves depicted in Figure 5-9). Data are shown as percentage of KPSS-induced maximum contraction. Vertical bars represent ± SEM. The calcium channel antagonists were added 20 min prior to endothelin-1 contraction. Presence or absence of a drug is indicated by (+) or (-), respectively. ET-1, endothelin-1; SK&F, SK&F 96365; NNC, NNC 55-0396; Nifed, nifedipine. *P<0.05 compared to vehicle (endothelin-1 alone), one way ANOVA followed by Dunnett’s post-test.

5.3.9. Relaxation of endothelin-1-induced contraction with calcium channel blockers

In a separate series of experiments, arteries were pre-contracted with endothelin-1 to assess the relaxant effects of various calcium channel
antagonists following pre-contraction induced by endothelin-1 (Figure 5-11). Over the same time course that the channel antagonists were tested, the plateau of endothelin-1 (10 nM)-induced contraction did not change in the time control group. SK&F 96365 caused concentration-dependent relaxation of rat cerebral arteries pre-contracted with endothelin-1 (10 nM). The relaxation potency of SK&F 96365 was similar in all cerebral arteries with no significant difference in the pEC$_{50}$ values observed (e.g., 4.9±0.11, n=3 in anterior cerebral artery). The endothelin-1 mediated contraction was almost completely relaxed in all arteries by 100 µM SK&F 96365. Nifedipine relaxed the endothelin-1-induced contraction with similar potency in all vessels (pEC$_{50}$ in anterior cerebral artery 7.7±0.14, n=4). Anterior cerebral artery, middle cerebral artery and posterior communicating artery were relaxed by about 65%, 75% and 77%, respectively, with nifedipine 1 µM while in basilar artery the contraction was relaxed only by 52%. In contrast, endothelin-1 pre-contraction was not effectively reversed by NNC 55-0396 (Figure 5-11). The arteries started to re-contract when the concentration of NNC 55-0396 was further increased.
Table 5-2: Endothelin-1 pEC$_{50}$ values in rat isolated cerebral arteries in the presence of various calcium channel antagonists

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ACA (n)</th>
<th>pEC$_{50}$</th>
<th>MCA (n)</th>
<th>pEC$_{50}$</th>
<th>Pcom (n)</th>
<th>pEC$_{50}$</th>
<th>BA (n)</th>
<th>pEC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>8.4±0.06</td>
<td>10</td>
<td>8.7±0.11</td>
<td>9</td>
<td>8.8±0.14</td>
<td>11</td>
<td>8.3±0.05</td>
</tr>
<tr>
<td>Nifedipine 1 µM</td>
<td>4</td>
<td>8.7±0.22</td>
<td>5</td>
<td>8.8±0.22</td>
<td>4</td>
<td>8.7±0.19</td>
<td>4</td>
<td>8.7±0.19</td>
</tr>
<tr>
<td>NNC 55-0396 1 µM</td>
<td>5</td>
<td>8.6±0.08</td>
<td>5</td>
<td>8.4±0.06</td>
<td>5</td>
<td>8.6±0.15</td>
<td>5</td>
<td>8.4±0.12</td>
</tr>
<tr>
<td>SK&amp;F 96365 10 µM</td>
<td>5</td>
<td>8.7±0.13</td>
<td>4</td>
<td>8.6±0.06</td>
<td>5</td>
<td>8.8±0.09</td>
<td>4</td>
<td>8.6±0.16</td>
</tr>
<tr>
<td>SK&amp;F 96365 30 µM</td>
<td>4</td>
<td>8.5±0.08</td>
<td>4</td>
<td>8.3±0.09</td>
<td>4</td>
<td>8.6±0.07</td>
<td>4</td>
<td>8.5±0.07</td>
</tr>
<tr>
<td>SK&amp;F 96365 10 µM + Nifedipine 1 µM</td>
<td>4</td>
<td>8.3±0.09</td>
<td>5</td>
<td>8.2±0.05</td>
<td>3</td>
<td>8.4±0.06</td>
<td>4</td>
<td>8.3±0.04</td>
</tr>
<tr>
<td>SK&amp;F 96365 30 µM + Nifedipine 1 µM</td>
<td>4</td>
<td>8.3±0.06</td>
<td>4</td>
<td>8.3±0.11</td>
<td>4</td>
<td>8.5±0.10</td>
<td>4</td>
<td>8.3±0.15</td>
</tr>
<tr>
<td>Calcium-free PSS</td>
<td>3</td>
<td>8.6±0.04</td>
<td>3</td>
<td>8.6±0.04</td>
<td>3</td>
<td>8.7±0.09</td>
<td>3</td>
<td>8.5±0.14</td>
</tr>
</tbody>
</table>

ACA, anterior cerebral artery; MCA, middle cerebral artery; Pcom, posterior communicating artery; BA, basilar artery, pEC$_{50}$, the negative logarithm of EC$_{50}$ (concentration of endothelin-1 that caused 50% maximum contraction); PSS, physiological salt solution; n, number of arteries (each from separate animals). The calcium channel antagonists were added 20 min before endothelin-1. *pEC$_{50}$ values are only estimates.
Table 5-3: The effects of various calcium channel antagonist pre-treatments on the $E_{max}$ of endothelin-1 in rat isolated cerebral arteries

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ACA $E_{max}$</th>
<th>%$E_{max}$</th>
<th>MCA $E_{max}$</th>
<th>%$E_{max}$</th>
<th>Pcom $E_{max}$</th>
<th>%$E_{max}$</th>
<th>BA $E_{max}$</th>
<th>%$E_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>122±2</td>
<td>100</td>
<td>119±2</td>
<td>100</td>
<td>117±4</td>
<td>100</td>
<td>108±2</td>
<td>100</td>
</tr>
<tr>
<td>Nifedipine 1 µM</td>
<td>101±4</td>
<td>83</td>
<td>96±7*</td>
<td>81</td>
<td>102±10</td>
<td>87</td>
<td>90±6</td>
<td>83</td>
</tr>
<tr>
<td>NNC 55-0396 1 µM</td>
<td>81±4*</td>
<td>66</td>
<td>87±4*</td>
<td>73</td>
<td>96±3*</td>
<td>82</td>
<td>89±9*</td>
<td>82</td>
</tr>
<tr>
<td>SK&amp;F 96365 10 µM</td>
<td>127±9</td>
<td>104</td>
<td>120±9</td>
<td>100</td>
<td>111±5</td>
<td>95</td>
<td>113±7</td>
<td>105</td>
</tr>
<tr>
<td>SK&amp;F 96365 30 µM</td>
<td>84±9*</td>
<td>69</td>
<td>70±3*</td>
<td>59</td>
<td>74±11*</td>
<td>63</td>
<td>83±2*</td>
<td>77</td>
</tr>
<tr>
<td>SK&amp;F 96365 10 µM + Nifedipine 1 µM</td>
<td>66±12*†</td>
<td>54</td>
<td>62±3*†</td>
<td>52</td>
<td>85±6*</td>
<td>73</td>
<td>85±9*</td>
<td>79</td>
</tr>
<tr>
<td>SK&amp;F 96365 30 µM + Nifedipine 1 µM</td>
<td>39±9*†</td>
<td>32</td>
<td>36±6*†</td>
<td>30</td>
<td>45±4*†</td>
<td>38</td>
<td>52±5*†</td>
<td>48</td>
</tr>
<tr>
<td>Calcium-free PSS</td>
<td>16±2*</td>
<td>13</td>
<td>13±3*</td>
<td>11</td>
<td>14±2*</td>
<td>12</td>
<td>24±1*</td>
<td>22</td>
</tr>
</tbody>
</table>

ACA, anterior cerebral artery; MCA, middle cerebral artery; Pcom, posterior communicating artery; BA, basilar artery; $E_{max}$, the maximum contractile effect of endothelin-1 expressed as % KPSS-induced maximum contraction; %$E_{max}$, expressed as % respective vehicle $E_{max}$. The calcium channel antagonists were added 20 min before endothelin-1 contraction. *P<0.05 compared to respective vehicle, one way ANOVA followed by Dunnett’s post hoc test. †P<0.05 compared to nifedipine 1 µM treatment, one way ANOVA followed by Bonferroni’s post hoc test between selected pairs.
Figure 5-11: Relaxant effects of calcium channel antagonists in cerebral arteries pre-contracted with endothelin-1.

Relaxation of the endothelin-1 induced contraction in rat isolated cerebral arteries using A. SK&F 96365; B. nifedipine; and C. NNC 55-0396. ET 1, endothelin-1; ACA, anterior cerebral artery; MCA, middle cerebral artery; Pcom, posterior communicating artery; and BA, basilar artery. Data are shown as percentage of KPSS-induced contraction. Vertical bars represent ±SEM; horizontal bars indicate EC\textsubscript{50}±SEM. The arteries were pre-contracted with 10 nM endothelin-1 and cumulative concentrations of each of the respective antagonists were added after the endothelin response had plateaued. n, number of arteries from different rats.
Figure 5-12: Relaxation of endothelin-1 pre-contracted cerebral arteries with combinations of calcium channel antagonists

Relaxation of endothelin-1 (10 nM)-pre-contracted middle cerebral arteries with calcium channel antagonists alone or in combination at low-high concentrations. A. Low concentration of SK&F 96365 (1 µM) and nifedipine (0.01 µM) and their combination; B. Low concentration of SK&F 96365 (1 µM) and high concentration of nifedipine (1 µM) and their combination; C. High concentration of SK&F 96365 (10 µM) and low concentration of nifedipine (0.01 µM) and their combination; and D. High concentration of SK&F 96365 (10 µM) and nifedipine (1 µM) and their combination. Data are shown as percentage of the contraction induced by 10 nM endothelin-1. Error bars are ±SEM. *P<0.05; one-way ANOVA followed by Dunnett’s post-test. n≥5 for each treatment group.
5.4. Discussion

In this *in vitro* study the effects of endothelin receptor activation and role of calcium channels in arterial segments isolated from different regions of the rat cerebral circulation were analysed. Endothelin-1 was shown to induce a potent vasoconstrictor response with no significant regional variation in sensitivity within the cerebral circulation. Endothelin-1 caused similar effects in mesenteric arteries. Bosentan, a dual endothelin ET\textsubscript{A} and ET\textsubscript{B} antagonist, shifted the contraction-response curve to the right. On the other hand, activation of endothelin ET\textsubscript{B} receptors using sarafotoxin S6c did not induce vasoconstriction in the cerebral arteries. The findings show that vasoconstriction of cerebral arteries induced by endothelin-1 may be dependent on activation of ET\textsubscript{A} receptors only. However, sarafotoxin S6c caused relaxation of pre-contracted cerebral arteries supporting the presence of endothelial ET\textsubscript{B} receptors (Nilsson *et al.*, 2008). Inhibition of endothelial nitric oxide production and removal of endothelium caused a variable increment in sensitivity to endothelin-1. This indicates a heterogeneous distribution of ET\textsubscript{A} and ET\textsubscript{B} receptors in cerebral arteries.

The interdependence between endothelin receptor activation and calcium mobilization involves complex mechanisms and appears to vary in different cells and tissues (Tykocki and Watts, 2010). The diversity of signals induced by endothelin-1 can be attributed to disparity in the level of expression of various channels involved in calcium mobilization (Neylon, 1999). The current study demonstrates that endothelin-1 induces contraction of cerebral arteries both by releasing calcium from intracellular stores and influx of extracellular calcium. However, the vasoconstrictor actions of endothelin-1 largely depend on an influx of calcium from extracellular sources as evidenced by the weak response (<22%) elicited by endothelin-1 in cerebral arteries in calcium-free solution. This shows that the calcium released from internal stores accounts for a small proportion of the calcium needed for endothelin-1-induced contraction of these arteries. On the other hand, the threshold concentration of endothelin-1 that caused vascular contraction was higher in calcium-free solution than in
calcium-containing buffer. This may indicate that low concentrations of endothelin-1 activate influx of extracellular calcium, while higher concentrations are needed to release calcium from internal stores. The finding is consistent with an earlier report by Komuro et al. (1997) that removal of extracellular calcium abolished effects of low concentrations of endothelin-1 in rabbit isolated aortic rings. However, it differs from a previous report that endothelin-1-induced contraction of rabbit basilar artery was exclusively dependent on influx of extracellular calcium (Kawanabe et al., 2002). The variation between this latter finding and our results may be due to species difference. In addition, endothelin may enhance the sensitivity of the vascular smooth muscle cell contractile machinery to calcium via the protein kinase C pathway, which might have contributed to the long-lasting contraction induced by endothelin (Labadia et al., 1997; Cain et al., 2002). In line with this, endothelin may also activate RhoA/Rho kinase and thus inactivate myosin light chain phosphatase leading to the maintenance of contraction for long periods (Scherer et al., 2002; Wynne et al., 2009).

