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FUNCTIONAL ACETYLCHOLINE AND SEROTONIN RECEPTORS IN BRAIN MICROCIRCULATION: IMPORTANCE OF SUBTYPE AND CELLULAR LOCALIZATION IN THE DETERMINATION OF FUNCTION

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for the degree of

DOCTOR OF PHILOSOPHY

For My Father

imagination is more important than knowledge

- Albert Einstein

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CONTRIBUTIONS OF CO-AUTHORS

In **Chapter 3** entitled "Functional acetylcholine muscarinic receptor subtypes in human brain microcirculation: identification and cellular localization", I performed all the reverse transcriptase polymerase chain reaction experiments and the majority of the second messenger assays under supervision of Dr. E. Hamel and with assistance from Dr. Z. Cohen. The isolation of human microvascular fractions was performed by Dr. Z. Cohen in the laboratory. Dr. Z. Cohen and Mrs Rita Ball under the supervision of Dr. D. Stanimirović (National Research Council, Canada) established the endothelial and smooth muscle cell cultures and performed some of the second messenger assays. Astrocyte cultures were kindly provided by Dr. V.W. Yong (McGill University). Dr. A. Olivier provided the human tissue samples. All analysis and paper writing were performed by myself and edited by Dr. E. Hamel, with critical review by Drs. D. Stanimirovic, and M. Moreno.

In **Chapters 4 and 5,** respectively entitled "Muscarinic -but not nicotinic- acetylcholine receptors mediate a nitric oxide (NO)-dependent dilation in brain cortical arterioles: a possible role for the M5 receptor subtype" and "5-HT_{1B} receptors mediate both constriction and nitric oxide-dependent dilation in bovine and human brain intracortical arterioles", I performed all experiments and analysis of data under the supervision of Dr. Edith Hamel. Drs. A Olivier and A. Sadikot provided the human tissue samples, and Mr. D. Bélanger (Abatoir Ecolait) generously donated bovine brain samples. The papers were written by myself and edited by Dr. E. Hamel.

ABSTRACT

The brain intraparenchymal microcirculation is regulated by various brain neuronal pathways, including the cholinergic and serotonergic systems. In this study, we determined the molecular identity, second messenger coupling, cellular distribution and/or vasomotor functions of the receptors for these two neurovascular systems in human and/or bovine brain microvasculature. Using reverse transcriptase-polymerase chain reaction (RT-PCR), we first identified heterogeneous muscarinic acetylcholine receptors (mAChRs) in microvessel and capillary fractions isolated from human cerebral cortex. Using a combination of RT-PCR and second messenger assays on human cerebromicrovascular cell cultures, we then identified functional M2 and M5 mAChRs in endothelial cells, the M1, M3 and possibly M5 in smooth muscle cells, and all five mAChRs in astrocytes. In order to identify the vasomotor function(s) mediated by these receptors, we developed a technique to test the effect of vasoactive neurotransmitters on isolated and pressurized human and/or bovine intracortical penetrating arterioles (diameter ~ 47μm), known to be innervated by brain neurovascular pathways. ACh dose-dependently dilated human and bovine intracortical arterioles at spontaneous tone or following pre-con\striction with serotonin (5-HT). In contrast, nicotine failed to significantly affect the diameters of the arterioles under similar conditions. Application of L-NNA (10^{-5} M), an inhibitor of nitric oxide synthase (NOS), elicited a gradual constriction (~ 40%) of the arterioles, indicating the presence of constitutive NO release in these vessels. It also significantly blocked the dilation induced by ACh in both human and bovine arterioles. The mAChR antagonists pirenzepine, 4-DAMP, and AF-DX 384 dose-dependently inhibited the dilation elicited by 10^{-5}M ACh with the following order of potency: 4-DAMP (pIC_{50} = 9.2 ± 0.1) >> pirenzepine (pIC_{50} = 6.7 ± 0.2) > AF-DX 384 (pIC_{50} = 5.9 ± 0.1), which best correlated with their affinities at the human M5 mAChR. Using the same technique, we demonstrated that 5-HT induces a dose-dependent constriction in human and bovine intracortical arterioles, a response which was potently inhibited (pIC_{50} value of 8.5 ± 0.1) by the 5-$HT_{1B/1D}$ receptor antagonist GR127935 in bovine vessels. Sumatriptan (a 5-$HT_{1B/1D/1F}$ agonist) induced a dual vasomotor response in both human and bovine microvessels which

consisted of a small but significant ($p< 0.05$) dilation at low concentrations, followed by a constriction at higher doses. Inhibition of NOS with L-NNA abolished the vasodilation to sumatriptan and potentiated its vasocontractile effect. Selective 5-HT_{1D} and 5-HT_{1F} receptor agonists failed to induce either a dilation or constriction. These results suggested that both the dilatory and contractile components of the sumatriptan vasomotor response are mediated by 5-HT_{1B} receptors. Interestingly, it was also shown in this study that the extent of the vasodilatory response to sumatriptan is inversely proportional to the size of the vessel diameter. Together, these results indicate that ACh and 5-HT, two important brain neuromediators, can induce direct and, in some cases, NO-dependent changes in the tone of intracortical arterioles, and that the vasomotor response can depend on the type and location of the receptors in the vessel wall.

RÉSUMÉ

La circulation intraparenchymateuse cérébrale est régulée par des voies neuronales variées, impliquant les systèmes cholinergiques et sérotoninergiques. Au cours de cette étude, nous avons déterminé, au niveau des microvaisseaux chez l'homme et/ou le boeuf, l'identité moléculaire des récepteurs pour ces deux systèmes de neurotransmetteurs, leur distribution cellulaire dans la paroi du vaisseau, ainsi que la nature des seconds messagers qui leur sont couplés et/ou leurs fonctions vasomotrices. Dans un premier temps, grâce à une approche moléculaire de RT-PCR, nous avons identifié la présence de différents récepteurs muscariniques à l'acétylcholine (mAChRs) dans des microvaisseaux et capillaires isolés de cortex cérébral humain. Par des dosages de seconds messagers et de RT-PCR sur des cultures de cellules microvasculaires d'origine cérébrale, nous avons montré la présence de récepteurs fonctionnels M2 et M5 sur les cellules endothéliales, M1 et M3, et possiblement M5 dans les cellules musculaires lisses, et tous les récepteurs muscariniques dans les astrocytes. Afin d'identifier les fonctions vasomotrices mises en jeu par ces récepteurs, nous avons développé une technique pour tester l'effet vasomoteur des neurotransmetteurs sur des artérioles pénétrantes intracorticales humaines et/ou bovines, isolées et pressurisées (diamètre ~ 47µm), celles-ci étant innervées par des systèmes de neurotransmetteurs. L'acétylcholine (ACh) dilate de façon dose-dépendante les artérioles intracorticales bovines et humaines au tonus de base ou après pré-constriction par la sérotonine (5-HT). En revanche, la nicotine ne modifie pas significativement le diamètre de ces artérioles dans des conditions similaires. L'application de L-NNA (10^{-5}M), un inhibiteur de la NO synthase (NOS), a produit une contraction progressive (-40%) des microartères, indiquant l'existence d'une libération constitutive de NO par ces vaisseaux. Le L-NNA a également inhibé significativement la dilatation des artérioles humaines et bovines induite par l'ACh. Les antagonistes des mAChRs, tels pirenzepine, 4-DAMP et AF-DX 384, inhibent de façon dose-dépendante la dilatation produite par l'ACh à la dose de 10^{-5}M. L'ordre de puissance des antagonistes est: 4-DAMP ($pIC_{50} = 9.2 \pm 0.1$) >> pirenzépine ($pIC_{50} = 6.7 \pm 0.2$) > AF-DX 384 ($pIC_{50} = 5.9 \pm 0.1$), et était corrélé au mieux avec leur affinité respective au récepteurs muscariniques de type M5 chez l'homme. En

utilisant la même technique, nous avons démontré que la 5-HT induit une vasoconstriction dose-dépendante dans les artérioles intracorticales humaines et bovines, qui a été puissamment inhibée ($pIC = 8.5 \pm 0.1$) dans les artérioles bovines par l'antagoniste des récepteurs $5\text{-}HT_{1B/1D}$, le GR 127935. Le sumatriptan (agoniste $5\text{-}HT_{1B/1D/1F}$) a induit une réponse vasomotrice différente au niveau des microvaisseaux tant bovins que humains. Son administration provoque une vasodilatation significative ($p<0.05$) quoique de faible intensité à de faibles concentrations, suivie d'une vasoconstriction à de plus fortes doses. L'inhibition de la NOS par le L-NNA a aboli la vasodilatation au sumatriptan et a potentialisé son effet vasoconstricteur. Les agonistes sélectifs des récepteurs $5\text{-}HT_{1D}$ et $5\text{-}HT_{1F}$ n'ont produit aucun effect vasomoteur. Ces résultats suggèrent que les deux composantes de la réponse vasomotrice au sumatriptan (dilatation et contraction) sont induites par les récepteurs $5\text{-}HT_{1B}$. Or, nous avons également montré dans cette étude que l'ampleur de la réponse vasodilatarice au sumatriptan est inversement proportionelle au diamètre des vaisseaux. L'ensemble de ces résultats indique que l'ACh et la 5-HT, deux neuromédiateurs cérébraux majeurs, peuvent induire des changements directs, et dans certains cas NO-dépendants, du tonus des artérioles intracorticales, et que la réponse vasomotrice peut dépendre du type et de la localisation des récepteurs dans la paroi vasculaire.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

4-DAMP:	4-diphenylacetoxy-N-methylpiperidine methiodide
5-HT:	5-hydroxytryptamine, serotonin
ACh:	acetylcholine
AF-DX384:	(±)-5,11-dihydro-11-{[(2-[(dipropylamino)methyl]-1-piperidinyl} ethyl)amino]carbonyl}-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one
AST:	fetal astroglial cell cultures
BBB:	blood brain barrier
cAMP:	cyclic adenosine monophosphate
CAP:	capillaries
CBF:	cerebral blood flow
cGMP:	cyclic guanosine monophosphate
CGRP:	calcitonin gene-related peptide
CICR:	calcium induced calcium release
CNS:	central nervous system
DAG:	diacylglycerol
DNA:	deoxyribonucleic acid
DRN:	dorsal raphe nucleus
EC:	endothelial cell cultures
EDHF:	endothelium derived hyperpolarizing factor
EDRF:	endothelium derived relaxing factor
GABA:	gamma aminobutyric acid
IP_3:	inositol-1,4,5-trisphosphate
L-NNA:	N^{ω}-nitro-L-arginine
LY344864:	(R)-(+)-N-(3-dimethlyamino-1,2,3,4-tetrahydro-9H-carbazol-6-yl)-4-fluorobenzamide
mAChR:	muscarinic acetylcholine receptors
MV:	microvessels
nAChR:	nicotinic acetylcholine receptors
NBM:	nucleus basalis of Meynert
NO:	nitric oxide
NOS:	nitric oxide synthase
NPY:	neuropeptide Y
PKC:	protein kinase C
PLC:	phospholipase C
PNS:	peripheral nervous system
PNU-109291:	(S)-(-)-1[1-[4-(4-methoxyphenyl)-1-piperaziny-1]ethyl]-N-methylisochroman-6-carboxamide
RNA:	ribonucleic acid
RT-PCR:	reverse transcriptase-polymerase chain reaction
SI:	substantia innominata
SMC:	smooth muscle cell cultures

PRELUDE

This thesis is not uniquely about brain nor circulation, but rather it is about the association of these two systems which culminates in the intricate and precise control of local blood flow in the brain. The focus is on the brain microcirculation, an area of high flow resistance and thus high vascular importance. In order for there to be a communication between brain and circulation, there has to be a means of sending and receiving information. We therefore examine neurotransmitters and their receptors as conveyors of this communication. While only two neurotransmitter systems are examined in detail with regards to their control of cerebral blood flow, many of the findings can be extrapolated to other systems.

There are only two ways to change cerebral blood flow, it can either be increased or decreased. These two opposing forces are well represented by the vasodilatory cholinergic system and the largely vasocontractile serotonergic system. Activation of these systems are known to result in neurogenic changes in cerebral blood flow in different regions of the brain. Underlying these changes are intricate intercellular, cellular, and intracellular mechanisms that work in synchrony to achieve this overall effect. Our molecular, biochemical and pharmacological findings shed light on some of these mechanisms at the level of blood vessels, vascular cells and receptors. By focusing at the junction between neurotransmitters and receptors, nerve terminals and vascular cells, neurons and vessels, this thesis provides novel information on how the brain controls its source of energy and life, and in doing so illustrates the complexity, subtlety and precision inherent in this organ.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE BRAIN INTRAPARENCHYMAL MICROCIRCULATION

Due to its high metabolic demand and its lack of metabolic storage, the brain is in constant demand for nutrients. These are largely provided by the intricate network of blood vessels which irrigate the whole brain. Blood enters the brain from two different sources, the vertebral arteries which combine to form the basilar artery and the carotid arteries. Both arteries supply a continuous segment of connected vessels at the base of the brain called the circle of Willis. The circle of Willis is the source of the major cerebral arteries which branch out into pial vessels that cover the entire surface of the whole brain. These vessels get progressively smaller until at some point they penetrate into the parenchyma of the brain and form the intraparenchymal or intracerebral circulation. All cerebral vessel structures previous to this junction are referred to as the extracerebral circulation.

The brain intracerebral microcirculation is composed mainly of arterioles, capillaries and venules located in the parenchyma of the brain (see Fig. 1). Penetrating arterioles branching at perpendicular angles from the surface pial vessels enter the brain via invaginations of the pia mater called the Virchow-Robin's space. Gradually the basement membrane of the pia and the penetrating arterioles coalesce and this space disappears. The arterioles then give rise to smaller arterioles and finally capillaries, forming an intricate meshwork that supplies all regions of the brain. The distribution of capillaries in the brain is heterogenous but not random as it appears that capillary density is not correlated with the number of neuronal or glial cell bodies, but rather with the number of synapses in the region (Dunning and Wolf, 1937). Additionally, it has been shown that brain areas with high energy demands have a higher density of blood

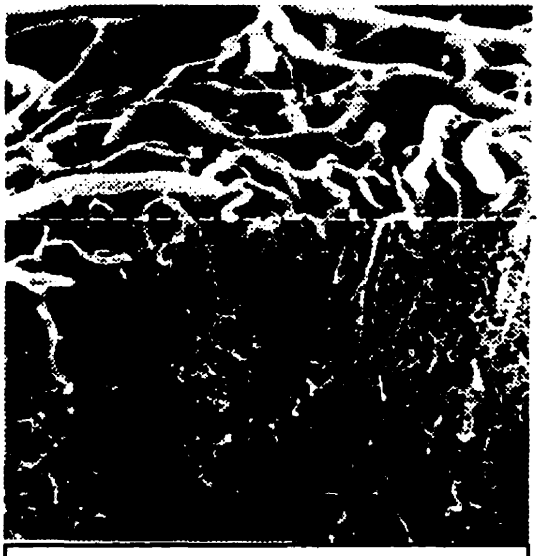

Fig. 1: Location of brain intracerebral microvessels (area below dotted line) with relation to pial (surface) vessels in human brain (from Duvernoy et al. 1983).

vessels than areas with lower demands (Sokoloff et al. 1977; Gobel et al. 1990) This is a strong anatomical indication of the relationship between neuronal function and metabolic requirements, an idea which has been and continues to be extensively studied and is the basis of the concept of metabolic regulation of cerebral perfusion (to be discussed later).

1.1.1 Cellular Anatomy of Blood Vessels

The cellular anatomy of the intracerebral microcirculation has features that are shared with other microvascular systems and others that are unique to its important role in the brain. The outer coating of arterioles, the *tunica adventitia*, is comprised mainly of collagen bundles, adventitial cells and nerve fibers. As vessels get progressively smaller, this layer tends to shrink and finally, at the level of the microarterioles and capillaries, disappears altogether (Roggendorf and Cervos-Navarro, 1977).

Interior to the *tunica adventia* lies the *tunica media*, a layer composed mainly of smooth muscle cells that are aligned in a tight spiral around the vessel. While the larger pial vessels are generally equipped with several layers of smooth muscle, arterioles contain at most two layers per circumference. In the smaller arterioles this layer can be composed of as little as one or two flattened smooth muscle cells (Roggendorf and Cervos-Navarro, 1977) while at the level of the capillaries the smooth muscle cells disappear completely and are replaced by pericytes (see Fig. 2).

The activity of smooth muscle cells provides vessels with the capacity to modify their diameter and thus regulate vascular tone. These changes occurring in numerous neighbouring vessels accumulate to affect the cerebral blood flow (CBF). Contraction of vascular smooth muscles in blood vessels results in the constriction of the vessel and a decrease in blood flow, while a relaxation will dilate the vessel and increase flow. The smooth muscle cells contain actin and myosin filaments which mediate contraction and relaxation of these cells through a complex series of biochemical events (see section 1.2.2.3).

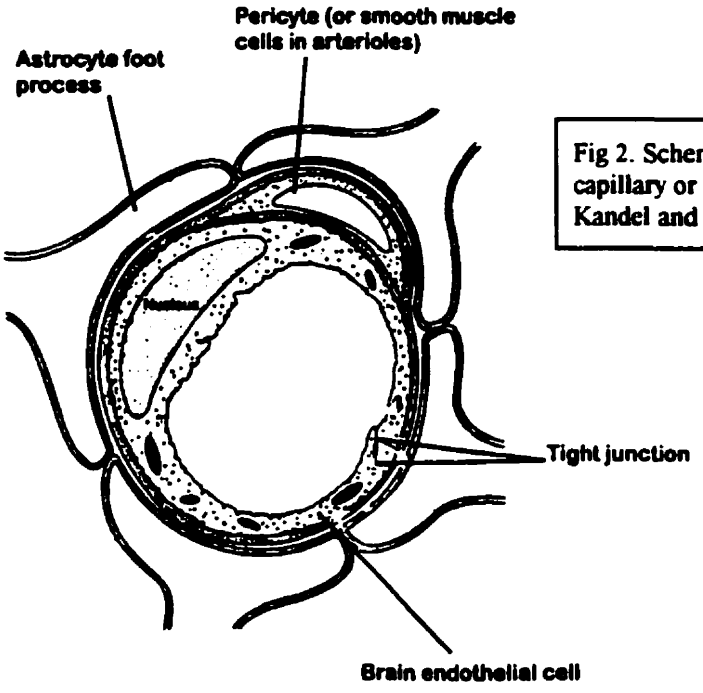

Fig 2. Schematic representation of a brain capillary or small arteriole. Adapted from Kandel and Schwartz, 1991.

Capillaries, unlike arterioles, do not contain a smooth muscle layer. They do contain however periendothelial cells called pericytes. Unlike smooth muscle cells, which constitute a separate layer, pericytes are encompassed within the same basement membrane as that of endothelial cells. Pericytes contain smooth muscle type contractile proteins and can develop into smooth muscle cells under certain conditions (Meyrick and Reid, 1978). They are known to be involved in endothelial development, blood-brain barrier (BBB) maintenance, and have also been suggested to mediate vasomotor reponses in capillaries, although direct evidence is still not available (Shepro and Morel, 1993).

The lumen of both arterioles and capillaries is lined with endothelial cells which form the *tunica intima*. Brain endothelial cells are different from other endothelial cells in that they contain few or no fenestrations, lack pinocytic activity and are joined together by tight junctions to form *zonae occludentes*. These morphological properties of brain endothelial cells, together with specialized cellular transport systems, form the basis of the blood brain barrier (BBB), a mechanism by which almost all but essential nutrients are excluded from

entering the brain parenchyma (Joo et al., 1996). The BBB is formed very early in development and is essential for maintenance of brain homeostasis. Interactions of different chemical mediators such as serotonin (5-HT), noradrenaline, histamine, bradykinin, arachidonic acid, leukotrines and calcitonin gene-related peptide (CGRP) with the endothelial cells have been shown to alter the permeability of the BBB (Wahl et al., 1988; Black et al., 1995; Moreno et al., 1999).

In the brain parenchyma, brain microvessels are surrounded by astrocytic endfeet which ensheath the vascular cylinder (Kacem et al., 1998), leading researchers to originally speculate that their role was uniquely structural. While the exact mechanisms are still unknown, perivascular astroglial cells have now been shown to be involved in angiogenesis, neurotransmitter uptake, and the development and maintenance of the blood-brain barrier (Tao-Cheng et al, 1987; Joo, 1996). Recently they have been implicated in the metabolic homeostasis of the brain, where they are thought to act as a relay for glucose transport between blood vessels and neurons (Tsacopoulos and Magistretti 1996). In their model, Magistretti and colleagues have proposed that glucose is taken up by the astrocytic endfeet and converted into lactate before being transported to neighbouring neurons as a primary source of energy. The driving force for this pathway appears to be the release of glutamate by neurons, a positive indicator of neuronal activity (Magistretti et al., 1999). Interestingly, this relationship could be more intricate since there is evidence that astrocytes might be involved in the control of local CBF (Paulson and Newman, 1987), possibly as a relay between neuronal activity and vessel dilation (for review see Harder et al, 1998). This fits perfectly with the astrocytic leaflets anatomical location between blood vessels and neurons, and their capacity to release vasodilatory compounds such as expoxyeicosatienoic acids upon glutamate release from neurons (Alkayed et al., 1997, Harder et al., 1998). Overall, it appears that the astrocytic environment created around microvessels serves to maintain as well as activate numerous processes involved in the nourishment of the brain (Joo, 1996).

1.2 CONTROL OF BLOOD FLOW IN THE BRAIN

1.2.1 Regulatory Mechanisms

Since the brain has virtually no capability to store metabolic energy it is dependent on its circulatory system to replenish its nutrients. If the blood flow is halted for more than a few minutes, irreversible damage will occur in the deprived area (for review see Choi, 1990; Iadecola, 1993). It is therefore paramount that the brain regulates its perfusion with accurate precision. This is especially pertinent for the smaller pial and parenchymal arterioles where the greatest fraction of total cerebrovascular resistance is sited (Stromberg and Fox 1972; Symon et al., 1973). There are three known mechanisms which work simultaneously to ensure that the brain can perform this function with utmost efficacy.

1.2.1.1 Metabolic Regulation

Roy and Sherrington (1890) were the first to suggest that changes in local blood flow were coupled with the metabolic demand of the cells surrounding microenvironment. An increase in neuronal activity in a region of the brain results in the release of metabolic coupling factor(s) by active cells which elicits dilation in the local cerebral blood vessels. By this mechanism, the brain would be able to control blood flow based on metabolic demand. To date the identity of such a coupling factor is unknown, although numerous candidates such as CO_2, extracellular pH, O_2, adenosine, adenine nucleotides, potassium (K^+), calcium (Ca^{2+}), (Kuschinsky and Wahl, 1978; Edvinsson et al., 1993) and most recently nitric oxide (NO; Iadecola, 1993; Estrada and DeFelipe, 1998; Lindauer et al., 1999), have been suggested.

1.2.1.2 Myogenic Regulation

When transmural pressure in an artery is altered, an active response termed autoregulation, is initiated to maintain a constant CBF protecting the brain from either ischemia at low blood pressure and edema at higher levels. There are several mechanisms by which this can performed, one of which is myogenic regulation (Folkow, 1964; see Paulson et al., 1990). The premise of the myogenic response is that smooth muscle in resistance arteries respond directly to alterations in perfusion pressure by contraction during increases and relaxation during

reductions in pressure. The myogenic response is also implicated in the maintenance of the basal or spontaneous tone of vessels. This is an intrinsic tone that vessels acquire that is dependent on pressure and/or flow in the vessel (Bevan and Laher, 1991). Within the physiological range of pressures, vessels will constrict to an increase, and dilate to a decrease in blood pressure. At very low and high pressures, however, this ability to regulate flow gradually disappears. Interestingly, under certain pathological conditions (eg hypertension), there can be shifts in the limits of this autoregulation (Paulson et al., 1990).

At the cellular level, this mechanism is thought to be mediated by sensors in the smooth muscle which are responsive to changes in pressure or flow and alter intracellular Ca^{2+} levels accordingly (Bevan and Laher, 1991, Edvinsson et al., 1993). Endothelial cells might be involved in this response, through the release of endothelial factors (Harder et al., 1989; Thorin et al., 1998) although some studies have shown that in vessels denuded of the endothelial layer, the myogenic response appears to be intact (Kuo et al., 1990; see Bevan and Laher, 1991). Thus both metabolic and pressure-induced changes in cerebrovascular tone involve intricate signaling and cellular mechanisms, which allow blood flow in an artery to be regulated based on cellular demand and/or transmural pressure.

1.2.1.3 Neurogenic Regulation

Another means by which the brain controls CBF has gained credibility over the recent years, namely, the direct neurogenic control via the release of neurotransmitters on or in the proximity of brain blood vessels (Edvinsson, 1975; Nakai et al., 1983). Neurotransmitters of different classes have been shown to exert specific vasomotor effects on cerebral blood vessels (especially extracerebral vessels) via interaction with receptors on the vessel wall or the surrounding neuropil (Paulson et al., 1990). This regulation is performed at two levels in the brain based on the type of vessels involved. Neurogenic control of the blood flow in the pial vessels is mainly performed by nerve fibres from the peripheral nervous system (PNS; Uddman and Edvinsson, 1989; Suzuki and Hardebo, 1993) including the cholinergic, serotonergic, noradrenergic, as well as peptidergic systems. The source of these innervation is usually from

the different ganglia in the PNS, such as the sphenopaletine, otic, or the superior cervical ganglia. In contrast with the surface pial vessels, neurogenic innervation of the brain intraparenchymal blood vessels has been suggested to be mediated by intrinsic nerve fibres (Nakai et al., 1983; Lou et al, 1987), thus indicating a possible functional role for brain neurons in controlling local perfusion (See Fig. 3). As in the extracerebral blood vessels, perivascular associations do not establish junctional contacts with vascular cells, but rather rely on diffusion of the vasoactive substance in the extracellular space before reaching its target receptors. These receptors could be localized directly on the vascular wall or the surrounding glial or neuronal cells. Interestingly, this neurogenic control has been shown in some cases to be "uncoupled" from metabolic demand, resulting in increased blood flow in regions without a corresponding increase in glucose utilization (Nakai et al., 1983; Kimura et al., 1990; Vaucher et al., 1997a). While there numerous types of nerve terminals innervating the brain microcirculation, this thesis will focus mainly on the cholinergic and serotonergic innervations due their proven role in altering blood flow in the brain (see sections 1.3.1.2 and 1.4.1.2).

1.2.2 Underlying Cellular Mechanisms

The control of CBF by any one of the above described mechanisms involves both inter- and intra-cellular signaling processes, which in some cases can overlap or be contradictory to each another. These processes are too numerous to be described in detail and in many cases not yet fully understood. However for the purposes of this thesis, the cellular mechanisms underlying nitric oxide (NO) production, receptor-mediated second messengers, and smooth muscle constriction/dilation deserve special mention since they are a recurring motif in this study.

1.2.2.1 Nitric Oxide and Cerebral Blood Flow

Endothelial cells play an active role in the control of vasomotor tone via the release of several agents, such as prostacyclins, prostaglandins, endothelins, endothelium-derived relaxing factor (EDRF, Furchgott and Zawadzki, 1980), and the still unidentified endothelium derived

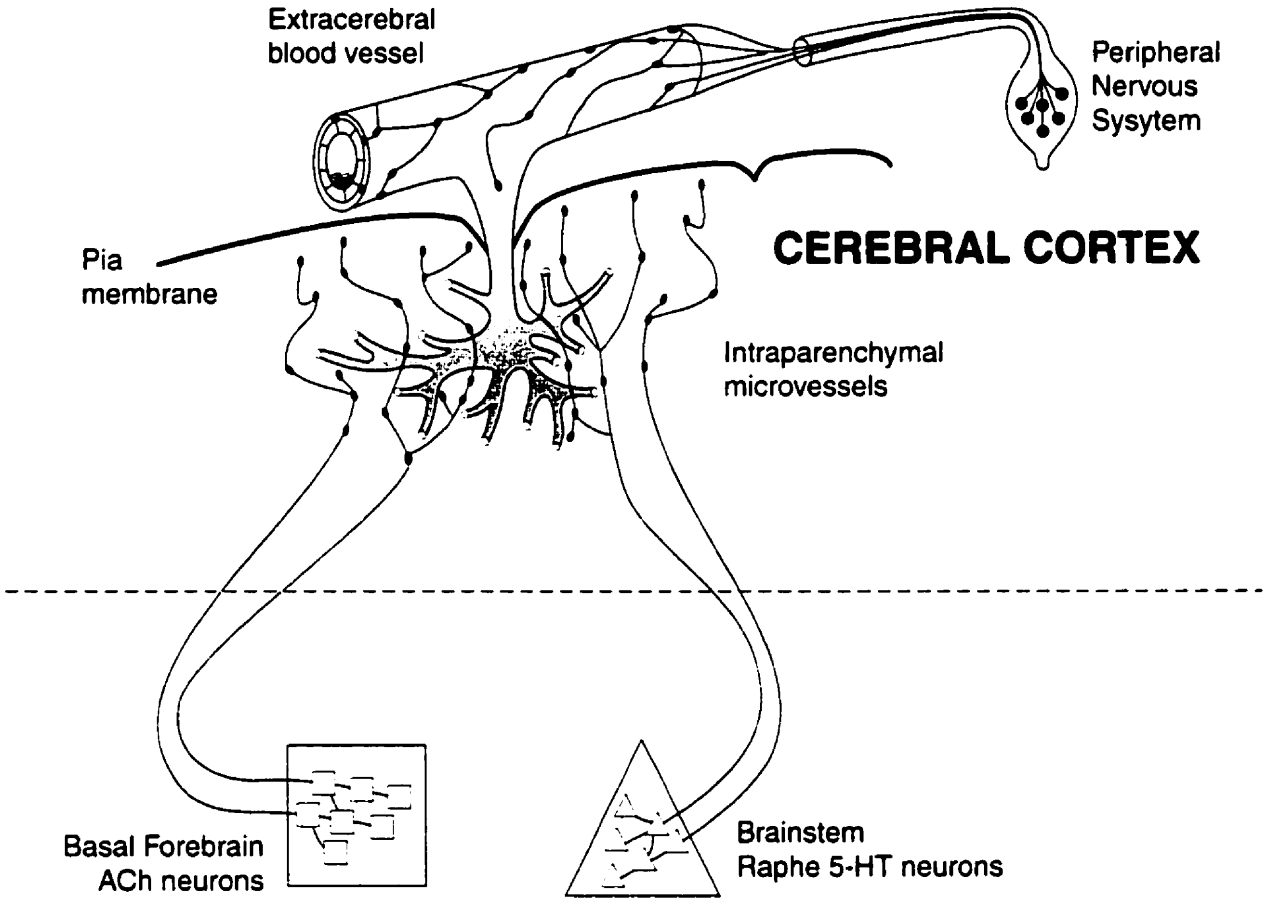

Fig. 3: Schematic representation of the neurogenic control of the intracerebral circulation by intrinsic ACh and 5-HT neurons. Note that the extracerebral blood vessels are innervated by the peripheral nervous system. Modified from Cohen et al., (1996).

hyperpolarizing factor (EDHF). Once released these compounds interact with the smooth muscle cells to mediate vasomotor effects. Of particular interest was the identification of NO (Palmer et al., 1987) or an NO derivative (Myers et al., 1990) as the EDRF. NO is a potent vasodilator that is synthesized by the conversion of L-arginine into NO and citrulline by an enzyme called nitric oxide synthase (NOS). There are three isoforms of NOS, the constitutively active endothelial (eNOS or NOS-3) and neuronal (nNOS or NOS-1) isoforms, and the inducible (iNOS or NOS-2) isoform. In the constitutive forms, NO is synthesized by a mechanism that requires the presence of Ca^{2+}/calmodulin, while the inducible form is Ca^{2+} independent (Watkins, 1995).

eNOS , localized mainly in endothelial cells, is constitutively active under basal conditions but may be further stimulated by increases in intracellular calcium (iCa^{2+}) levels, such as in response to receptor mediated agonists (eg acetylcholine). NO exerts its vascular effect by diffusing into smooth muscle cells where it activates soluble guanylyl cyclase increasing cGMP levels which results in reduced intracellular Ca^{2+} and hence relaxation of smooth muscle cells (Lincoln and Cornwell, 1991; Faraci and Brian, 1994; see section 1.2.2.3). More recently it has been shown that NO can also induce relaxation by lowering the Ca^{2+} sensitivity of the contractile apparatus (Lee et al., 1997) or by directly activating K^+ channels mostly of the Ca^{2+} dependent type and hyperpolarizing the smooth muscle cell (Bolotina et al., 1994; Koh et al., 1995; see Fig. 4). Due to NO's tonic production by endothelial cells, removal of the endothelial layer or inhibition of NOS results in the constriction of blood vessel and decreased blood flow (Rosenblum et al., 1990; Faraci, 1991; Kimura et al., 1994; Fergus et al., 1996). Thus NO is involved in maintaining the spontaneous tone of blood vessels.