Pre-treatment with nifedipine 1 µM, a concentration that is reported to maximally inhibit the L-type VOCC (Iwamuro et al., 1998; Navarro-Gonzalez et al., 2009), caused suppression of the contraction induced by higher concentrations of endothelin-1 in cerebral arteries but did not affect the low concentration responses. This suggests that L-type VOCC may not play a significant role in the influx of extracellular calcium that is needed for contraction induced by lower concentrations (<1 nM) of endothelin-1. Interestingly, inhibition of the T-type VOCC with NNC 55-0396 1 µM decreased the contraction response induced by low as well as high concentrations of endothelin-1. The observation that the responses to low concentrations of endothelin-1 (unaffected by nifedipine) were inhibited by NNC 55-0396 demonstrates that T-type VOCC may be activated by low concentrations of endothelin-1 in the rat cerebral arteries. Kuo et al. (2010) also demonstrated a mibebradil- and NNC 55-0396-sensitive response in smaller branches of rat basilar artery and there is an increasing body of evidence that T-type VOCC are expressed in cerebral vasculature (Abd El-Rahman et al., 2013; Harraz and
Welsh, 2013). While the results from this study revealed a potential role of T-type VOCC in endothelin-1-induced vasoconstriction of cerebral arteries, rigorous experimental investigation is needed for further understanding of the actual role of T channels in cerebral arteries.

The nifedipine (L) or NNC 55-0396 (T)-insensitive component of the contraction induced by endothelin-1 was blocked by SK&F 96365, a putative non-VOCC inhibitor, suggesting that the residual response may be mediated by non-VOCC activation. It is worth noting that SK&F 96365 (1-10 µM) reportedly blocked multiple VOCC including T-type channels in HEK 293 cells (Singh et al., 2010). But there are substantial reservations about the findings of VOCC blockade in HEK 293 cells compared with native tissue. Therefore, this drug was not used in this study for the purpose of blocking a specific calcium channel - rather it was used to block the voltage-insensitive component of calcium influx or the non-VOCC-mediated calcium entry. SK&F 96365 caused concentration-dependent inhibition of the endothelin-1-induced contraction of the cerebral arteries demonstrating roles of the non-VOCC in addition to the L- and T-type VOCC. A previous study by Saleh et al. (2009) reported that endothelin-1 causes activation of canonical transient receptor potential type-1 (TRPC1) channels in rabbit coronary artery myocytes. TRPC1 channels are often loosely referred to as ROCC or SOCC in order to highlight that they may be gated due to receptor activation or secondary to depletion of intracellular calcium storage. Complete inhibition of endothelin-1-induced contraction, however, shows that high concentrations of SK&F 96365 (100 µM) non-specifically block other calcium channels including those located on intracellular stores.

Addition of nifedipine (0.01-1 µM) to cerebral vessels pre-contracted with a high concentration (10 nM) of endothelin-1 resulted in marked relaxation. The maximum relaxation caused by nifedipine 1 µM was about 74% in the middle cerebral artery. In contrast, NNC 55-0396 (0.1-1 µM) did not cause significant relaxation of the arteries pre-contracted with endothelin-1. This implies that L-type VOCC, which can be activated secondary to depolarization caused by opening of other calcium channels, play greater role in the maintenance phase
of contraction caused by higher concentrations of endothelin-1 in rat cerebral arteries, as previously suggested (Neylon, 1999; Navarro-Gonzalez et al., 2009). SK&F 96365 (1-100 µM) also caused a concentration-dependant relaxation of arteries pre-contracted with endothelin-1 with a complete reversal observed at 100 µM.

Activation of G-protein-coupled endothelin ET₄ receptors stimulates phospholipase C which hydrolyses the membrane bound phosphatidylinositol 4, 5 bisphosphate (PIP₂) into inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ and DAG then cause release of calcium from intracellular stores (sarcoplasmic/endoplasmic reticulum) and opening of non-VOCC directly and/or indirectly through depletion of intracellular calcium stores (Motte et al., 2006). The findings of the current study indicate that the initiation and maintenance phases of endothelin-1-induced contraction may involve a different profile of calcium channel activation. The contraction induced by low concentrations of endothelin-1 (<1 nM) may be initiated by release of calcium from intracellular stores as well as activation of non-VOCC, which in turn cause membrane depolarization that gradually leads to opening of the low voltage-activated calcium channels (T-type VOCC) which normally open at membrane potential of -65 to -45 mV (Kuo et al., 2011). Higher concentrations of endothelin-1 caused opening of L-type VOCC and the maintenance phase of contraction caused by high concentrations of endothelin-1 largely depends on influx of calcium through L-type VOCC and the non-VOCC play a lesser role (Figure 5-13).

Combination treatment with nifedipine and SK&F 96365 revealed an interaction between VOCC and non-VOCC in mediating the contraction of cerebral arteries induced by endothelin-1. Pre-treatment with either nifedipine (1 µM) or SK&F 96365 (10 µM) alone did not significantly inhibit responses to low concentrations of endothelin-1, but their combination significantly decreased the overall responses, including those induced by low endothelin-1 concentrations, as illustrated in Figure 5-10. The synergy observed between the antagonists in
inhibiting the endothelin-1-induced contraction may illustrate interdependence of VOCC and non-VOCC in mobilization of the extracellular calcium.

The relative contribution of non-VOCC and VOCC is altered during the contraction phase. Thus, NNC 55-0396 sensitivity was decreased in pre-contracted arteries. In contrast, arteries pre-contracted with endothelin-1 were more sensitive to nifedipine and SKF 96365. Thus, nifedipine, caused about 74% relaxation of endothelin-1 pre-contraction, in contrast to the weak inhibitory effect (<25%) on endothelin-1 contraction when used as pre-treatment, and SK&F 96365 caused complete concentration-dependent relaxation of the pre-contracted arteries. In addition, in endothelin-1 pre-contracted arteries additive responses were obtained when the arteries were relaxed using combinations of nifedipine and SK&F 96365. This is in contrast to the pre-treatment findings and suggests that nifedipine and SK&F 96365 relax the endothelin-1-pre-contracted arteries through independent inhibition of the VOCC and non-VOCC. It can be implied that relatively low concentrations of these calcium channel antagonists may be more effective in reversing rather than preventing contraction, a finding that may point to the therapeutic management of cerebral vasospasm.

In conclusion, the potent ETₐ receptor-mediated vasoconstrictor effect of endothelin-1 involves mobilization of calcium through activation of non-VOCC as well as L-and T-type VOCC. Combination of nifedipine and SK&F 96365 demonstrated synergistic inhibition of endothelin-1-induced vasoconstriction when added prior to endothelin. Interestingly, nifedipine and SK&F 96365 were more effective in relaxing the contraction induced by endothelin-1 than preventing it through pre-treatment. This work suggests that non-VOCC may work in concert with VOCC in mediating induction of vasoconstrictor effects of endothelin-1 in cerebral arteries and that the L-type VOCC antagonists are not sufficient to fully relax endothelin-1 pre-contracted cerebral arteries.
Figure 5.13: A schematic representation of the contribution of different calcium channels in the contractile response to endothelin-1.

The results calculated from the area under the concentration-response curve (expressed as %), contrasting the effect of adding antagonists before or during endothelin-1 contraction. The contributions were allocated according to i) the effect of the L-type VOCC antagonist nifedipine (1 µM), the T-type VOCC antagonist NNC 55-0396 (1 µM) on the endothelin-1 concentration-response curve and the residual response unaffected by either agent; and ii) the contribution from L-type VOCC and non-VOCC antagonists to the relaxation of endothelin-1 (10 µM) pre-contracted middle cerebral arteries. VOCC; voltage-operated calcium channels.
Chapter 6

Cerebrovascular effects of vasoactive substances *in vivo*
6.1. Introduction

Cerebral autoregulation (or pressure autoregulation) is defined as the ability of the cerebral circulation to maintain adequate cerebral blood flow in spite of changes in perfusion pressure due to variation in peripheral blood pressure within the range of 60 to 150 mmHg (Paulson et al., 1990; van Beek et al., 2008). The main goal of autoregulation is to maintain cerebral blood flow in face of varying perfusion pressure by changing the cerebrovascular resistance. The relationship between cerebral blood flow, perfusion pressure and vascular resistance can be expressed using Ohm’s law which states that flow [current] is directly proportional to pressure [voltage] but inversely proportional to the vascular resistance (Cipolla, 2009; Partington and Farmery, 2014).

\[ \text{CBF} = \frac{\text{CPP}}{\text{CVR}} \]

Where, CBF, cerebral blood flow; CPP, cerebral perfusion pressure; and CVR, cerebral vascular resistance.

Cerebral perfusion pressure is the difference between mean arterial pressure (MAP) and intracranial pressure (ICP) or central venous pressure, whichever is highest.

\[ \text{CPP} = \text{MAP} - \text{ICP} \]

The normal intracranial pressure is maintained within 5-13 mmHg in adults with only minor variation associated with arterial pulse pressure or change in body posture (Partington and Farmery, 2014).

Under high blood pressure conditions, destructive increases in cerebral blood flow are prevented by vasoconstriction and when the blood pressure decreases, cerebral blood flow is maintained through vasodilatation of the cerebral arteries. In addition cerebral blood flow is also dependent on the metabolic needs of the brain (flow-metabolism coupling). Brain regions with higher metabolic activities require more blood flow than other areas. The process of
Vasoactive agents in vivo

autoregulation involves various mechanisms including vascular and neural systems to provide a beat-to-beat control over the cerebral arterial diameter. The interplay between various factors in the regulation of cerebral blood flow is illustrated in Figure 6-1.

![Diagram showing factors affecting cerebral blood flow and the effects of vasoactive drugs]

**Figure 6-1: Major physiological factors affecting cerebral blood flow and the effects of vasoactive drugs.**

Cerebral blood flow (CBF) is principally dependent on mean arterial pressure (MAP), intracranial pressure (ICP) and cerebral vascular resistance (CVP). Autoregulation maintains CBF by varying the cerebrovascular resistance (CVR) in spite of changes in MAP within the autoregulatory range. Innervation of the cerebral arteries control the vascular tone, and thus the CVR. Vasoactive drugs circulating in blood may affect CBF through direct effect on CVR or indirectly by altering MAP through their effects on cardiac output (CO) and/or total peripheral resistance (TPR). Some drugs may also augment the neural effects on CBF.

The large pial arteries may not play a significant role in maintaining cerebral blood flow since their diameters do not change in response to changes in peripheral blood pressure (Giller et al., 1993). This assumption has been employed in transcranial Doppler sonography which measures the blood flow velocity in major arteries such as the middle cerebral artery as a surrogate
marker of cerebral blood flow. Therefore, small branches of the large cerebral arteries, such as branches of the middle cerebral artery, and arterioles may be directly involved in the regulation of cerebral blood flow through compensatory vasoconstriction or vasodilatation in response to decreases or increases in blood pressure, respectively. Previous studies suggested that changes in cerebral vascular resistance caused by systemic administration of vasoactive substances such as adrenaline and angiotensin II are induced by the changes in arterial blood pressure, not direct effects of the agents on cerebral arteries (Olesen, 1972). Most vasoactive agents circulating in blood have limited access to cerebral vascular smooth muscle cells due to the presence of the blood-brain barrier in the cerebral circulation (Pardridge, 2012). However, it has not been clear whether vasoactive substances are exclusively prevented by the blood-brain barrier from affecting the cerebral circulation. Further, instantaneous onset of autoregulation by a change in blood pressure may augment any vascular effect of the drug in the cerebral circulation. It has been reported that autoregulation, as determined by continuous oximetry analysis, returns the oxygen saturation disturbed by changes in arterial blood pressure to its basal level in about 40-45 seconds (Ekström-Jodal, 1970). This study attempted to dissect the direct versus indirect effects of vasoactive drugs by administering them into systemic circulation via femoral vein or directly into the cerebral circulation through the internal carotid artery. Drug administration through the internal carotid artery can initially allow the distribution of vasoactive drugs to the cerebral circulation undiluted which then cause localized effects on cerebral arteries before affecting the peripheral circulation. Thus, drugs administered via carotid artery should change the diameter of cerebral arteries before affecting blood pressure, unlike simultaneous responses in cerebral artery and blood pressure induced by intravenous administration of the drugs.

The aim of this study was to analyse the changes in diameter of rat small cerebral arteries, as well as in mean arterial blood pressure, caused by the infusion of vasoactive substances through the femoral vein or via direct delivery into the cerebral circulation through the internal carotid artery.
6.2. Materials and methods

6.2.1. Anaesthesia and pre-surgical preparation

The experiments were performed in accordance with the Australian code for the care and use of animals for scientific purposes (8th edition, 2013, National Health and Medical Research Council, Canberra) and were approved by The University of Melbourne Animal Ethics Committee. Male or female Long Evans rats (300-350 g) were deeply anaesthetized by intramuscular injection of a mixture of ketamine (60 mg/kg) and xylazine (5 mg/kg). Throughout the experiment, the body temperature was maintained at 37°C using a heat pad regulated by feedback from a rectally inserted thermometer. The depth of anaesthesia was regularly monitored and confirmed by lack of reaction to toe-pinch tests, absence of blood pressure fluctuations in response to pinching and regular observations of the animal for pupil dilatation. The anaesthesia was administered every 30 min or as required.

6.2.2. Cannulation of arteries and veins

The right femoral artery was cannulated with a blunt-ended catheter (inner/outer diameter: 0.28/0.61 mm, Microtube Extrusions, NSW, Australia) and used for blood pressure monitoring. The right femoral vein was cannulated and used for systemic administration of the drugs. The external carotid artery was used for direct administration of drugs into the cerebral circulation. The right common carotid artery, right external carotid artery and right internal carotid artery were isolated and carefully separated from the adjacent vagus nerves. The external carotid artery was clipped with a microvascular clip near the bifurcation. A small puncture was made on the external carotid artery and a PE-10 catheter was retrogradely inserted and advanced to the bifurcation point of internal and external carotid arteries without blocking blood flow through the internal carotid artery as shown in Figure 6-2. The catheter was then secured in place by tying with a 6.0 silk suture and then applying a tissue glue in order to avoid slipping back due to arterial pressure. The surgical wound
area was sutured to avoid incidence of bleeding before the animal was turned over into the prone position for cranial window preparation.

![Carotid artery cannulation](image)

**Figure 6-2: Carotid artery cannulation.**

A catheter with a smaller tubing connected to the tip was inserted through the external carotid artery and advanced to the point of the bifurcation of internal and external carotid arteries without blocking the blood flow through the internal carotid artery. Suture and tissue glue were used to secure the tubing in place and prevent movements.

### 6.2.3. Cranial window preparation

After cannulation, the head of the animal was shaved and symmetrically fixed by ear bars, front teeth and nosebar in a stereotactic frame (David Kopf® Small animal stereotactic instrument, Model 900, Tujunga, USA). The skin overlying the periosteum of the skull was removed with sharp scissors. The periosteum was wiped away with gauze and the skull was covered with a squirt of hydrogen peroxide in order to minimize bleeding. A small surgical cautery was used whenever the bleeding was not stopped by the above means. A small area on the left temporal bone (approximately 3 mm x 3 mm area: 0.80 mm posterior...
and 1.5 mm lateral relative to bregma) was gradually thinned with a silicon carbide grinding stone (Dremel 84922 silicon carbide grinding stone 4.8 mm, Dremel, USA) attached to a drill (Dremel 300 Series variable speed rotary tool, Dremel, USA) until the meningeal arteries could be observed through the skull (Figure 6-3). A rubber polishing point (Dremel 462 Rubber Polishing Cone Point, Dremel, USA) and/or a scalpel blade was then used to smooth the skull surface in order to reduce reflection during the imaging process. The area was continuously rinsed with sterile saline to avoid overheating of the skull and to improve visualization of the field. Care was taken during the procedure to avoid application of too much pressure that may crack the skull. The eyes of the animal were covered with a transparent ophthalmic lubricant ointment to prevent damage during the surgical procedure.

6.2.4. Imaging and image analysis

A standard Olympus stereo microscope fitted with a 10x magnification objective was used to visualize the brain vessels through the skull with the help of a Polychrome V microscope illumination system (TILL Photonics LLC, Pleasanton, CA, USA) as shown in Figure 6-3. A clear gel was applied to the skull as an immersion medium into which the microscope objective was placed. The image was captured with a Neo scientific CMOS camera (Andor Technology Ltd, Belfast, UK) controlled by Metamorph® imaging software (Universal Imaging Corporation, West Chester, PA). The arteries were differentiated from the veins by the direction of their bifurcation (towards the midline for arteries) and by the constriction induced when the animals inhaled a 100% oxygen for about 60 s. Images with 500 x 500 pixel frame were captured during the experiments and stored for offline analysis. The images were analysed using ImageJ software (U.S. National Institutes of Health, Bethesda, MA, USA). The image stacks were initially averaged with the Grouped ZProjector plug-in and the diameters of the arteries were obtained by selecting a line in the ‘analyse’ menu that perpendicularly crossed through the vessel from edge to edge, followed by selection of the ‘measure’ menu in the software. Data were then copied to a spreadsheet for further statistical analyses.
Figure 6-3: Cranial window preparation and imaging.
After the skin was removed from the skull, the cranial bone was gradually thinned using a silicon carbide grinding stone attached to a driller and the area was continuously flushed with saline to prevent heating of the skull (left panel). The arteries were visualized with a stereo microscope fitted with a 10x magnification objective lens (right panel). The image was captured with a Neo scientific CMOS camera (Andor Technology Ltd, Belfast, UK) controlled by Metamorph® imaging software (Universal Imaging Corporation, West Chester, PA).

6.2.5. Drug administration and haemodynamic monitoring

The animal was allowed to stabilize for at least 30 min post-surgery before drugs were infused via the femoral vein or carotid artery using a syringe infusion pump (Harvard Apparatus Inc., Holliston, MA). The blood pressure and heart rate of the animal were continuously monitored during the experiment using a pressure transducer (Transpact, Abbott Critical Care Systems, Sligo, IRE) connected to femoral artery catheter and the data were recorded using
PowerLab data acquisition system and Chart software (AD Instruments, Pty. Ltd, Bella Vista, NSW, Australia). A stock concentration of each drug was made in normal saline and was administered by continuous infusion via the femoral vein or carotid artery. The doses were progressively increased by changing the infusion rate after the blood pressure response had plateaued following each previous dose rate. The rates of infusion used in the protocols were initially determined by running pilot experiments wherein the drugs were administered by step-by-step increment of the infusion rate until the blood pressure had been changed by 15-20 mmHg. Drug administration was stopped when the mean arterial pressure was increased to a maximum of 150-160 mmHg or decreased to a minimum of 50 mmHg. Sufficient time was allowed for the effects of every drug to wear off before another drug was tested. At the end of the experiments, the rats were euthanized by an overdose of intravenous injection of pentobarbitone (>100 mg/kg).

6.2.6. Experimental protocols

Rats were randomly assigned into groups of 5-7 for administration of phenylephrine (0.3-10 µg/kg/min), prenalterol (0.03-10 µg/kg/min), formoterol (0.03-3 µg/kg/min), vasopressin (0.1-10 ng/kg/min) or saline (0.003-1 ml/kg/min) via the femoral vein or carotid artery. The animals received the drugs in a randomized order allowing sufficient time between the drugs for parameters to return to baseline. For femoral vein administration, the drugs were infused at the same rate (0.003-1 ml/kg/min) with each rate lasting for about 2 min before increasing to the next higher rate. However, the dose rate varied for individual drugs. Only two doses of each drug were used for intra-carotid administration. A separate group of animals received infusion of the drugs at the same dose rates as intra-carotid infusion via the femoral vein to compare the time-course of effects between peripheral (through femoral vein) and local (through carotid artery) administration. The blood pressure and heart rate were continuously monitored via a cannula inserted in the femoral artery. Simultaneously, the images of the cerebral arteries corresponding to the maximum effect of a specific doses of each drug were obtained through the
cranial window before the infusion was started, before each increment of the rate and before the infusion was stopped at the end of the experiment.

6.2.7. Statistical analysis

All data were expressed as mean ± SEM and the analysis was performed using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). Values before drug infusion were taken as baseline and the percentage changes of the mean arterial blood pressure (MAP) and the cerebral artery diameter were calculated for each animal. An unpaired t test was used to compare the intravenous and intra-arterial effects of drugs between animals as required. \( P<0.05 \) was considered significant in all cases.
6.3. Results

6.3.1. Effects of intravenous vasoactive agents on blood pressure and cerebral arterial diameter

The effects on blood pressure and cerebral artery diameter of phenylephrine (a selective α₁-adrenoceptor agonist drug), vasopressin (a nonspecific V₁ and V₂ receptor agonist peptide), prenalterol (a selective β₁-adrenoceptor agonist drug) and formoterol (a selective β₂-adrenoceptor agonist drug) were investigated in anaesthetized rats. The baseline mean arterial blood pressure and heart rate diameters of cerebral arteries of the rats are shown in Table 6-1.

An equivalent rate of infusion of normal saline (0.003-1 ml/min; in half-log unit increments) given through the femoral vein did not change the mean arterial pressure and heart rate of the rats (Figure 6-5A). Phenylephrine (0.3-10 µg/kg/min) induced a dose-dependent increase in mean arterial blood pressure. The mean arterial blood pressure was increased by 81±7% after intravenous infusion of phenylephrine 10 µg/kg/min, but there was no change in heart rate (Figure 6-6A). Similarly, intravenous administration of vasopressin (0.1-10 ng/kg/min) increased the rat arterial blood pressure as shown in Figure 6-7A. Vasopressin 10 ng/kg/min increased the mean arterial pressure by 88±6% before the infusion was stopped, but the heart rate was not significantly changed. Infusion of prenalterol (0.03-0.3 µg/kg/min) increased the mean arterial pressure by 21±5%. The heart rate was significantly increased by 29±6% after infusion of prenalterol 10µg/kg/min (Figure 6-8A). The blood pressure was decreased by about 24±9% after intravenous administration of formoterol. Formoterol 0.3 µg/kg/min increased the heart rate by 10±0.6% (Figure 6-9A).

The diameter of small distal branches of the left middle cerebral artery were monitored through the cranial window as the drugs were infused. Images were taken before the drug infusion was increased to the next higher rate or dose, or before stopping the last dose (Figure 6-4). The average baseline outer
diameter of the arteries ranged from 50-70 µm (Table 6-1). The diameter of rat cerebral arteries was unaffected by intravenous infusion of normal saline (up to a maximum rate of 1 ml/min; $P>0.05$, compared to baseline) (Figure 6-5B). Phenylephrine, vasopressin and prenalterol decreased cerebral artery diameter. The rat cerebral arteries were dilated by infusion of $\beta_2$-adrenoceptor agonist formoterol. Phenylephrine 10 µg/kg/min constricted the arteries by 22±3% of the baseline diameter (Figure 6-6B), while the maximum constriction induced by vasopressin 10 ng/min was 20±5% (Figure 6-7B). It appeared that the cerebral artery started to constrict before mean arterial pressure increased upon infusion of vasopressin 1 ng/kg/min - increases in blood pressure were observed only after the dose rate was increased to 3.2 ng/kg/min. Prenalterol 10 µg/kg/min decreased the cerebral artery diameter by 8±4% at the maximum infusion rate (Figure 6-8B). The arteries were dilated by 13±5% after infusion of formoterol (0.03-0.3 µg/kg/min) (Figure 6-9B).

Table 6-1: Baseline values of rat blood pressure, heart rate and cerebral artery diameter before intravenous administration of the drugs.

<table>
<thead>
<tr>
<th></th>
<th>S  (n=6)</th>
<th>PE (n=7)</th>
<th>PR (n=7)</th>
<th>FO (n=6)</th>
<th>AVP (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>98±6</td>
<td>97±3</td>
<td>77±2</td>
<td>87±3</td>
<td>76±4</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>328±19</td>
<td>343±18</td>
<td>337±5</td>
<td>323±11</td>
<td>350±18</td>
</tr>
<tr>
<td>D (µm)</td>
<td>50±4</td>
<td>70±15</td>
<td>67±14</td>
<td>63±13</td>
<td>68±14</td>
</tr>
</tbody>
</table>

S, saline; PE, phenylephrine; PR, prenalterol; FO, formoterol; AVP, vasopressin; MAP, mean arterial pressure; HR, heart rate; D, cerebral artery outer diameter; and n, number of rats in each group. Data are shown and mean±SEM.
Vasoactive agents \textit{in vivo}

Figure 6-4: Representative images of cerebral arteries illustrating the effects of vasoactive drug infusion

The images show the diameter of cerebral arteries before and after intravenous infusion of phenylephrine 10 $\mu$g/kg/min (top) and formoterol 0.3 $\mu$g/kg/min (bottom) in different rats.
Figure 6-5: Effects of saline on blood pressure, heart rate and cerebral artery outer diameter.