In addition to the endothelium, NO from other sources can also play a role in control of CBF. Ultrastructural studies have shown that nitrergic neurons are frequently found in apposition to intraparenchymal microvessels (Iadecola et al., 1993; Estrada et al., 1993; Regidor et al., 1993; Tong and Hamel, 1999). Additionally functional studies using 7-nitroindazole (7-NI), which preferentially inhibits nNOS, have indicated that in the cerebral cortex, NO derived

from perivascular nerves can have an effect on vasomotor tone (Zhang et al., 1995, Cholet et al., 1997). NOS positive neurons have also been suggested to play a role in control of CBF and in the coupling of blood flow to neuronal activation (Iadecola, 1993; Estrada and DeFelipe, 1998; Lindauer et al., 1999).

The non-constitutive iNOS was initially identified in macrophages where it is viewed as an effector molecule in immunological reactions. It is calcium-independent and is thought to be activated by certain cytokines and lipopolysaccarides mainly under pathophysiological conditions. iNOS produces larger quantities of NO for a longer duration of time than constitutive forms of NOS. It is now known to be present in other types of cells such as smooth muscle, endothelial, microglia and astroglia, however, it appears that iNOS is not the primary source of NO under normal physiological conditions (Faraci and Brian, 1994; Watkins, 1995; Christopolous and El-Fakahany, 1999).

1.2.2.2 Receptor-Mediated Mechanisms

The overwhelming majority of receptor mediated mechanisms that will be described in this thesis are of the G protein coupled receptor family. These receptors are characterized by a single protein with seven transmembrane hydrophobic domains connecting three intracellular and three extracellular loops. It is known that the primary site of interaction of the receptors with G proteins are at the cytoplasmic domains, in particular the third cytoplasmic loop (LeVine, 1999). There are several different types of G proteins, and more than one can be activated by any individual receptor (Hosey, 1992). Signaling in G proteins is mediated by the dissociation of the Gα subunit from the Gβδ subunits. These subunits then proceed to activate the appropriate effector proteins, which in the case of the receptors in this study, involves primarily the activation of the inositol phosphate pathway or the modulation of adenylate cyclase activity (see Fig. 4).

The inositol phosphotase pathway is activated by receptor subtypes that are linked, through G proteins G_q, G_{11}, G_{14}, or G_{16}, to phospholipase C (PLC). When activated, PLC results in

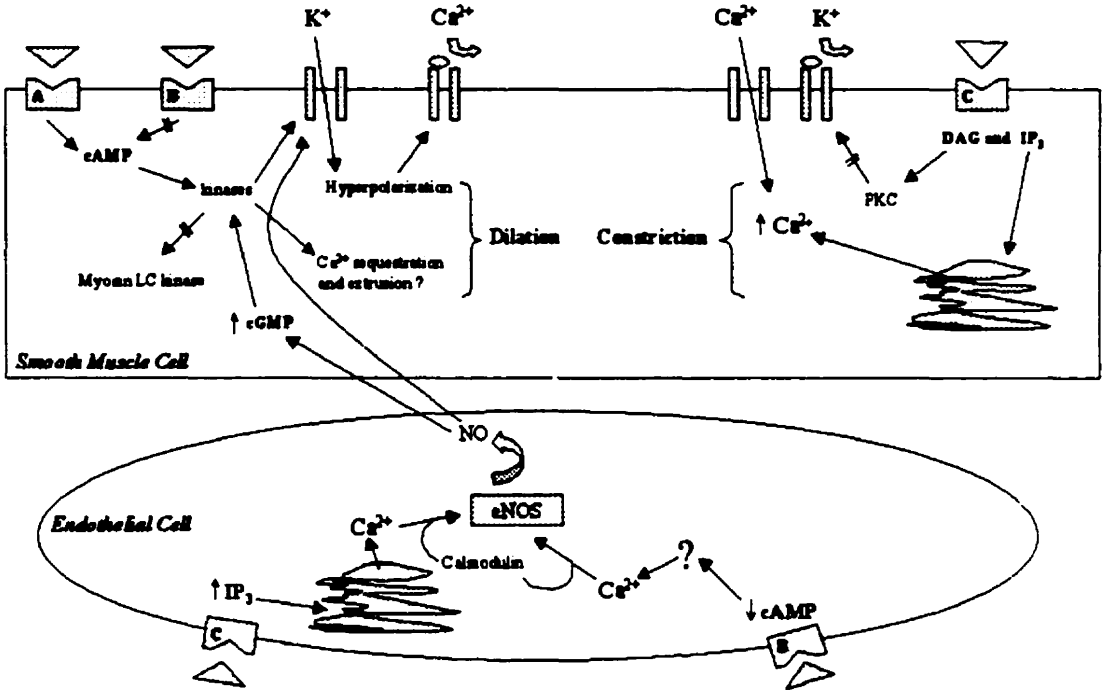

Fig 4: Receptor mediated mechanisms in endothelial and smooth muscle cells. **A** receptors stimulate adenylate cyclase and hence cAMP production (eg. 5-HT_7). **B** receptors attenuate adenylate cyclase activity (eg. M2 or 5-HT_{1B}). **C** receptors stimulate PLC activity (eg. M1, M5 and 5-HT_{2A}).

the hydrolysis of phosphoinositide (PI) and the generation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG stimulates protein kinase C (PKC) which can then phosphorylate a whole series of proteins involved in signal transduction such as phospholipase D and phospholipase A2. In smooth muscle cells, activation of PKC can induce contraction through interaction with actin-based regulatory mechanisms (Horowitz et al, 1996) as well as by suppressing the activity of Ca^{2+} or ATP-activated K^+ channels (Minami et al., 1993; Hatakeyama et al., 1995) thus depolarizing the cell. IP_3 on the other hand releases Ca^{2+} from the endoplasmic (or in muscle, sarcoplasmic) reticulum by binding to receptors on the membrane of these intracellular compartments (Felder, 1995). An increase in iCa^{2+} levels can result in different effects depending on the cell type. For example in vascular smooth muscle, an augmentation of iCa^{2+} results in contraction (see section 1.2.2.3). In endothelial cells

however, it could activate of the Ca^{2+}/Calmodulin-dependent eNOS, which would lead to NO production and subsequent dilation (Hosey 1992; see Fig 4).

The stimulation of adenylate cyclase or guanylate cyclase respectively results in increased cAMP and cGMP production in the cell. This in turn activates cAMP and cGMP kinases which can, by phosphorylating different channels, enzymes or membrane pumps, ultimately affect Ca^{2+} levels in the cell. While the exact mechanisms are still no fully determined, it is thought that increases in cAMP or cGMP levels result in the reduction of Ca^{2+} entry through increased opening of large conductance Ca^{2+}-activated K^+ channels and hyperpolarization. They may also increase Ca^{2+} sequestration in intracellular stores and Ca^{2+} extrusion from the cell (for review see Raeymaekers and Wuytack, 1993; Bolton et al., 1999) presumably by increasing sodium-Ca^{2+} exchange. In smooth muscle cells, NO-mediated increases in guanylyl cylclase activity results in relaxation of vessels, while decreases in cAMP production by G protein coupled receptors have been shown to induce constriction, primarily via the augmentation of Ca^{2+} levels (Ebersole et al., 1993) and, secondarily, by increasing myosin light chain kinases sensitivity to Ca^{2+} (McDaniel et al., 1994; see below). Interestingly, a similar increase in iCa^{2+} levels in endothelial cells would activate NO production via eNOS and possibly result in dilation (Ullmer et al., 1995). However to date, it is uncertain if and how cAMP affects iCa^{2+} levels in endothelial cells (Nobels and Abbot, 1998).

1.2.2.3 Constriction and dilation of smooth muscle

Muscle cells are endowed with repeated interspaced actin and myosin filaments that contract and relax by the movement of the myosin microfilaments on the actin cables. The constriction is driven by the hydrolysis of ATP in a cyclical biochemical reaction whereby myosin attaches to actin forming an actomyosin complex, followed by a pulling action by the myosin head (or S1 region), and finally the detachment of myosin from actin. Activation of this process is mediated by an increase in iCa^{2+} which can occur by numerous mechanism, one of which is the depolarization of the muscle cell and the opening of voltage-dependent Ca^{2+} channels. Interestingly, an increase in iCa^{2+} has been suggested to stimulate a process known as calcium-

induced calcium release (CICR) which would result in even more Ca^{2+} entering the cell (Bolton et al., 1999), although this has yet to be conclusively demonstrated in vascular smooth muscle (Ganitkevich and Isenberg, 1995; Kamishima and McCarron, 1996).

One of the major differences between skeletal muscle and smooth muscle is that in the latter, Ca^{2+}-induced constriction is not regulated by a troponin-tropomyosin mechanism. Instead, increased iCa^{2+} levels in smooth muscle activate Ca^{2+}/calmodulin dependent kinases which phosphorylate myosin light chains allowing them to move on actin cables. Interestingly, cAMP levels have been shown to modulate the sensitivity of the myosin light chain kinase to Ca^{2+}-calmodulin and hence can directly influence smooth muscle contractility (see Fig. 5) (McDaniel et al., 1994; Stryer, 1988).

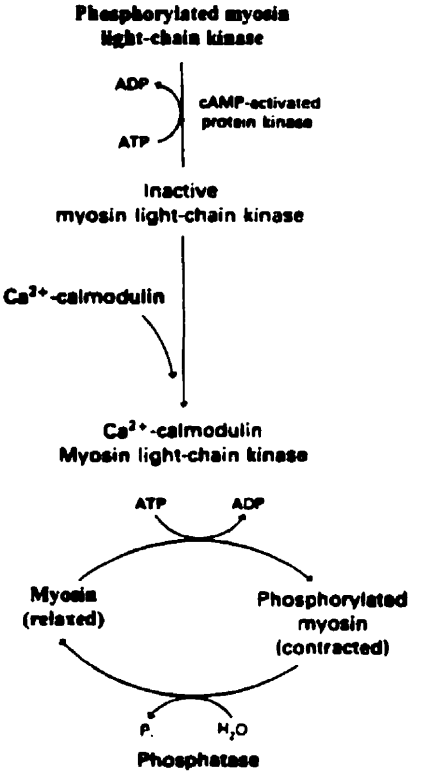

Fig. 5: The contraction of smooth muscle is mediated by phosphorylation of myosin. Ca^{2+}-calmodulin activates a specific kinase that phosphorylates the myosin light-chains, leading to contraction. cAMP mediates a cascade that phosphorylates the light-chain and lowers its affinity to Ca^{2+}-calmodulin (from Stryer, 1988).

Initiation and maintenance of muscle constriction is an active process that requires the expenditure of energy. Muscle relaxation and the subsequent dilation of blood vessels occurs largely by the reduction of iCa^{2+} levels resulting in the inhibition or reversal of some, or all, of the above described contractile mechanisms. Additionally there appears to be specific mechanisms by some substances such as cAMP and cGMP which can affect the activity of the myosin light chain and other contractile or cytoskelatal proteins to promote relaxation (McDaniel et al., 1994).

1.3 ACETYLCHOLINE AND CONTROL OF BRAIN MICROCIRCULATION

1.3.1 The Cholinergic Innervation of Brain Microvasculature

1.3.1.1 The Basal Forebrain and Intracortical Cholinergic Neurons

The cerebral cortex is innervated primarily (in rodents) or almost exclusively (primates) by cholinergic neurons originating from the a region of the brain called the basal forebrain (see Fig. 3). Cholinergic neurons from this region form a more or less continuous band of cells which can be divided into different areas based on size, location and arrangement within various nuclear regions. Since the basal forebrain is topographicaly organized; different areas project to distinct regions of the brain. The medial septal nucleus neurons project primarily to the hippocampus, with some projections to the cingulate cortex (Sofroniew et al., 1990). The vertical limb and the horizontal limb of the diagonal band of broca regions project to the cingulate cortex, the olfactory bulb and entorhinal cortex (Sofroniew et al., 1990; Zaborzsky et al., 1991). Finally neurons within the nucleus basalis of Meynert (NBM; also known as the CH4 group) and substantia innominata (SI) project to the entire neocortex, as well as the basolateral amygdala (Lamour et al.,1982; Rye et al., 1984; see Page and Sofroniew, 1996). These neurons are known to play a key role in cognitive functions such as attention, learning and memory as evidenced in lesion studies in rodents and primates (for review see Page and Sofroniew, 1996), as well as in patients with Alzheimer's disease where there is a severe degeneration of these cholinergic neurons (Whitehouse et al., 1982; Arendt et al., 1985; Geula et al., 1998).

The other source of cholinergic innervation in the cortex is from intrinsic bipolar cholinergic neurons which in rodents, contributes roughly one third of the cholinergic innervation (Eckenstein et al., 1988). These cells are evenly distributed across the cortical layers and exhibit very characteristic long dendrites spanning almost throughout the full extent of the cerebral cortex. Interestingly, they have also been shown to co-localize with vasoactive intestinal polypeptide (VIP) (Eckenstein and Baughman, 1984; Chédotal et al., 1994a, Bayraktar et al., 1997), a neuropeptide with vasomotor properties.

1.3.1.2 Innervation of Brain Intraparenchymal Microvessels by Cholinergic Neurons

There is now physiological and anatomical evidence for basal forebrain cholinergic neurons involvement in the control of the CBF. This would imply that neurons from the basal forebrain interact, either directly or indirectly, with the brain microvasculature.

Working independently, Lacombe's group in France and Sato's group in Japan were the first to report the effect of basal forebrain stimulation on CBF. They noted that both chemical (Biesold et al., 1989) and electrical (Lacombe et al., 1989; Biesold et al., 1989) stimulations of the basal forebrain increase ipsilateral cortical CBF up to 200% of the prestimulus control flow (for review see Sato and Sato, 1995). These increases were predominantly in the frontoparietal and occipital corticies, regions to which the basal forebrain projects (see Sato and Sato, 1995). They were also shown to be dependent on the cortical release of ACh (Kurosawa et al., 1988; Arnerić et al., 1988), but were not secondary to increased glucose utilization (Kimura et al., 1990; Vaucher et al., 1997a; Barbelivien et al., 1999), blood pressure variations caused by anaesthesia, or to changes in the diameter of the surface pial vessels (Adachi et al, 1992a). Lesioning of the SI results in decreases in blood flow in the parietal, frontal and occipital cortices, an indication that this response is constitutive (Gomi et al., 1991; Peruzzi et al., 1996). Interestingly, it appears that this neurovascular effect can be mediated directly and/or be relayed through local neurons (Iadecola et al., 1987; Linville et al., 1993a; Barbelivien et al., 1999), such as NOS neurons (see below).

The increase in CBF observed after electrical stimulation of the SI is attenuated by intravenous administration of NOS inhibitors (Adachi et al., 1992b; Raszkiewicz et al., 1992). The source of NO, however, is still unclear since there is evidence for innervation of these vessels by NOS positive neurons (Iadecola et al., 1993, Estrada et al., 1993; Regidor et al., 1993) as well as evidence for the involvement of endothelial NO (Zhang et al., 1995). The NO neurons could serve as relay neurons, since they are innervated by basalocortical cholinergic nerve terminals (Vaucher et. al, 1997b; Tong and Hamel, 1999) and express acetylcholine receptors (Moro et al., 1995a; Smiley et al., 1998). It is therefore quite possible that NO from

both the microvascular endothelial cells and the perivascular neurons are involved in the control of blood flow, with preferential activation for one or the other under different conditions (Iadecola and Zhang, 1996).

Intravenous application of antagonists for the muscarinic (Biesold et al., 1989; Dauphin et al., 1991a) and nicotinic (Biesold et al.,1989; Linville et al., 1993b) ACh receptors significantly attenuated the basal forebrain-induced dilation, implicating both these acetylcholine receptor types in this response. The nicotinic component was suggested to be due to blockade of nicotinic receptors in the basal forebrain itself, and not in the cortex (Linville et al., 1993b). Muscarinic and nicotinic receptor antagonists which cannot cross the blood brain-barrier were ineffective in attenuating the response (Biesold et al., 1989; Uchida et al., 1997), suggesting that the implicated receptors exist on either the adventitial side of the vessel wall, or in the surrounding cerebral tissue. Interestingly, both muscarinic and nicotinic receptors have indeed been identified in brain cortical microvascular fractions (Estrada et al., 1983; Grammas et al., 1983; García-Villalón et al., 1991; Kalaria et al., 1994), including in human (Kalaria et al., 1994; Linville and Hamel, 1995), although their function is still undetermined.

The physiological evidence detailed above provides support for a neural link between the basal forebrain and intraparenchymal microvessels that is dependent both on muscarinic and nicotinic ACh receptors, as well as on the synthesis and release of NO. Indeed, there is corroborating anatomical evidence of intimate associations between cholinergic terminals and intracortical blood vessels (Eckenstein and Baughman 1984; Arnerić et al., 1988; Chédotal et al, 1994a/b) which originate primarily from the basal forebrain (Vaucher and Hamel, 1995), although in the rat there is also a contribution from the intrinsic bipolar cortical cholinergic neurons (Galea et al., 1991; Chédotal et al., 1994a). Perivascular basal forebrain cholinergic terminals can directly abut on microvascular smooth muscle and endothelial cells (see Chédotal et al., 1994b; Vaucher and Hamel, 1995) thus allowing direct neurovascular interactions via acetylcholine receptors present on the microvessel wall (see section 1.3.3). This, however, does not rule out indirect interactions via perivascular astroglia or cortical

interneurons (Chédotal et al., 1994b; Vaucher and Hamel, 1995), such as the cholinoceptive NO neurons (Moro et al., 1995a; Smiley et al., 1998).

1.3.2 The Acetylcholine Receptors

Acetylcholine is involved in the regulation of numerous and diverse physiological activities from muscle contraction to heart rate to memory retention. It is therefore not surprising that the receptors to ACh are also diverse in structure and function. Signal transduction mediated by ACh receptors (AChRs) occurs through two very different classes of receptors, nicotinic and muscarinic, identified initially by their high affinity for nicotine and muscarine. Interestingly, these two types of receptors are totally unrelated in terms of structure and function. Nicotinic receptors (nAChRS) are ion channels, while muscarinic receptors (mAChRs) belong to the family of seven trans-membrane domain G-protein coupled receptors. The recent advent of new molecular biological tools has revealed that the extent of diversity within these two families of receptors is broader that originally suspected on the basis of pharmacology alone.

1.3.2.1 Nicotinic Receptors

The nAChR is a ligand-gated cation-selective ion channel composed of five subunits (Dani, 1993) namely α, β, δ, δ, and ϵ. The α and β units can be further subdivided into nine ($\alpha_{1\text{-}9}$) and four ($\beta_{1\text{-}4}$) subunits, respectively (Alexander and Peters, 1998). nAChRs have been identified in neurons and in striated muscle, where they mediate the synaptic transmission at the neuromuscular junction. In muscle, the functional pentameric unit typically consists of two α_1 units and one of each β_1, δ and depending on the developmental stage, either δ, or ϵ. The primary role for skeletal muscle nAChRs is to open a cation channel in order to induce a depolarization of the membrane. Unlike in striated muscle, the presence of nicotinic receptors in vascular smooth muscle cells has not been conclusively demonstrated (Tang et al., 1999).

Neuronal nAChRs, located in both the peripheral and central nervous system are much more

diverse in structure than those in muscle. Numerous combinations of the $\alpha_{(2-8)}$ or $\beta_{(2-4)}$ have been shown to form functional channels with different pharmacological and physiological properties (Albuquerque et al., 1996; Gotti et al., 1997). This diversity is necessary considering that these receptors can be localized both pre- and post-synaptically. Additionally neuronal nAChRs have a high permeability for Ca^{2+} suggesting a possible role in neuronal plasticity and learning (Dani, 1993). This is in line with the dementia observed in Alzheimer's disease where there is a substantial loss of nicotinic receptors (Araujo et al, 1988; Perry et al., 1995). nAChRs have also been implicated in many other neuropathologies such as epilepsy, schizophrenia and Tourette's syndrome, in addition to their well known roles in the modification of behaviour and smoking addiction (Gotti et al., 1997).

1.3.2.2 Muscarinic Receptors

Five different muscarinic receptor (mAChR) subtypes have been cloned (M1, M2, M3, M4, M5; Bonner et al., 1987, 1988; Peralta et al., 1987), although only the first four can be conclusively characterized pharmacologically in whole tissue functional studies, as defined by the Committee on Receptor Nomenclature and Drug Classification of the International Union of Pharmacology (NC-IUPHAR) nomenclature recommendations (Caulfield and Birdsall, 1998). Recently, a gene for a putative sixth receptor, m6, has been cloned, but due to patent restrictions, no details are available on this new member's function or pharmacology (see Eglen et al., 1999). mAChRs mediate the inhibitory and excitatory effects of ACh in many regions of the body such as the heart, glands, smooth muscle, blood vessels and the brain. In addition to being varied in their location, mAChR receptors vary in terms of their pharmacology, second messenger profile, and function.

1.3.2.2.1 Pharmacology and Second Messengers

Muscarinic receptors are difficult to study due to the paucity of highly discriminatory compounds whether they be selective mAChR agonists or antagonists (Caulfield and Birdsall, 1998). Identification of mAChRs therefore has to be performed on a comparative basis between antagonists with different affinity profiles for the different receptor subtypes (see

Table 1 for the affinities of some of the more commonly used mAChR antagonists). In the case of the first three receptor subtypes, however, this is made easier by the presence of somewhat selective compounds for each type. For example, the M1 subtype can be identified by its high affinity for pirenzepine and 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) and its low affinity for AF-DX 384 ((±)-5,11-dihydro-11-{[(2-[(dipropylamino)methyl]-1-piperidinyl}ethyl)amino]carbonyl}-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one). In contrast the M2 receptor has a high affinity for AF-DX 384 and a low affinity for pirenzepine. The M3 receptor, like M1, has a high affinity for 4-DAMP, however, it exhibits a comparatively lower affinity for pirenzepine.

Unlike the other mAChRs, the M4 and M5 receptors were initially identified by molecular biology and pharmacologically characterized in transfected cells (Dorje et al., 1991). Many of the earlier studies grouped these receptors along with the M1, M2 or M3 subtypes. Recently studies have provided more complete pharmacological profiles of these subtypes (see Caulfied and Birdsall, 1998) however, they still remain difficult to discriminate (Eglen and Watson, 1996). This applies in particular to the M5 subtype, which until very recently was identified by the lowercase appellation (i.e. m5) denoting its lack of full characterization according to NC-IUPHAR guidelines. Functional studies of this receptor have been limited to transfected cells since no tissue expressing a predominance of the native M5 has been identified. From these studies, it was shown that M5 is the mAChR that couples the most potently with NOS activation (Wang et al. 1994, 1996). The recent discovery of a human melanoma cell line that expresses the M5 receptor (Kohn et al., 1996) will greatly aid in the further characterization of this receptor

The second messenger profile of the mAChRs can be another means of discriminating between the different mAChR subtypes. The inositol phosphate pathway is activated by the M1, M3 and M5 receptor subtypes through pertussis toxin-insensitive $G_{q/11}$ proteins which stimulate phospholipase C (PLC). The evenly number mAChRs, M2 and M4, couple to adenylate cyclase inhibition through the pertussis toxin sensitive protein G_i (Felder, 1995; see Table 1).

Table 1: AFFINITY AND 2nd MESSENGER PROFILES FOR THE FIVE MUSCARINIC RECEPTORS*

Antagonists	M1	M2	M3	M4	M5
Atropine	9.0-9.7	9.0-9.3	8.9-9.8	9.1-9.6	8.9-9.7
4-DAMP †	8.6-9.2	7.8-8.4	8.9-9.3	8.4-9.4	8.9-9.0
Pirenzepine †	7.8-8.5	6.3-6.7	6.7-7.1	7.1-8.1	6.2-7.1
AF-DX384 †	7.3-7.5	8.2-9.0	7.2-7.8	8.0-8.7	6.3
Himbacine	7.0-7.2	8.0-8.3	6.9-7.4	8.0-8.8	6.1-6.3
Methoctramine	7.1-7.8	7.8-8.3	6.3-6.9	7.4-8.1	6.9-7.2
p-F-HHSiD	7.2-7.7	6.0-6.9	7.8-7.9	7.5	7.0
2nd Messenger	↑IP_3	↓cAMP	↑IP_3	↓cAMP	↑IP_3

*Based on articles by Alexander and Peters (1998) and Caulfield and Birdsall (1998).
†Antagonists used in this thesis

1.3.2.2.2 Function(s)

It now is apparent that the mAChRs span a large range of functions in different cells types. mAChRs mediate different responses in heart, glands, brain, as well as numerous other organs. This section will provide an overview of the major areas in which mAChRs have been identified. A more detailed account of these receptors with regards to the circulatory system, in particular the cerebrovascular one, will be provided in the following section (1.3.3).

• Smooth and Heart Muscle

mAChRs have been implicated in the mediation of smooth muscle function. For example M1 receptors are known to mediate the contraction of the canine saphenous vein (see Caulfield and Birdsall, 1998) and cat pial vessels (Dauphin et al., 1991b). The M3 on the other hand has been suggested as the receptor mediating contractile function of guinea-pig ileum, as well as the endothelium dependent vasodilation of vascular smooth muscle (see below). There are other examples of mAChRs mediating smooth muscle function, mainly contraction, in the uterus, urinary bladder, gut, airways and lungs; however, the exact subtypes involved are still not fully identified (see Eglen and Watson, 1996). In heart muscle, the M2 receptors seem to be involved in the reduction of the heart rate by inhibition of voltage gated Ca^{2+} channels and activation of inwardly rectifying K^+ channels (reviewed by Caulfield, 1993).

• Neuronal

mAChRs are important regulators of neuronal function. In the peripheral nervous system, the M1 receptor is known to mediate the depolarization of rat isolated superior cervical ganglions by the inhibition of the voltage-gated M-type K^+ channels (Bernheim et al., 1992). Pre-synaptic M4 receptors seem to modulate the release of neuronal NO involved in the dilation of the rabbit anococcygeus muscle (Gross et al., 1997). Similarly, pre-synaptic M2 mAChRs have been implicated in the control of vessel tone by modulation of neurotransmitter release from perivascular nerve terminals (Liu and Lee, 1999; Casado et al, 1994; Alonso et al, 1991). Several other examples of both pre- and post-synaptic actions by mAChRs exist, however the exact receptor subtype(s) involved are still unknown (Caulfield and Birdsall, 1998).

In the central nervous system, mAChRs have been identified in many regions of the brain and suggested to be involved in memory mechanisms, as evidenced by reports of disruption of memory processing by centrally-active antimuscarinic compounds (see Caulfield 1993). This would be consistent with the idea that loss of cholinergic inputs to the hippocampus and cortex in Alzheimer's disease is responsible for memory disorders, a view supported in animal studies which reported disruption of cognitive functions after lesions of neurons in the basal forebrain (Ridley et al., 1986). The hippocampus expresses the five mAChR subtypes (see Brann et al., 1988; Tice et al., 1996) which appear to be functional as evidenced by recordings of muscarinic postsynaptic action potentials in the septo-hippocampal pathway (Pitler and Alger, 1992) as well as the involvement of the hippocampal post-synaptic M2 receptor in neuronal plasticity (see Segal and Auerbach, 1997). Finally, the M1 mAChR, along with nerve growth factor, may serve to augment neurite outgrowth and modulate secretion of amyloid precursor protein (APP; Nitsch et al., 1992; Buxbaum et al., 1992, see Eglen et al., 1999). M1 agonists may thus putatively play an important role in the prevention of the formation of beta-amyloid plaques, a key component of Alzheimer's disease. Centrally active mAChRs may also play roles in other disorders besides Alzheimer's disease, as they have been targeted for the treatment of Schizophrenia and pain (Eglen et al., 1999)

• Glands

Stimulation of mAChRs produces secretion from exocrine glands of various types such as tear glands, sweat glands, salivary glands and pancreas. While there is evidence for several mAChRs involvement in this response, the M3 receptor is the only one that has been conclusively identified by pharmacology, immunoreactivity and molecular biology in glandular tissue (see Caulfield, 1993; Caulfield and Birdsall, 1998).

1.3.3 Acetylcholine Receptors and the Cerebral Vasculature

If, as physiological and anatomical studies indicate (see section 1.3.1.2), direct functional interactions between cholinergic neurons and blood vessels take place, then receptors for ACh should exist in the brain microvasculature. Indeed, there is evidence for the presence of both

types of ACh receptors in the brain microcirculation, their role however is still not well defined mainly due to the technical limitations inherent in studying brain intraparenchymal microvessels.

1.3.3.1 Nicotinic Receptors

Unlike at the neuromuscular junction, where the localization of nAChRs is well defined, there is very little evidence for nicotinic receptors on vascular tissue. Recently, electrophysiological responses to nicotine in isolated rat vascular smooth muscle cells have been recorded (Tang et al., 1999) and nAChRs were identified by binding and *in situ* hybridization in human as well as bovine endothelial cell cultures, albeit from peripheral vessels (Macklin et al., 1998). nAChRs binding sites (Kalaria et al, 1994) have also been reported in both porcine and human brain pial and intraparenchymal vessels. These last findings however, can be questioned since the microvessel isolation technique does not exclude nerve terminals and astroglial processes still attached to the vessels. Furthermore and most surprisingly, the levels of nicotinic binding sites observed in microvessels were very high with respect to those found in brain tissue. This finding therefore needs to be confirmed before a definite conclusion can be reached.

Nicotinic receptors have been shown to induce endothelium-independent vasodilation in canine and cat cerebral arteries (Toda, 1976; Ayajiki et al., 1994; Toda et al., 1995) presumably via the release of NO from perivascular nerve terminals attached to the isolated vessels (Toda et al., 1995, Zhang et al., 1998). It is thus conceivable that similar mechanism exists at the level of the intracerebral microcirculation, since cholinergic terminals have been shown to contact perivascular NO neurons (Vaucher et al., 1997b; Tong and Hamel, 1999). This association could explain the nicotinic component of the dilation induced by the basal forebrain induced stimulation (see section 1.3.1.2) and the increase in CBF observed after intravenous injection of nicotine in rats (Uchida et al., 1997).

1.3.3.2 Muscarinic Receptors

The majority of information regarding the vascular function of muscarinic receptors comes

from studies on extracerebral and peripheral vessels, many of which were performed before the cloning and the establishment of a pharmacological profile of individual mAChRs. It is known from these studies that stimulation of vascular mAChRs will in most cases produce a vasodilation, although vasoconstrictions have also been observed. The variability in this response can depend on parameters such as the species, the concentration of ACh, the presence of an intact endothelial layer, the mAChR subtypes involved, and their location in the vessel wall. For example high concentrations (10^{-5} to 10^{-3} M) of ACh will activate M1 and/or M2 mAChRs to induce a vasoconstriction of smooth muscle in extracerebral or peripheral arteries (Van Charldorp et al., 1988, Dauphin et al., 1991b, Jaiswal et al., 1991; Shimizu et al., 1993). Interestingly, while the M1 receptor appears to function directly at the level of the vascular smooth muscle, there is evidence that in porcine cerebral arteries the M2 receptor acts presynaptically by modulating the release of NO (Liu and Lee, 1999) or noradrenaline (Alonso et al., 1991; Casado et al., 1994) from perivascular fibers.

The endothelial M3 receptor subtype on the other hand, is thought to mediate the dilation of both extracerebral and peripheral vessels to low (10^{-9} to 10^{-6} M) ACh concentrations (Dauphin and Hamel, 1990; Shimizu et al., 1993; Casado et al., 1994). This vasodilation is presumed to occur via the release of EDRF or NO, which subsequently diffuses into smooth muscles to induce relaxation (see section 1.2.2.1. and Fig 4). When the endothelial layer is removed, cerebral blood vessel will not dilate to ACh, but, in some cases, will constrict at high concentrations (Shimizu et al., 1993, for review see Dauphin and McKenzie, 1995).

At the level of the intraparenchymal microcirculation, mAChR have been evidenced (Peroutka et al., 1980; Estrada et al, 1983; Moro et al. 1995b), and specific mAChR subtypes identified (García-Villalón et al., 1991; Linville and Hamel, 1995; Levey et al., 1995; Smiley et al., 1998). In man, early reports on cerebromicrovascular mAChRs have been unconvincing (Ferrari-Dileo and Potter, 1985; O'Neill et al., 1988), whereas more recent radioligand binding studies suggested the presence of heterogenous mAChRs in microvessels and capillaries isolated from human cerebral cortex (Linville and Hamel, 1995). However, the relative

absence of subtype-selective ligands for mAChRs made it difficult to conclusively identify their nature, and the complexity of the cell types contained within isolated microvascular fractions further hampered their localization to a specific cellular compartment within the vessel wall (Linville and Hamel, 1995; Moro et al., 1995b).

In terms of function, Dacey and Bassett (1987) have shown that ACh induced vasodilation of isolated rat penetrating arterioles preconstricted by either 10^{-4}M $PGF_{2\alpha}$ or 10^{-6}M 5-HT. This dilation was inhibited by atropine, a non-selective muscarinic receptor antagonist. Using a different technique on brain slices, Sagher et al. (1993) were able to show a similar cholinergic (ACh 10^{-5}M) vasodilation following preconstriction with norepinephrine (10^{-7} M). No attempts were made to characterize the mAChR subtype(s) involved or the role of endothelial cells and NO in this response. Such studies are essential for a better understanding of acetylcholine's effect on cerebral microvascular tone, and hence CBF under both normal and pathological conditions.

1.4 SEROTONIN AND THE CONTROL OF BRAIN MICROCIRCULATION

1.4.1 The Serotonergic Innervation of Brain Microvasculature

1.4.1.1 The Raphe Nuclei

The brainstem raphe nuclei are the origin of most of the vast serotonergic network found in the brain with projections to the cerebral cortex, caudate-putamen, substantia nigra, hippocampus, amygdala, thalamus, and hypothalamus. These neurons are involved in a variety of different activities such as sleep, locomotion, sexual behaviour, mood and pain (Jacobs and Azmitia, 1992). It now appears that 5-HT neurons, in particular those in the dorsal raphe nucleus (DRN) which projects largely to the cortex, are also be involved in the control of local CBF (see Fig. 3; Cohen et al., 1996).

1.4.1.2 Innervation of Brain Intraparenchymal Microvessels by the Raphe Nucleus

A direct link between central 5-HT neurons and the control of brain microcirculation was first suggested by Reinhard et al., (1979) who showed that lesions of the raphe nuclei would

decrease the 5-HT content of rat brain microvessels. This finding was further supported by studies demonstrating significant changes in CBF upon electrical or chemical stimulation of the DRN (Goadsby et al., 1985a,b; Bonvento et. al, 1989; Underwood et al., 1992; Cao et al., 1992). The overall change in blood flow upon raphe stimulation was a decrease in CBF, however, in some cases an increase in blood flow was observed (Goadsby et al., 1985; Underwood et al., 1992; Cudennec et al., 1993). These changes in blood flow were found to be dependent on 5-HT release, as the response could be abolished by the application of 5-HT_1 (methysergide) or 5-HT_2 (ketanserin) receptor antagonists (Cao et al., 1992). Lesioning of the raphe nuclei, however, did not result in any significant changes in CBF, an observation which led to the suggestion that the effect of 5-HT on cerebral perfusion is phasic and not tonic (Underwood et al., 1992; Cudennec et al., 1993). Furthermore, the changes in blood flow were shown to be, at least in part, independent from changes in glucose metabolism (Bonvento et al., 1991; Cudennec et al., 1993), thus suggesting a direct effect at the level of the local intraparenchymal blood vessels.

The dual vascular response upon DRN stimulation has been attributed to factors such as the initial tone of the blood vessels (Rosenblum and Nelson 1990), the subregion within the DRN stimulated (Underwood et al. 1992), and the method used to measure blood flow (see Cohen et al., 1996). The recent identification of numerous 5-HT receptor subtypes in brain microvascular tissue and cell types could also be an important factor in this variability (Cohen et al., 1999).

In addition to the functional studies, there is also ultrastructural evidence for the association of serotonergic terminals with brain intraparenchymal microvasculature (Descarries et al.,1975; Itakura et al., 1985). An analysis of the neurovascular serotonergic interactions in different brain regions, suggested a correspondence between the frequency and proximity of perivascular 5-HT terminals and areas showing significant blood flow changes upon stimulation of central 5-HT neurons (Cohen et al., 1995), thus implying that these associations could be functional. The 5-HT nerve terminals would in some cases make direct contact with

the basement membrane of microvessels (Itakura et al., 1985; Cohen et al., 1995) but more frequently with the surrounding astroglial leaflets (Cohen et al., 1995), two compartments known to possess 5-HT receptors (Hirst et al., 1997; Cohen et al., 1999). There are, however, a large number of 5-HT terminals that interact with neuronal elements within the perivascular space. It is possible that 5-HT acts on intermediary neurons to indirectly influence the release of other vasoactive substances such as ACh, or NO. This could also contribute to the dual response observed upon the stimulation of the central 5-HT neurons (Cohen et al., 1996).

1.4.1.3 Other sources of 5-HT in the brain

The blood circulating platelets are an alternative source of 5-HT that can affect vasomotor tone. Indeed it has been shown that 5-HT release from platelets can vasoconstrict cerebral arteries (Lopez de Pablo et al., 1991). However under normal physiological conditions, release of 5-HT from platelets is negligible and most likely not a factor in the control of cerebral perfusion.

1.4.2 The Serotonin Receptors

The recent re-classification of 5-HT receptor subtypes based on their functional (drug-related), transductional (receptor-coupling) and structural (primary amino acid sequence) properties, has lead to the characterization of seven receptor classes, 5-HT_1 5-HT_2, 5-HT_3, 5-HT_4, 5-ht_5, 5-ht_6, and 5-HT_7 with each class being further divided into sub-classes (e.g. 5-HT_{2A}, 5-HT_{2B}, etc., Hoyer et al., 1994). The 5-ht receptors identified by only molecular biology retain the lowercase appellation until their functional and transductional requirements are characterized. All 5-HT receptors are G-protein coupled, with the exception of 5-HT_3 subtype, which is a ligand-gated ion channel. In the interest of brevity and practicality, only the 5-HT receptors classes that have been shown to be involved in the mediation of vascular function will be discussed in detail. This consists mainly of the 5-HT1, 5-HT2 and 5-HT7 family of receptors. For more information about any of the 5-HT receptors, please refer to Table 2 and the review articles by Hoyer et al. (1994) and Hoyer and Martin (1997).

TABLE 2: 5-HT RECEPTORS PHARMACOLOGY, BIOCHEMISTRY AND LOCALIZATION IN CEREBROMICROVASCULAR TISSUE*

Receptor	Agonist	Antagonist	Effector	EC	SMC	AST
$5\text{-}HT_{1A}$	8-OH-DPAT, 5-CT	WAY100135	⇓cAMP			
$5\text{-}HT_{1B}$	Sumatriptan SB216641	GR127935 GR55562	⇓cAMP	-/+ †	++++	++
$5\text{-}HT_{1D}$	Sumatriptan PNU 109291	GR127935	⇓cAMP	++	+	++
$5\text{-}ht_{1E}$.	5-HT		⇓cAMP			
$5\text{-}ht_{1F}$	LY344864		⇓cAMP	-	-	+
$5\text{-}HT_{2A}$	α-Me-5-HT	Ketanserin MDL100907	⇑IP_3	-	-	++++
$5\text{-}HT_{2B}$	α-Me-5-HT BW723C86	SB204741 SB200646	⇑IP_3	+++	++++	++++
$5\text{-}HT_{2C}$	α-Me-5-HT	Mesulergine SB200646	⇑IP_3			

(cont. on next page)

*Based on review articles by Hoyer et al., (1994) and Hoyer and Martin (1997). Expression in microvascular tissue from Bouchelet et al., (1997), and Cohen et al., (1999). EC: Endothelial Cell Cultures, SMC: Smooth Muscle Cell Cultures, AST: Fetal Astroglial Cell Cultures, (-) denotes absence of PCR products for the receptor subtype, (+, ++, +++,++++) present, based on intensity and expression in cell lines. †(-/+) absent in endothelial cell cultures from capillaries, but present in endothelial cells of microvessels (Riad et al., 1998).

TABLE 2 (Cont.): 5-HT RECEPTORS PHARMACOLOGY, BIOCHEMISTRY AND LOCALIZATION IN CEREBROMICROVASCULAR TISSUE

Receptor	Agonist	Antagonist	Effector	SMC	EC	AST
$5\text{-}HT_3$	2-Me-5-HT m-chlorophenyl-biguanide	granisetron ondansetron tropisetron	Ligand gated cation channel			
$5\text{-}HT_4$	BIMU 8, RS67506 ML10302	GR113808 SB204070	↑cAMP			
$5\text{-}ht_{5A}$	5-HT, 5-CT	LSD	⇓cAMP ?			
$5\text{-}ht_{5B}$	5-HT, 5-CT	LSD	?			
$5\text{-}ht_6$	5-HT, 5-MeOT	LSD Methiothepine	↑cAMP			
$5\text{-}HT_7$	5-HT, 5-MeOT 5-CT	Methiothepine Mesulergine	↑cAMP	++	+++	++

1.4.2.1 5-HT$_1$ Receptors

There are now five identified 5-HT$_1$ receptors, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-ht$_{1E}$, and 5-HT$_{1F}$. They are all characterized by their high affinity (nanomolar range) for 5-HT, and are seven transmembrane $G_{i/o}$ protein coupled receptors which preferentially link with the attenuation of adenylate cyclase activity (see Uphouse, 1997).

1.4.2.1.1 The 5-HT$_{1A}$ receptor

The 5-HT$_{1A}$ receptor is located predominantly in the brain and is known to mediate mood and anxiety. This receptor can be found both pre- and post-synaptically, and in its prejunctional localization, can be a somatodendritic autoreceptor mediating 5-HT release from raphe neurons (Sprouse and Aghajanian, 1987). It can also function as a heteroreceptor inhibiting the release of ACh from enteric cholinergic neurons (Fozard and Kilbinger, 1985). In addition to attenuating adenylate cyclase activity via the pertussis-toxin sensitive protein, G_i, it has also been shown to couple to inositol phosphate production at high concentrations of 5-HT (Fargin et al., 1989). Although this receptor does not appear to mediate vascular tone, 5-HT$_{1A}$ receptor agonists are known to produce a decrease in heart rate and blood pressure by activation of central 5-HT receptors (Doods et al., 1988; Dreteler et al., 1990).

1.4.2.1.2 The 5-HT$_{1B}$ receptor

Originally thought to be specific to rats and some other rodents, the 5-HT$_{1B}$ receptor was previously identified in man as the 5-HT$_{1D\beta}$ receptor gene (Adham et al., 1992, Hartig et al., 1996). While the two receptor share a high homology, they have different pharmacological profiles (Hamblin et al. 1992). In the brain, this receptor can be found in the basal ganglia, most notably in the globus pallidus and the pars reticularis of the substantia nigra (Bruinvels et al., 1993, 1994). The 5-HT$_{1B}$ receptor is present both pre- and post-synaptically, and in the former case has been shown act as both as a terminal autoreceptor controlling the release of 5-HT (Pineyro et al., 1995; Roberts et al., 1996) as well as a hetero-receptor mediating the release of ACh, glutamate and noradrenaline (see Martin and Humphrey, 1994). This receptor is coupled to inhibition of adenylate cyclase, but has also been shown to mediate increases in

intracellular Ca^{2+} levels (Ebersole et al., 1993; Zgombick and Branchek, 1998). In blood vessels, it can mediate the constriction, and possibly the dilation, of vascular smooth muscle (see section 1.4.3.2). In the extracerebral circulation, it is largely viewed as the target of the anti-migraine drug sumatriptan, a selective 5-$HT_{1B/1D/1F}$ agonist, although its exact role in migraine treatment still remains to be established.

1.4.2.1.3 The 5-HT_{1D} receptor

This receptor, formally known as the 5-$HT_{1D\alpha}$ in humans, is found mainly in non-rodent species, although it also exists in low amounts in rodents (Bruinvels et al., 1993, 1994). It has been localized both pre- and post-synaptically and could possibly function as either an auto- or hetero-receptor (Pineyro et al., 1996). This receptor shares a very similar pharmacological profile with the 5-HT_{1B} receptor and in earlier studies, receptors identified as 5-HT_{1D} would be inclusive of both the 5-HT_{1B} and 5-HT_{1D} receptor subtypes (Hoyer and Martin, 1997). This could partially explain the identification of the 5-HT_{1D} receptor in the mediation of vasomotor effects, a property that was later attributed to the 5-HT_{1B} receptor (Hamel et al., 1993b; Bouchelet et al., 2000). The 5-HT_{1D} subtype can serve as a somatodendritic as well as a terminal autoreceptor on raphe neurons and their terminals, and has been implicated in the inhibition of trigeminal firing (Moskowitz, 1993). The identification of a functional role for this receptor will be greatly facilitated by the recent discovery of a selective agonist (PNU-109291; Ennis et al., 1998).

1.4.2.1.4 The 5-ht_{1E} and 5-HT_{1F}

Little is known about the 5-ht_{1E} and 5-HT_{1F} receptors, the most recent additions to the 5-HT_1 family. Both attenuate adenylate cyclase activity, while the 5-HT_{1F} has also been shown to activate IP_3 formation in some cases (Adham et al, 1993). The precise function for both receptors is not known, although the 5-HT_{1F} receptor has been implicated in migraine pathophysiology, where it appears to regulate the release of peptides from trigeminovascular sensory afferents (Johnson et al., 1998; Mitsikostas et al., 1999). The recent identification of selective agonists for 5-HT_{1F} (Phebus et al., 1997; Razzaque et al., 1999) should expedite the

identification of a functional role for this receptor.

1.4.2.2 5-HT$_2$ Receptors

There are three recognized 5-HT$_2$ receptor subtypes 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$, all of which are preferentially coupled to phospholipase C by $G_{q/11}$. Receptor activation would therefore lead to stimulation of phosphoinositide metabolism and increase in IP_3 levels. The affinity of these receptors to 5-HT is in the micromolar range.

1.4.2.2.1 The 5-HT$_{2A}$ receptor

This receptor was formally known as the 5-HT$_2$ receptor and is widely distributed in the peripheral and central nervous system. Its precise role in the CNS remains unclear, although it has been shown to be involved in motor and sleep mechanisms (Sharpley et al., 1990; Martin and Humphrey, 1994). In the periphery, it plays a large role in the mediation of constriction in vascular as well as non-vascular smooth muscle, although the extent of its role can vary from species to species. There is also evidence for a role in the release of other neurotransmitters such as ACh, adrenaline, dopamine, and vasopressin (Feniuk et al., 1981; Muramatsu et al., 1988a,b; Rittenhouse et al, 1990; see Martin and Humphrey, 1994).

1.4.2.2.2 The 5-HT$_{2B}$ receptor

This receptor was originally classified as the 5-ht$_{2F}$ due to its identification in the stomach fundus, where it mediates fundic smooth muscle contraction. It is also known to be involved in the constriction of other smooth muscle, such as the longitudinal muscle in the human intestine (Baxter et al., 1994). More recently, this receptor has been identified in rat heart, lung, kidney and discrete regions of the human but not rat brain (Foguet et al., 1992; Kursar et al. 1994; Schmuck et al., 1994). This receptor also has been localized in blood vessels (Ullmer et al, 1995; Bouchelet et al., 1997) where it could possibly mediate an endothelium-dependent vasodilation via NO release (Ellis et al., 1995; Ullmer et al., 1995; Schmuck et al., 1996).

1.4.2.2.3 The 5-HT_{2C} receptor

Pharmacologically very similar to the 5-HT_{2B} receptor, this receptor (formally known as 5-HT_{1C}) has been implicated in brain functions that affect eating, anxiety and locomotion (Martin and Humphrey, 1994). A possible role in the control of vasomotor tone has been ascribed to this receptor, however, this is most likely due to the inability to discriminate this subtype from the 5-HT_{2B} receptor, since it appears that there is no expression of this receptor in blood vessels (Ullmer et al., 1995; Schmuck et al., 1996; Bouchelet et al., 1997) .

1.4.2.3 The 5-HT_7 Receptors

These receptors have been identified both in the brain and smooth muscle and are known to increase adenylate cyclase activity via G_s upon stimulation. They are thought to be implicated in the circadian rhythm as evidenced by their presence in the suprachiasmatic nucleus (Inouye and Shibata, 1994). They are also known to mediate vasodilation in both vascular and non-vascular smooth muscle (Ullmer et al., 1995; Carter et al., 1995). Recently, 5-HT_7 receptors have been implicated in the dilation of canine cerebral arteries and suggested as a potential target in migraine prophylaxis (Terron et al., 1998).

1.4.3 Serotonergic Receptors and Cerebral Microvasculature

1.4.3.1 Identification

Despite strong anatomical and physiological evidence for the innervation of cerebral microvessels by serotonergic neurons (see section1.4.1), there has not been, until very recently, much information available about the nature or location of 5-HT receptors in brain microvasculature. Early studies provided evidence for 5-HT_1 receptors in human brain microvessels (O'Neill et al., 1988), and 5-HT was shown to stimulate cAMP in human brain endothelial cell cultures (Bacic et al., 1991). More recently, Cohen et al., (1999) demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) and second messenger assays, the presence of numerous 5-HT receptors in brain microvascular tissue and cell cultures. The results from this study (summarized in Table 2) when viewed in regards to receptors that mediate vasomotor function, illustrated the presence of functional 5-HT_{1B} and 5-HT_7

receptors and the absence of 5-HT_{2A} receptors in smooth muscle cells cultures. Using antibodies for the 5-HT_{1B} receptor, Riad et al. (1998) demonstrated the presence of this receptor in the endothelial cells and smooth muscle of human brain microarterioles, and its absence from endothelial cells in capillaries, prompting speculation as to whether it can be involved in arteriolar endothelium-dependent vasodilation.

1.4.3.2 Function

As with ACh, technique limitations have hindered the characterization of the function of 5-HT on brain intraparenchymal vessels. The sole exception is an *in vitro* study on isolated brain intracerebral arterioles by Dacey and Basset (1987), demonstrating a small dose-dependent constriction to 5-HT in rat intracerebral arterioles. In order to obtain a better picture of the effect of 5-HT on microvascular tone, it is necessary to extrapolate information from studies on the extracerebral and peripheral circulation. In this regard, 5-HT can mediate both constriction and dilation of blood vessels depending on receptor subtypes and anatomical localization, as well as in some cases, the size of the vessel.

1.4.3.2.1 Constriction

The dominant vasomotor response to 5-HT is constriction, and it is mediated in most cases by either the 5-HT_{1B} and/or the 5-HT_{2A} receptor depending on the species and vascular segment studied. Indeed, constriction in cerebral blood vessels can be mediated primarily by 5-HT_{2A} receptors as in the rat, sheep and rabbit, or by 5-HT_{1B} as in human and guinea pig vessels. In the monkey, dog, bovine and ovine vessels, the response appears to be mediated by a combination of both the 5-HT_{1B} and 5-HT_{2A} (for review see Lincoln, 1995). In some cases the extent of participation of each type of receptor can vary based on different parameters. In this regard, it has been shown that constriction to 5-HT in smaller vessels (Teng et al., 1998) and vessels with vascular tone (MacLean et al., 1994) tends to be preferentially meditated by the 5-HT_{1B} receptor as opposed to the 5-HT_{2A}. In human cerebral microvessels, as in human pial vessel (Hamel et al,. 1993a; Kaumann et al., 1993; Bouchelet et al., 1996), there is an absence of 5-HT_{2A} receptors and a strong expression of 5-HT_{1B}

receptors (Cohen et al., 1999). While there are no functional blood flow studies specific to the brain intraparenchymal microcirculation, infusion of sumatriptan, a 5-$HT_{1B/1D/1F}$ agonist, is known to result in an overall decrease in blood flow, implying at least a partial role for 5-HT_1 receptor in the control of CBF (Kobari et al., 1993; Fukuda et al., 1999). It should also be noted that in a very recent study on the extracerebral blood vessels, sumatriptan's vasoconstrictor properties were shown to be mediated exclusively by 5-HT_{1B} and not the 5-HT_{1D}, or 5-HT_{1F} subtypes (Bouchelet et al., 2000).

1.4.3.2.2 Dilation

In addition to its constricting properties 5-HT has, in some cases and under certain conditions, been shown to dilate blood vessels in both cerebral and peripheral blood vessels. While the exact mechanisms and receptor subtypes implicated in this dilation are still unclear, the physiological consequence of this response could be particularly important in pathological conditions such as migraine headache (see Fozard, 1995; Terron, 1998).

• Endothelium Dependent

Studies have demonstrated an NO and/or endothelium-dependent dilation to 5-HT and/or its agonists in peripheral blood vessels (Cocks and Angus, 1983; Schoeffter and Hoyer, 1990; Whiting and Cambridge 1995). There are two candidates for the 5-HT receptor mediating this response. In several studies this dilation was shown to be meditated by a 5-HT_1 receptor located in the endothelium (Moldering et al., 1989; Whiting and Cambridge, 1995; Lamping, 1997), most likely of the 5-HT_{1D} (or 5-HT_{1B}) subtype (Schoeffter and Hoyer 1990; Gupta, 1992). Other studies have focussed mainly on the 5-HT_{2B} receptor (Schmuck et al, 1996, Fozard and Kalkman 1994) as the mediator of this type of dilation. Since the 5-HT_{1D}, 5-HT_{1B} and the 5-HT_{2B} have been identified in brain microvascular endothelial cells (Cohen et al., 1999, Bouchelet et al., 1997) and have been shown to couple to second messenger mechanisms that can lead to NO production in endothelial cells (Zgombick et al., 1993, Ishida et al., 1998), it is possible that any of these receptors is involved in the mediation of vasodilation at the level of the brain microcirculation.

•Endothelium Independent:

5-HT has also been shown to elicit dilation in endothelium-denuded cerebral arteries. This response has been attributed to the 5-HT_7 receptor localized in the smooth muscle layer (see Terron, 1998). The identification of 5-HT_7 in the smooth muscle cell cultures from human brain microvasculature (Cohen et al., 1999), permits speculation that it may also play a vasomotor role in this vascular segment.

1.4.3.2.3 Influence of Vessel Size

One interesting phenomenon regarding 5-HT's vasomotor properties is that the type of response can be highly dependent on the size of the vessel. Early studies on pial vessels from cat demonstrated that large cerebral blood vessel typically constrict in response to 5-HT whereas smaller vessel tend to dilate (Harper and MacKenzie, 1977; Auer et al., 1985). Differences in tone (Harper and MacKenzie, 1977), blood brain permeability (Auer et al., 1985) and receptor distribution (Lamping, 1997) are some of the proposed explanations for this phenomena.

1.5 HYPOTHESIS AND OBJECTIVES

Hypothesis: Multiple acetylcholine and serotonin receptors are present within different cellular compartments of the brain microcirculation and have the ability to result in different vasomotor functions.

In order to examine the above hypothesis, we propose the following objectives:

i) To identify, by RT-PCR and second messenger assays, the muscarinic receptor subtypes present in human brain microvascular fractions and in microvascular and astroglial cells in culture.

ii) To set up an *in vitro* technique that permits the study of intraparenchymal brain microvessels under normal physiological conditions.

iii) To utilize this technique to investigate the vasomotor response(s) of the cerebral microcirculation to cholinergic and serotonergic stimulation, and to pharmacologically characterize the receptor(s) involved in the response(s) and examine the role of NO in their mediation

PREFACE TO CHAPTER 2

The basal forebrain is a major, if not exclusive, source of cholinergic innervation to the cerebral cortex and it plays an important role in the regulation of cerebral blood flow (CBF) within specific subdivisions of the neocortex. Recent anatomical studies have shown that this cholinergic input also reaches the microarterioles and capillaries located within the rat and human cortical parenchyma. The presence of such neurovascular associations is compatible with several physiological studies in the rat showing that electrical or chemical stimulation of the basal forebrain results in considerable increases in CBF ipsilateral to the side of stimulation (for review see Sato and Sato, 1995) which appear to be independent of changes in glucose metabolism. Blockers for muscarinic or nicotinic receptors, and for nitric oxide synthase are able to attenuate this response. These observations lead to the hypothesis that neurally released ACh most likely exerts a vasodilatory effect on the microvascular bed with NO as an intermediary. In this respect, muscarinic and/or nicotinic receptors have been shown to be involved in the vasodilation elicited by basal forebrain stimulation, and it has been suggested that some receptors are likely to be present on intraparenchymal vessels. Indeed, the presence of mAChRs in brain microcirculation had been demonstrated (Estrada et al., 1983) and in some cases multiple subtypes identified, using radioligand binding techniques (Garcia-Villalon et al., 1991; Linville et al., 1995). Some of the receptors corresponded to those pharmacologically characterized as mediators of the cholinergic vasoconstriction or dilation in extracerebral arteries. However, due to the paucity of selective ligands, and the inability to discriminate between different compartments in the brain microvasculature, the results from these studies were contradictory and inconclusive.

In this chapter we present the results from a study that uses the selective RT-PCR technique to identify the different mAChR subtypes present in human brain intraparenchymal microvessels and capillary fractions. Further, the receptors were localized in the different compartments of the brain microvessel wall through the use of endothelial, smooth muscle and astroglial cell cultures. Finally, to ensure that the receptor proteins were present and functional, second messenger assays were performed on the different cell types.

CHAPTER 2

FUNCTIONAL ACETYLCHOLINE MUSCARINIC RECEPTOR SUBTYPES IN HUMAN BRAIN MICROCIRCULATION: IDENTIFICATION AND CELLULAR LOCALIZATION

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ABSTRACT

Acetylcholine (ACh) is an important regulator of local cerebral blood flow. There is, however, limited information available on the possible sites of action of this neurotransmitter on brain intraparenchymal microvessels. In this study, we used a combination of molecular and functional approaches to identify which of the five muscarinic acetylcholine receptors (mAChRs) is present in human brain microvessels and their intimately associated astroglial cells. Microvessel and capillary fractions isolated from human cerebral cortex were found by reverse transcriptase-polymerase chain reaction to express m2, m3 and occasionally, m1 and m5 receptor subtypes. In order to localize these receptors to a specific cellular compartment of the vessel wall, cultures of human brain microvascular endothelial (EC) and smooth muscle (SMC) cells were used together with cultured human brain astrocytes (AST). EC invariably expressed m2 and m5 receptors, and occasionally the m1 receptor, SMC exhibited messages for all except the m4 mAChR subtypes, whereas messages for all five muscarinic receptors were identified in AST. In all three cell types studied, ACh induced a pirenzepine-sensitive increase (62-176%, $p \leq 0.05$-0.01) in inositol trisphosphate, suggesting functional coupling of m1, m3 and/or m5 mAChRs to a phospholipase C signalling cascade. Similarly, coupling of m2 and/or m4 mAChRs to adenylate cyclase inhibition in EC and AST, but not in SMC, was demonstrated by the ability of carbachol to significantly reduce (44-50%, $p \leq 0.05$-0.01) the forskolin-stimulated increase in cAMP levels. This effect was reversed by the mAChR antagonist AF-DX 384. The results indicate that microvessels are able to respond to neurally released ACh and that mAChRs, distributed in different vascular and/or astroglial compartments, could regulate cortical perfusion and, possibly, blood-brain barrier permeability, functions which could become jeopardized in neurodegenerative disorders such as Alzheimer's disease.

Key words: cerebral blood vessels, cerebral blood flow, endothelium, smooth muscle, astrocyte, basal forebrain

Running Title: Muscarinic receptor subtypes in human brain microvasculature

INTRODUCTION

Acetylcholine (ACh) is an important regulator of cerebral blood flow (CBF) in man and in many other species (for review see Sato and Sato, 1995; Dauphin and MacKenzie, 1995). Intracortical and/or basalocortical cholinergic nerve fibers have been shown to intimately associate with microarterioles and capillaries in the rat (see Vaucher and Hamel, 1995) and human (Tong and Hamel, 1997) cerebral cortex. Interestingly, cholinergic-mediated increases in local CBF have been observed in the frontal and parietal subdivisions of the cerebral cortex upon stimulation of basal forebrain cholinergic neurons and muscarinic ACh receptors (mAChRs), some of which presumably associated with the local microcirculation, have been implicated in this response (for review see Sato and Sato 1995). Cerebromicrovascular mAChR binding sites have indeed been reported (Estrada et al, 1983) and, more recently, multiple mAChR subtypes have been identified in intracortical microvessels and capillaries, including in man (García-Villalón et al., 1991; Linville and Hamel, 1995). However, the absence of highly selective subtype-specific ligands for all mAChRs made it difficult to conclusively identify the exact receptor subtype involved. Furthermore, these radioligand binding studies in tissue homogenates could not localize mAChRs to a specific cellular compartment of the isolated microvascular fractions (Linville and Hamel, 1995; Moro et al., 1995).

In this respect, in addition to direct interactions with microvascular smooth muscle and endothelial cells, ACh could also affect local brain perfusion and other microvascular functions through perivascular astrocytes, suggested as intermediaries in the neurogenic control of the microcirculation by cholinergic mechanisms (Vaucher and Hamel, 1995). Interestingly, astrocytic processes associated with bovine and rat brain capillaries have been shown to express mAChRs (Moro et al., 1995, Luiten et al., 1996), and could thus be involved in the local regulation of cortical perfusion through the release of vasoactive mediators (Alkayed et al., 1997).

ACh exerts its effects through any one of the five cloned mAChR subtypes with downstream effects of either inositol phosphate activation (m1, m3, and m5) or attenuation of adenylate cyclase activity (m2 and m4) (for review see Felder, 1995). In large cerebral arteries, activation of mAChRs can result in different vascular responses depending on which mAChRs subtype is stimulated. Pharmacological studies have identified the M3 mAChR subtype as the mediator of the ACh-induced endothelium-dependent dilation (Dauphin and Hamel, 1990; García-Villalón et al., 1991), while the M1 subtype has been implicated in cerebral vasoconstriction (Dauphin et al, 1991; Shizumi et al. 1993). At the level of the brain microcirculation, ACh induces vasodilation of intracortical arterioles in rat (Dacey and Basset, 1987), bovine and human (Elhusseiny et al., 1998). However, the subtype(s) and location of the receptor(s) involved in this and other putative ACh-mediated responses are not known.