Effects of equivalent rate of intravenous saline administration on A) mean arterial pressure (MAP) and heart rate (HR); and B) cerebral artery outer diameter (D) in the rat. Normal saline was infused through the femoral vein in anaesthetised rats in increasing rate, each rate lasting for 2 min. Data are expressed as % change from the baseline MAP, HR or D. Vertical bars are ±SEM. n, number of animals in the group.
Figure 6-6: Effects of phenylephrine on blood pressure, heart rate and cerebral artery outer diameter.

Effects of intravenous phenylephrine administration on A) mean arterial pressure (MAP) and heart rate (HR); and B) cerebral artery outer diameter (D) in the rat. Phenylephrine was infused through the femoral vein in anaesthetised rats in increasing doses, each dose rate lasting for 2 min. Data are expressed as % change from the baseline MAP, HR or D. Vertical bars are ±SEM. n, number of animals in the group.
Figure 6-7: Effects of vasopressin on blood pressure, heart rate and cerebral artery outer diameter.

Effects of intravenous vasopressin administration on A) mean arterial pressure (MAP) and heart rate (HR); and B) cerebral artery outer diameter (D) in the rat. Vasopressin was infused through the femoral vein in anaesthetised rats in increasing dose, each dose rate lasting for 2 min. Data are expressed as % change from the baseline MAP, HR or D. Vertical bars are ±SEM. n, number of animals in the group.
Figure 6-8: Effects of prenalterol on blood pressure, heart rate and cerebral artery outer diameter.

Effects of intravenous prenalterol administration on A) mean arterial pressure (MAP) and heart rate (HR); and B) cerebral artery outer diameter (D) in the rat. Prenalterol was infused through the femoral vein in anaesthetised rats in increasing dose, each dose rate lasting for 2 min. Data are expressed as % change from the baseline MAP, HR or D. Vertical bars are ±SEM. n, number of animals in the group.
Figure 6-9: Effects of formoterol on blood pressure, heart rate and cerebral artery outer diameter.

Effects of intravenous formoterol administration on A) mean arterial pressure (MAP) and heart rate (HR); and cerebral artery outer diameter (D) in the rat. Formoterol was infused through the femoral vein in anaesthetised rats in increasing dose, each dose rate lasting for 2 min. Data are expressed as % change from the baseline MAP, HR and D. Vertical bars are ±SEM. n, number of animals in the group.
6.3.2. Intravenous versus intra-arterial effects of drug infusion on blood pressure and cerebral arterial diameter

In an attempt to ascertain the direct effects on the cerebral arteries, drugs were directly infused into the cerebral circulation through the carotid artery. Only two doses of each drug were given in order to minimize the redistribution of the drugs to the peripheral circulation and thereby change the blood pressure before affecting cerebral arteries. Separately, the drugs were infused at the same dose rate via the femoral vein to compare with the intra-carotid artery administration. The baseline mean arterial blood pressure and heart rate of each group before infusion of the drugs are shown in Table 6-2. The drugs were infused at the following dose rates, each lasting for 2 min, or until the maximum limit of blood pressure was reached: phenylephrine (3 and 10 µg/kg/min), vasopressin (3 and 10 ng/kg/min) and formoterol (0.3 and 1 µg/kg/min).

Intravenous and intra-arterial phenylephrine (3 µg/kg/min) increased the blood pressure by 32±10% and 14±4%, respectively, but no statistically significant difference was found between the two routes (P>0.05, unpaired t test). Similarly, the increase in mean arterial pressure caused by phenylephrine (10 µg/kg/min) administration was comparable by intravenous (81±14%) and intra-arterial (80±10%) administration (Figure 6-10A). The respective increase in blood pressure induced by vasopressin (3 ng/kg/min) was 12±4% and 17±1% when administered intravenously and intra-arterially. Similarly, at the 10 ng/kg/min dose rate, vasopressin caused an increase in mean arterial pressure of 69±12% by intravenous infusion and 84±9% after intra-arterial infusion (Figure 6-11A) - no significant difference was found between the two routes (P>0.05). Intravenous and intra-arterial infusion of formoterol (0.3 µg/kg/min) decreased the blood pressure by 1±1% and 7±3%, respectively. The blood pressure was decreased by similar extent when the dose of formoterol was increased to 1 µg/kg/min (intravenous, -37±7%; and intra-arterial, -43±13%) as shown in Figure
6-12A. Rat blood pressure was unaffected by the same rate of saline infusion - intravenous or intra-arterial.

Table 6-2: Baseline rat blood pressure and heart rate before intra-femoral vein or intra-carotid artery administration of the drugs

<table>
<thead>
<tr>
<th></th>
<th>S (n=5-7)</th>
<th>PE (n=5-7)</th>
<th>FO (n=5-7)</th>
<th>AVP (n=5-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPi.v.(mmHg)</td>
<td>85±2</td>
<td>92±7</td>
<td>98±6</td>
<td>86±2</td>
</tr>
<tr>
<td>MAPi.a.(mmHg)</td>
<td>74±6</td>
<td>83±5</td>
<td>89±7</td>
<td>77±3</td>
</tr>
<tr>
<td>HRi.v. (bpm)</td>
<td>348±22</td>
<td>346±12</td>
<td>322±37</td>
<td>335±19</td>
</tr>
<tr>
<td>HRi.a. (bpm)</td>
<td>337±6</td>
<td>352±23</td>
<td>334±15</td>
<td>354±17</td>
</tr>
</tbody>
</table>

S, saline; PE, phenylephrine; PR, prenalterol; FO, formoterol; AVP, vasopressin; MAP, mean arterial pressure; HR, heart rate; i.v., intravenous; i.a., intra-arterial; and n, number of rats in each group.

Both phenylephrine and vasopressin induced constriction of rat cerebral arteries when administered through the femoral vein or carotid artery. The changes in arterial diameter were similar for both intravenous and intra-arterial phenylephrine (3 µg/kg/min) infusion (-8±3% for i.v.). At the higher dose of phenylephrine (10 µg/kg/min), greater constriction of cerebral arteries was obtained upon direct infusion into the cerebral circulation via the carotid artery (-30±3%) than systemic administration through femoral vein (-16±2%, P<0.05, unpaired t test) (Figure 6-10B).

Vasopressin (3 ng/kg/min) caused comparable decreases in cerebral arterial diameter by intravenous (-3±2%) and intra-arterial routes of infusion (-13±5%, P>0.05, unpaired t test). Likewise, vasopressin (10 ng/kg/min) induced a similar degree of constriction by intravenous (-22±3%) and intra-arterial administration (-36±6%) (Figure 6-11B).

Infusion of formoterol (0.3 µg/kg/min) increased the cerebral arterial diameter by 8±9% and 6±4% when given by intravenous and intra-arterial routes,
respectively. A further dilatation was induced by increasing the dose of formoterol to 1 µg/kg/min, either intravenous (37±7%) or intra-arterial (41±3%) (Figure 6-12B), but there was no statistically significant difference between the two routes. No observable change in rat cerebral artery diameter was induced by infusion of saline at the same volume rate as other drugs (P>0.05, compared to baseline).
Figure 6-10: Intravenous versus intra-arterial effects of phenylephrine on blood pressure and cerebral artery outer diameter.

Comparison of the effects of intravenous (via femoral vein) and intra-arterial (via carotid artery) phenylephrine administration on A) mean arterial pressure (MAP); and B) cerebral artery outer diameter (D) in the rat. Phenylephrine was infused at two dose rates (3 and 10 µg/Kg/min), each dose lasting for 2 min. Data are expressed as % change from the baseline MAP or D values. Vertical bars are ±SEM. *P<0.05, unpaired t test. n, number of animals in the group.
Figure 6-11: Intravenous versus intra-arterial effects of vasopressin on blood pressure and cerebral artery outer diameter.

Comparison of the effects of intravenous (via femoral vein) and intra-arterial (via carotid artery) vasopressin administration on A) mean arterial pressure (MAP); and B) cerebral artery outer diameter (D) in the rat. Vasopressin was infused at two dose rates (3 and 10 ng/Kg/min), each dose lasting for 2 min. Data are expressed as % change from the baseline MAP or D values. Vertical bars are ±SEM. n, number of animals in the group.
Figure 6-12: Intravenous versus intra-arterial effects of formoterol on blood pressure and cerebral artery outer diameter.

Comparison of the effects of intravenous (via femoral vein) and intra-arterial (via internal carotid artery) formoterol administration on A) mean arterial pressure (MAP); and B) cerebral artery outer diameter (D) in the rat. Formoterol was infused at two dose rates (0.3 and 1 µg/Kg/min), each dose lasting for 2 min. Data are expressed as % change from the baseline MAP or D values. Vertical bars are ±SEM. n, number of animals in the group.
6.3.3. Time-course of effects of vasopressor agents on blood pressure and cerebral artery diameter

The mean arterial pressure and cerebral artery diameter were measured every 30 seconds to analyse the time-course effect of the drugs at two rates (low- and high-rate) of infusion each lasting for 2 min. The blood pressure and arterial diameter were only slightly affected by the first phase of phenylephrine (3 µg/min) (Figure 6-13A), vasopressin (3 ng/min) (Figure 6-14A) and formoterol (0.3 µg/min) (Figure 6-15A). Increasing the infusion rate by half an order of magnitude (half-log\(_{10}\) unit increments) markedly affected both the blood pressure and vessel diameter. The onset of action was similar for both intravenous and intra-arterial administration of the drugs. Each drug appeared to cause a similar pattern of change (slope of the curves) in mean arterial pressure and cerebral artery diameter after intravenous or intra-arterial administration. Phenylephrine caused a significantly greater maximum constriction by intra-carotid than by intravenous administration (Figure 6-13B). However, the maximum effects on cerebral artery diameter were similar by either intravenous or intra-arterial administration for both vasopressin (Figure 6-14B) and formoterol (Figure 6-15B)
Figure 6-13: The time-course of effects of intravenous and intra-arterial phenylephrine on blood pressure and cerebral artery outer diameter.

The time-course effect of phenylephrine infusion via femoral vein or carotid artery on A) mean arterial pressure (MAP); and B) cerebral artery outer diameter (D) in the rat. Phenylephrine was infused at 3 and 10 µg/kg/min rates and the measurements taken every 30 s. Data are expressed as % change from the baseline MAP or D. Vertical bars are ±SEM. *P<0.05, unpaired t test between intravenous and intra-arterial routes for each time point. n, number of animals in each group.
Figure 6-14: The time-course of effects of intravenous and intra-arterial vasopressin on blood pressure and cerebral artery outer diameter.

The time-course effect of vasopressin infusion via femoral vein or carotid artery on the rat mean arterial pressure (MAP) and cerebral artery outer diameter (D) in the rat. Vasopressin was infused at 3 and 10 µg/kg/min rates and the measurements taken every 30 s. Data are expressed as % change from the baseline MAP or D. Vertical bars are ±SEM. n, number of animals in each group.
Figure 6-15: The time-course of effects of intravenous and intra-arterial formoterol on blood pressure and cerebral artery outer diameter.

The time-course effect of formoterol infusion via femoral vein or carotid artery on A) mean arterial pressure; and B) cerebral artery outer diameter (D) in the rat. Formoterol was infused at 0.3 and 1 µg/kg/min rates and the measurements taken every 30 s. Data are expressed as % change from the baseline MAP or D. Vertical bars are ±SEM. n, number of animals in each group.
6.4. Discussion

In this study, the blood pressure and cerebrovascular responses to systemically administered vasoactive agents in anaesthetised rats were investigated. The cranial window was made to visualize the vessels through a ‘thinned’ but intact skull without releasing the intracranial pressure. The current results demonstrate that administration of vasoactive drugs in vivo affects both the peripheral blood pressure and diameter of cerebral arteries. The changes in calibre of cerebral arteries appear to be indirect autoregulatory responses to changes in peripheral blood pressure induced by vasoactive agents.

Phenylephrine and vasopressin increase arterial blood pressure by inducing vasoconstriction of peripheral arteries through activation of $\alpha_1$-adrenoceptors and $V_1$ receptors, respectively. Formoterol decreases the blood pressure by activating $B_2$-adrenoceptors that mediate dilatation of peripheral arteries. Prenalterol (a $B_1$-adrenoceptor agonist) does not cause significant effects in peripheral arteries, but moderately increases blood pressure due to its positive chronotropic and inotropic effects (Hedberg et al., 1982). In this study, intravenous administration of phenylephrine and vasopressin induced comparable increases in arterial blood pressure with simultaneous constriction of the cerebral arteries, while formoterol induced hypotension along with cerebral vasodilatation. Prenalterol had weak effects on blood pressure as well as diameter of the rat cerebral arteries. Cerebral arteries can be constricted or dilated either by direct actions of vasoactive agents or in response to changes in peripheral blood pressure, or both. In vitro studies have shown the presence in rat cerebral arteries of $\alpha_1$-adrenoceptors mediating constriction and $B_1$ and $B_2$-adrenoceptors mediating dilatation as discussed in Chapter 4. Vasopressin also induces a potent constriction of cerebral arteries in vitro (see Chapter 3).

There were simultaneous and parallel changes in blood pressure and cerebral arterial diameter induced by intravenous administration of the agents. As a result, it is not possible to distinguish whether the cerebrovascular effects are caused by direct actions of the vasoactive drugs on cerebral arteries or are
autoregulatory responses induced by blood pressure changes. Further, the
direct vascular effects of the drugs may be concealed due to the autoregulatory
responses since the agents were administered over a wide range of doses for
long period of time. Thus, higher doses of the vasoactive drugs were directly
infused through the carotid artery so that any direct effects of the drugs on
cerebrovascular smooth muscle would be observed before affecting the blood
pressure due to redistribution to the peripheral circulation. Again, there were
no differences in the onset time of the effects of the drugs on blood pressure
and diameter changes between intravenous and intra-carotid administration.
There were no significant differences in the magnitude of responses between
the two routes except that intra-arterial phenylephrine (10 µg/kg/min) caused
a significantly greater constriction of cerebral arteries than intravenous
administration.

These findings show that the cerebrovascular reactions to systemic vasoactive
drug administration are predominantly indirect autoregulatory responses to the
changes in peripheral blood pressure or perfusion pressure rather than direct
effects on the arterial smooth muscle cells. The presence of a selective blood-
brain barrier in cerebral arteries prevents most substances circulating in the
blood from accessing the vascular smooth muscle cells (discussed in section
1.3). While phenylephrine, formoterol or prenalterol can exert their effect on
cerebral vasculature in vitro, their direct in vivo effect in cerebral arteries may
be prevented by the blood-brain barrier. Phenylephrine does not cross the
blood-brain barrier under normal conditions, but higher blood pressure may
disrupt the integrity of the blood-brain barrier such that circulating drugs can
access the cerebrovascular smooth muscle and induce vasoconstriction as
discussed below. Similarly, vasopressin causes a potent constriction of isolated
cerebral arteries but its direct effect on cerebral vasculature in vivo may
limited by the blood-brain barrier (Zaidi and Heller, 1974). Infusion of
vasopressin has been reported to cause endothelium-dependent dilatation of
large cerebral arteries but increase the resistance of small arteries due to
activation of cerebral autoregulation in response to increased arterial pressure
(Faraci and Heistad, 1990). However, the results from this study do not reflect
direct effects of vasopressin on cerebral arteries since no difference was found between intravenous and intra-carotid administration of vasopressin. Nevertheless, the possibility of overlap between the autoregulatory response and direct constriction induced by the drugs cannot be excluded. Differences in the degree of vasoconstriction induced by phenylephrine and vasopressin in spite of similar changes in arterial blood pressure may indicate additional effects related to direct actions of the agents on the cerebral vasculature.

Elevated arterial blood pressure may disrupt the function of the blood-brain barrier so that vasoactive agents may directly affect the tone of cerebral arteries (Ito et al., 1980; Tsai et al., 1989; Cole et al., 1991). Previous studies have shown that acute vasopressor-induced elevation of blood pressure or chronic hypertension increased the permeability of the blood-brain barrier (Domer et al., 1980; Johansson, 1980). For example, infusion of phenylephrine (30 µg/kg/min) for 5 min induced a severe increase in rat blood pressure (from 122 to 187 mmHg) that was associated with disruption of the blood-brain barrier that led to the leakage of fluorescent albumin (Mayhan, 1991). Similarly, vasopressin and adrenaline caused disruption of the blood-brain barrier after haemorrhagic circulatory arrest in pigs (Semenas et al., 2014). In this study, rat blood pressure was increased to about 150-160 mmHg by phenylephrine and vasopressin. This may be an additional reason why intra-carotid administration of vasopressin tended to cause a slightly greater constriction of cerebral artery than intravenous administration in spite of similar effects on blood pressure induced by the two drugs. However, it has been shown that there were more disruptions of the blood-brain barrier after a rapid increase than after stepwise increase in blood pressure (Baumbach and Heistad, 1985). Thus, the contribution of the direct effects of vasoactive drugs to overall cerebrovascular responses as a consequence of blood-brain barrier disruption was minimized in this study because the drugs were infused gradually in increasing doses. The extent of leakage through a pressure-disrupted blood-brain barrier may be variable for different vasoactive agents and requires further investigation.
Additionally, this study emphasizes the greater role of small calibre cerebral arteries rather than large cerebral arteries in the regulation of cerebral blood flow in face of changing systemic blood pressure as previously indicated (Olesen, 1972; Auer et al., 1987). Small branches of the rat middle cerebral arteries responded to changes in arterial pressure by constriction or dilatation, which are critical elements of cerebral autoregulation. The size dependence of responses in cat pial arteries was previously reported by Kontos et al. (1978), but only vessels larger than 200 µm in diameter responded to changes in arterial blood pressure. Small arteries of less than 100 µm dilated when the pressure was less than 90 or greater than 170 mmHg. The difference between the previous study and our results may be related to species and/or methodological differences. Kontos et al. used a cranial window in the cat where the intracranial pressure was artificially controlled through catheter inserted through the skull. However, the cranial window in this study was prepared in rat without breaking the cranial bone, thus maintained a natural intracranial pressure. This study also allowed the normal variation of intracranial pressure due to changes in arterial blood pressure.

The current study has further implications to the clinical practice wherein vasoactive drugs are sometimes administered through the carotid artery in order to obtain localized effects in the cerebral circulation, or to minimize their systemic adverse effects. For example, intra-arterial nimodipine or nicardipine has been used to prevent or reverse the cerebral vasospasm following subarachnoid haemorrhage (Biondi et al., 2004). It has been shown that nicardipine induces angiographic cerebral vasodilatation as well as hypotension which indicates that a decrease in arterial pressure may have induced the cerebrovascular dilatation by activating autoregulation (Linfante et al., 2008). Thus, intra-carotid administration of vasoactive drugs may not cause local effects in the cerebral circulation unless the agents are highly lipophilic and cross the blood-brain barrier (Pluta et al., 1997).

In summary, systemic administration of vasoactive drugs primarily affects the systemic arterial pressure which then induces the autoregulatory response in
cerebral arteries. Small calibre cerebral arteries may play a significant role in the regulation of cerebral blood flow.
Chapter 7

Effects of vasopressor agents on human cerebral oxygen saturation during anaesthesia
7.1. Introduction

7.1.1. Background to clinical problem

In healthy subjects, a stable cerebral blood flow of approximately 50 ml/100 g/min is maintained in the face of considerable fluctuations in peripheral arterial blood pressure (Lassen, 1959). This autoregulation occurs within the mean arterial blood pressure range of 60-150 mmHg, and adjusts perfusion for normal physiological changes in brain function including intellectual work, sensory stimulation, muscle movement and sleep (Lassen, 1974). However, when mean arterial pressure is outside the autoregulatory range, changes in cerebrovascular resistance are unable to compensate for extreme mean arterial pressure fluctuations, and perfusion becomes pressure-passive. The lower limit of the autoregulatory blood pressure threshold varies significantly in individuals (Drummond, 1997), and is shifted to the right (higher blood pressure) in disease states such as hypertension and diabetes (Bentsen et al., 1975). In episodes of acute hypotension due to haemorrhage or overuse of vasoactive drugs, cerebral hypoperfusion may occur, potentially leading to cerebral ischaemia and infarction (Agnoli et al., 1968; MacKenzie et al., 1979). Further, the autoregulation can be disrupted by certain disease conditions such as head injury, acute ischaemic stroke, brain tumour or haematoma (Paulson et al., 1989; Paulson et al., 1990; Carnevale and Lembo, 2010).

The concept of cerebral perfusion pressure is central to cerebral protection, and states that cerebral blood flow is determined primarily by the pressure gradient across the cerebral vascular bed (Moraine et al., 2000). The driving force for flow equates to the difference between mean arterial pressure and intra-cranial pressure or central venous pressure. The normal value of intracranial pressure in adults who are in the supine position ranges from 7-10 mmHg, but can be elevated in pathological conditions such as traumatic brain injury. Maintaining cerebral blood flow through prevention of hypotension and by lowering elevated intracranial pressure is a priority in the management of acute brain injury, particularly in traumatic brain injury and subarachnoid
haemorrhage. Current guidelines recommend mean arterial pressure to be maintained above 90 mmHg (Bratton et al., 2007), but ensuring the intracranial pressure should be less than 20 mmHg (Kirkman and Smith, 2014).

General anaesthesia may alter cerebral autoregulation with effects on cerebral vascular reactivity and cerebral perfusion pressure. The lower limit of autoregulation may be increased by both volatile anaesthetic agents (Dagal and Lam, 2009) and by hypocapnia (Meng and Gelb, 2015). In some patients excessive hypotension may occur during surgery placing them at risk of cerebral ischaemia. This is of particular concern when anaesthetized patients are placed into an upright “beach chair” or “barber’s chair” position, as commonly employed in arthroscopic shoulder procedures. The “beach chair” position is associated with significant hypotension, due to gravitational pooling of blood in the lower parts of the body (Buhre et al., 2000). The risk of cerebral hypoperfusion and ischaemic injury with the beach chair position was first reported in a case series by Pohl and Cullen (2005). There has since been significant discussion relating to the safe management of patients in the beach chair position and importantly how to monitor for cerebral ischaemia (Cullen and Kirby, 2007). The seriousness of this problem was illustrated by a recent death of a 50 year old man after undergoing surgery in the beach chair position (The Australian, 2013).

In recent years the monitoring of cerebral oxygen saturation using near-infrared spectroscopy (NIRS) has emerged as a potential monitor of intraoperative cerebral blood flow during anaesthesia in the chair position (the principle of NIRS oximetry is discussed in the following section). It has been shown that patients undergoing shoulder surgery in the beach chair position had lower cerebral oxygen saturation compared to the lateral decubitus position (Laflam et al., 2015). Unlike transcranial Doppler measurements that are based on determination of the red blood cell velocity, cerebral oximetry measures haemoglobin oxygen saturation within the frontal lobe microcirculation. Though NIRS provides an indirect measurement of tissue perfusion, continuous
monitoring of regional cerebral oxygen saturation however has the potential to detect episodes of silent ischaemia during surgery.

A current approach to protect patients during beach chair surgery is to increase cerebral perfusion pressure with vasopressor drug administration (Murphy and Szokol, 2011). Clinically used drugs include the sympathomimetics noradrenaline, phenylephrine and ephedrine, and the vasoactive peptide vasopressin. Whilst noradrenaline and phenylephrine are effective in increasing arterial blood pressure, recent studies indicate these drugs may also induce cerebral oxygen desaturation (Brassard et al., 2009; Meng et al., 2011; Soeding et al., 2013). Vasopressor agents have the potential to constrict both systemic and cerebral arteries. In normal patients, however, the unique structural blood-brain barrier prevents most blood-borne substances, including catecholamines, from accessing the cerebral vascular smooth muscle and brain parenchymal tissue. In pathological conditions such as traumatic brain injury or subarachnoid haemorrhage the permeability of the blood-brain barrier to circulating drugs may be altered (Hardebo and Owman, 1980; Myburgh, 2005). The question arises whether all vasopressor drugs adversely alter regional cerebral perfusion, either via direct constriction of cerebral arteries, or indirectly by changing arterial blood pressure, and importantly in relation to differences in the doses of each agent used. This study employed a NIRS oximetry technique to analyse the effect of vasopressor agents on cerebral oxygen saturation.