In the present study, we used reverse transcriptase-polymerase chain reaction (RT-PCR) to identify the mAChRs expressed in human brain microvessels and capillaries. In order to examine the localization and operational behaviour of the mAChRs within the vessel wall, we evaluated receptor mRNA expression in cultures of human brain microvascular (i.e. endothelial, and smooth muscle) and astroglial cells, and assayed their ability to couple to their expected second messenger signalling pathways in cultured cells. The results show that human intracortical microvessels and capillaries are endowed with multiple functional mAChR subtypes which exhibit a distinct cellular localization within the vessel wall. These receptors could mediate vasomotor responses in intracortical microvessels, either directly by activating receptors on smooth muscle cells, or indirectly via receptors expressed on the endothelium or the perivascular astrocytes.

MATERIALS AND METHODS

Isolated microvessels and capillaries

Human brains (n=6, average post-mortem delay 16.2 ± 2.2hr, 4 males, 2 females) were

obtained at autopsy from subjects who died from diseases not affecting the CNS, with approval from the Institutional Research Ethics Committee, either from the Brain Bank of the Douglas Hospital Research Centre (Verdun, Québec, Canada) or the department of Pathology at the Royal Victoria Hospital (Montréal, Québec, Canada). Intracortical microvessels (MVs) and capillaries (CAPs) were isolated as described previously (Linville and Hamel, 1995). Briefly, after homogenisation and centrifugation over 15% dextran, MVs and CAPs were harvested by filtration through a 151μm and subsequently a 53μm nylon mesh screen, respectively. Previous light microscopic examination of similarly prepared MVs and CAPs fractions have indicated their high level of purity, as also shown by the enrichment (>30 fold) in the endothelial marker enzymes γ-glutamyl transpeptidase (γ-GTP) and alkaline phosphatase (AP) over cerebral tissue (see Linville and Hamel, 1995).

Cell Cultures

Cultures of human brain endothelial (EC) and smooth muscle (SMC) cells were produced with approval from the Institutional Research Ethics Committee, from intracortical CAPs and MVs harvested from biopsies obtained from patients (n=6) undergoing surgery for the treatment of temporal lobe epilepsy (Stanimirović et al., 1996; Abounader et al.,1998). In short, SMC cultures were generated from cortical homogenates that were sequentially filtered through a 350 μm and 112 μm mesh. The resistance MVs were then dislodged from these meshes with cold cell culture medium M199 and dissociated with type IV collagenase (1mg/ml; 15 min). After 4-5 weeks in culture, more than 85% of cells stained positively for the smooth muscle marker α-actin, whereas about 10% of cells demonstrated endothelial cell morphology and incorporated acetylated low density lipoproteins (Ac-LDL) (Abounader et al., 1998). Six primary cultures of SMC from different biopsies were used in this study.

Human microvascular and capillary EC cultures were grown from CAPs obtained after the 112 μm filtrate had been further resuspended in M199 medium and sieved through a 20μm mesh screen. EC colonies were isolated with cloning rings and grown to confluence. More than 95% of cells in these cultures were immunopositive for Factor VIII-related antigen,

incorporated Ac-LDL and expressed high levels of cerebral endothelium-specific enzymes, γ-glutamyl-transpeptidase and alkaline phosphatase. Morphological, phenotypic, biochemical and functional characteristics of these EC cultures have been described earlier (Stanimirović et al., 1996). Both primary and passaged (3-7 times, n=6) EC cultures were used in this study.

Cultures of fetal (10-18 weeks of gestation) human brain astrocytes (AST) (n=5) were provided by Drs. J. Antel and W. Yong (Montreal Neurological Institute, Montreal, QC, Canada) and prepared according to well established procedures (for details, see Abounader et al., 1998). Brains were trypsinized (0.25%), homogenized and filtered through a 130μm mesh. Cell pellets were resuspended in a 95% Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, and plated onto poly-l-lysine coated tissue culture dishes. Contaminating neurons were eliminated by trypsinization and passaging (2-5 times). More than 95% of the cells in these cultures were immunopositive for the glial fibrillary acidic protein (GFAP).

RT-PCR

RNA Isolation and Reverse Transcription

Total RNA was extracted by homogenization with a Polytron (for whole blood vessel preparations) or passage through a syringe (for cell cultures) in the TRIzol Reagent (1ml per 50-100mg tissue, Gibco BRL), according to Chomczynski and Sacchi (1987). RNA (2-20μg) was treated with DNase I (3 U, Gibco BRL) to remove residual genomic DNA, extracted with phenol/chloroform, ethanol precipitated, and the quantity and purity assessed by the A_{260}/A_{280} ratio. Synthesis of single stranded cDNA was performed by reverse transcription (1hr, 42°C) in a reaction mixture (final volume 50μl) containing 1X reaction buffer (Gibco BRL), 2mM DTT, 30-60 U RNase Inhibitor (Promega), random hexamers (200 ng/μg RNA, Gibco BRL), and 1-3 U of avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega). To control for genomic DNA contamination, one half of the primed RNA was used to generate non-RT samples by incubation in a reaction mixture lacking AMV-RT.

Primers

Specific primers were designed according to published sequences of the cloned human mAChR genes for m1, m2 (Peralta et al., 1987) m3, m4 (Bonner et al., 1987) and m5 (Bonner et al., 1988), and synthesized using Applied Biosystems synthesizer. The m1 and m3 primers were flanked with the T7 (5'-GGT AAT ACG ACT CAC TAT AGG GCG A-3') and SP6 (5'-CTC GGA TTT AGG TGA CAC TAT ATG AAT AC-3') RNA polymerase promoter sequences for use in other experiments. Primer sequences and length of amplified regions were as follows: m1, TGA GGG CTC ACC AGA GAC T (forward 844-862), GTC CAG GTG AGG ATG AAG G (reverse 1218-1236) (Δ393); m2, ACA AGG AAG GAT AGT GAA GCC (forward 808-828), CAT CTC CAT TCT GAC CTG AAG (reverse 1146-1166) (Δ 359); m3, AGA TGG ACC AAG ACC ACA G (forward 1178-1196), CAG AGT GGC TTC CTT GAA G (reverse 1572-1590) (Δ413); m4 GAA GGA GAA GAA AGC CAA G (forward 1502-1520), ATC TCA ATG GCT GTC ACA C (reverse 1855-1873) (Δ372); m5 (TCT GTT CAG ATC CTG CTT G) (forward 992-1010), TGC TGG AGA CAG AAG GTA GT (reverse 1327-1346) (Δ 355). The primers were tested for specificity by performing RT-PCR in *Spodoptera frugiperda* (Sf-9) cells infected with recombinant bacculoviruses individually expressing one of the five human mAChRs (graciously provided to us by BioSignal Inc., Montréal, QC, Canada).

PCR Amplification and Sequencing

PCR amplifications were carried out in a final volume of 50μL containing 3mM $MgCl_2$, 0.5 mM dNTP, 2.5μL DMSO, 0.5μM of each primer, 3-5μL of experimental template and 2U of Taq polymerase. An MJ Research thermocycler was used with the following program: an initial denaturation step of 95°C for 5 min, 40 cycles (except in three out of more than 20 PCR runs for the m2 subtype where 35 (n=1) and 37 (n=2) cycles were performed) of 94°C for 40 sec, 56°C for 40 sec, and 72°C for 60 sec and a final extension at 72°C for 5 min. PCR products were electrophoresed on 1.2% agarose gels and photographed under fluorescent UV illumination. All samples were normally tested in triplicates, but occasionally in duplicates. A positive control consisting of the human cerebral cortex which contains all five mAChRs

(Costa et al. 1995) was used to verify the efficiency of the amplification. RT and non-RT RNA were similarly treated for PCR amplification. PCR products corresponding to each mAChR were sequenced from representative tissue to verify homology with the cloned human receptor genes. For the m1 (SMC), m2 (EC) and m3 (SMC) mAChRs, PCR products were first digested with appropriate restriction enzymes and inserted into PGEM (Promega) or Bluescript plasmids by ligation with T4 DNA ligase. Competent DH5 (PGEM) and XL1 (Bluescript) cells, respectively, were transformed and prepared for sequencing using mini (5' to 3') followed by midi (Qiagen) preparations. Nucleotide sequence analysis was performed using the Sanger dideoxy-nucleotide chain termination method and Sequenase (U.S. Biochemical) in an automated sequencer (ALF, Pharmacia). The m4 (AST) and m5 (AST) PCR products were purified (Qiagen QIAquick) and sequenced directly using direct automated fluorescent sequencing (W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, CT).

Second Messenger Assays

Inositol Tris-Phosphate Formation (IP_3)

IP_3 formation in cells subjected to mAChR stimulation was assessed on confluent EC, SMC or AST cultures grown in petri dishes and prelabelled with [^{3}H]myo-inositol (1-5μCi/ml) for 24 hr in serum and inositol free medium M199, as previously described (Stanimirovic et al., 1996). The cells were washed and exposed to ACh (1mM) alone or following a 15 min pre-incubation with the M1 preferring mAChR antagonist, pirenzepine (1mM) (Dörje et al., 1991), which was shown in preliminary experiments to have no significant effect (104 ± 24% of basal levels) on phosphoinositides production. In some cases, the mAChR agonist carbachol (200μM) was also used. The stimulation was carried out in the presence of 10mM LiCl (Berridge et al., 1982) for 15 min and then terminated by addition of 10% TCA. Cells were scraped, centrifuged, and the supernatants were repeatedly extracted with ethyl ether. IP_3 was separated from the aqueous phase by ion exchange chromatography. The columns (DOWEX-AG 1 X 8-formate) were washed with 20 volumes of 60 mM ammonium formate/5mM sodium tetraborate followed by the consecutive elution of inositol mono-, bis-,

and tris-phosphate by 8 volumes of 0.2 M ammonium formate/0.1 M formic acid, 0.4 M ammonium formate/0.1 formic acid, and 1M ammonium formate/0.1M formic acid, respectively. IP_3 production was expressed as a function of protein content in cell extracts. All results are expressed in mean ± standard deviation (S.D.), and statistical differences were compared by ANOVA followed by a Newman-Keuls comparison test.

cAMP attenuation

Inhibition of adenylate cyclase through mAChR stimulation was evaluated by measuring cAMP levels in confluent cells exposed to forskolin (1μM, 15 min) alone or in the presence of either the mAChR agonist carbachol (200μM, 15 min) alone or carbachol and the M2/M4 mAChR antagonist AF-DX 384 (1×10^{-5}M) (Dörje et al., 1991) pre-incubated with the cells for a 15 min period. AF-DX 384 was found in preliminary experiments to have no significant effect on cAMP production in cultured microvascular cells (81 ± 23% of basal levels). Levels of cAMP were measured by commercially available enzyme immunoassay kit (Amersham Life Science) and calculated as a function of cell protein content. All results are expressed as mean ± standard deviation (S.D.) and statistical differences were evaluated by ANOVA followed by a Newman-Keuls comparison test.

RESULTS

Expression of mAChRs in isolated microvascular fractions and cells in culture

As can be appreciated from Figure 1, the primers were found to be highly selective and to yield PCR products of the expected size exclusively when the amplification was performed on the Sf-9 cells infected with the corresponding cloned human mAChR. In all experiments, amplification of PCR products for the various mAChRs was not a result of genomic DNA contamination since they were exclusively detected in samples that had been reverse transcribed (+ RT lanes in Figs.1-3, see also legends to these figures).

Multiple types of mAChRs were detected in MVs and CAPs isolated from human cerebral

cortex (Fig. 2). In MVs, message for m3 mAChR was detected in a majority of preparations, whereas message for m1 mAChR was seen only occasionally. In CAPs, mRNAs for m2 and m3 mAChR subtypes were consistently amplified, and PCR products corresponding in size to the m1 and m5 receptors were also present, albeit in fewer preparations. Expression of the m4 mAChRs was not detected in either of the isolated microvascular fractions (see Table 1).

When studied in microvascular and astroglial cell cultures, PCR products for the m2 and m5 mAChRs were invariably amplified in EC (Fig. 3) and message for the m1 receptor subtype was detected in some preparations (Table 1). In contrast, mRNAs for the m3 mAChR subtype were not detected in any EC cultures, including the primary EC culture. In primary cultures of microvascular SMCs, strong PCR signals could be demonstrated by gel electrophoresis for the m1, m2, m3 and m5 mAChRs, but not for the m4 subtype (Fig. 3 , Table 1). In cultures of fetal human brain AST, expression of the five cloned human AChRs was evidenced with their expression level being generally very strong, as evaluated by gel electrophoresis (Fig. 3, Table 1). Sequence analysis of the selected PCR products showed that they unambiguously corresponded to the cloned human mAChR with the following accuracy: m1 (99%), m2 (98%), m3 (99%), m4 (98%), and m5 (99%). This slight variation in sequence is fully acceptable for single-strand sequence analysis.

Second Messenger Assays

IP_3 Assay

Addition of ACh (1mM) to EC, SMC and AST cell cultures resulted in a significant increase in IP_3 production. The increase over basal levels was comparable in EC (62.6 ± 10.8%; $p<0.01$) and SMC (63.5 ± 18.0%; $p<0.05$), and somewhat higher in AST (176.2 ± 19.5%; $p<0.01$) (Fig.ure 4). A similar increase (120 ± 34.3%) was obtained with 200 μM carbachol when tested in AST (not shown). In all three cell types, the ACh-induced IP_3 production was significantly ($p<0.05$) inhibited to near basal levels by pre-incubation with the mAChR antagonist pirenzepine (1mM) (Fig. 4).

cAMP Assay

Forskolin (1μM) resulted in an 8-18 fold increase ($p<0.01$) in cAMP levels in EC, SMC and AST in culture. The cholinergic agonist carbachol (200μM) significantly reduced the forskolin-stimulated cAMP levels by 50 ± 23.4% ($p<0.05$) in EC and 44 ± 10.2% ($p<0.01$) in AST. Addition of the mAChR antagonist AF-DX 384 (10^{-5}M) significantly antagonized the inhibitory effect of carbachol in both cell types, returning cAMP levels to 82.6 ± 7.1% ($p<0.05$) and 98.6 ± 11.4% ($p<0.01$) of forskolin-stimulated cAMP levels, respectively. In contrast, carbachol and AF-DX 384 had no significant effect on the forskolin-mediated increase in cAMP levels in SMC cultures (n=3) (Fig. 5B).

DISCUSSION

This study demonstrates that heterogeneous mAChR subtypes are localized in specific cellular compartments of the human brain microcirculation. Together MVs and CAPs isolated from post-mortem human cerebral cortex were found to express a combination of mAChRs, but to lack the m4 subtype. When receptor expression was assessed in more details in microvascular and astroglial cell cultures generated from surgery specimen or fetal brain, the results indicated that human brain EC are endowed with m2 and m5 mAChR subtypes, SMC with m1, m3 and possibly m5 mAChRs, while all five mAChRs were found in human fetal brain AST. The results with mAChR antagonists further indicated that their second messenger coupling efficacy corresponded to that reported previously in endothelial and smooth muscle cell cultures from peripheral blood vessels of man (Hawley et al. 1995), or in rat glial cell cultures (Cohen and Almazan, 1994). Altogether, the present results suggest that intracortical microvessels have the ability to respond to changes in central cholinergic neurotransmission either directly through receptors in the vessel wall itself or, indirectly, by interacting with receptors located on the perivascular astroglial cells.

The presence of specific binding sites for the mAChR antagonist [^{3}H]quinuclidinylbenzilate in collagenase-treated capillaries and microvessels (Estrada et al. 1983), provided the first

convincing evidence that mAChRs are associated with vascular elements of the brain microcirculation. Although more than one population of mAChRs was later suggested (García-Villalón et al, 1991; Linville and Hamel, 1995), there was disagreement as to which mAChR subtypes were present in the brain microvasculature. García-Villalón and colleagues exclusively identified a pharmacological M4 mAChR in bovine microvascular fractions while others concluded, based on pharmacological characterization and correlation analyses with the cloned mAChRs, that the M1, M3 and possibly the m5 receptors were most likely the cerebromicrovascular receptors present, not only in bovine, but also in human cerebromicrovascular fractions (Linville and Hamel, 1995). In the present study, the high sensitivity and selectivity of the RT-PCR approach together with the demonstration of functional second messenger signalling pathways in the cell cultures provided unequivocal support for the presence of cerebromicrovascular m1, m3 and m5 mAChRs, and further allowed to identify endothelial m2 mAChRs. In contrast, m4 mAChRs were found to be exclusively astroglial, a finding which could possibly explain its detection by radioligand binding studies in isolated bovine microvessels (García-Villalón et al., 1991), since these also contain a proportion of attached astroglial cells (see Linville and Hamel, 1995). Most interestingly, the present findings in the human intracortical microvascular bed closely match those reported in a recent RT-PCR study which showed expression of m1, m2, m3, and m5 mAChRs in the rat basilar artery (Phillips et al., 1997). This remarkable similarity in the composition of mAChRs between the rat major cerebral arteries and the human intracortical microvessels strongly suggests a high level of conservation for vascular mAChRs across species and between intra- and extra-parenchymal blood vessels. It may further imply comparable functions for these receptors in their respective cerebrovascular beds.

RT-PCR results obtained in the EC cultures showed the presence of mAChRs in these cells with a consistent and predominant expression of the m2 and m5 mAChRs, a finding which was corroborated by their coupling to the expected downstream signalling pathways, but which is at variance with previous studies that could not detect mAChRs in brain capillaries, albeit in the rat (Moro et al., 1995; Luiten et al. 1996; Badaut et al. 1997). However, the immunocytochemical demonstration of endothelial m2 mAChRs in rat, primate and human brain capillaries (Levey et al., 1995; Smiley et al. 1998) would argue to the contrary and

rather support the present findings. The exclusive mRNA expression of the m2 subtype and the ability of the mAChR antagonist AF-DX 384 (Dörje et al., 1991) to block the inhibitory effect of carbachol on cAMP production (the downstream effect of m2 activation), unequivocally indicated the existence of functional endothelial m2 mAChRs. The apparent discrepancy between the present results and the inability of previous radioligand binding studies to detect m2 mAChRs in isolated human cerebral microvasculature (Linville and Hamel, 1995) could possibly be explained by the fact that radioligand binding studies, unlike PCR, require that a sizeable amount of the receptor protein be present in order to be detectable. In fact, based on the present results, m2 receptors are located in the endothelial but not in the muscular compartment (see below) of the intraparenchymal vascular tissue. It is thus possible that m2 binding sites in a single layer of endothelial cells would have been masked by more abundant mAChR subtypes present in both the endothelial and smooth muscle cells, or the smooth muscle layer(s) alone. This putative problem of relative abundance between the different mAChR subtypes has been circumvented in the present study by the use of the highly sensitive PCR technique and cell cultures of the different cellular components of the vessels wall.

With regards to mAChRs associated with the phosphoinositide pathway, the findings that only the m5 subtype was consistently expressed in human brain EC and that pirenzepine, which at the concentration used will affect not only m1 but also the other mAChR subtypes (Dörje et al., 1991), effectively inhibited the IP3 response induced by ACh in these cultures, further indicated the presence of endothelial m5 mAChR proteins. No molecular evidence, however, was found in primary and passaged human cerebromicrovascular EC cultures for the m3 mAChR subtype. This finding suggests that its mRNA detection in isolated CAPs is probably due to other cells intimately associated with this isolated microvascular fraction, such as pericytes and/or astrocytes (see below), rather than to repeated passaging of the cells (Tracey and Peach, 1992). In large cerebral arteries of various species, the M3 mAChR (Dauphin and Hamel, 1990; García-Villalón et al., 1991; for review see Dauphin and Mackenzie, 1995) and the m5 subtype (Hamel et al.,1994; Phillilps et al., 1997) have been suggested as possible

mediators of the endothelium-dependent, nitric oxide (NO)-mediated dilatation induced by ACh. As ACh is also a potent dilator of isolated intracortical arterioles in rat (Dacey and Basset 1987), bovine and human, in which NO-mediated mechanisms appear to be involved (Elhusseiny and Hamel, 1998), the present results suggest that the m5 mAChR is a likely candidate for mediating this response at the microvascular level in man. This receptor is known to share substantial similarities with the m3 mAChR subtype, both in terms of pharmacology (Dörje et al., 1991) and second messenger profile (Felder, 1995*)*. Furthermore, the m5 receptor has been reported to stimulate NO production when transfected in cells (Wang et al., 1994), a property which could be of interest for a receptor putatively involved in an endothelium-dependent vasodilatory response. Additional studies with m5 mAChR selective compounds will be of great help in clarifying this issue when they become available.

Isolated MVs, which unlike CAPs are enriched with vessels with a smooth muscle layer, were found to express m1 and m3 mAChR subtypes, a finding corroborated by the expression of these same receptors in microvascular SMC cultures. Although a role for these receptors in human brain microcirculation still has to be defined, both subtypes have been implicated in the ACh-induced vasoconstriction of endothelium denuded vessels, with the M1 being particularly important at the level of cerebral blood vessels (for review see Dauphin and Mackenzie, 1995). In spite of the detection of mRNA for the m2 receptor in SMC, carbachol systematically failed to significantly block the forskolin-induced cAMP production in these cells while being very effective in EC and AST cultures. This observation suggests that the m2 mRNA is either amplified from the small population of contaminating endothelial cells present in the primary SMC cultures, or that functional m2 mAChRs exist in a very limited population in SMC, too small to produce a significant effect on the cAMP production in a biochemical assay. The presence of m5 mAChRs in both EC and SMC cultures similarly indicate that further investigations will be needed to verify whether the m5 mAChR is genuinely expressed in SMC or if its detection in these cells is due to the small proportion of contaminating endothelial cells. However, as for other microvascular receptors (Riad et al., 1998), it is also possible that the same receptor subtype is expressed in both the EC and SMC

compartments.

Our finding that human brain AST express all five mAChRs extends previous pharmacological and molecular studies which showed the presence of m2, m3, and m5 mRNAs in human astrocytoma cells (Guizzetti et al., 1996), and m1 and m3 in murine astroglial cells in culture (André et al., 1994). In addition to the m1, m2, m3, and m5 subtypes, we further show expression of the m4 mAChR in fetal human brain astrocytes. Its presence in adult human brain astrocytes should be further confirmed, as fetal brain tissue and astrocytes may express different receptor populations than those seen in the adult (Van der Zee et al., 1993; Hohmann et al., 1995), a possibility raised by the absence of m4 mAChR message in either isolated MVs or CAPs which originated form adult brain. Astroglial cells fulfil a variety of functions within the central nervous system (Van der Zee et al., 1993; Guizzetti et al., 1996) and the multiplicity of mAChRs associated with these cells could be a reflection of such a polyvalence. More specifically, perivascular astroglial cells have been involved in the regulation of microvascular tone, metabolic homeostasis, and/or blood-brain barrier permeability (Tao-Cheng et al., 1987; Tsacopoulos and Magistretti, 1996; Alkayed et al., 1997), all of which could be regulated by ACh since a large proportion of neurovascular cholinergic nerve terminals are in fact associated with the perivascular astrocytes (Vaucher and Hamel, 1995).

Physiological Implications

This study reports the presence of functional cerebromicrovascular and astroglial mAChRs in human cerebral cortex, corresponding in part to those able to mediate vasomotor responses in other cerebrovascular beds (for review see Dauphin and MacKenzie, 1995). Cortical microvessels and capillaries have been identified as a target for basalocortical cholinergic nerve terminals in both rat (Vaucher and Hamel, 1995) and human (Tong and Hamel,1997). The present results further indicate that these intraparenchymal vessels are equipped with adequate receptors to directly modulate blood flow in response to cholinergic stimulation, in agreement with many physiological studies involving stimulation of basal forebrain neurons

(for review see Sato and Sato, 1995). Furthermore, dysfunctions in this system, like those seen during neurodegenerative disorders such as Alzheimer's disease, could impact on the regulation of the local cortical microcirculation and result in inadequate perfusion, capillary morphology and/or blood brain barrier permeability.

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Table 1: Summary of mAChR receptors expression in human intracortical microvessels (MVs) and capillaries (CAPs), microvascular endothelial (EC) and smooth muscle (SMC) cells, as well as astroglial cells (AST) in culture.

	m1	m2	m3	m4	m5
CAPS	+/-	++	++	-	+/-
MV	+/-	-	++	-	-
EC	+/-	++	-	-	++
SMC	++	++	++	-	++
AST	+++	+++	+++	++	+++

PCR results were obtained from six (or five for AST) separate human donors and are expressed as the amount of samples which showed a positive PCR product, with the following scale: (-): 0; (+/-) :1-2; (++): 3-4; (+++): 5-6. In final results analysis, only receptors that appeared in 50% or more of samples were considered to be conclusively present in the tissues.

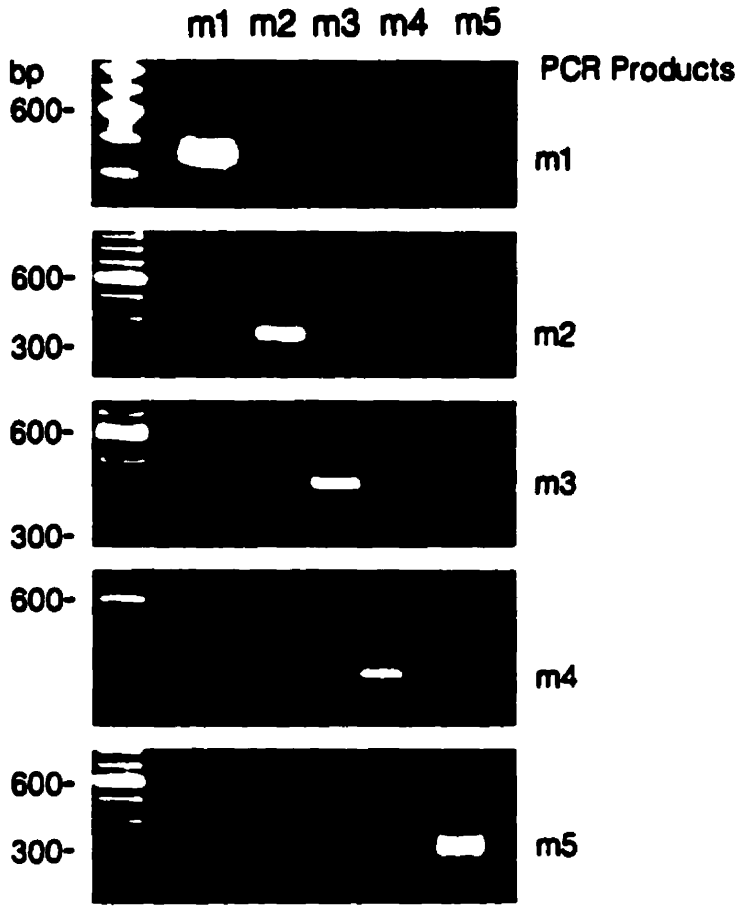

FIG. 1. Specificity of selected primers for their respective human mAChR. Agarose gel electrophoresis showing that the selected oligonucleotide primers yield reverse transcription-polymerase chain reaction (RT-PCR) products exclusively in Sf-9 cells infected with the corresponding human mAChR subtype. No products were amplified from the non-RT preparation (not shown).

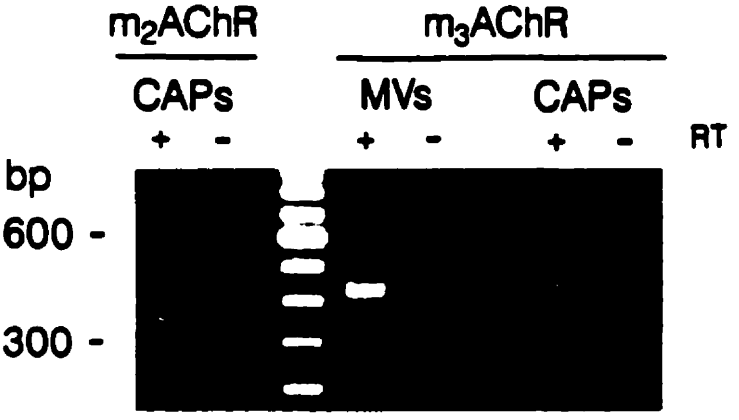

FIG. 2. Identification of mAChR in human MV and CAP isolated from postmortem cerebral cortex. Gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) products from representative samples show that the m2 and m3 mAChR are present in a majority of CAP fractions, whereas only the m3 subtype was clearly identified in the MV. Only PCR products present in greater than or equal to 50% of samples tested (n = 6) are illustrated. The PCR reactions were performed on RT (+) and non-RT (−) samples.

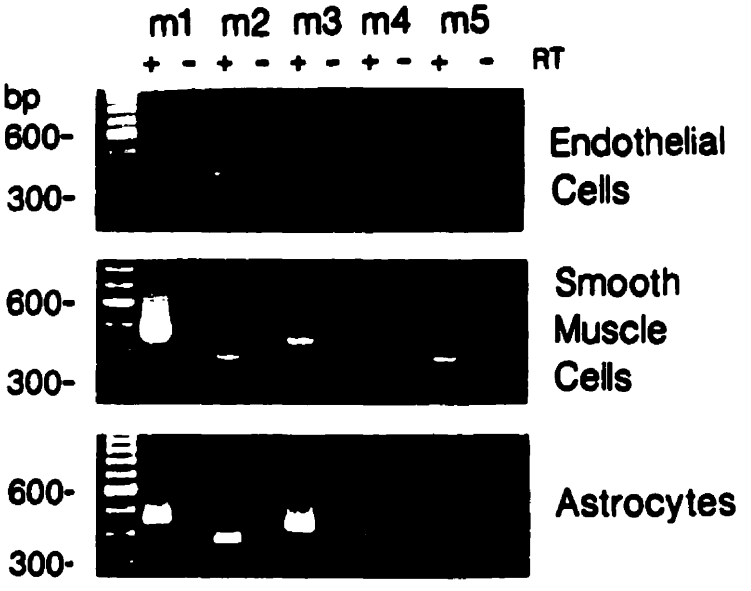

FIG. 3. Representative gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) products for the mAChR expressed in human brain microvascular and astroglial cells in culture. Only products that were present in greater than or equal to 50% of cell cultures are shown. Cultured EC express m2 and m5, SMC express messages for all mAChR except the m4 subtype, and AST, all five mAChR. The PCR reactions were performed on RT (+) and non-RT (–) samples.

Figure 4: Stimulation of IP_3 production in EC (A), SMC (B) and AST (C) by ACh (1mM) and inhibition of the response by the mAChR antagonist pirenzepine. Values are mean ± S.D. of triplicate determinations from one representative experiment. A total of three different experiments were performed and yielded similar results. * and **: $p \leq 0.05$ and $p \leq 0.01$, respectively, as compared to basal levels (taken as 100%, open box), *: $p \leq 0.05$ as compared to ACh alone.