7.1.2. Principle of cerebral oximetry

In cardiac surgery, NIRS monitoring is becoming routine management (Douds et al., 2014) and it is increasingly being used during shoulder surgery. It is a noninvasive technique based on the use of near infrared light (700-1000 nm) reflectance spectroscopy. The NIRS optode, consisting of a light emitter and detector(s) separated by 3-4 centimetres, is placed on the forehead overlying the prefrontal cortex. Near-infrared light is transmitted through the scalp and is finally reflected back to the detectors which measure the intensity (Figure 7-1). The reflected light is scattered and its intensity is attenuated as it passes
through the scalp due to oxygen-dependent absorption by certain chromophores, such as haemoglobin, myoglobin and cytochrome oxidase (Delpy and Cope, 1997). Oxygenated and deoxygenated haemoglobin have different absorption spectra, thus their relative concentration can be determined based on the degree of differential near-infrared light absorption. This can be used as a marker to monitor changes in cerebral blood flow (Ferrari et al., 2004; Highton et al., 2010).

The NIRS equipment available to date varies in the analysis of the reflected NIR light. Currently, there are four methods used in NIRS oximetry - continuous wave, frequency domain, time resolution and spatially resolved spectroscopy. In the continuous wave technique, the NIR light is emitted at a constant frequency and amplitude and the changes in light intensity are related to changes in haemoglobin concentration (Scholkmann et al., 2014). The frequency domain technique employs an amplitude modulated light (50 to 500 MHz) and relates the changes in amplitude and phase of the back-scattered signal to changes in haemoglobin concentration (Delpy and Cope, 1997). Time resolution spectroscopy involves emission of a short pulse NIR (<100 picoseconds) and calculates the haemoglobin concentration based on temporal delay of the light propagated through the tissue (Wabnitz et al., 2010). The spatially-resolved spectroscopy (SRS) technique uses a complex algorithm that determines the absolute concentration of haemoglobin and oxyhaemoglobin based on localized attenuation of the NIR light. The light attenuation is measured as a function of distance using multiple detectors; i.e., the slope of NIR light attenuation vs distance from the light source is used to determine the absolute ratio of oxyhaemoglobin to deoxyhaemoglobin concentration (Highton et al., 2010). Thus, regional cerebral oxygen saturation (rScO₂) can be calculated using the following relationship:
Vasopressor agents and cerebral oxygenation

\[ rScO_2 = \frac{[O_2Hb]}{[O_2Hb+HHb]} \times 100\% , \]

where, \( O_2Hb \) is oxyhaemoglobin and \( HHb \) is deoxyhaemoglobin.

Using NIRS for monitoring cerebral oxygen has several limitations. The most common drawback of this technology is the possibility of interference from extracranial tissues such as the scalp. To overcome this problem, multiple light emitting and detector systems are used to identify extracranial reflectance. A special algorithm that subtracts the light absorbed by the scalp from the entire absorption is used to determine the oxygen-dependent absorption of NIR light by the brain tissues (Figure 7-1) (Zheng et al., 2013). Nevertheless, NIRS oximetry a non-invasive method that offers a direct and monitoring of cerebral oxygen saturation. It can be used to continuously monitor the dynamic changes in cerebral oxygen saturation induced by postural changes or vasoactive drug administration in neurocritical care.

The aim of this study was to compare the effects of clinically-used vasopressor drugs on cerebral oxygen saturation in anaesthetised subjects during both supine and beach chair positioning. A primary question was to identify whether vasopressor drug infusion could prevent postural hypotension associated with head-up positioning, and secondly identify the effect of each drug on the incidence of cerebral oxygen desaturation in the supine and beach chair positions.
Figure 7-1: Application of near infrared spectroscopy (NIRS) for monitoring regional cerebral oxygen saturation.
The setup consists of a 2-channel Equinox (Model 7600) oximetry device set (top left) with the optode placed on each side of the forehead in a “beach chair”-seated subject (top right). The components of the optode - a NIR light source and dual detectors - are shown (lower left). The first detector located closer to the source detects light reflected from the superficial tissue (skin/skull/dura), whilst the second sensor located 3-4 cm further from the emitter detects light reflected from deep brain tissues (lower right).
7.2. Materials and methods

7.2.1. Patient recruitment

After institutional approval was obtained from the Melbourne Health and Clinical Trial Registration Ethics Committee (ANZCTR No 12610001075077), 24 patients undergoing elective shoulder arthroscopy in the upright position were recruited for this study. A written informed consent was obtained from each patient. Patients were excluded if there was a history of cerebrovascular event, significant cardiac disease (New York Heart Association symptoms class 3, or pacemaker), carotid endarterectomy, uncontrolled hypertension, uncontrolled diabetes, contraindications to interscalene block or a body mass index (BMI) ≥35.

7.2.2. Anaesthesia and patient preparation for experiments

Patients were randomized to receive an infusion of either saline, noradrenaline (Levophed®, Hospira Ptd Ltd, VIC, Australia) or vasopressin (Pitressin®, Link Medical Products Pty. Ltd, NSW, Australia) following induction of general anaesthesia. A standardized sevoflurane general anaesthetic technique, combined with interscalene brachial plexus anaesthesia, was used in all patients (Figure 7-2). On arrival to the operating theatre, patients received warmed Hartmann’s solution 15 ml/kg intravenously (i.v.) and were sedated with midazolam 0.05 mg/kg. Interscalene brachial plexus anaesthesia was performed under ultrasound guidance, using 0.75% ropivacaine (30-35 ml). The radial artery was directly cannulated for continuous arterial pressure monitoring. The pressure transducer was positioned at the level of the tragus throughout surgery to reflect cerebral perfusion pressure. Near-infrared spectroscopy (NIRS) optode sensors (EQUINOX 7600, Nonin Medical Inc, Plymouth, MA USA) were placed bilaterally on each forehead 1-2 cm above the brow, and sensor edges taped to prevent interference from ambient light. Cerebral oxygen saturation (ScO₂) was measured from both left and right sensors. Systemic pulse oximetry, ECG, and bispectral index monitoring for
Vasopressor agents and cerebral oxygenation

depth of anaesthesia (BIS, Aspect Medical Systems, Newton, MA, USA) were applied.

The ScO₂ and haemodynamic variables were measured with patients inspiring room air, and after pre-oxygenation as shown in Figure 7-3. General anaesthesia was induced intravenous administration of fentanyl (1 mg/kg), propofol (1.5-2.0 mg/kg) and atracurium (0.5 mg/kg), and the trachea intubated. Patients were ventilated with oxygen and the end-tidal carbon dioxide maintained at 4.7-5.3 kPa. Anaesthesia was maintained with sevoflurane by targeting end tidal concentration (ET$_{SEVO}$) at 1.5 % and BIS value at 40-60%. ScO₂, and haemodynamic parameters were continuously monitored. Temperature was monitored via a nasopharyngeal probe.

7.2.3. Experimental protocols

After induction of anaesthesia, the patient was observed for a period of 5-10 min during which time mean arterial pressure, heart rate and anaesthetic depth stabilised, before the study drug was infused (Figure 7-2). The drugs were diluted with sterile saline in a 60 ml syringe to make the following concentrations: noradrenaline 20 µg/ml or vasopressin 0.3 units/ml (1 unit vasopressin corresponds to ~17 µg). The vasopressor drug, or control saline was infused by increasing the rates in 5 steps to a maximum dose over 15 min. At maximum infusion rate, noradrenaline infusion was 0.3 µg/kg/min and vasopressin 4 milliunits/kg/min. Measurements were taken before the infusion was started and just before the infusion rate was increased to the next higher level. The patient was then quickly placed in the beach chair position while the last infusion was continued (Figure 7-2).
Figure 7-2: Study protocol for patients randomized to receive either saline, noradrenaline or vasopressin infusion.

Interscalene brachial plexus anaesthesia was initially performed under ultrasound guidance while the patient was spontaneously breathing. General anaesthesia was induced after the patient was pre-oxygenated. About 5-10 min was allowed for haemodynamic stabilization before the infusion was started. A vasopressor drug or saline was continuously administered with gradual increment in rate of infusion, each rate lasting for 2.5 min. The patient was then placed into the beach chair position (BCP) while the drug or saline was being infused at the maximum rate. The MAP, HR, ScO2, BIS, ETCO2, ETSevo, Paw and temperature were continuously monitored while the patient was lying supine as well as in the beach chair position. The diagram is only informative about the sequence of activities and not drawn to scale. MAP, mean arterial pressure; HR, heart rate; ETCO2, end tidal carbon dioxide concentration; ETSevo, end tidal sevoflurane concentration; BIS, bispectral index value; and Paw, airway pressure.
7.2.4. **Intervention**

A prolonged decrease in mean arterial pressure below 60 mmHg was treated with metaraminol (sympathomimetic amine) 500 µg intravenous bolus, and/or atropine (muscarinic antagonist) 300 µg intravenous bolus injection if bradycardia (heart rate below 45 beats/min) was present. A sustained mean blood pressure greater than 110 mmHg was treated by initially decreasing the infusion and if necessary, treating with an intravenous glyceryl trinitrate (25 µg bolus). A further criterion for intervention was a significant decrease in ScO₂ (more than 25% decrease below the baseline, or absolute value below 50%).

7.2.5. **Statistical analysis**

Data are presented as numbers or mean±SEM. Mean arterial blood pressure and heart rate measurements were taken after the induction of anaesthesia were used as baseline recordings. The effects of treatments on mean arterial pressure and heart rate were expressed as the difference between measured values and the post-induction baseline (Δ baseline). For cerebral oxygen saturation (ScO₂) data are expressed as percentage change (ΔScO₂%) relative to the values before induction of anaesthesia (basal) in order to demonstrate the effects of anaesthesia on cerebral oxygen saturation compared to spontaneous breathing before anaesthesia. Data analysis was performed using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). Statistical comparison between groups was performed using unpaired t test or one-way ANOVA followed by Tukey’s post-test for intergroup comparison. Dunnett’s post-test was used whenever treatment groups were compared to the saline group. P<0.05 was considered significant in all cases.
7.3. Results

7.3.1. Patient characteristics and intra-operative parameters

The patients involved in this study were admitted for a right or left shoulder arthroscopic repair surgery. The patient characteristics are shown in Table 7-1. There was no significant difference in the age, body weight, body mass index (BMI) between the groups taking saline, noradrenaline or vasopressin \((P>0.05)\). Similar doses of propofol were used to induce general anaesthesia in all groups. The end tidal carbon dioxide concentration \((E_{TCO2})\), end tidal sevoflurane concentration \((E_{TEVO})\), bispectral index value (BIS), airway pressure (Paw) and body temperature were maintained constant throughout the experiment and no significant difference was found between the control (saline) and treatment groups for each of these variables \((P>0.05)\) (Table 7-2).
Figure 7-3: A representative measurement of haemodynamic and anaesthesia parameters recorded in a study patient.

A representative monitor showing haemodynamic and anaesthesia parameters including ECG trace, heart rate, blood pressure, % oxygen administered and end tidal sevoflurane and carbon dioxide concentrations.

Table 7-1: Characteristics of the patients randomly assigned to saline, noradrenaline or vasopressin infusion groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>Noradrenaline</th>
<th>Vasopressin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female (n)</td>
<td>6/3</td>
<td>5/2</td>
<td>6/2</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45 (19-69)</td>
<td>39 (22-62)</td>
<td>34 (20-47)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.0±3.7</td>
<td>80.1±4.6</td>
<td>77.1±2.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.0±0.8</td>
<td>25.5±0.9</td>
<td>24.6±0.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Age mean (range). BMI, body mass index (body weight/height²; n, number of patients in each group.
Table 7-2: Intraoperative details of the patients assigned to saline, noradrenaline and vasopressin groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline (n=9)</th>
<th>Noradrenaline (n=7)</th>
<th>Vasopressin (n=8)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propofol (mg)</td>
<td>173±12</td>
<td>171±16</td>
<td>181±7.1</td>
<td>0.82</td>
</tr>
<tr>
<td>ETSEVO (%)</td>
<td>1.6±0.03</td>
<td>1.6±0.04</td>
<td>1.6±0.04</td>
<td>0.51</td>
</tr>
<tr>
<td>ETCO2 (mmHg)</td>
<td>35.8±0.2</td>
<td>36.0±0.4</td>
<td>36.0±0.3</td>
<td>0.38</td>
</tr>
<tr>
<td>BIS</td>
<td>36.7±1.5</td>
<td>39.9±2.9</td>
<td>37.2±1.8</td>
<td>0.37</td>
</tr>
<tr>
<td>T (°C)</td>
<td>35.6±0.1</td>
<td>36.0±0.1</td>
<td>35.8±0.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Paw (cmH₂O)</td>
<td>16.8±0.8</td>
<td>16.5±0.6</td>
<td>15.0±0.4</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ETCO₂, end tidal carbon dioxide concentration; ETSEVO, end tidal sevoflurane concentration; BIS, bispectral index value; T, body temperature; and Paw, airway pressure. P, comparison between all groups, one-way ANOVA.

7.3.2. Effects of vasopressor agents on mean arterial blood pressure and heart rate

There was no significant difference in the mean arterial pressure before induction of general anaesthesia between the groups receiving saline (104±9), noradrenaline (99±5) or vasopressin infusion (96±4 mmHg) (P>0.05). Induction of general anaesthesia considerably decreased the blood pressure (by 40 mmHg in saline group) without significant effect on heart rate. Following induction of anaesthesia, mean arterial pressure and heart rate stabilised with similar values in each group (Table 7-3). After stabilization, infusion of saline caused no change in heart rate and mean arterial pressure in the supine position compared to the baseline (Figure 7-4A). Infusion of noradrenaline (0.003-0.3 µg/kg/min) significantly increased mean arterial pressure to about 109 mmHg (P<0.05), along with a significant decrease in heart rate in the supine position (P<0.05), as shown in Figure 7-5A. Infusion of vasopressin 0.4-4 milliunits/kg/min (3-28.5 ng/kg/min) did not cause significant changes either in blood pressure or heart rate (Figure 7-6A).
The mean arterial pressure of the saline group significantly decreased as soon as the patients were moved from the supine into the beach chair position \( (P<0.05) \), requiring an intervention with an injection of metaraminol (500 µg), but the heart rate was not immediately changed. The mean arterial pressure gradually recovered after intervention to the values in the supine position. In the noradrenaline treatment group, the mean arterial pressure was slightly but insignificantly decreased by moving the patients to the upright position \( (P>0.05) \). Noradrenaline maintained the post-beach chair mean arterial pressure with similar values to those of pre-anaesthetic, but significantly greater than the mean arterial pressure in the corresponding saline-treated group \( (P>0.05) \) (Figure 7-5A). There was a further decrease in mean arterial pressure without immediate change in heart rate in the vasopressin treatment group after moving the patients into the beach chair position. The decrease in blood pressure following the change to the beach chair position in patients taking vasopressin was gradually reversed after intervention with metaraminol injection (Figure 7-6A).
Table 7-3: Summary of MAP, heart rate and ScO$_2$ before and after anaesthesia in the supine and beach chair positions.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Saline (n=9)</th>
<th>Noradrenaline (n=7)</th>
<th>Vasopressin (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (Air)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>104±3.0</td>
<td>99±1.9</td>
<td>96±1.4</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>74±4.3</td>
<td>69±3.8</td>
<td>76±5.7</td>
</tr>
<tr>
<td>ScO$_2$ AIR (%)</td>
<td>74±2.0</td>
<td>72±0.8</td>
<td>76±1.8</td>
</tr>
<tr>
<td>Baseline (BL) (anaesthesia)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>64±2.3</td>
<td>70±3.8</td>
<td>69±2.1</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>74±3.7</td>
<td>66±2.6</td>
<td>75±4.6</td>
</tr>
<tr>
<td>ScO$_2$ BL (%)</td>
<td>78±3.0</td>
<td>79±1.1</td>
<td>82±1.8</td>
</tr>
<tr>
<td>Post-infusion (INF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔMAP$_{BL-INF}$ (mmHg)</td>
<td>-2±1.3</td>
<td>39±8.3*</td>
<td>1±2.1</td>
</tr>
<tr>
<td>ΔHR$_{BL-INF}$ (bpm)</td>
<td>-8±1.3</td>
<td>-17±3.0*</td>
<td>-11±2.5</td>
</tr>
<tr>
<td>ΔScO$<em>2$$</em>{BL-INF}$ (%)</td>
<td>-2±0.7</td>
<td>-6±1.1</td>
<td>-9±1.1</td>
</tr>
<tr>
<td>Beach chair position (BCP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔMAP$_{BL-BCP}$ (mmHg)</td>
<td>-16±2.7</td>
<td>29±5.3*</td>
<td>-17±2.8</td>
</tr>
<tr>
<td>ΔHR$_{BL-BCP}$ (bpm)</td>
<td>-6±3.7</td>
<td>-2±8.3</td>
<td>2±1.8</td>
</tr>
<tr>
<td>ΔScO$<em>2$$</em>{BL-BCP}$ (%)</td>
<td>-6±1.3</td>
<td>-2±1.1</td>
<td>-11±1.8*</td>
</tr>
<tr>
<td>Intervention</td>
<td>yes$^a$</td>
<td>yes$^b$</td>
<td>yes$^a$</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. MAP, mean arterial pressure; HR, heart rate; ScO$_2$, cerebral oxygen saturation; and Δ, change from baseline (BL) to post-infusion (INF) or “beach chair” position (BCP). $^a$All patients received metaraminol 500 µg injection in BCP. $^b$Infusion rate was decreased in 4 patients in the BCP. $^*P<0.05$ compared to saline, one-way ANOVA followed by Dunnett’s post hoc test.
7.3.3. **Effects of vasopressor agents on cerebral oxygen saturation**

The cerebral oxygen saturation (ScO$_2$) was continuously monitored in the control (saline), noradrenaline and vasopressin treatment groups both in supine and beach chair positions. The ScO$_2$ values obtained from the two optodes placed on the right and left sides of the forehead overlying the prefrontal cortex were averaged and compared with the pre-anaesthetic values. There was no significant difference in the post-induction baseline ScO$_2$ values between the control and treatment groups ($P>0.05$) (Table 7-3). The ScO$_2$ was stable in the group receiving saline infusion while the patients were in the supine position, but it was significantly decreased ($\Delta$ScO$_2$ -6%, $P<0.05$) by placing the patient into the beach chair position (Figure 7-4B). The decrease in ScO$_2$ observed in the saline group following beach chair positioning was gradually reversed after intervention with metaraminol injection. In patients receiving noradrenaline infusion, the ScO$_2$ showed a slight but statistically insignificant decrease when the patient was placed in the supine position. However, there was no further desaturation upon placing the patients into the beach chair position, as shown in Figure 7-5B. The post-beach chair ScO$_2$ level, which was similar to the post-anaesthetic baseline value, was maintained stable for the entire period of surgery without requiring intervention. In the patient group treated with vasopressin infusion, the ScO$_2$ in the supine position demonstrated a progressive decrease (Figure 7-6B). There was an additional decrease in the ScO$_2$ after the patients were seated into the beach chair position. The reduction in the ScO$_2$ following beach chair positioning was gradually reversed with metaraminol intervention.
Figure 7-4: Effects of saline infusion on MAP and HR and ScO$_2$ in anaesthetised patients in supine and beach chair positions.

The effects of saline infusion on mean arterial pressure (MAP; A) and heart rate (HR; A) and cerebral oxygen saturation (ScO$_2$; B) in anaesthetized human subjects in supine and beach chair positions. After post-anaesthetic stabilization, the normal saline solution was continuously infused. The infusion rate was increased over 15 min (2.5 min allowed for each rate). The patient was then placed into the beach chair position (BCP) and the infusion continued at the maximum rate (shown by the horizontal arrow). Measurements for ScO$_2$, MAP and HR were taken before the infusion was started and after every dose was administered. Data are expressed as changes compared to the post-induction baseline (BL) values before treatment for MAP and HR (Δ baseline) and to the basal values (before induction) for ScO$_2$ (Δ ScO$_2$ %). *P<0.05 compared to BL, one-way ANOVA followed by Dunnett’s post hoc test. Number of subjects in the group =9.
Figure 7-5: Effects of noradrenaline on MAP, HR and ScO₂ in supine and beach chair positions during anaesthesia.

The effects of noradrenaline infusion on mean arterial pressure (MAP; A) and heart rate (HR; A) and cerebral oxygen saturation (ScO₂; B) in anaesthetized human subjects in supine and beach chair positions. After post-anaesthetic stabilization, noradrenaline was continuously infused. The infusion rate was increased over 15 min (2.5 min allowed for each rate). The patient was then placed into the beach chair position (BCP) and the infusion continued at the maximum rate (shown by the horizontal arrow). Measurements for ScO₂, MAP and HR were taken before the infusion was started and after every dose was administered. Data are expressed as changes compared to the post-induction baseline (BL) values before treatment for MAP and HR (Δ baseline) and to the basal values (before induction) for ScO₂ (Δ ScO₂ %). Vertical bars are ±SEM.

Number of subjects in the group = 7.
Figure 7-6: Effects of vasopressin on MAP, HR and ScO$_2$ in supine and beach chair positions during anaesthesia.

The effects of vasopressin infusion on mean arterial pressure (MAP; A) and heart rate (HR; A) and cerebral oxygen saturation (ScO$_2$; B) in anaesthetized human subjects in supine and beach chair positions. After post-anaesthetic stabilization, vasopressin was continuously infused. The infusion rate was increased over 15 min (2.5 min allowed for each rate). The patient was then placed into the beach chair position (BCP) and the infusion continued at the maximum rate (shown by the horizontal arrow). Measurements for ScO$_2$, MAP and HR were taken before the infusion was started and after every dose was administered. Data are expressed as changes compared to the post-induction baseline (BL) values before treatment for MAP and HR (Δ baseline) and to the basal values (before induction) for ScO$_2$ (Δ ScO$_2$ %). Vertical bars are ±SEM. *P<0.05 compared to BL, one-way ANOVA followed by Dunnett’s post hoc test. Number of subjects in the group =8.
7.4. Discussion

This study has compared the effects of noradrenaline and vasopressin on blood pressure and cerebral oxygen saturation in anaesthetised patients in supine and beach chair positions. Hypotension was present in both positions, with occurrence of cerebral desaturation upon placement of the patients in the beach chair position. Continuous administration of noradrenaline increased the blood pressure and prevented incidence of cerebral oxygen desaturation during anaesthesia both in supine and beach chair positions, unlike vasopressin which had minimal effects on blood pressure but led to further cerebral oxygen desaturation. The findings from the current study suggest that vasopressor therapy can maintain cerebral perfusion pressure and thus prevent occurrence of cerebral oxygen desaturation during beach chair anaesthesia, but individual agents may induce differential effects on the cerebral circulation causing either beneficial or harmful outcomes.

General anaesthesia in the beach chair position has been known to be a precarious practice because it is associated with significant hypotension and decreased blood supply to the brain. A real-time monitoring of cerebral blood flow along with blood pressure helps to detect episodes of cerebral ischaemia associated with intraoperative hypotension. Cerebral oximetry is a rather direct method of monitoring cerebral oxygenation and helps to detect any episode of cerebral ischaemia. In the current study, NIRS oximetry technique was used to continuously monitor regional cerebral oxygen saturation in anaesthetised patients in supine and beach chair positions. Induction of anaesthesia significantly decreased the patients' mean arterial pressure from pre-anaesthetic values of about 100 to under 70 mmHg (~30%). Placing the patient into the beach chair position further decreased the blood pressure by about 20 mmHg. Similar findings have previously been reported where positioning anaesthetised patients in the upright position significantly decreased the mean arterial pressure, but the blood pressure was not affected by placing sedated patients (control) in the beach chair position (Soeding et al., 2011). Excessive decrease in arterial blood pressure during beach chair anaesthesia compromises
the cerebral perfusion pressure posing a risk of ischaemic brain damage in the patients. Despite decreases in mean arterial pressure in anaesthetised patients in the supine position, cerebral oxygen saturation was not significantly decreased which may be due to the existence of cerebral autoregulation or decreased oxygen extraction as a result of anaesthesia-induced decrease in neural metabolism (Meng et al., 2013). It has been indicated that most anaesthetic agents in clinical use, including sevoflurane, do not interfere with the cerebral autoregulation (Summors et al., 1999). Thus, intraoperative hypotension in supine position is as such may not have a great risk of ischaemic brain damage. However, placing anaesthetised patients into the beach chair position induces a further decrease in arterial blood pressure associated with a significant cerebral oxygen desaturation. Severe and prolonged cerebral oxygen desaturation or hypoxia can lead to ischaemic brain damage. Thus, severe decreases in blood pressure should be prevented to maintain cerebral perfusion while anaesthetised patients are seated in upright position, similar to the recommended management of traumatic brain injury (Bratton et al., 2007).