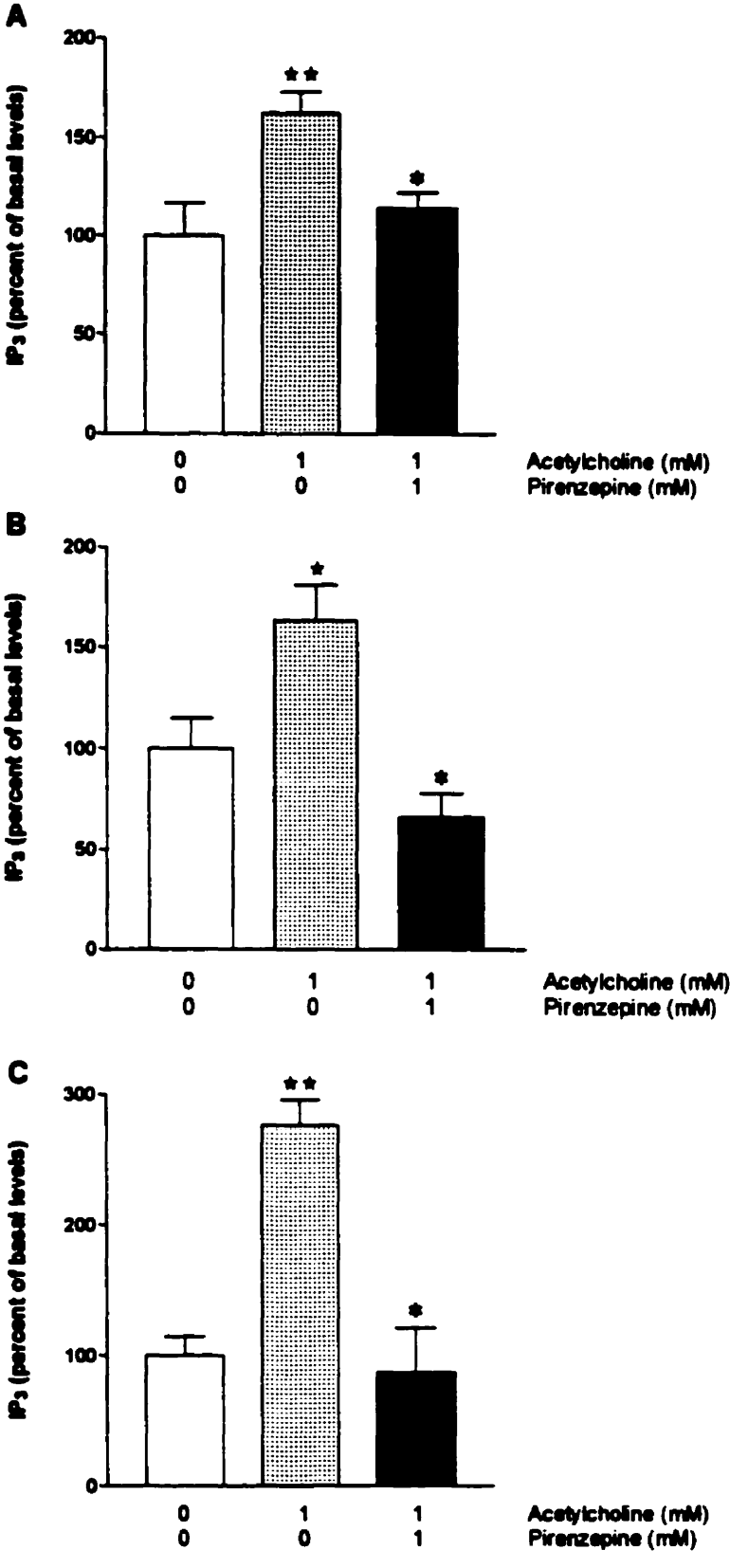
A
B
C
IP3 (percent of basal levels)
200
150
100
50
0
300
200
100
0
**
*
0 1 1
0 0 1
Acetylcholine (mM)
Pirenzepine (mM)

Fig. 4

Figure 5: Effect of carbachol and AF-DX 384 on the forskolin-induced production of cAMP in EC (A), SMC (B), and AST (C). EC and AST, but not SMC appear to have a functional mAChR pharmacologically related to the m2 and/or m4 subtype. Representative results (mean ± S.D.) from one of three individual experiments performed in triplicates. * and **: $p \leq 0.05$ and $p \leq 0.01$, respectively, as compared to forskolin alone; * and **: $p \leq 0.05$ and $p \leq 0.01$, respectively, as compared to carbachol and forskolin added together without antagonist.

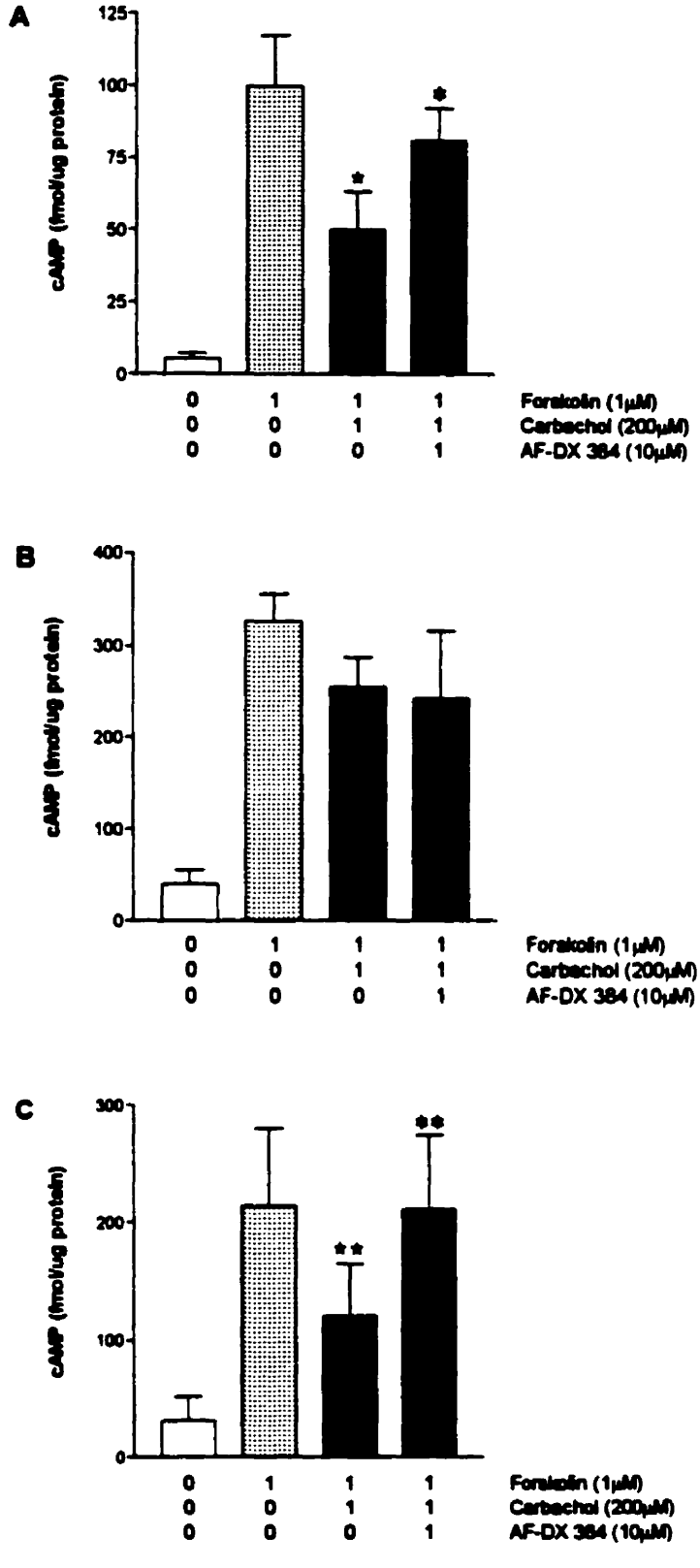
A
cAMP (fmol/ug protein)
125
100
75
50
25
0
*
*
0 1 1 1 Forskolin (1μM)
0 0 1 1 Carbachol (200μM)
0 0 0 1 AF-DX 384 (10μM)
B
cAMP (fmol/ug protein)
400
300
200
100
0
0 1 1 1 Forskolin (1μM)
0 0 1 1 Carbachol (200μM)
0 0 0 1 AF-DX 384 (10μM)
C
cAMP (fmol/ug protein)
300
200
100
0
**
**
0 1 1 1 Forskolin (1μM)
0 0 1 1 Carbachol (200μM)
0 0 0 1 AF-DX 384 (10μM)

Fig. 5

PREFACE TO CHAPTER 3

Having established in the previous chapter that multiple mAChRs are present in different compartments of the vessel wall, the logical next step was to explore if they were implicated in the regulation of microvascular tone and hence CBF.

ACh typically vasodilates vessels in the extracerebral and peripheral circulation, although a vasoconstriction has also been shown in some species under certain conditions. The vasodilation is presumed to occur via the release of an endothelium-dependent relaxing factor, namely NO, which subsequently diffuses into smooth muscles to induce muscle relaxation. Most of this information however, is based on pharmacological studies performed on large cerebral vessel types. Technical limitations have hindered the *in vitro* study of the intracerebral arterioles, in spite of the fact that they contribute significantly to regional CBF. To date, only two techniques have been devised that can perform this function. Both techniques were used to examine the response of brain microvessels to cholinergic stimulation at a basic level. Dacey and Bassett (1987) have shown that ACh induced vasodilation of isolated rat penetrating vessels only when they were preconstricted by either $PGF_{2\alpha}$ or 5HT. Using a different technique on brain slices, Sagher et al. (1993) were able to show a similar cholinergic vasodilation upon preconstriction with norepinephrine. In both studies, vessels had to be pre-constricted for an observable dilation to occur. No attempts were made to characterize receptor subtypes involved in the response or the role of endothelial cells and/or NO in the vasodilation. Studies which are essential for a better understanding of the cholinergic influence on CBF under both normal and pathological conditions.

In order to address these concerns, we developed a technique that permitted the study of isolated brain intracortical arterioles *in vitro* and used it to examine the effect of cholinergic stimulation of bovine and human brain intracortical arterioles. A dose-response curve for ACh on these arterioles was obtained, and the specific mAChR subtypes involved were pharmacologically identified using mAChR receptor antagonists. Additionally, the role of NO and nAChRs in the ACh response was examined. The results are presented in this chapter.

CHAPTER 3

MUSCARINIC -BUT NOT NICOTINIC- ACETYLCHOLINE RECEPTORS MEDIATE A NITRIC OXIDE (NO)-DEPENDENT DILATION IN BRAIN CORTICAL ARTERIOLES: A POSSIBLE ROLE FOR THE M5 RECEPTOR SUBTYPE

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ABSTRACT

Increases in cortical cerebral blood flow are induced by stimulation of basal forebrain cholinergic neurons. This response is mediated in part by nitric oxide (NO) and reportedly involves both nicotinic and muscarinic receptors, some of which possibly located in the vessel wall. In the present study, we investigated the vasomotor response(s) elicited by acetylcholine (ACh) on isolated and pressurized bovine and/or human intracortical penetrating arterioles and we pharmacologically characterized the receptor involved in this response. ACh (10^{-11} to 10^{-4} M) dose-dependently dilated bovine and human intracortical arterioles at spontaneous tone (respective pD_2 values of 6.4 ± 0.3 and 7.2 ± 0.3 and E_{Amax} of 65.0 ± 26.8 and 43.2 ± 30.1% of the maximal dilation obtained with papaverine) and, in bovine, following preconstriction with serotonin (pD_2 = 6.3 ± 0.1, E_{Amax} = 80.0 ± 17.9 % of the induced tone). In contrast, nicotine (10^{-8}- 10^{-4} M) failed to induce any vasomotor response in bovine vessels whether at spontaneous or pharmacologically-induced tone. Application of the nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine (L-NNA) (10^{-5} M) elicited a gradual constriction (~20%) of the arterioles, indicating the presence of constitutive NO release in these vessels. L-NNA (10^{-5}M -10^{-4}M) also significantly blocked the dilation induced by ACh. The mAChR antagonists pirenzepine, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), and (±)-5,11-dihydro-11-{[(2-[(dipropylamino)methyl]-1-piperidinyl}ethyl)amino]carbonyl}-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one (AF-DX 384) dose-dependently inhibited the dilatation induced by ACh (10^{-5}M) with the following rank order of potency: 4-DAMP (pIC_{50} = 9.2 ± 0.3) >> pirenzepine (pIC_{50} = 6.7 ± 0.4) > AF-DX 384 (pIC_{50} = 5.9 ± 0.2). These results suggest that ACh can induce a potent, dose-dependent and NO-mediated dilation of bovine and/or human intracortical arterioles, most likely via interaction with a mAChR that best corresponds to the M5 subtype.

Running title: NO-dependent cholinergic dilation in brain intracortical arterioles

Key words: intracortical microcirculation, human, bovine, microvessels, cerebral blood flow, endothelium

INTRODUCTION

The brain intraparenchymal microcirculation consists mainly of arterioles, small venules and capillaries. These vessels are known to be innervated by fibers originating from brain intrinsic neurons (Reis and Iadecola, 1989; Lou et al., 1987) which can impart vasomotor as well as other functions via the release of neurotransmitters. For instance, intracortical and/or basalocortical cholinergic nerve fibers have been shown to intimately associate with arterioles and capillaries in the rat (Arneric et al., 1988; Vaucher and Hamel, 1995) and human (Mesulam et al., 1992; Tong and Hamel, 1999) cerebral cortex. These perivascular cholinergic nerve fibers may play a role in the control of local cerebral blood flow (CBF) as suggested by the cortical increases in CBF following stimulation of basal forebrain neurons (Biesold et al., 1989; Lacombe et al., 1989). Both nicotinic and muscarinic acetylcholine receptors - some of which possibly localized at the microvascular level - as well as nitric oxide (NO) have been implicated in these local changes in CBF (for review see Sato and Sato, 1995).

Stimulation of muscarinic acetylcholine receptors (mAChRs) in cerebrovascular tissue can induce either a vasodilation or a vasoconstriction depending on the species, the concentration of acetylcholine (ACh), the presence of the endothelial cell layer, the subtypes of mAChRs present and their localization in the vessel wall. For instance, activation of smooth muscle mAChRs with a pharmacological profile corresponding to the M1 subtype induces constriction of cerebral arteries (Dauphin et al., 1991). In contrast, endothelial mAChRs pharmacologically similar to the M3 mAChR subtype seem to mediate dilation of cerebral blood vessels (Dauphin and Hamel, 1990; Shimizu et al., 1993; Dauphin and MacKenzie, 1995). This endothelial-dependent dilation is mediated by NO or an NO derivative (for review see Faraci and Brian, 1994). With respect to brain intraparenchymal circulation, ACh has been shown to dilate isolated rat cerebral arterioles (Dacey and Bassett, 1987) as well as microvessels in hippocampal slice preparations (Sagher et al., 1993). Except to show that this response was atropine-sensitive, therein involving mAChRs (Dacey and Bassett, 1987), the

mechanism(s) and the receptor subtype(s) implicated still remain to be established.

Radioligand binding studies have demonstrated the presence of both nicotinic (Kalaria et al., 1994) and muscarinic ACh receptors (Estrada et al., 1983; Linville and Hamel, 1995) in brain microvessels of several species, including man. Additionally, we showed by radioligand binding studies, RT-PCR and second messenger assays that isolated human brain microvessels are endowed with heterogenous mAChR subytpes (Linville and Hamel., 1995; Elhusseiny et al., 1999). Functional M2 and M5 mAChRs have been identified in human cerebromicrovascular endothelial cell cultures (Elhusseiny et al., 1999), allowing speculation that an endothelium-dependent ACh vasodilation could be mediated by either of these mAChRs.

In this study, we examined the effects of ACh and nicotine on isolated and pressurized bovine and/or human penetrating intracortical arterioles. The putative role of NO in mediating spontaneous tone of these intracortical resistance microvessels and the ACh-induced dilation was assessed with the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA). In addition, the mAChR subtype mediating the ACh-induced dilation was characterized using mAChR antagonists with a selective profile of activity at the various mAChR subtypes, namely 4-DAMP, pirenzepine and AF-DX 384 (Dörje et al., 1991; Alexander and Peters, 1998 ; Caulfield and Birdsall, 1998). Parts of these results have appeared in abstract form (Elhusseiny and Hamel, 1998).

MATERIALS AND METHODS

Intracortical penetrating arterioles were isolated from bovine (frontoparietal cortex, Abattoir Forget and Abattoir Ecolait, Terrebonne, Québec, Canada) and human (biopsies of temporal and frontal cortex obtained at surgery for the treatment of epilepsy and with approval from the Institution Research Ethics committee) cerebral cortex following a similar procedure to that described by Dacey and Duling, 1982. Briefly, brain slices (1 - 2 mm thick) were cut parallel to the brain surface with the overlaying pial layer still intact. The slices were pinned down in a petridish in cold MOPS solution (4°C , pH 7.4 ± 0.1) with a basic composition (in

mM) of NaCl, 144; KCl, 3.0; $CaCl_2$, 2.5; $MgSO_4$, 1.5; glucose, 5; pyruvate, 2.0; ethylenediaminetetraacetic acid (EDTA), 0.02; morpholinopropanesulfonic acid (MOPS), 2.0; and NaH_2PO_4, 1.2 (Dacey and Bassett, 1987). The pial membrane was reflected from the brain surface exposing the underlying penetrating vessels, which were then isolated by gentle tugging. Upon removal, the vessels were carefully suctioned along with dissection fluid into a modified pasteur pipette to be transported into a cold holding chamber until later use for cannulation.

Vessels were maintained *in vitro* in an arteriograph chamber system (Living Systems Instrumentation, Burlington, Vermont) modified for continuous on-line monitoring and maintenance of temperature (37 ± 1°C) and pH (7.4 ± 0.1), and filled with a de-bubbled MOPS/albumin (albumin 1%) buffer. Arterioles were placed in the chamber and were cannulated and secured (with ultrafine string, Living Systems) at one end to a glass micropipette (20-30μm diameter) filled with a MOPS/albumin solution and attached to tubing leading to a pressure-servo micropump (Living Systems Instrumentation).

Albumin is required on the inside of the arterioles to stabilize and maintain the endothelial cell layer (Muller et al., 1993). A small amount of flow was applied to the pipette by the pressure-servo system to wash out any remains of blood or other substances from the vessel lumen. The distal end of the vessel was then sealed and secured to the opposing glass micropipette (~ 40μm diameter) with ultrafine string. Vessels were pressurized to 60mmHg (Dacey and Bassett, 1987), superfused (6 ml/min) with MOPS solution and allowed to stabilize and acquire spontaneous tone (45 min - 1 hr) before any experimentation. Vessels were magnified (400-800 fold) on an inverted microscope (Leica, Canada) coupled to a video caliper (Imagen Instrumentation, Trenton NJ, USA); and on-line measurement of intraluminal diameters was performed with a closed circuit video system (National Electronics). All compounds were added to the superfusing MOPS solution and thus were applied, at the desired concentrations, extraluminally for a period of 3 minutes. Smooth muscle reactivity was tested with 70mM K^+ in MOPS. At the end of each experiment, papaverine (10^{-4}M) was added to maximally dilate

the vessels.

Graded concentrations of ACh (10^{-11} to 10^{-4}M) and nicotine (10^{-8} to 10^{-4}M) were added sequentially to isolated bovine or human arterioles either at spontaneous tone and, in bovine, after induction of a pharmacological tone (amounting to 76.0 ± 11.7% of the spontaneous tone) with serotonin (5-HT, 10^{-6} M). The nitric oxide synthase (NOS) inhibitor L-NNA (10^{-5} and/or 10^{-4}M) was used (30 min pre-incubation) to study the role of NO on vessel tone, and in the ACh-mediated dilation of bovine and human arterioles at spontaneous tone or following preconstriction of vessels with 10^{-6}M 5-HT (in bovine only).

To characterize the subtype(s) of mAChRs involved in the ACh-induced dilation, inhibition curves with a fixed concentration of ACh (10^{-5}M) were generated in the presence of increasing concentrations of selected mAChR antagonists applied to a single vessel segment. This method was chosen over the classical Schild analysis because the latter requires a series of repeated full dose-response curves, a criterion which could not be performed in a consistent manner in these delicate arterioles (unpublished observation). Bovine arterioles were thus successively pre-incubated (15 min) with different concentrations of the discriminative mAChR antagonists 4-DAMP (10^{-11}-10^{-7}M; RBI, Natick, MA, USA), pirenzepine (10^{-9}-10^{-5}M; RBI, Natick, MA, USA), and AF-DX 384 (10^{-8}-10^{-5}M; generously supplied by Dr. H Doods, Boehringer Ingelheim Pharma KG, Biberach, Germany), preconstricted with 5-HT (10^{-6}M; Sigma) and then exposed to ACh (10^{-5}M). A control for the reproducibility of the ACh-mediated response was performed in an identical manner as described above except that no mAChR antagonist was added.

Agonists dose-response curves were generated and the agonists maximal response (recorded E_{Amax}) and potency (pD_2 values or -log of EC_{50}) determined. The efficacy of the mAChR antagonists in blocking the ACh-mediated dilation was determined by calculating the pIC_{50} values (negative logarithm of the molar antagonist concentration which induces 50% inhibition of the ACh response) from the antagonists concentration-response curves. The

antagonists vascular potencies were compared by rank order of potency (Dörje et al., 1991; Alexander and Peters, 1998; Caulfield and Birdsall, 1998) and regression analyses to their published affinities at the cloned human mAChRs (Dörje et al., 1991).

Calculations and Statistical Analysis

All results are expressed as the mean ± S.D. The significance in changes of diameter between control values at spontaneous tone and at different time points following application of L-NNA (Fig. 1) was determined by repeated measures ANOVA followed by a Dunnett comparison test. The significance of the differences between the dose-response curves of ACh and ACh with L-NNA (10^{-5} M and 10^{-3}M; Fig. 2) was determined by one-way ANOVA with a Bonferonni comparison between sets of data, while the difference between the pD_2 values was analyzed by unpaired Students *t* test. The means of two measurements for ACh and ACh with L-NNA in preconstricted arterioles (Fig. 3) was determined by unpaired Students *t* test for independent observations. One way ANOVA followed by a Dunnett comparison test was used to analyze the inhibitory effects of muscarinic receptor antagonists on the ACh dilation (Fig. 4 upper panel). In all cases, a $p<0.05$ was considered significant. All statistics and curve fittings (non-linear regression analysis for sigmoidal dose-response curves) were performed with the software GraphPad Prism (GraphPad Software Inc).

RESULTS

At 37°C, pH 7.4 and 60 mmHg intraluminal pressure, the vessel luminal diameter at spontaneous tone was 46.9 ± 19.5 µm in bovine (n=56) and 47.4 ± 19.1 µm in human (n=14). This spontaneously developed tone represented constrictions of 37.8 ± 12.4 % (n=17) in bovine and 41.0 ± 7.6 % (n=5) in human arterioles from the passive diameter as determined by application of papaverine (10^{-4}M). Application of the non-selective NOS inhibitor L-NNA (10^{-5}M) resulted in a gradual and significant decrease in diameter which amounted to 20.6 ± 13.3% in bovine (n=10, $p<0.05$, ANOVA) and 19.0 ± 14.0% in human (n=4, $p<0.05$,

ANOVA) from the spontaneous tone. This vasocontractile response developed gradually over a 25 min period and, in bovine vessels, was demonstrated by a vehicle control, not be due to any other time dependent changes in the vessel (Fig. 1).

At spontaneous tone, ACh (10^{-11} to 10^{-4}M) dilated bovine and human intracortical arterioles (Fig. 2), while nicotine (10^{-8} to 10^{-4}M), failed to significantly affect bovine arteriole diameters (Fig. 2, *top panel*). The ACh pD_2 values for bovine and human arterioles were 6.4 ± 0.3, (n=8) and 7.2 ± 0.3, (n=9), respectively, and the E_{Amax} corresponded to 65.0 ± 26.8 % and 43.2 ± 30.1%, respectively, of the maximal dilation obtained with papaverine (10^{-4}M). L-NNA dose-dependently inhibited the ACh-mediated dilation in bovine vessels, with a significant shift of the curve to the right at 10^{-5}M (pA_2 value of 5.4 ± 0.4; $p<0.001$), and a virtually complete abolition at 10^{-4}M ($p< 0.001$, Fig. 2 *top panel*). In human vessels, 10^{-5}M L-NNA was sufficient to significantly abolish the ACh dilation ($p< 0.05$, Fig. 2, *bottom panel*).

In preconstricted bovine arterioles, ACh (10^{-8} to 10^{-4}M) also elicited a dose-dependent dilation (pD_2= 6.3 ± 0.1, E_{Amax} = 80.0 ± 17.9 % of 5-HT- induced tone) (Fig. 3) and, again, nicotine (10^{-8} to 10^{-4}M) was devoid of any vasomotor effects. In such vessels, the dilation induced by ACh (10^{-5}M) was potently inhibited (86.7 ± 18.2 %, $p<0.05$) by 10^{-5}M L-NNA (Fig. 3).

Repeated (up to 4 times) application of ACh (10^{-5} M) to preconstricted bovine arterioles resulted in a reproducible dilation with no significant differences in efficacy between trials (see Fig.4A). 4-DAMP potently inhibited the ACh-mediated dilation at concentrations as low as 10^{-9}M, with almost complete blockade (95.5 ±7.7 %, $p<0.01$) of the response at 10^{-7}M (Fig. 4B). In contrast, the mAChR antagonist pirenzepine significantly inhibited the ACh-induced vasodilation only at relatively high concentrations with a maximal inhibition (84.4 ± 1.9%, $p<0.01$) obtained at 10^{-5}M pirenzepine (Fig. 4C). The M2/M4 mAChR antagonist AF-DX 384 was also a weak inhibitor of the ACh-induced dilation (Fig. 4D). A significant ($p<0.05$) decrease was only observed at AF-DX 384 concentrations of 10^{-6} M and higher, with maximal

inhibition (77.5 ± 5.6%, $p<0.01$) occurring at 10^{-5}M. Calculated pIC_{50} values from the inhibition curve for each antagonist (Fig. 4, *bottom panel*) yielded the following rank order of potency: 4-DAMP (pIC_{50}= 9.2 ± 0.3) >> Pirenzepine (pIC_{50}= 6.7 ± 0.4) > AF-DX 384 (pIC_{50}= 5.9 ± 0.2). Although limited to three values, linear regression analyses indicated a positive and significant correlation ($p<0.05$) between antagonist potencies and published affinities only with the M5 mAChR (r= 0.997, slope = 0.80 ± 0.06).

DISCUSSION

The present study demonstrates that ACh can induce an NO-mediated dilation in bovine and human intracortical arterioles *in vitro*. The data further indicate that this response is mediated uniquely, at least in bovine microvessels, by muscarinic receptors since nicotine failed to affect microvascular tone. Moreover, by using discriminative mAChR antagonists which exhibit a profile of affinity for the different subtypes (Caulfield and Birdsall, 1998), their rank order of potency suggests that the ACh-induced microvascular dilation is mediated by a receptor pharmacologically similar to the M5 mAChR subtype. Altogether these findings suggest that ACh released perivascularly can regulate the tone of neighboring microvessels and thus modify local CBF.

A consistent and reproducible dose-dependent dilation was induced by ACh in both bovine and human intracortical arterioles. In agreement with other studies in rat intracerebral arterioles (Dacey and Bassett, 1987; Sagher et al., 1993), and in bovine and human major cerebral arteries, (Fischer-Nakielski and Schrör; 1990; Tsukahara et al., 1989; Conde et al., 1991) we did not observe any vasoconstriction with ACh, a response previously reported in intact cat (Dauphin et al., 1991) and endothelium-denuded mouse (Shimizu et al., 1993) pial vessels. Dacey and Basset, 1987 were the first to demonstrate an ACh-mediated dilation of pressurized intracerebral arterioles, albeit in rat preconstricted vessels. ACh (10^{-5} M) also dilated norepinephrine-preconstricted intracerebral microvessels studied in a brain slice preparation (Sagher et al., 1993). In the present study, both bovine and human intracortical

arterioles dilated to ACh at spontaneous tone and, in the bovine, following preconstriction with 5-HT. The failure of ACh to vasodilate rat pressurized intracerebral arterioles without a pharmacologically-induced tone (Dacey and Bassett, 1987), might be partly explained by the weaker spontaneous tone in these rat vessels, as compared to those in bovine and human. Indeed, the percent of constriction from passive diameter to spontaneous tone was slightly but significantly ($p<0.05$, by unpaired t-test) larger in bovine (38%) and human (41%), than that reported (30%) in the rat (Dacey and Duling, 1982). However, the possibility that the cholinergic response may vary based on species, vessel types, or be unmasked under certain conditions (Shimizu et al., 1993) should not be excluded. In this respect, we found that ACh did not dilate rat intracerebral lentiform arteries regardless whether or not they were preconstricted (unpublished data, 1998).

The finding that the pressure-induced spontaneous tone developed by the arterioles *in vitro* is sufficient to allow neuromediator-induced dilation to take place is compatible with the suggestion that, under resting tone *in vivo*, perivascular release of ACh may result in local vasodilation of brain microvessels. Interestingly, the potency of ACh in inducing dilation was very similar at both spontaneous and pharmacological tone, an indication of the fidelity of the ACh response under different conditions of microvascular tone. Such a property could be highly relevant *in vivo* due to the presence of multiple neuromediators with vasoactive properties in the perivascular space. Additionally, the affinity values obtained for ACh in inducing dilation in intracortical arterioles (pD_2 values of 6.4 and 7.2 in bovine and human, respectively) compared very well with those (6.1 to 7.5) reported in other vessels, such as the pial and major cerebral blood vessels of several species, including man (Dauphin et al., 1991; Tsukahara et al., 1989; Edvinsson et al., 1977; Kanamaru et al., 1989).

The ACh-induced dilation in intracortical arterioles at spontaneous tone (bovine and human) or following preconstriction (bovine) was potently inhibited by the L-NNA, a strong indication of the involvement of NO in the mediation of this response. We cannot ascertain the exact source (endothelial or neuronal) of NO in our arterioles due to the lack of selective

inhibitors for the various forms of NOS and the difficulty in removing the endothelium of these small arterioles without damaging the smooth muscle layer. However, it has previously been shown that the ACh dilatory response is abolished in endothelium-denuded human (Conde et al., 1991; Kanamaru et al., 1989) and bovine cerebral arteries (Fischer-Nakielski and Schrör, 1990). Additionally, physiological studies have indicated that endothelial NO is involved in the hyperemia observed after basal forebrain stimulation in rats (Zhang and Iadecola, 1995). We therefore suggest that in these isolated arterioles, endothelial NO synthesis and release is the most likely contributor of the ACh mediated dilation. Further, our finding that inhibition of NOS with L-NNA elicited a spontaneous contractile response in both human and bovine intracortical arterioles agrees with previous studies which suggested that cerebrovascular tone is in part mediated by constitutively active endothelial NOS (Rosenblum et al., 1990; Faraci, 1991; Kimura et al., 1994; Fergus et al., 1996).

The NO - dependent dilation described above could be mediated by either one of two types of ACh receptors identified in brain microvasculature; nicotinic (Kalaria et al., 1994) and/or muscarinic (Estrada et al., 1983). The failure of nicotine to elicit any vasomotor response(s) in bovine arterioles, in addition to the potent inhibition and/or abolition of the ACh response by selective mAChR antagonists (especially 4-DAMP), suggests that vascular nicotinic receptors are not involved in the ACh-mediated dilation. This finding would agree with the previous suggestion that the efficacy of nicotinic antagonists in attenuating the cortical vasodilation elicited by stimulation of the basal forebrain (Biesold et al., 1989) is most likely mediated by neuronal mechanisms, such as the inhibition of nicotinic receptors located on basal forebrain neurons (Linville et al., 1993) or on perivascular nerve terminals releasing NO or CGRP, as documented in large cerebral arteries (Ayajiki et al., 1994).

In comparison, selective mAChR antagonists blocked the ACh-mediated dilation with varying degrees of efficacy depending on their selectivity for the different mAChR subtypes (Dörje et al., 1991; Alexander and Peters, 1998; Caulfield and Birdsall, 1998). The mAChR antagonist 4-DAMP was the most potent inhibitor of the ACh dilation. It exhibited a potency

which matched quite closely that reported for this compound at the M1 (potency = 8.6-9.2), M3 (potency = 8.9-9.3) and M5 (potency = 8.9-9.0) mAChRs (Dörje et al., 1991; Alexander and Peters, 1998; Caulfield and Birdsall, 1998). However, the respective M1 and M2/M4 mAChR antagonists pirenzepine and AF-DX 384 inhibited the ACh-mediated dilation at concentrations much higher than those expected from their affinities at the respective receptors, thus allowing to exclude the participation of M1, M2 and M4 mAChR subtypes in this response. Together, the antagonists rank order of potency (which best corresponded to the that of the M5 subtype) (Alexander and Peters, 1998) and the significant correlation between their microvascular potencies and those at the M5 mAChR subtype (Dörje et al., 1991; Alexander and Peters, 1998; Caulfield and Birdsall, 1998), suggested that the M5 subtype as the most likely candidate to mediate the NO-dependent dilation in bovine intracortical arterioles. The availability of a selective agonist or antagonist at this site will be necessary to unequivocally confirm this suggestion.

Such an hypothesis, however, is supported by recent findings which showed expression of M2 and M5 mAChR messages in cultures of human cerebromicrovascular endothelial cells, the latter subtype having previously been identified in isolated bovine intracortical microvessels by radioligand binding studies (Linville and Hamel, 1995), and suggested as a candidate for mediating an endothelial- dependent cholinergic vasodilation in these vessels (Elhusseiny et al., 1999). Indeed, the M5 is potently coupled to NO synthesis and release (Wang et al., 1994), and shares pharmacological similarities to the M3 mAChR, a subtype previously suggested to mediate the endothelium-dependent dilation of large cerebral (Dauphin and Hamel, 1990) and small pial (Shimizu et al., 1993) arteries. These characteristics, together with the ubiquitous presence of M5 mAChR in peripheral (Phillips et al., 1997) and cerebral vascular tissues has already led to the suggestion of its role in the ACh-mediated dilation (Hamel et al., 1994; Linville and Hamel, 1995; Elhusseiny et al., 1999; Phillips et al.,1997).

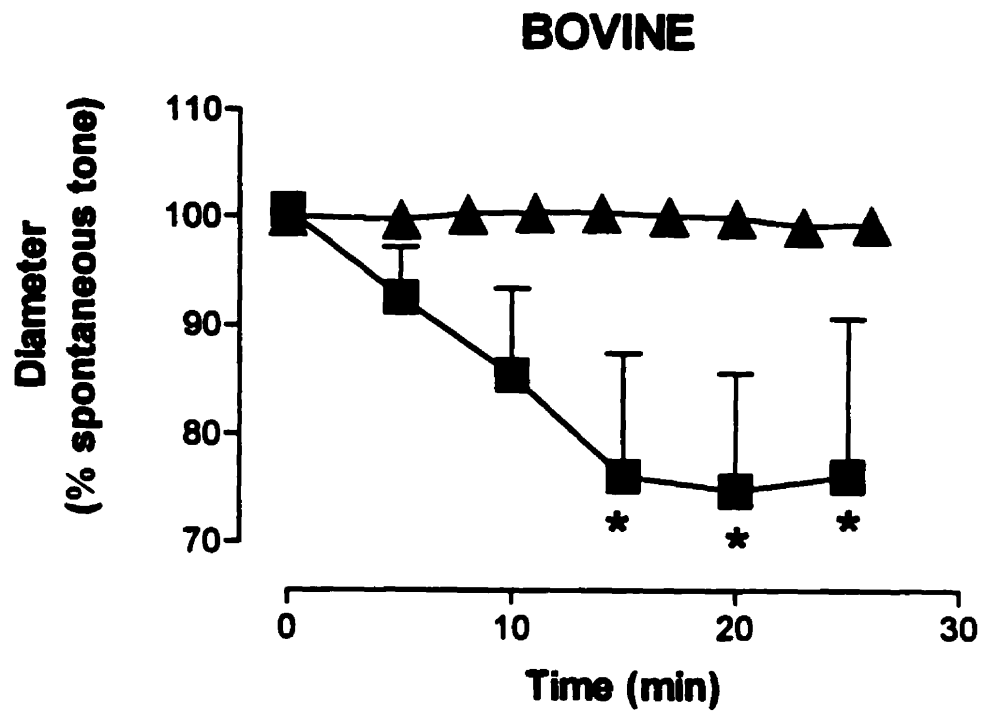
In conclusion, this study provides new and important insights on the physiological and pharmacological properties of vessels that are known to contribute significantly to local cerebrovascular resistance. The data show that brain intracortical arterioles dilate via an NO-

dependent mechanism in response to perivascular ACh application, putatively by direct activation of an endothelial mAChR corresponding best to the M5 subtype. As these vessels are located within the cortical subdivisions in which changes in cerebral blood flow have been observed following stimulation of the basal forebrain in the rat (for a review see Sato and Sato, 1995), we hypothesize that the cholinergic-induced changes in flow can occur, in part, directly at the level of the microvessels. These findings may have implications for the regulation of CBF in pathological conditions, such as Alzheimer's disease, which alter brain cholinergic basal forebrain neurons and their cortical neurovascular projections (Tong and Hamel, 1999).

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada (MA-9967). The authors are indebted to Dr. M. Ward (McGill University) , Drs KS Lee and BR Duling (University of Virginia) for their invaluable assistance in the development of techniques. The authors would also like to thank Dr A. Olivier for provision of human tissues, Mr D. Bélanger from Ecolait Ltée for the generous gift of bovine brains and Ms Linda Michel for preparing the manuscript.

Figure 1: Representation of the progressive decrease over time in the diameter of bovine (*top panel*) and human (*bottom panel*) intracortical arterioles (n=4 per group) following application of L-NNA (10^{-5} M) to the perfusion solution (■). For comparison, the lack of effect of vehicle (▲) is illustrated in bovine arterioles (n=3) over the same time period. (*: $p < 0.05$).


BOVINE
Diameter
(% spontaneous tone)
110
100
90
80
70
*
*
*
0
10
20
30
Time (min)

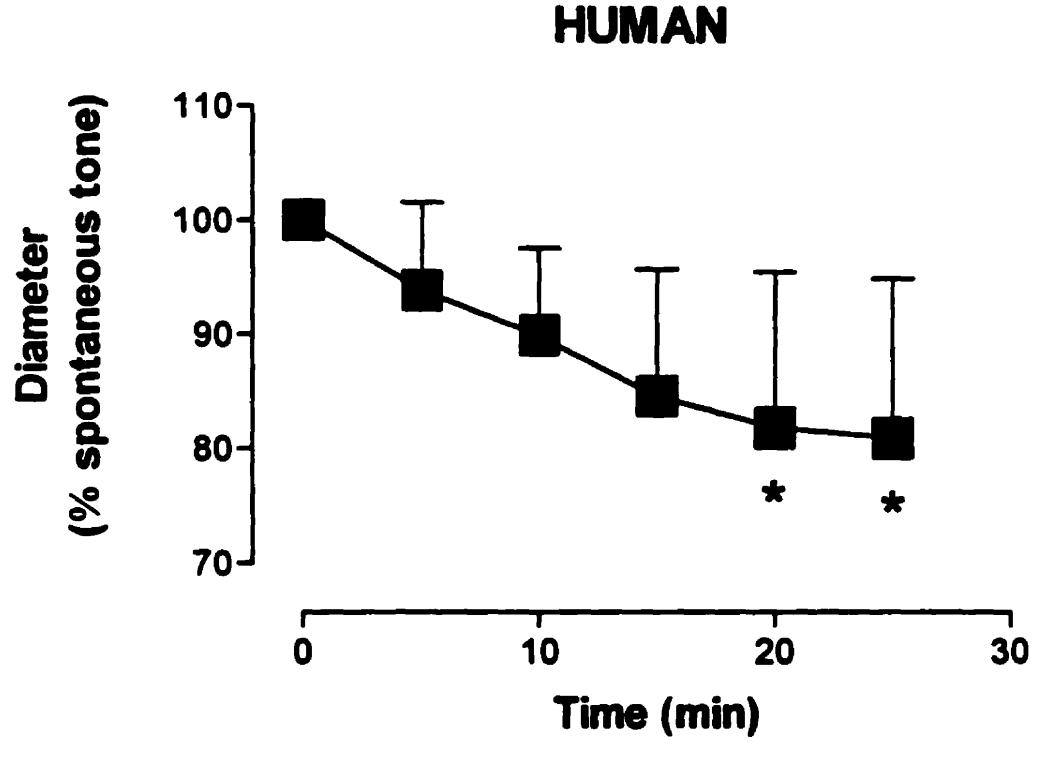
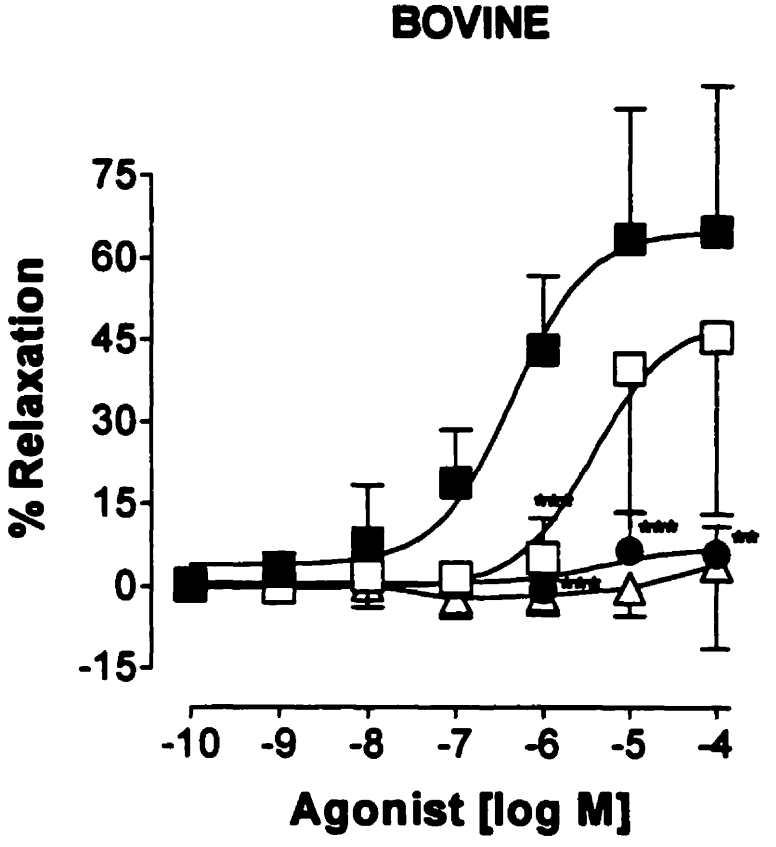

HUMAN
Diameter
(% spontaneous tone)
110
100
90
80
70
*
*
0
10
20
30
Time (min)

Fig. 1

Figure 2: Concentration-dependent dilation elicited by acetylcholine in bovine (*top panel*, n= 4-10) and human (*bottom panel*, n= 5-9) intracortical arterioles at spontaneous tone, in the absence (■) and presence of 10^{-5}M L-NNA (□) or 10^{-4}M L-NNA (●). The acetylcholine-induced dilation in bovine arterioles was inhibited by L-NNA in a dose-dependent manner (10^{-5}-10^{-4}M) while it was virtually abolished by 10^{-5}M L-NNA in human arterioles (*: $p< 0.05$, **: $p< 0.01$, ***: $p<0.001$). The lack of vasomotor response on increasing concentrations of nicotine (Δ, n=5) is also illustrated in bovine intracortical arterioles. Results are expressed as percent relaxation of spontaneous tone relative to papaverine.


BOVINE
% Relaxation
75
60
45
30
15
0
-15
-10
-9
-8
-7
-6
-5
-4
Agonist [log M]

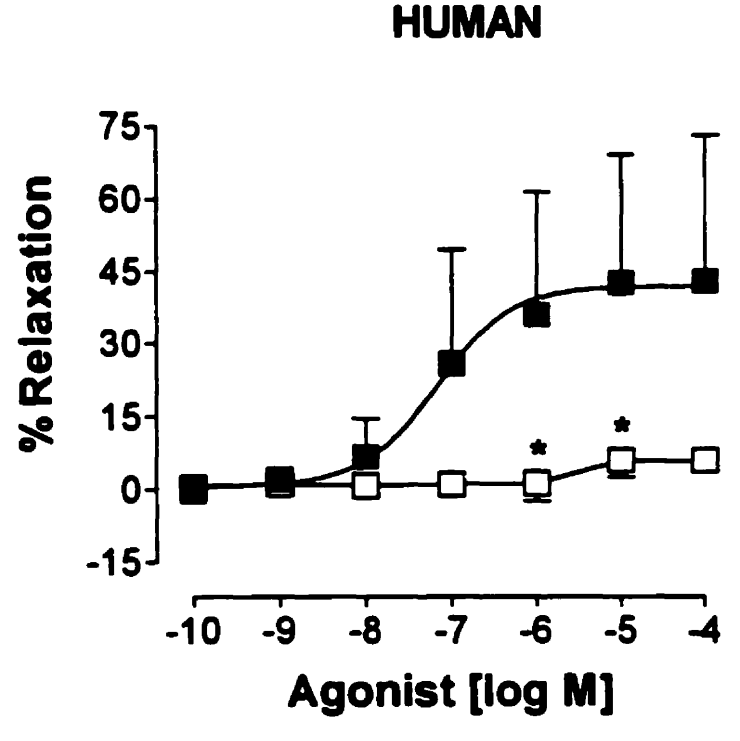

HUMAN
% Relaxation
75
60
45
30
15
0
-15
-10
-9
-8
-7
-6
-5
-4
Agonist [log M]

Fig. 2

Figure 3: Concentration-response curves of the effects of acetylcholine (■) and nicotine (Δ) in bovine intracortical arterioles preconstricted with 10^{-6}M 5-HT. The inhibitory effect of 10^{-5}M L-NNA (□) on the dilation induced by 10^{-5}M acetylcholine is also illustrated. (n = 5, ✳: $p < 0.05$).

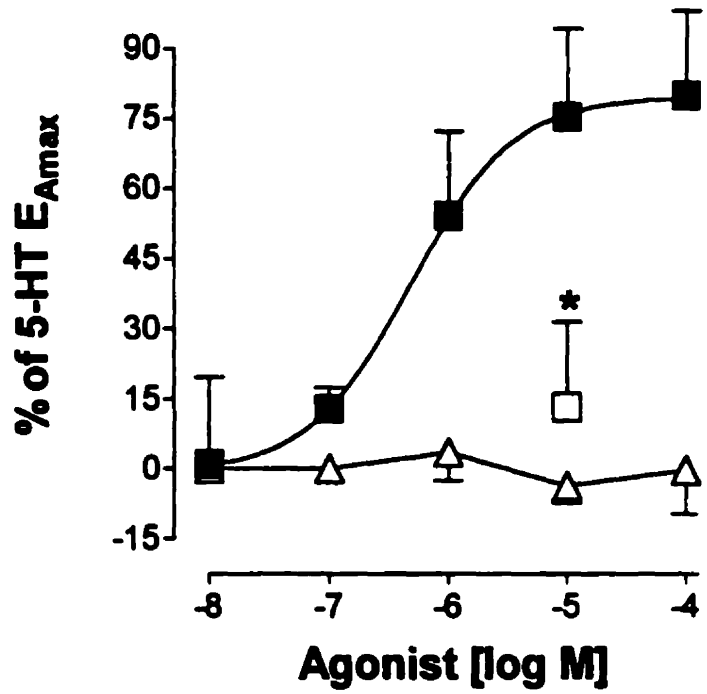


Fig. 3

Figure 4: *A*: Inhibition of the acetylcholine-induced dilation in 10^{-6} M 5-HT-preconstricted bovine intracortical arterioles following addition of 10^{-5} M acetylcholine in the absence (**A**, Control) and presence of increasing concentrations of the muscarinic receptor antagonists 4-DAMP (**B**), pirenzepine (**C**) or AF-DX 384 (**D**). Results are expressed as the means ± SD of 3 to 6 independent experiments (*: $p< 0.05$, **: $p<0.01$). *B*: Inhibition curves of the 10^{-5}M acetylcholine-induced dilation in bovine intracortical arterioles by the muscarinic receptor antagonists pirenzepine (■), 4-DAMP (▲) or AF-DX 384 (●). Results are expressed as a percent ± SD of the maximal dilation induced by acetylcholine in the absence of antagonists. The respective pIC_{50} values for these antagonists are given in the text.

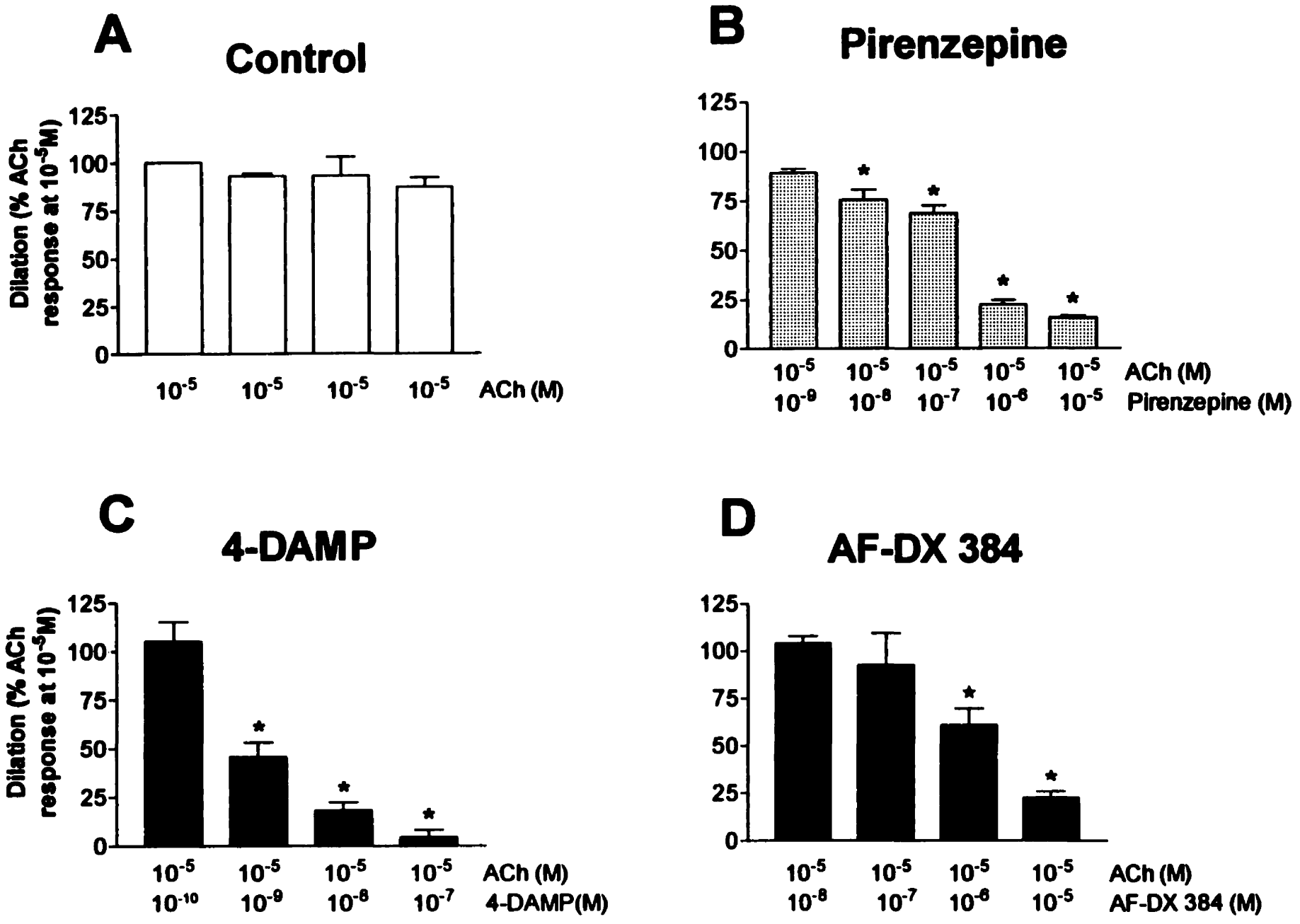

A
Control
Dilation (% ACh response at 10^{-5}M)
125
100
75
50
25
0
10^{-5} 10^{-5} 10^{-5} 10^{-5} ACh (M)
B
Pirenzepine
125
100
75
50
25
0
10^{-5} 10^{-5} 10^{-5} 10^{-5} 10^{-5} ACh (M)
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} Pirenzepine (M)
C
4-DAMP
Dilation (% ACh response at 10^{-5}M)
125
100
75
50
25
0
10^{-5} 10^{-5} 10^{-5} 10^{-5} ACh (M)
10^{-10} 10^{-9} 10^{-8} 10^{-7} 4-DAMP(M)
D
AF-DX 384
125
100
75
50
25
0
10^{-5} 10^{-5} 10^{-5} 10^{-5} ACh (M)
10^{-8} 10^{-7} 10^{-6} 10^{-5} AF-DX 384 (M)

Fig 4A

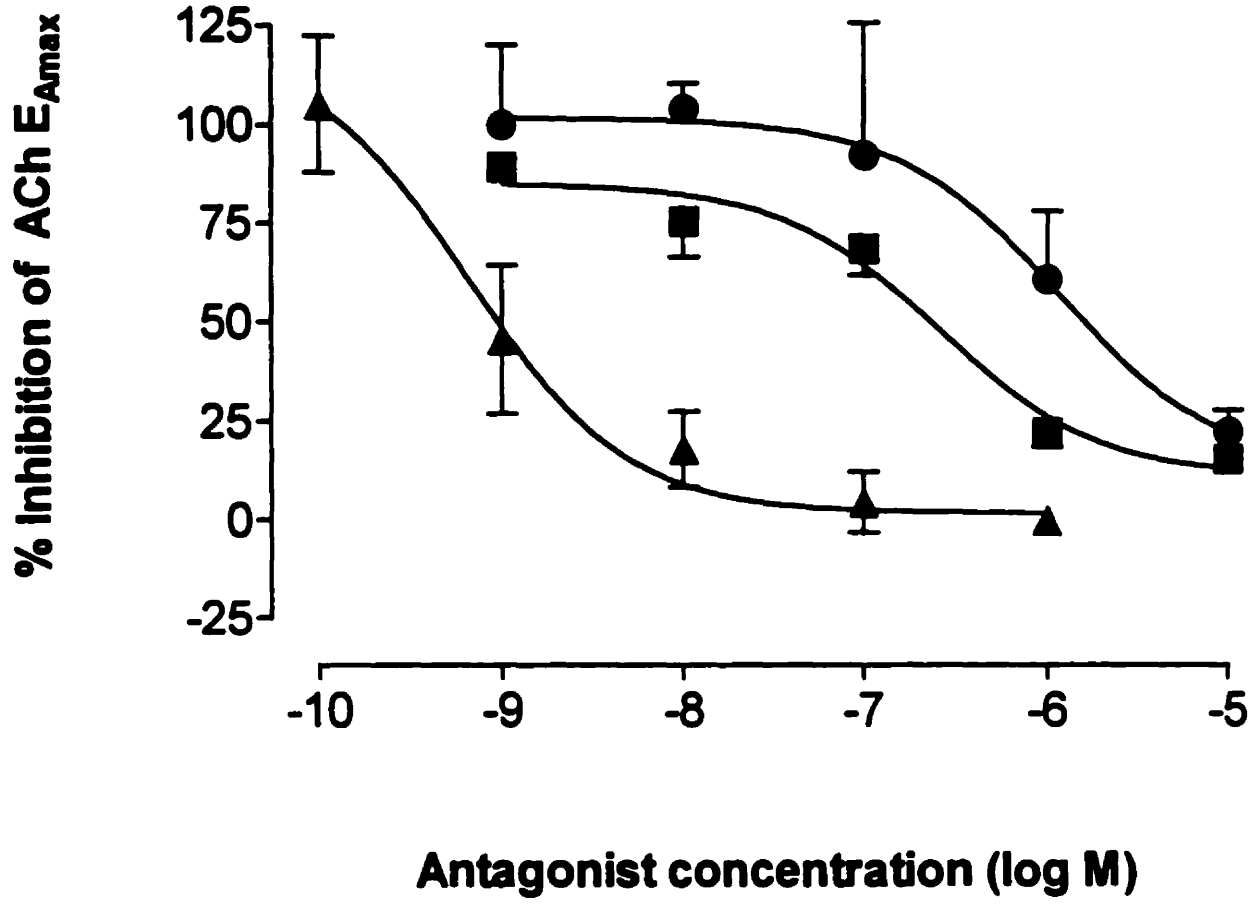

% Inhibition of ACh E_{Amax}
125
100
75
50
25
0
-25
-10
-9
-8
-7
-6
-5
Antagonist concentration (log M)

Fig. 4B

PREFACE TO CHAPTER 4

We demonstrated in the previous chapter that ACh will dilate brain intraparenchymal arterioles which *in vivo* could translate into an increase in CBF in the brain. Studies have indicated that it is possible for the brain to lower CBF via neuronal mechanisms as well. In particular, physiological and anatomical studies on the brain serotonergic system suggest that this neurotransmitter can play a role in this regard. Recently, Cohen et al. (1999) demonstrated that the human brain intraparenchymal microcirculation is endowed with numerous 5-HT receptor subtypes, indicating the possibility that they can directly mediate function to 5-HT release. It still is unknown however, if and how these receptors can alter the vasomotor tone of microvessels. This question is addressed in this chapter.

Using the same technique as the one described in chapter 3, we investigated the effect(s) of 5-HT and its subtype selective 5-HT_1 agonists and antagonist on human and bovine intracortical arterioles. We then investigated the role of NO in some of the observed responses. Finally, we compared the effect of 5-HT in intracortical arterioles with those seen in pial vessels and drew a correlation between response and vessel size.

CHAPTER 4

5-HT_{1B} RECEPTORS MEDIATE BOTH CONSTRICTION AND NITRIC OXIDE-DEPENDENT DILATION IN HUMAN AND BOVINE BRAIN INTRACORTICAL ARTERIOLES

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Key Words: Serotonin, cerebral blood flow, endothelium, smooth muscle, sumatriptan, migraine, arteries

Running Title: 5-HT_{1B} receptors mediate constriction and dilation of intracerebral arterioles

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ABSTRACT

Stimulation of brainstem serotonergic neurons induces changes in regional cerebral blood flow (CBF), possibly via direct activation of serotonin (5-HT) receptors localized in brain microvessels. To date, however, little is known about these microvascular receptors. In this study, we assessed *in vitro* the effect(s) of 5-HT receptor activation on microvascular tone of pressurized intracortical arterioles (~ 40 - 50 μm) isolated from bovine and human cerebral cortex. 5-HT, subtype selective 5-HT_1 receptor agonists, a selective 5-HT_1 receptor antagonist (GR127935), and the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA) were used to identify the 5-HT_1 receptor subtype(s) and mechanisms involved in the observed vasomotor responses. Bovine pial vessels were studied for comparitive purposes. At spontaneous tone, 5-HT induced a dose-dependent constriction in human and bovine arterioles (pD_2 = 7.3 ± 0.2 and pD_2 = 6.9 ± 0.1, respectively), a response potently inhibited by GR127935 (pIC_{50} = 8.5 ± 0.1). In both species, the 5-HT_1 receptor agonist sumatriptan mediated a biphasic response consisting of a small but significant dilation at low concentrations, followed by a constriction at higher doses (pD_2 for contraction = 6.9 ± 0.1 and 6.6 ± 0.2 in human and bovine vessels, respectively). In contrast, the selective 5-HT_{1D} (PNU-109291) and 5-HT_{1F} (LY344864) receptor agonists failed to elicit any vasomotor effect. In both species, pre-incubation with the L-NNA (10^{-5}M) abolished the sumatriptan-induced dilation and significantly shifted the dose-response curve of the constriction phase to the left. In bovine pial vessels, 5-HT and sumatriptan elicited potent constrictions (pD_2 = 7.2 ± 0.1 and 6.6 ± 0.1, respectively), and a weak dilation was occasionally observed at low sumatriptan concentrations. An overall significant negative correlation was observed between vessel diameter and the extent of the dilatory response to sumatriptan. These results indicate that stimulation of 5-HT_{1B} receptors in brain vessels can induce a constriction and/or an NO-mediated dilation depending on the size and, possibly, the existing tone of the vessel.

INTRODUCTION

Serotonergic fibers from peripheral and central neurons are known to respectively innervate blood vessels in the extra- and intracerebral circulation, and to exert direct vasomotor effects (for review see Lincoln, 1995; Cohen et al., 1996). In brain intracortical microvascular bed, the source of perivascular 5-HT nerves has been identified as the raphe nucleus (Reinhard et al., 1979) which upon stimulation can induce either an increase (vasodilation) or a decrease (vasoconstriction) in cerebral blood flow (CBF) (for review Cohen et al., 1996). This dual response has been attributed to several factors such as the initial tone of the blood vessels (Rosenblum and Nelson 1990), the region of the dorsal raphe nucleus stimulated (Underwood et al. 1992), and the method used to measure blood flow (see Cohen et al., 1996). However, the recent identification of numerous 5-HT receptor subtypes (some of which known to mediate dilation or constriction) in human brain microvascular tissues and cells in culture (Cohen et al., 1999), raises the possibility that these different vasomotor responses can be due to activation of different receptor populations. The presence of specific 5-HT receptors in distinct endothelial and/or smooth muscle compartments of the microcirculation further points to the possibility that they both can regulate vasomotricity, as was shown previously in other vascular beds following removal of the endothelial cell layer (Faraci and Heistad, 1992; MacLean et al., 1994).

Of the various receptors identified in the brain microcirculation, the 5-HT_{1B} (formally identified in man as the 5-$HT_{1D\beta}$) subtype is of particular interest in light of its known vasomotor role in both the extracerebral and peripheral circulation. This receptor has been implicated in the contraction (Hamel and Bouchard, 1991; Hamel et al., 1993b; Kaumann et al., 1993) and endothelium-dependent relaxation (Schoeffter and Hoyer 1990, Gupta 1992) of blood vessels. In brain cortical blood vessels, the 5-HT_{1B} receptor protein has been localized to both the smooth muscle and endothelial cells of large microvessels (Riad et al. 1998) but not to the endothelial cells of capillaries (Riad et al. 1998; Cohen et al., 1999) implying a possible role for the endothelial receptor in larger microvessels. To date the only recorded *in vitro* response to 5-HT application in intraparenchymal arterioles has been

constriction (Dacey and Basset, 1987). This contrasts with early studies which documented, using the cranial window technique, that brain surface arterioles and small pial arteries (<200μm) would dilate while larger arteries (>200μm) would constrict, in response to 5-HT administration (Harper and Mackenzie 1977, Edvinsson et al., 1978, Auer et al., 1985).

In the present study, we examined the response(s) of bovine and human brain intracortical arterioles to exogenously applied 5-HT. We further used a selective 5-HT_1 receptor antagonist and subtype selective 5-HT_1 receptor agonists to identify the receptor subtype(s) involved in the observed vasomotor responses. Since some of 5-HT_1 receptors are specifically targeted by acute antimigraine drugs (e.g. the triptans), the present data may have implications in the treatment of this disease.