Maintaining blood pressure with prophylactic administration of a vasopressor agent can prevent the occurrence of cerebral oxygen desaturation during beach chair anaesthesia. The right choice of a vasopressor agent may be of paramount importance to obtain a favourable therapeutic outcome. Here, the ‘suitability’ of two vasopressor agents frequently used in neurocritical care, noradrenaline and vasopressin, for prevention of cerebral oxygen desaturation during the beach chair anaesthesia was investigated in patients. Infusion of noradrenaline increased the blood pressure which was maintained both in the supine and beach chair positions. Noradrenaline prevented cerebral oxygen desaturation induced by placing the patient in the beach chair position.

The effects of noradrenaline on cerebral blood flow may be a consequence of its effects on peripheral blood pressure. The direct effects of systemically administered noradrenaline on cerebral arteries and arterioles is limited due to the presence of blood-brain barrier in these vessels (MacKenzie et al., 1976). In the peripheral circulation, noradrenaline increases arterial blood pressure by
multiple mechanisms owing to its effects on both α- and β-adrenoceptors. Activation of α1-adrenoceptors located on arteries causes constriction that leads to increased peripheral resistance, while α1-adrenoceptor-mediated constriction of the veins increases the venous return and improves cardiac preload. Additionally, noradrenaline stimulates the heart by activating cardiac β-adrenoceptors increases cardiac output. Overall, noradrenaline increases the arterial blood pressure and maintains cerebral perfusion. Thus, continuous infusion of noradrenaline has beneficial effects in preventing severe hypotension and cerebral oxygen desaturation induced by seating the patient in the upright beach chair position commonly used in shoulder surgery.

Noradrenaline had a tendency, though statistically insignificant, to decrease ScO2 in supine position. Other studies have found similar decreases in cerebral oxygen saturation after noradrenaline administration in healthy subjects (Brassard et al., 2009; Ogoh et al., 2011). Another study reported that noradrenaline increased mean arterial pressure in humans without affecting the middle cerebral blood flow velocity, a surrogate measure of cerebral blood flow (Moppett et al., 2008). The likelihood that noradrenaline may cause constriction of the cerebral microcirculation is very low because it cannot access the cerebrovascular smooth muscle unless the blood-brain barrier is disrupted (Moller et al., 2004). Further, the constriction effect of noradrenaline in human isolated cerebral arteries is very weak (Toda and Fujita, 1973). The possible reason for the discrepancy between apparent cerebral desaturation and unchanged cerebral blood flow observed after noradrenaline administration may be related to interferences with the cerebral oximetry, such as constriction of extracerebral arteries. Recent studies have indicated that constriction of cutaneous arteries induced by noradrenaline influences the NIRS measurement of cerebral oxygen saturation (Sorensen et al., 2012; Ogoh et al., 2014).

Infusion of vasopressin did not increase the blood pressure in the supine position. Neither did vasopressin significantly affect the heart rate in anaesthetised subjects. However, it decreased cerebral oxygen saturation in the supine position. Vasopressin did not prevent a further decrease in blood
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pressure induced by placing the patient in the beach chair position. The decrease in ScO$_2$ was sustained in the beach chair position. This shows that systemically infused vasopressin may cause direct vasoconstrictor effects in human cerebral arteries. Similar results were recently reported wherein vasopressin (0.07 unit/kg bolus) increased the mean arterial pressure by 26 mmHg (31%) without altering heart rate in supine position, which was reversed in the beach chair position, and induced cerebral oxygen desaturation. The cerebral desaturation induced in the beach chair position was more pronounced in patients treated with vasopressin than in untreated (control) patients (Cho et al., 2013).

The lack of effect on mean arterial pressure found in this study upon infusion of vasopressin may be because relatively lower doses were used. Higher doses of vasopressin were not considered for fear that certain serious cardiac adverse effects such as myocardial ischaemia may develop (Maturi et al., 1991). Similar findings were previously reported where a stepwise infusion of vasopressin at 0.2-5 pmol/kg/min (0.2-5 ng/kg/min) did not affect blood pressure in healthy subjects, but induced a significant pressor response in patients with autonomic insufficiency (Williams et al., 1986). Vasopressin (10-20 units bolus followed by 0.1 units/min) is recommended for the management of intraoperative hypotension (Treschan and Peters, 2006), but its benefit in preserving cerebral perfusion is uncertain. It appears that low doses of vasopressin have minimal effects on mean arterial pressure in healthy subjects but may increase blood pressure in hypotensive conditions that are associated with impaired sympathetic nerve activity and low plasma vasopressin levels such as in septic shock (Montani et al., 1980; Landry et al., 1997; Holmes et al., 2001; Dunser et al., 2003; Holmes et al., 2004).

The prolonged decrease in ScO$_2$ without significant change of mean arterial pressure observed in patients receiving vasopressin infusion, however, is interesting. A study in pig models of cardiopulmonary resuscitation found that administration of vasopressin induced a sustained decrease in cerebral oxygenation (Bein et al., 2005). Vasopressin may have caused selective
constriction of the cerebral microcirculation without significantly affecting the tone of peripheral resistance arteries. Peptides injected into the peripheral circulation may cross the blood-brain barrier passively or via specific transporters located on the endothelial cells (Banks, 2015). Whether significant levels of vasopressin cross an intact blood-brain barrier and accumulate on the interstitial side to cause constriction of cerebral arteries in healthy subjects is not clearly known. An in situ brain perfusion study in pigs showed influx of vasopressin into the brain indicating the existence of a carrier-mediated transport located on the luminal side of the blood-brain barrier in cerebral microcirculation (Zlokovic et al., 1990). However, the effects of vasopressin on permeability of cerebral arteries and microcirculation have not been studies in other species including humans.

Both vasodilator and vasoconstrictor effects of vasopressin have been previously reported in cerebral arteries obtained from various species (Katusic et al., 1984; Lluch et al., 1984; Martin de Aguilera et al., 1990; Suzuki et al., 1993). The complexity of the cerebrovascular response to vasopressin has been demonstrated by Takayasu et al. (1993) who showed that vasopressin induces a triphasic response in rat isolated cerebral arterioles. Low concentrations of vasopressin ($10^{-12}$-$10^{-11}$ M) dilate, but moderately higher concentrations of vasopressin ($10^{-10}$-$10^{-8}$ M) constrict the arteries. A further increase in vasopressin concentration ($>10^{-8}$ M) completely dilated the arterioles. Systemic administration of vasopressin can possibly cause constriction of cerebral arteries owing to its carrier-mediated transportation across the blood-brain barrier. Thus, the benefit of using vasopressin in the management of hypotension induced by general anaesthesia is questionable. Rather, vasopressin may increase the risk of cerebral desaturation in hypotensive states.

In conclusion, maintaining arterial blood pressure during beach chair surgery can minimize the risk of cerebral desaturation, however the choice of vasopressor agent should be considered carefully. Noradrenaline can effectively
prevent cerebral desaturation but vasopressin has no benefit, or even increase the risk of desaturation during general anaesthesia in the beach chair position.
Chapter 8

Summary and conclusions
The brain has a unique blood flow that is specially regulated in order to maintain oxygen and nutrient supply over a range of peripheral blood pressure (50-150 mmHg). This is achieved by networks of superficial and penetrating arteries and arterioles that are controlled by the peripheral nerves, as well as neural inputs from cortical and subcortical areas. The endothelial lining of the blood vessels in the brain also plays an important role in regulating the tone of cerebral arteries by releasing vasoconstrictor and vasodilator mediators. Additionally, the vasogenic responses, pressure- or stretch-induced constriction, of cerebral arteries, may also play important roles in the regulation of cerebral blood flow. This study characterised the cerebrovascular responses to in vitro pharmacological stimulation in rat isolated arteries, in vivo effects of systemically administered vasoactive agents in rat models, as well as effects of vasopressor administration on cerebral oxygen saturation during anaesthesia in patients.

Rat cerebral arteries demonstrated variable responses to vasoactive agents depending on their regional location in the rat cerebral circulation. Arteries isolated from the anterior parts of the rat cerebral circulation were highly sensitive to the constrictor mediators such as noradrenaline, vasopressin and serotonin unlike arteries of the posterior cerebral circulation, which did not respond or were only weakly constricted by many vasoconstrictor agents. On the contrary, vasodilator agents relaxed arteries isolated from the posterior cerebral circulation with greater potency than those isolated from anterior regions of the rat cerebral circulation. The variations in the pharmacological responses of cerebral arteries may be related to regional heterogeneity in the distribution of the receptors mediating vasoconstriction and vasodilatation.

This study characterised the mechanisms involved in adrenoceptor-mediated vascular responses in rat cerebral arteries. Stimulation of $\alpha_1$-adrenoceptors induced a contractile response in arteries of the anterior rat cerebral circulation, namely the anterior and middle cerebral arteries, but not in posterior communicating and basilar arteries. However, the $\beta$-adrenoceptor-mediated relaxant responses were found in all arteries taken from different
regions of the rat cerebral circulation. The relaxation response is mediated by both \( \beta_1 \)- and \( \beta_2 \)-adrenoceptors that are distributed differentially in smooth muscle or endothelial cells depending on the location of the arteries. \( \beta \)-adrenoceptors are located on smooth muscle of the middle cerebral artery, while they are located on the endothelium of the basilar artery. Activation of the endothelial \( \beta \)-adrenoceptors in the rat basilar artery induces release either NO or EDH depending on the type of agonist used. Noradrenaline primarily induces release of NO, but it may also release a small proportion of EDH. Isoprenaline, however, favours the release of EDH along with a small proportion of NO. These variations may be related to differences in the intrinsic activity of the agonists on adrenoceptors.

Some of the vasoactive mediators including endothelin-1 and noradrenaline have been implicated in the pathogenesis of cerebrovascular diseases such as ischaemia or vasospasm that often occurs following subarachnoid haemorrhage. In light of this, the link between endothelin receptor stimulation and activation of different calcium channels was analysed in this study. The potent contraction of rat cerebral arteries induced by endothelin-1 was found to be largely dependent on influx of \( \text{Ca}^{2+} \) from the extracellular space. Endothelin-1 activates both voltage-operated and non-voltage operated calcium channels to induce contraction of cerebral arteries. The non-voltage-operated calcium channels play an important role in induction of the contraction by low endothelin-1 concentrations. T-type (low-voltage-operated) calcium channels are also activated by low concentrations of endothelin-1 during the early phase of contraction, but L-type (high-voltage-operated) calcium channels are activated by higher concentrations of endothelin-1 and are involved in the maintenance phase of the contraction.

The effects of systemically administered vasoactive agents on the diameter of cerebral arteries were investigated \textit{in vivo} in the rat using the cranial window technique to visualize the cerebral arteries. Phenylephrine (an \( \alpha_1 \)-adrenoceptor vasoconstrictor agonist) and vasopressin (a vasoconstrictor peptide) increased arterial blood pressure with simultaneous constriction of the cerebral arteries,
whereas formoterol (a $\beta_2$-adrenoceptor agonist vasodilator) decreased blood pressure and dilated the cerebral arteries. The extent and time-course of the blood pressure and cerebral arterial responses were similar when the drugs were systemically administered either through the femoral vein, or directly into the cerebral circulation via the carotid artery. Thus, the cerebrovascular effects of systemic vasoactive drug administration are indirect autoregulatory responses induced by changes in the arterial pressure. Nonetheless, phenylephrine and vasopressin may also elicit negligible effects on cerebral arteries. Under normal conditions, the blood-brain barrier limits most blood-borne substances from accessing the vascular smooth muscle, but its permeability may be affected when the blood pressure is elevated.

Finally, the clinical benefit of vasopressor therapy in protecting cerebral oxygen desaturation during anaesthesia in the ‘beach chair’ position was investigated in patients. Excessive hypotension associated with placing an anaesthetised patient in the beach chair position poses a risk of cerebral oxygen desaturation. Noradrenaline prevented cerebral oxygen desaturation by maintaining arterial pressure in the beach chair position. However, vasopressin did not prevent hypotension during anaesthesia in the beach chair position, but exacerbated the cerebral oxygen desaturation. A further cerebral oxygen desaturation may result from the direct constrictor effect of vasopressin on the cerebral microcirculation. The findings demonstrate that some vasopressor agents may have deleterious effects on cerebral blood flow possibly leading to ischaemic brain damage. Thus, the effects of vasopressor drugs on the cerebral circulation should be rigorously investigated before they are applied to provide haemodynamic support under conditions where the cerebral blood flow is compromised.

In summary, this study found variable effects of vasoactive drugs in cerebral arteries and demonstrated the need to understand the mechanisms behind the effects induced by individual drugs. Future studies are required to test more drugs and investigate their effects under different cerebrovascular diseases. This will help to identify the systems affected by pathological conditions, as
well as to design rational therapy for effective treatment of cerebral blood flow disorders including stroke.
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