MATERIALS AND METHODS

Intracortical penetrating arterioles were isolated from human (biopsies of temporal and/or frontal cortex from patients undergoing surgery for the treatment of epilepsy obtained with approval from the Institution research ethics committee) and bovine (frontoparietal cortex, Abattoir Ecolait, Terrebonne, QC, Canada) cerebral cortex following a procedure similar to that previously described in detail (Elhusseiny and Hamel, 2000; Dacey and Duling, 1982). Briefly, penetrating arterioles (average intraluminal diameter ~47μm) were dissected from cortical slices (1 - 2 mm thick) cut parallel to the surface of the brain and placed in cold MOPS solution (4°C, pH 7.4 ± 0.1) with a basic composition (in mM) of NaCl, 144; KCl, 3.0; $CaCl_2$, 2.5; $MgSO_4$, 1.5; glucose, 5; pyruvate, 2.0; ethylenediaminetetraacetic acid (EDTA), 0.02; morpholinopropanesulfonic acid (MOPS), 2.0; and NaH_2PO_4, 1.21 (Dacey and Basset, 1987). For comparison, pial vessels (intraluminal diameters ~ 180μm) were isolated from the surface of the bovine cerebral cortex and prepared in the exact same manner as the intracortical vessels.

Arterioles were cannulated on a glass micropipette (20-30μm diameter) at one end and sealed

to another glass micropipette (~ 40μm diameter) on the other end in an arteriograph chamber system (Living Systems Instrumentation, Burlington, Vermont) modified for continuous on-line monitoring and/or maintenance of temperature (37±1°C) and pH (7.4±0.1), and filled with a de-bubbled MOPS/albumin (albumin 1%). Pial vessels were cannulated with the exact same procedure as arterioles except that the glass micropipette diameters were larger (~80μm). A pressure-servo micropump (Living Systems Instrumentation) was used to maintain intraluminal pressure at 60mmHg (Dacey and Basset, 1987). Vessels were then superfused (6 ml/min) with MOPS solution and allowed to stabilize and acquire basal tone (45 min - 1 hr). On-line measurements of intraluminal vessel diameters were performed using a closed circuit video system (National Electronics, Taiwan) coupled to a video caliper (Imagen Instrumentation, Trenton, New Jersey, USA). All compounds were added to the superfusing MOPS solution and thus were applied extraluminally for a period of 3 minutes. Smooth muscle reactivity was tested with 70mM K^+ in MOPS. At the end of each experiment, the smooth muscle relaxant papaverine (10^{-4}M) was added to maximally dilate the vessels.

Graded concentrations of 5-HT were added to the superfusion solution bathing isolated intracortical arterioles (bovine and human) or pial arteries (bovine) at basal tone. In order to identify the receptor subtype(s) involved in the 5-HT-mediated vasocontractile response, bovine intracortical arterioles were exposed to 10^{-6}M 5-HT following pre-incubation (15 min) with different concentrations (10^{-10} - 10^{-7}M) of the selective $5HT_1$ receptor antagonist GR127935 (N-[methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methly-4'-(5-methly-1,2,4-oxadiazol3-yl)[1,1-biphenyl]-carbozamide hydrochloride; Skingle et al., 1996; GlaxoWellcome, Greenford, UK). The reproducibility of the 5-HT contraction was verified by sequential application (four times) of 10^{-6}M 5-HT to the vessel segments and recording any change in the resulting response. In addition, dose-response curves (10^{-11} or 10^{-10} - 10^{-5} M) of selective $5\text{-HT}_{1B/1D/1F}$ (sumatriptan), 5-HT_{1D} (PNU-109291: (S)-(-)-1[1-[4-(4-methoxyphenyl)-1-piperaziny-1]ethyl]-N-methylisochroman-6-carboxamide, Ennis et al., 1998; Pharmacia and Upjohn, Kalmazoo, MI) and 5-HT_{1F} (LY344864: (R)-(+)-N-(3-

dimethlyamino-1,2,3,4-tetrahydro-9H-carbazol-6-yl)-4-fluorobenzamide, Phebus et al., 1997; Eli Lilly, Indianapolis, IN) receptor agonists were generated in bovine and/or human intracortical arterioles. The role of nitric oxide (NO) in the sumatriptan-induced dilation of bovine and human arterioles was assessed by pre-incubation (30 min) of the vessels with the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA, 10^{-5} M) before generation of the dose-response curves. As a comparison, the vasomotor responses of small bovine pial vessels to increasing concentrations of 5-HT and sumatriptan were also evaluated.

Calculations and Statistical Analysis

Agonists maximal response (expressed as percent change in vessel diameter from spontaneous or basal tone) and potency (pD_2 values or -log of EC_{50}) were determined, and the efficacy of the 5-HT_1 receptor antagonist GR127935 expressed as a pIC_{50} value (negative logarithm of the molar antagonist concentration which induces 50% inhibition of maximal response) calculated from the antagonist concentration curve.

Values are expressed as the mean ± S.E.M. The statistical differences between the means of two measurements were determined by either paired or unpaired Students *t* test for independent observations as described in the legend to each figure. One way ANOVA followed by a Dunnett comparison test was used to analyze the inhibitory effects of the antagonist on the 5-HT-induced constriction, and a one way ANOVA followed by a Bonferroni comparison test was used to test the effect of L-NNA inhibition on the sumatriptan response. In all cases, $p<0.05$ was considered significant. All statistics were performed on the statistical software Prism (GraphPad Software inc.)

RESULTS

At basal tone, 5-HT (10^{-11} to 10^{-4} M) constricted human and bovine intracortical arterioles, in a dose- dependent manner (Figs. 1A and 1B) with respective pD_2 values of 7.3 ± 0.2 and 6.9 ± 0.1 and maximal responses accounting for 10.5 ± 1.5 % and 19.9 ± 3.1 % from

spontaneous tone. Repeated (four times) application of 10^{-6}M 5-HT to bovine arterioles did not affect the constrictor response to 5-HT (Fig 2, *top panel*). However, increasing concentrations of the 5-HT_1 receptor antagonist GR127935 significantly ($p<0.01$) inhibited the 5-HT-induced constriction (Fig 2, *bottom panel*) with a pIC_{50} value of 8.5 ± 0.1 and a maximal inhibition (94.9 ± 2.2%) at 10^{-7}M GR127935 (Fig 2, *bottom panel inset*).

The 5-HT_1 receptor agonist sumatriptan elicited a biphasic response consisting of a dilation at low concentrations followed by a constriction. The dilation, while small, was shown to be significantly different from baseline diameters at 10^{-9} and/or 10^{-8}M sumatriptan in human and bovine arterioles (Figs 1A and 1B). In both species, sumatriptan elicited constrictions with respective potencies (pD_2 values = 6.9 ± 0.0 and 6.6 ± 0.2) and maximal effects from spontaneous (4.9 ± 2.3% and 12.7 ± 1.7 %) which were significantly lower ($p<0.05$) than those induced by 5-HT. In bovine arterioles, application of the selective 5-HT_{1D} and 5-HT_{1F} receptor agonists PNU-109291 and LY344864, respectively, did not produce any significant changes in vessel diameters (Fig. 1B).

In both species, pre-incubation of the vessel segments with the NOS inhibitor L-NNA (10^{-5}M) abolished the vasodilation observed at low sumatriptan concentrations (Figs. 3A and 3B). In these L-NNA-treated vessels, the sumatriptan dose-response curve was significantly shifted to the left in human and bovine vessels (respective pD_2 of 7.7 ± 0.2 , $p<0.001$ and 7.1 ± 0.1, $p<0.01$). In human vessels, blockade of NO synthesis by L-NNA (10^{-5}M) also resulted in a significantly higher maximal contractile response to sumatriptan (21.2 ± 6.7% as compared to 4.9 ± 2.3%, $p<0.05$).

Finally, in order to identify if the sumatriptan-induced dilation is also present in extracerebral blood vessels, the effect of 5-HT and sumatriptan was investigated in bovine small pial vessels (diameter = 179 ± 34, range 63 - 343μm; Fig 4). In these vessels, the predominant response to 5-HT and sumatriptan was a constriction of equivalent intensity (25.9 ± 2.5% and 22.6 ± 4.1%, respectively, from spontaneous tone) but sumatriptan was significantly less potent than

5-HT (respective pD_2 values of 6.6 ± 0.1 and 7.2 ± 0.02, $p<0.001$). A small but not significant dilation to low sumatriptan concentrations was also observed in about half (55.6%) of the pial vessels studied. The dilation was more pronounced in small size vessels than in large ones. An overall significant negative correlation ($p<0.05$, $r = 0.6$, slope = -0.03 ± 0.01: Fig.5) was observed between vessel diameter at spontaneous tone (both intracortical and pial vessels) and increased diameter at 10^{-9}M sumatripan. This observation suggested that the dilation to sumatriptan was inversely related to vessel size with a break point at 201μm (see Fig. 5).

DISCUSSION

The present *in vitro* findings demonstrate for the first time that 5-HT constricts human and bovine brain intracortical arterioles via a 5-HT_{1B} receptor as identified by the use of subtype selective 5-HT_1 receptor agonists and antagonist. Moreover, they show that low concentrations of the 5-HT_1 receptor agonist sumatriptan resulted in a small but significant NO-dependent dilation that was overridden by constriction at higher concentrations. Together with our previous localization of 5-HT_{1B} receptor in the endothelial and smooth muscle cells of intracortical arterioles, the present results suggest that the NO-dependent dilation is mediated by an endothelial 5-HT_{1B} receptor and that its magnitude depends on the size of the vessel; small vessels will tend to dilate more than large ones, with vessels larger than 200μm showing primarily, if not exclusively, a constriction.

The overall potency for 5-HT in intracortical vessels compared very well with that reported in extracerebral blood vessels from both human and bovine brain (Edvinsson et al., 1978; Parsons et al., 1989; Hamel and Bouchard 1991; Hamel et al., 1993b; Bouchelet et al., 2000). In the only other *in vitro* study on isolated brain intracerebral arterioles, albeit in the rat, Dacey and Basset (1987) demonstrated a small vasoconstriction to 5-HT, followed by a vasodilation at high 5-HT concentrations ($>10^{-6}$M) that they attributed to changes in pH. In the present study, we did not observe a significant and reproducible dilation to 5-HT at any concentrations. This finding may appear at variance with previous studies which showed that

5-HT can act as a vasodilator under certain conditions (Cohen et al., 1983; Cocks and Angus, 1983; Potenza et al., 1998), especially in small size cerebral (Harper and MacKenzie, 1977; Edvinsson et al., 1978; Auer et al., 1985) and peripheral (Lamping 1997) arteries and arterioles. However, the 5-HT_1 receptor agonist sumatriptan systematically and significantly dilated bovine and human intracortical arterioles at low concentrations while at higher doses, it elicited contraction, observations suggesting that a 5-HT_1 receptor is involved in both vasomotor responses.

The participation of 5-HT_{1B} receptors in the 5-HT-mediated microvascular constriction is supported by the ability of the selective 5-HT_1 receptor antagonist GR127935 to potently antagonize this response, with an affinity (pIC_{50} = 8.5) compatible with that reported for this compound at the 5-HT_{1B} receptors in large cerebral arteries (Skingle et al., 1996; Teng et al., 1998; Bouchelet et al.,2000). Additional evidence for the involvement of 5-HT_{1B} in the arteriolar vasoconstriction was provided in bovine microvessels by the failure of selective 5-HT_{1D} (PNU-109291) and 5-HT_{1F} (LY344864) receptor agonists - which exhibit affinities in the nM range for their respective receptor subtypes (Ennis et al., 1998; Phebus et al., 1997) - to mimic this response at concentrations as high as 10^{-5}M. The high level of expression of 5-HT_{1B}, and the virtual or complete absence of 5-HT_{1D} of 5-HT_{1F} receptor mRNAs in smooth muscle cells derived from human intracortical microvessels (Cohen et al, 1999), are also fully compatible with the present findings and further support that 5-HT_{1B} receptors are the mediators of the 5-HT vasocontractile response. This conclusion agrees with previous studies in peripheral (Ennis et al. 1998; Johnson et al., 1998; Phebus et al., 1997) and extracerebral (Bouchelet et al.,2000) blood vessels.

However, a novel finding from the present study was that low concentrations of sumatriptan exerted dilation of human and bovine brain intracortical arterioles, a response which was reversed by inhibition of NO synthesis by L-NNA. Interestingly, an NO- or endothelial-dependent component in the vasomotor response to 5-HT and/or sumatriptan has been documented in cerebral (Faraci and Heistad, 1992) and peripheral (Cocks and Angus, 1983;

Cohen et al., 1983; Schoeffter and Hoyer, 1990; Whiting and Cambridge 1995) blood vessels and suggested to be mediated by an endothelial 5-HT_1 (Molderings et al., 1989; Whiting and Cambridge, 1995; Lamping, 1997), most likely 5-HT_{1B} or 5-HT_{1D} (Schoefter and Hoyer 1990; Gupta et al., 1992), receptor subtype. As neither the selective 5-HT_{1D} or 5-HT_{1F} receptor agonist, PNU109209 or LY344864, could elicit a vasodilatory response in our preparation, we conclude that the dilation of intracortical arterioles, like the vasoconstriction, is mediated by 5-HT_{1B} receptors. Such statement also agrees with our ultrastructural localization of 5-HT_{1B} receptor protein in endothelial cells of large intraparenchymal microvessels (Riad et al., 1998). Similarly, endothelial 5-HT_{1B} receptors have recently been identified in coronary arteries (Ullmer et al., 1995; Ishida et al., 1998; Nilsson et al., 1999b), in which they promote NO production (Ishida et al., 1998).

The dilatory response was not restricted to intracortical vessels, as some pial vessels also dilated to low sumatriptan concentrations. In fact, the vasomotor response to low sumatriptan concentrations was inversely proportional to the diameter of the vessel at basal tone. Intracortical and/or pial vessels with diameters less than 200μm were more likely to dilate, while larger vessels typically constricted. Interestingly, a similar correlation between vessel diameter and vascular response has been reported in pial vessels, with a breakpoint between dilation and constriction evaluated around 200μm (Edvinson et al., 1978; Harper and MacKenzie 1977; Auer et al., 1985). Differences in tone (Harper and MacKenzie, 1977), blood-brain barrier permeability (Auer et al., 1985) and 5-HT receptor subtypes (Lamping, 1997) between large and small vessels have been proposed as possible explanations for this effect. Alternatively, it could be argued that as vessels get larger, they contain more layers of smooth muscle cells endowed with contractile 5-HT_{1B} receptors (Hamel et al., 1993a; Bouchelet et al., 1996; Riad et al., 1998; Cohen et al., 1999; Nilsson et al., 1999a,b), which eventually pushes the equilibrium between endothelial (Riad et al. 1998, Nilsson et al., 1999a,b) and smooth muscle receptors towards constriction. 5-HT failed to induce a significant dilation of intracortical arterioles, possibly because it interacts with additional,

sumatriptan-insensitive 5-HT receptors in the vessel wall (Hamel et al., 1993b; Cohen et al., 1999; Bouchelet et al., 2000; Kauman et al., 1993) that mask the vasodilator response. It could also be that, like recently reported in rat mesenteric vessels (Potenza et al., 1998), the relaxant potency and efficacy of sumatriptan are greater than those of 5-HT, thus allowing the vasodilatory response to be more readily detected.

Notably, the sumatriptan-induced vasoconstrictions occurred at slightly but significantly higher concentrations and were smaller in magnitude than those elicited by 5-HT in bovine and human microvessels. In contrast, the maximal response to 5-HT and sumatriptan in the larger bovine pial vessels were of comparable intensity. It thus appears that the dilatory response in the larger pial vessels is not readily detectable, possibly due to the predominance of smooth muscle over endothelial 5-HT receptors (Nilsson et al., 1999a). In this respect, it should be noted that in intracortical arterioles, the maximal constriction to sumatriptan was calculated as the change from spontaneous tone, i.e. the baseline diameter of the vessel. If calculated as the change from the maximal dilation, then the maximal constrictions to sumatriptan (9.3 ± 2.2 and 15.0 ± 1.85 % from dilation, respectively in human and bovine vessels) are not statistically different from those of 5-HT (10.5 ± 1.5% and 19.9 ± 3.1) from spontaneous tone, observations which indicate that it is the dilatory response which produces an apparent reduction in the magnitude of the sumatriptan-induced contraction. The fact that the maximal vasoconstrictions between sumatriptan in the presence of L-NNA (to abolish dilation) and 5-HT are comparable (14.2 ± 3.2 vs 19.9 ± 3.1%, respectively from spontaneous tone) in bovine vessels, provides additional support to this conclusion. These observations would indicate that most if not all of the contractile response to 5-HT is mediated by the 5-HT_{1B} receptors in bovine intracortical arterioles, findings at variance with studies on bovine major cerebral arteries in which there is a contribution of 5-HT_{2A} receptors in the 5-HT-induced contraction (Hamel et al., 1993b; Kaumann et al., 1993; Bouchelet et al., 2000). However they are in agreement with the absence of 5-HT_{2A} receptors in human brain vessels (Hamel and Bouchard, 1991; Hamel et al.1993b; Kauman et al., 1993) and microvessels (Cohen et al., 1999), and with the observation that in ovine and/or bovine small cerebral

arteries (Teng et al., 1998) as well as in vessels with vascular tone (MacLean et al., 1994), constriction to 5-HT tends to be preferentially mediated by the 5-HT_{1B} as opposed to the 5-HT_{2A} receptor. The latter two conditions apply exquisitely well to the intracortical vessels used in the present study and further support a predominant if not exclusive role of a 5-HT_{1B} receptor in the contractile response.

Incubation with L-NNA shifted the contraction curve for sumatriptan to the left and in human arterioles significantly increased the maximal response. Sumatriptan (Whiting and Cambridge, 1995) and 5-HT-induced vasoconstrictions have previously been shown to increase upon endothelial denudation or application of NOS inhibitors (Cocks and Angus, 1983; Sercombe et al., 1985; Faraci and Heistad, 1992). We attribute the potentiating effect of NOS inhibition of the sumatriptan-induced microarteriolar contractile response to the inhibition of NO production and release following activation of endothelial 5-HT_1 receptors (Whiting and Cambridge, 1995), the reduction in the constitutive endothelial NO synthesis by intracerebral arterioles (Kimura et al., 1994; Fergus et al., 1996; Elhusseiny and Hamel, 2000), and/or the release of endothelin by NOS inhibition (Thorin et al., 1998). The more pronounced potentiating effect of L-NNA in human than bovine arterioles could be explained by possible species differences in 5-HT_{1B} receptor density in smooth muscle and endothelial cells, or by the recently documented differences in NOS sensitivity to L-NNA between bovine and human intracortical arterioles (see Elhusseiny and Hamel, 2000).

In conclusion, this study provides the first functional evidence for a dual role of 5-HT_{1B} receptors in human and bovine brain microvessels. Identification of a dual response, which appears to depend on vessel size, could be an intrinsic feedback mechanism aimed at preventing excessive 5-HT-induced responses in smaller vessels. It also provides a basis for previous physiological findings which reported increases or decreases in local CBF upon stimulation of the raphe nucleus (see Cohen et al., 1996). It is thus possible that, in addition to the subregions of the raphe being stimulated and the intrinsic tone of the vessels, the equilibrium between endothelial (dilatory) and muscular (contractile) 5-HT_{1B} receptors might

be an important contributing factor to the final adaptation of local blood flow to neuronal activity following raphe stimulation and 5-HT release. Further, since the present finding in microarterioles can be extrapolated to larger pial vessels which are also endowed with endothelial 5-HT_{1B} receptors (Nilsson et al., 1999a), the identification of an NO producing 5-HT_{1B} receptor becomes particularly interesting in light of the suggested role for endothelial NO-generating receptors in the aetiology of migraine headache (Schmuck et al., 1996, Fozard and Kalkman 1994). It could be argued that NO production by endothelial 5-HT_{1B} receptors is altered during migraine attacks, with NO reaching levels that might be detrimental. Furthermore, since the vasodilation occurs at low sumatriptan concentrations, the high headache recurrence rate attributed to sumatriptan (Schoenen, 1997) could conceivably be a direct consequence of circulating low bioactive levels of this compound, which would no longer constrict vessels and ease the pain, but rather promote dilation and, possibly, headache recurrence.

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Figure 1: Concentration-dependent vasomotor responses to 5-HT (■), and the selective 5-HT_1 receptor agonists sumatriptan (▲), PNU-109291 (▼), and LY344864 (●) in human (A: n=3-8) and/or bovine (B: n=5-13) intracortical arterioles. (*: $p<0.05$, **: $p<0.01$ by paired *t*-test from control).

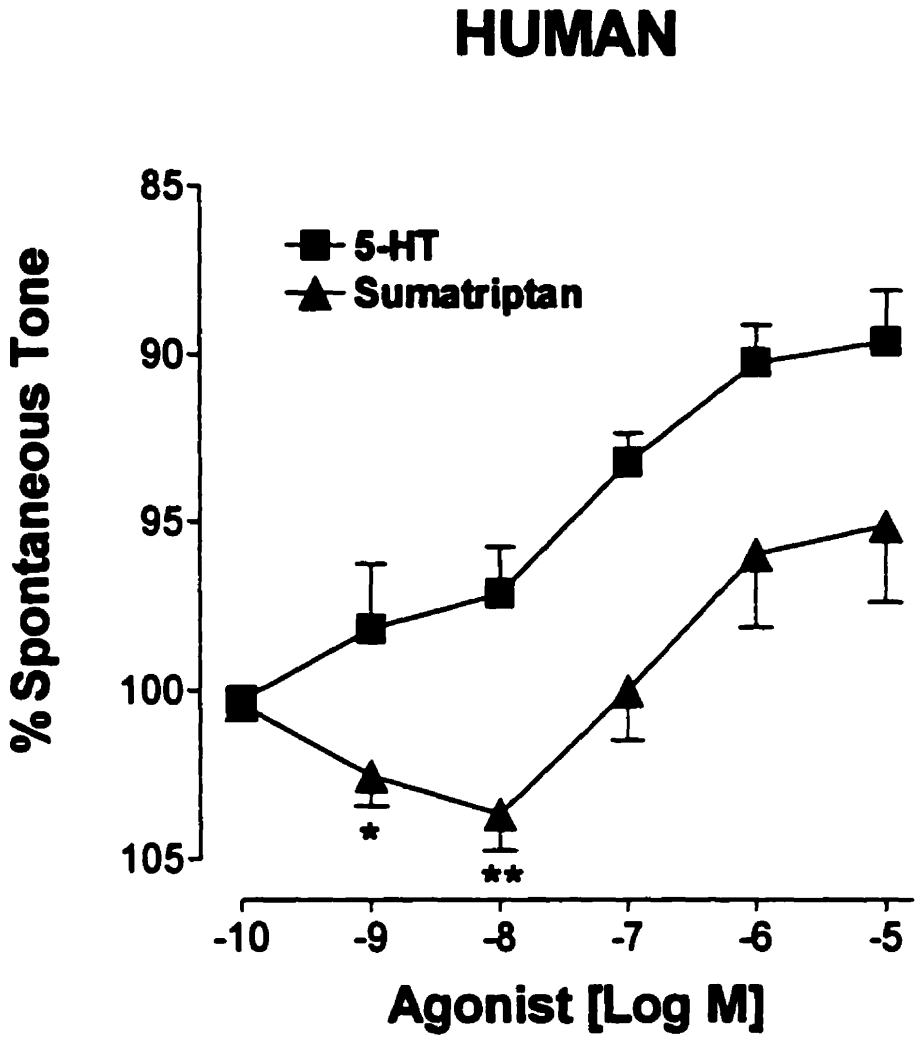

HUMAN
% Spontaneous Tone
85
90
95
100
105
5-HT
Sumatriptan
*
**
-10
-9
-8
-7
-6
-5
Agonist [Log M]

Fig. 1A

BOVINE

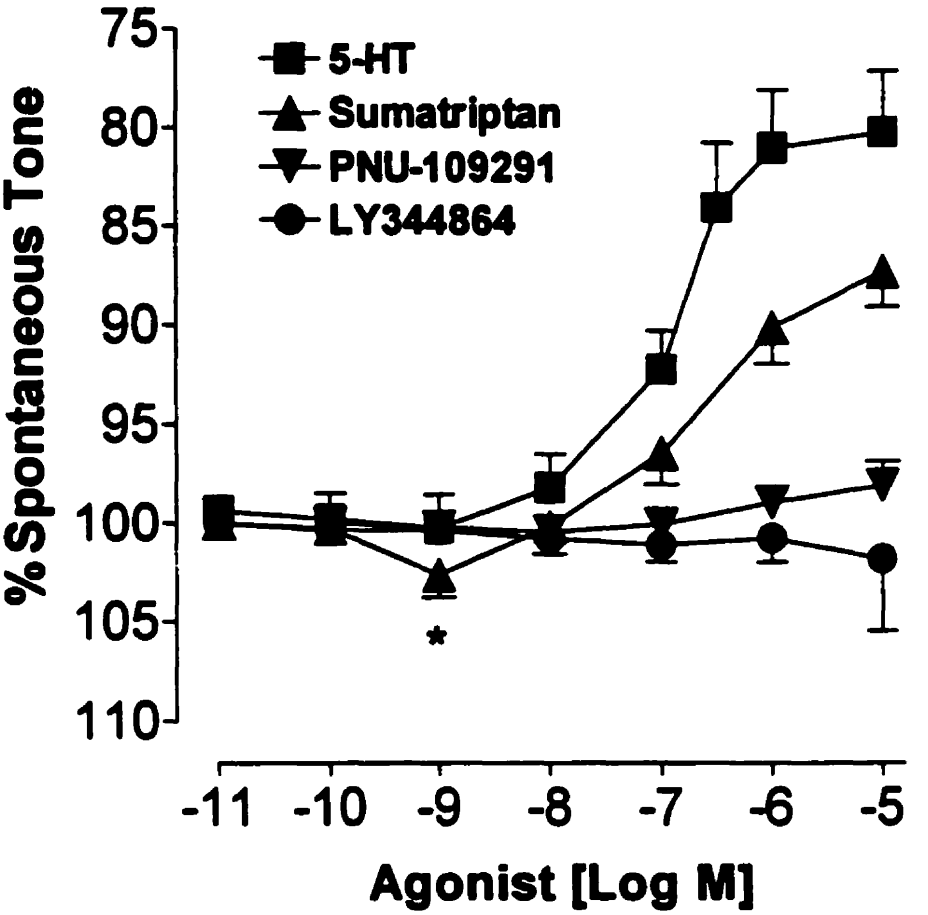
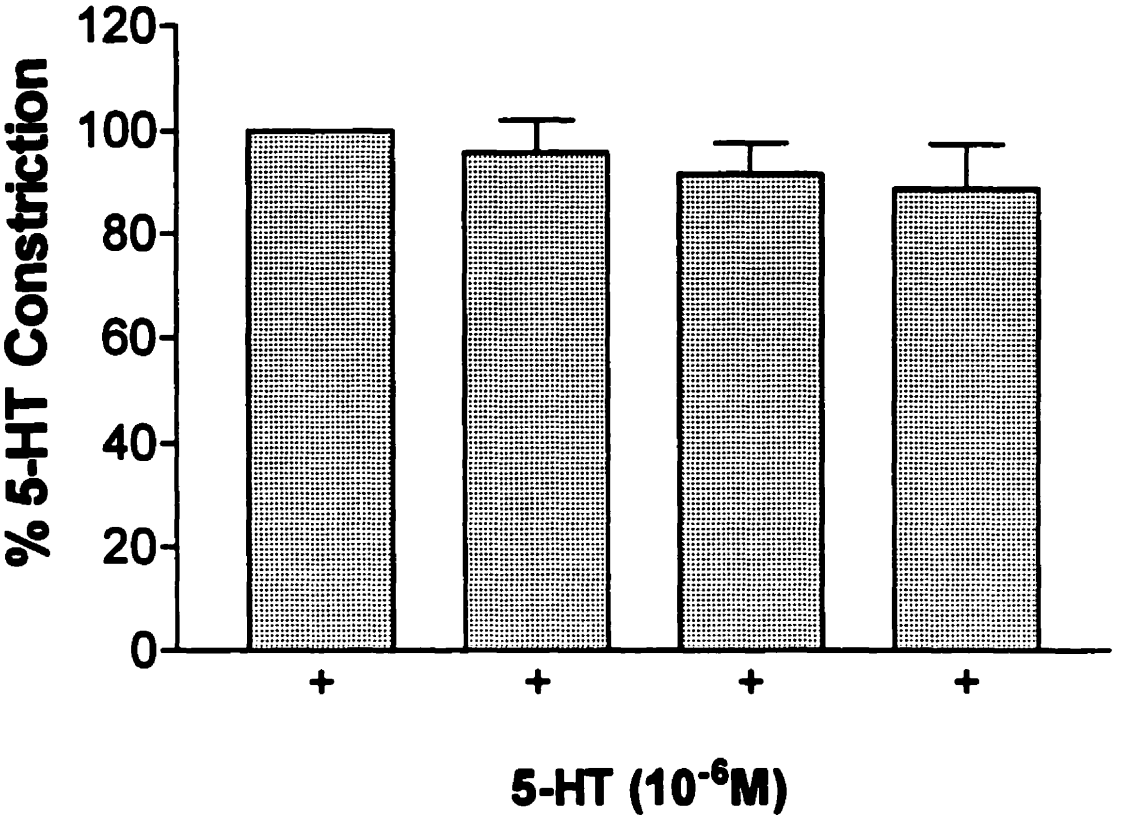


Fig. 1B

Figure 2: Repeated constriction upon application of 5-HT (10^{-6}M) to bovine intracortical arterioles in the absence (*top panel*), and presence (*bottom panel*) of graded concentrations of the 5-HT_1 receptor antagonist GR127935. Inset: inhibition concentration curve for GR127935 (n=5, **: $p<0.01$, by ANOVA)


120
100
80
60
40
20
0
% 5-HT Constriction
+
+
+
+
5-HT (10-6M)

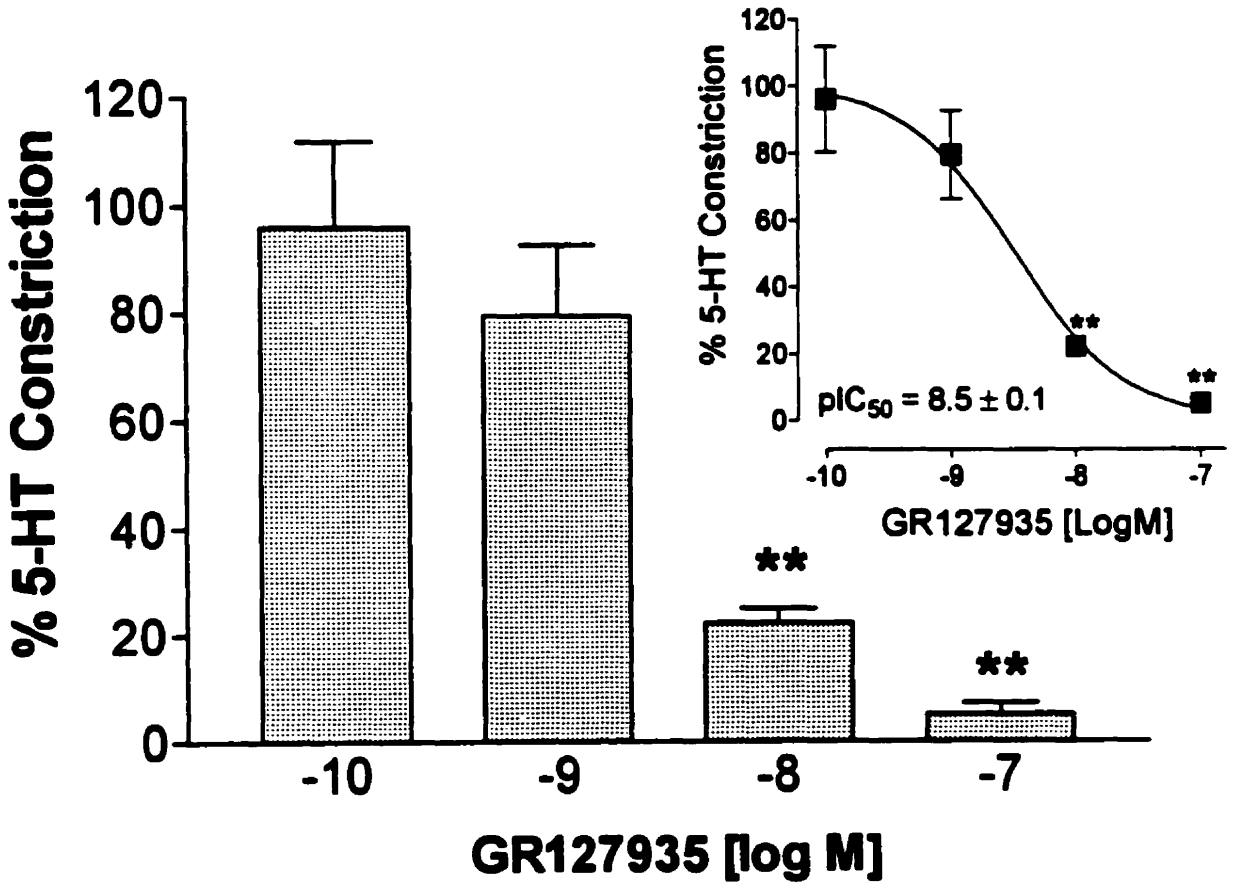

120
100
80
60
40
20
0
% 5-HT Constriction
-10
-9
-8
-7
GR127935 [log M]
**
**
% 5-HT Constriction
pIC50 = 8.5 ± 0.1
GR127935 [LogM]

Fig 2

Figure 3: Effect of 10^{-5}M L-NNA (Δ) on the sumatriptan (▲)-mediated vasomotor responses in (A), human (n= 5 - 8, *: $p< 0.05$, ***: $p<0.001$, by ANOVA) and (B), bovine (n= 5 - 12, *: $p< 0.05$, by paired *t*-test)intracortical arterioles.

HUMAN

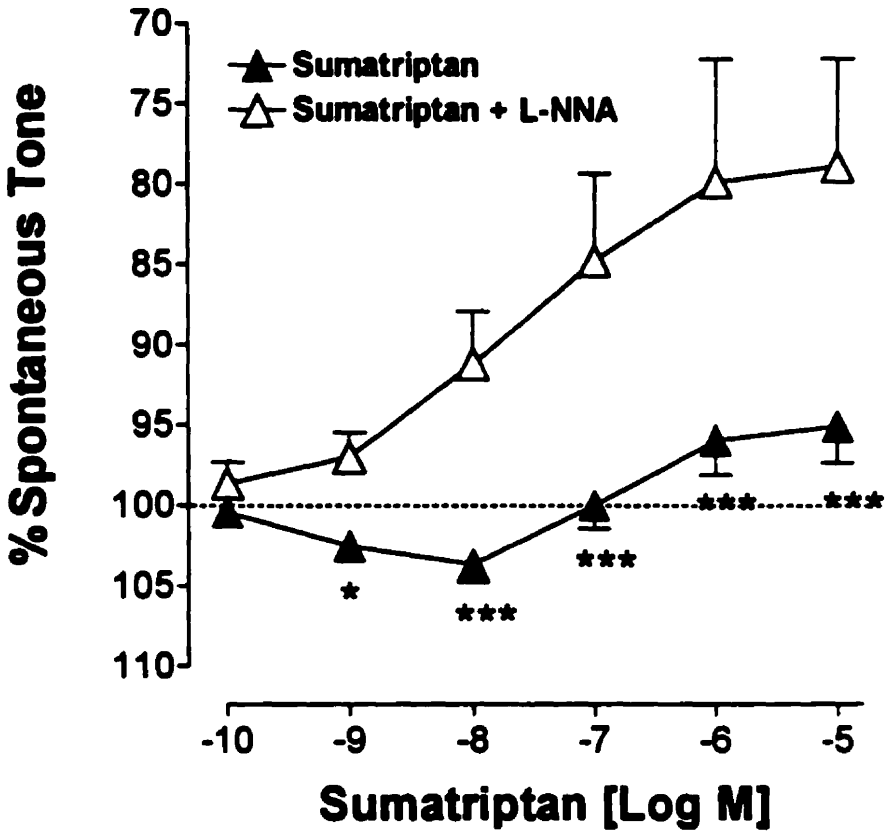

% Spontaneous Tone
70
75
80
85
90
95
100
105
110
Sumatriptan
Sumatriptan + L-NNA
*
***
***
***
***
-10
-9
-8
-7
-6
-5
Sumatriptan [Log M]

Fig. 3A

BOVINE

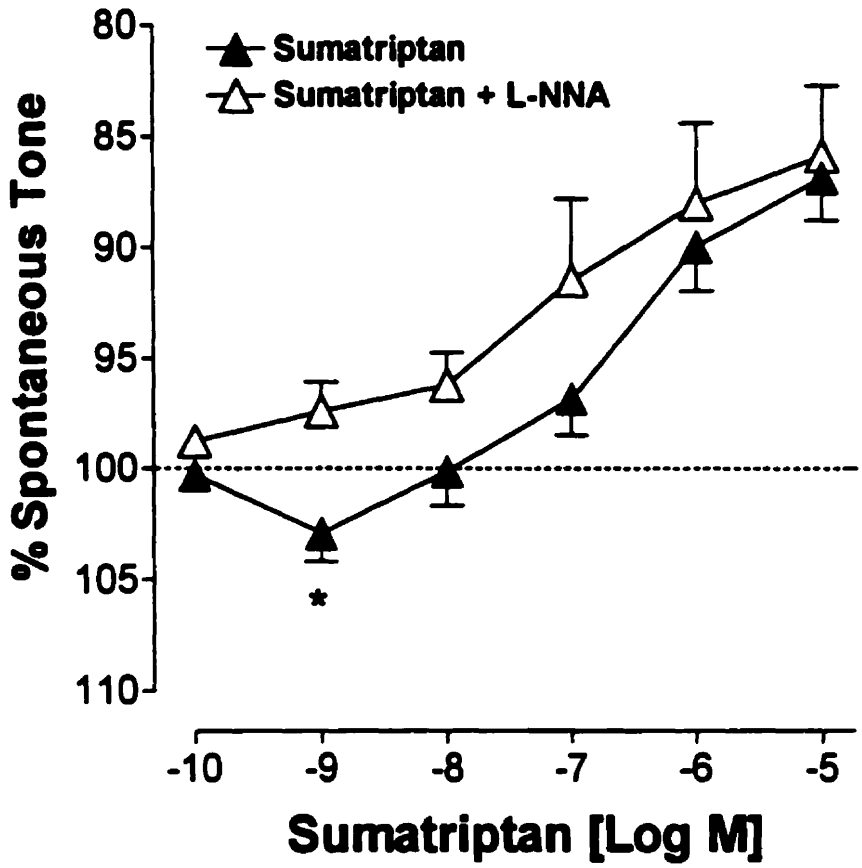


Fig. 3B

Figure 4: Dose-response curves to 5-HT (■; n=9) and sumatriptan (▲; n=6) in bovine pial vessels.

Bovine Pial Vessels

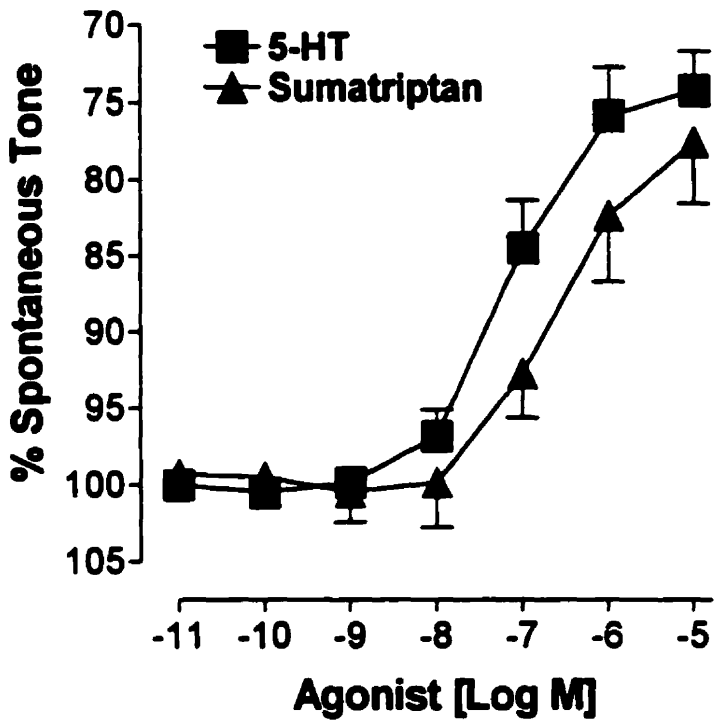


Fig. 4

Figure 5: Correlation analysis between the vasomotor response to 10^{-9}M sumatriptan and the diameter of both pial (n=7, ○) and intracortical (n=9, □) vessels ($r=0.6$, $p < 0.05$).

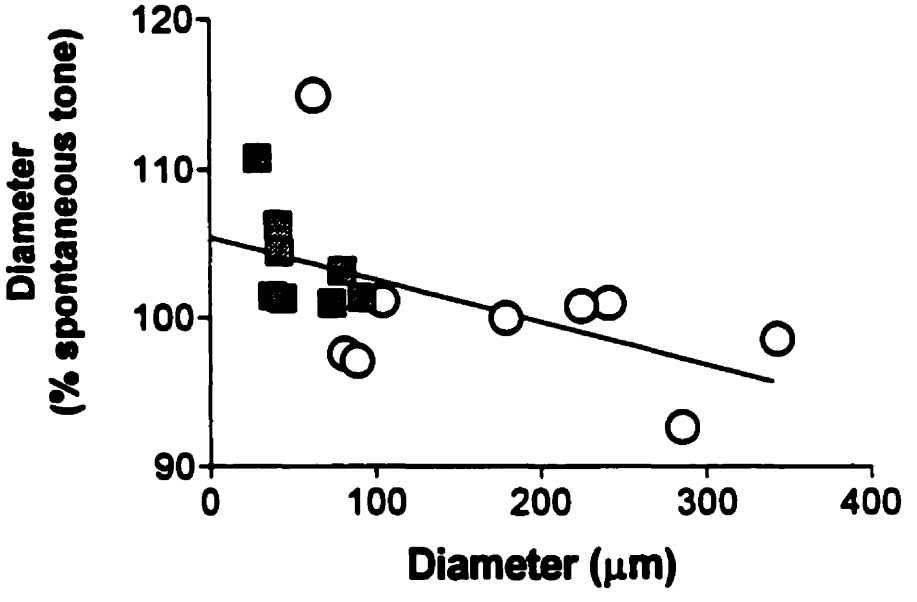

120
110
100
90
0
100
200
300
400
Diameter
(% spontaneous tone)
Diameter (μm)

Fig. 5

CHAPTER 5

GENERAL DISCUSSION

5.1 MAJOR FINDINGS

1) Microvessels and capillaries from the human brain intraparenchymal microcirculation are endowed with multiple mAChRs subtypes. These receptors are localized and functional in endothelial cells (M2, M5), smooth muscle cells (M1, M3 and possibly M5) and astrocytes (all five) as demonstrated by RT-PCR and signaling assays in different cell types.

2) ACh is a potent dilator of human and bovine penetrating intracortical arterioles. This response is mediated by muscarinic, but not nicotinic receptors. Application of NOS inhibitor L-NNA decreased the spontaneous tone of arterioles and abolished the ACh dilation. The ACh dilation is also inhibited by application of mAChRs antagonists of different selectivity. From a comparison of antagonist affinities, it is suggested that an M5 mAChR is most likely mediating the NO-dependent dilation to ACh.

3) 5-HT elicits constriction of bovine and human penetrating intracortical arterioles. By using selective 5-HT receptor antagonist and agonists, this response was shown to be mediated by 5-HT_{1B} receptors. Sumatriptan, a selective 5-$HT_{1B/1D/1F}$ agonist induces an NO-dependent dilation at low concentrations followed by a vasoconstriction at high concentrations in both human and bovine intracortical arterioles. Both responses are mediated by 5-HT_{1B} receptors respectively located in the endothelial and smooth muscle cells. The type and extent of the vasomotor response to sumatriptan was correlated with the size of the vessel.

5.2 METHODOLOGICAL CONSIDERATIONS FOR THE REACTIVITY STUDIES

It should be stated that the largest obstacle to the pharmacological and functional characterization of brain intraparenchymal microcirculation has been the absence of a reliable and affordable technique for the *in vitro* study of these vessels. This has left a gap in the efforts to identity the function of many of receptor subtypes identified in brain microcirculation, not the least of which are ACh and serotonin.

Brain intracerebral microvessels are very small, delicate and due to their location deep in the brain parenchyma, difficult to access (Sagher et al., 1993). Any technique that attempts to study these vessels must take all these factors into account. We therefore had two approaches to studying these vessels, namely, brain slices (as per Sagher et al., 1993) and vessel cannulation (as per Dacey and Basset, 1987). While pharmacological results presented in this thesis are based exclusively on the vessel cannulation technique, our limited experience with the slice preparations deserves mention.

The slice preparation consisted of a superfused slice (300μm) of rat brain placed under a microscope connected to a CCD camera and a computerized digital image analysis system (Metamorph; Universal Imaging Corp). Microvessels ranging from 12 -30 μm in diameter were located in the slice, and their responses to different neurotransmitter recorded by video microscopy. Although an excellent means of visualizing small arterioles and capillaries, this technique had two major shortcomings which made it impractical for our purposes. The first problem was that vessels could not be pressurized, hence the experiments were not performed on the spontaneous tone of the vessel, a factor that is important in mimicking *in vivo* conditions. This also meant that vessels were flaccid and would take very long time to recover sufficiently after application of contractile or dilatory compounds. The second problem was that applied agonists or antagonists could interact with various cellular (neuronal and glial) elements in the slice in addition to the vessels of interest. The influence of non-vascular cells on vascular tone could therefore not be entirely excluded. Nonetheless, despite its misgivings, this method is still an excellent means to study vessels that cannot be isolated due to their location or small size (eg. microarterioles and capillaries), as well as a means to directly record the effect of neuronal activity on brain microvasculature (as per Fergus and Lee, 1997).

The isolated vessel technique on the other hand (see Fig. 1), is much more demanding in terms of manual dexterity and vessel viability, which places limitations on the size of the vessels used (>30μm). However, in this technique, vessels are both isolated and pressurized, permitting pharmacological manipulations directly on the vessel's spontaneous tone. This allowed

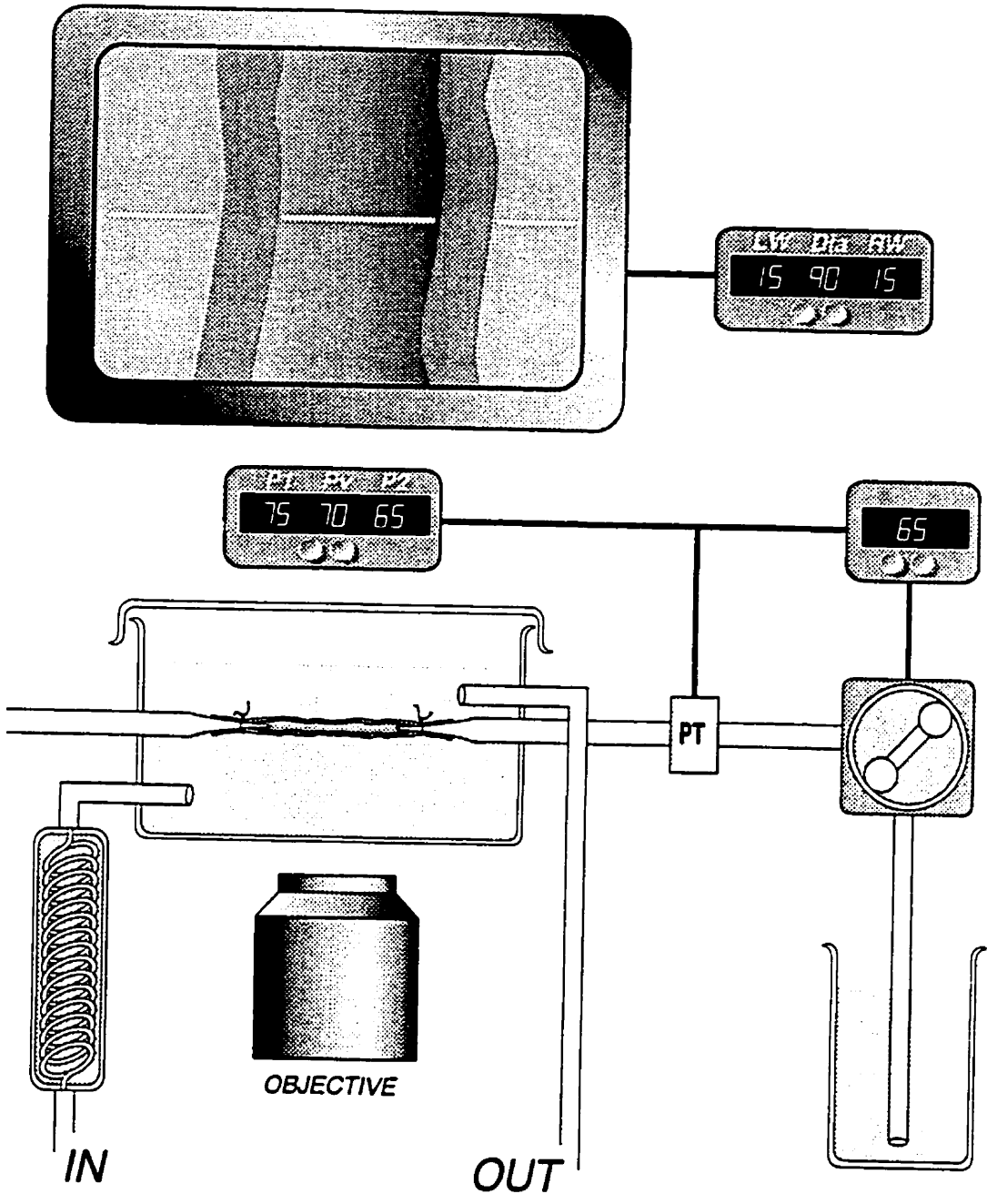


Figure 1: Schematic representation of the isolated vessel arteriograph chamber technique (Living Systems Instrumentation, Burlington, Vermont). Contrary to this figure, which illustrates cannulation at both ends, the arterioles used in the present study were cannulated and secured to the cannula proximal to the pressure transducer (PT), and sealed and secured to the cannula at the distal end. All compounds were added to inflowing superfusing MOPS solution. See the material and methods section of chapter 3 for more details. Modified from original figure provided by Dr. M. Ward (Royal Victoria Hospital, McGill University, Montréal, Québec, Canada)

experimentation on vessels in conditions that are analogous to those that exist *in vivo*. The finding that the pressure-induced spontaneous tone developed by the arterioles *in vitro* is sufficient to allow neuromediator-induced dilation is compatible with the suggestion that, under resting tone *in vivo*, perivascular release of ACh or sumatriptan may result in local vasodilation of brain microvessels

There were, however, shortcomings to this technique which were mostly due to the size and fragility of the vessels used. In this respect, it was not possible to perform exhaustive experimentation on the vessels for long periods of time. Additionally, mounting of vessels required a great deal of care and training, and experiments could only be performed one vessel at a time. Finally, we were unable to remove the endothelial layer without damaging the smooth muscle layer, an observation previously noted by others utilizing these small type of vessels (Dacey and Basset 1987).

5.3 CORRELATION OF RECEPTORS WITH PHYSIOLOGICAL FUNCTION

5.3.1 The Acetylcholine Receptors

The use of RT-PCR with primers for the five mAChRs, on isolated microvascular tissue segments as well as cultures of endothelial, smooth muscle and astroglial cells, was a powerful tool in the identification and cellular localization of mAChRs in the cerebral microvasculature. This was coupled to second messenger signaling assays demonstrating the presence of functional receptor protein. The identification of multiple mAChRs in the brain microvasculature is compatible with other pharmacological or immunohistochemical studies demonstrating the presence of different mAChRs in bovine, primate and human cerebromicrovascular fractions (Estrada et al., 1983; Garcia-Villalon et al., 1991; Linville et al., 1995; Smiley et al., 1998). Furthermore, the specific identification of the M1, M2, M3 and M5 in microvascular tissue, matched exquisitely well with a recent RT-PCR study which identified these same receptors in the rat basilar artery (Phillips et la., 1997). This remarkable similarity in mAChR populations strongly suggests a high level of conservation of vascular mAChRs between intra- and extra-cerebral arteries and across species.

In smooth muscle cultures (SMC), message for the M1, M2, M3 and M5 receptors were identified by RT-PCR. We concluded, however, that the detection of M2 and possibly M5 receptors were most likely due to a small population of contaminating endothelial cells inherently present in all SMC cell cultures used, because these two receptors were either not coupled to their signaling pathway, or expressed at barely detectable levels in these cells (see Chapter 2 for details). In this regard, the M1 and M3 receptors appear as the only confirmed smooth muscle mAChRs. It is interesting to note that the isolated MV fractions, which are highly enriched with the smooth muscle component, predominantly expressed these two receptor subtypes as well.

Both the M1 and M3 receptors have previously been shown to mediate constriction in vascular smooth muscle upon ACh application (Dauphin et al., 1991b; Ren et al., 1993). The M1 in particular has been shown to mediate constriction to high concentration of ACh in cerebral arteries in the cat (Dauphin et al., 1991b) and upon endothelium removal in the mouse (Shimizu et al., 1993). It is perceived that these constrictions are mediated by an increase in Ca^{2+} via the G protein activated IP_3 pathway (see section 1.2.2.2). In agreement with this hypothesis we demonstrate the activation of this pathway by muscarinic receptors in SMC upon ACh stimulation.

However, despite the expression of heterogenous and functional mAChRs in SMC, these receptors did not translate into vasomotor function since at no point in the bovine or human isolated arterioles was a vasoconstriction observed, even at high concentrations of ACh. Although we could not remove the endothelial layer of these small arterioles, inhibition of NOS did not unmask an underlying constriction of these vessels, as is the case in some rat cerebral vessels (Lagaud et al.,1999). The lack of a contractile response could be due to species differences, since ACh typically does not constrict either bovine or human extracerebral vessels (Tsukahara et al., 1989; Kanamaru et al., 1989; Fischer-Nakielski and Schrör, 1990; Conde et al., 1991). Alternatively, it could be due to the types of vessels being used (intracerebral vs extracerebral). In any case, it appears that constriction to ACh is not

common under physiological conditions, and when it does occur, it is usually under either extreme conditions of high ACh concentrations, or after endothelial damage.

The ACh-induced dilation was inhibited by L-NNA in both human and bovine arterioles, a strong indication of the involvement of NO in the mediation of this response. We cannot ascertain the exact source (endothelial or neuronal) of the NO, due to the difficulty in removing the endothelium from these small arterioles. However, it has previously been shown that the ACh dilation is abolished in endothelium-denuded human (Conde et al., 1991; Kanamaru et al., 1989) and bovine (Fischer-Nakielski and Schrör, 1990) cerebral arteries. We therefore suggest that ACh dilates intracortical arterioles through the synthesis and release of NO in endothelial cells. This is supported by physiological data indicating that the hyperemia observed after stimulation of the basal forebrain involves endothelial NO (Zhang et al., 1995).

For ACh to have its dilatory effect, it must stimulate a receptor in the endothelium whose downstream effect is NOS activation. Selective mAChR antagonist with varying degrees of efficacy for the different mAChR subtypes (Dörje et al., 1991; Caulfield and Birdsall, 1998; see Table 1) indicated, by rank order of potency and correlation analysis, that the M5 receptor subtype is the most likely mediator of the ACh dilation. This correlates very well with our identification of the M5 subtype in human microvascular endothelial cell cultures (EC), and its ability to stimulate IP_3 production which can lead to NOS activation (see section 1.2.2.2). Furthermore the ubiquitous expression of M5 receptors in the cerebral and peripheral arteries of the rat (Phillips et al., 1997), in addition to its very potent link to NO generation (Wang et al.1994, 1996), provides strong evidence for a role of this receptor in the mediation of the ACh dilation. The future development of a selective M5 ligands, however, is necessary to confirm this hypothesis.

This finding might appear to counter previous studies that had pointed to the endothelial M3 subtype as the mediator of ACh dilation in peripheral (Ren et al. 1993; Chiba and Tsukada, 1996) and cerebral circulation (Dauphin and Hamel, 1990; Shimizu et al., 1993). However,

the M3 and M5 mAChRs are pharmacologically very similar and a revised correlation analysis with antagonist affinity values for the M1 to M5 mAChRs that have more recently been published (Dörje et al., 1991; Caulfield and Birdsall 1998), indicated that pharmacological analysis performed in these studies could correlate just as well with the M5 subtype (see Hamel et al., 1994). In addition, we failed to identify the expression of the M3 subtype in EC, even in primary cell cultures where loss of receptors due to passaging can be excluded, making it an unlikely candidate for this response.

The other cholinergic receptors that could play a role in the ACh dilation are the nAChRs. However, nicotine, when tested on bovine intracortical arterioles at concentrations that would activate all subtypes of nAChRs known to be expressed in brain tissue (Lindstrom et al., 1996), had no vasomotor effects. This was further supported by the complete blockade of ACh dilation by the muscarinic receptor antagonist 4-DAMP, a finding which would not be observed if a significant population of nicotinic receptors were also involved. While both these observations would indicate that microvascular nAChRs are not participating in the ACh dilation, they do not exclude the possibility of a presynaptic neuronal nAChR involvement, as shown in the peripheral and extracerebral circulation (Toda et al., 1995, Zhang et al., 1998).

It is also important to note that a large proportion of neurovascular cholinergic nerve terminals are in fact associated with the perivascular astrocytes (Chédotal et al., 1994b; Vaucher and Hamel, 1995) Perivascular astroglial cells have been shown to be involved in the regulation of microvascular tone, metabolic homeostasis, and/or the development and maintenance of the blood-brain barrier permeability (Tao-Cheng et al., 1987; Tsacopoulos and Magistretti, 1996; Alkayed et al., 1997). All of the above could be regulated *in vivo* by ACh, since we demonstrate that all five mAChRs are expressed in astroglial cell cultures. In *in vitro* studies such as this one, however, the role of astroglial receptors is most likely negligible.

5.3.2 The Serotonin Receptors

In very similar RT-PCR studies to the one described above, Bouchelet et al. (1997) and Cohen et al. (1999), demonstrated the presence of multiple functional 5-HT receptors in endothelial, smooth muscle and astroglial cell cultures from human brain tissue (See Table 2). The smooth muscle cells strongly expressed the 5-HT_{1B} and the 5-HT_{2B} receptors. Based on its cellular localization and coupling, the authors suggested that smooth muscle 5-HT_{1B} receptor is the most likely mediator of constriction in these human brain microvessels.

Using sumatriptan (a 5-$HT_{1B/1D/1F}$ agonist), we demonstrated that a 5-HT_1 receptor is indeed implicated in the constriction to 5-HT in both bovine and human intracortical arterioles. More specifically, subtype selective 5-HT receptor agonists showed that the 5-HT_{1B} receptor subtype is mediating a significant portion, if not all, of the 5-HT constriction in bovine arterioles. This is in line with other studies implicating this receptor in the constriction of human and bovine cerebral vessels (Hamel and Bouchard, 1991; Hamel et al., 1993b; Bouchelet et al., 1996; Bouchelet et al., 2000). Unlike in humans, where the 5-HT_{2A} subtype is not present in the brain microvasculature (Cohen et al., 1999), we cannot totally exclude the possibility that 5-HT_{2A} receptors also contribute to the 5-HT induced constriction of bovine arterioles. However, it has recently been shown in species that express both the 5-HT_{1B} and the 5-HT_{2A} receptors, that the 5-HT_{1B} tends to be the predominant, if not the exclusive constrictor of small vessels (Teng et al., 1998) and vessels with induced tone (MacLean et al., 1994). Two conditions that fully apply to the intracortical arterioles in this study.

Unexpectedly, low concentrations of sumatriptan also mediated an NO-dependent dilation in both bovine and human arterioles. Based on the use of selective agonists, we hypothesize that this dilation is mediated by the 5-HT_{1B} subtype. A similar NO- and/or endothelial- dependent dilation of cerebral (Faraci and Heistad, 1992) and peripheral (Cocks and Angus, 1983; Cohen et al., 1983; Schoeffter and Hoyer, 1990; Whiting and Cambridge 1995) blood vessels by 5-HT and/or sumatriptan has previously been documented. This response was shown to be

most likely mediated by an endothelial 5-HT_1 receptor (Moldering et al., 1989; Whiting and Cambridge, 1995; Lamping, 1997) most likely corresponding to the 5-HT_{1D} or 5-HT_{1B} subtype (Schoeffter and Hoyer 1990; Gupta, 1992). Our conclusion that the endothelial 5-HT_{1B} receptors are responsible for the NO-induced dilation to sumatriptan is further supported by the presence of 5-HT_{1B} receptor protein and mRNA in the endothelial cells of intraparenchymal microvessels, but not capillaries (Riad et al., 1998; Cohen et al., 1999). The 5-HT_{1B} subtype has also been identified in large cerebral (Nilsson et al., 1999a) and coronary (Ullmer et al., 1995; Ishida et al., 1998; Nilsson et al., 1999b) artery endothelial cells, where it has been shown to be linked, at least in peripheral vessels, to NO production (Ishida et al., 1998; McDuffie et al., 1999). The specificity in the localization of this receptor, and its ability to stimulate NO, make it a good candidate for the mediation of endothelium-dependent dilation. Furthermore, we found that this dilation was more prominent in smaller vessels than in larger ones, possibly due the fact that larger vessels contain more layers of smooth muscle cells than smaller ones, and hence are more likely to push the equilibrium in favour of the contractile 5-HT_{1B} receptors in the smooth muscle, and away from the dilatory endothelial 5-HT_{1B} receptor.

5.3.3 Correlation With *In Vivo* Physiological Studies

It should be stated from the onset that *in vivo* responses in CBF are infinitely more complex than those evoked in *in vitro* isolated vessels, since they can also include numerous other components such as astroglial, neuronal or blood borne factors. Nonetheless, the results from our *in vitro* studies on ACh and 5-HT are well reflected in the *in vivo* physiological studies. That ACh dilates intracortical arterioles in a NO-dependent manner via a receptor most similar to the M5 mAChR, correlates exquisitely well with the NO- and mAChR- dependent increases in CBF seen upon stimulation of the basal forebrain (see Sato and Sato, 1995). Similarly, the dual response observed in intracortical arterioles upon stimulation of 5-HT receptors, is in line with the DRN stimulation studies that show either increases or decreases in CBF depending on conditions (see Cohen et al., 1996).

The only possible inconsistency between the physiological and *in vitro* studies is that nAChRs known involvement in the basal forebrain induced dilation contrasted with the lack of response to nicotinic activation in brain intracortical arterioles. One explanation for this discrepancy could be that the nAChRs mediating this effect are located on perivascular nerve terminals, in a manner similar to that which occurs in the extracerebral circulation (Toda et al., 1995; Zhang et al., 1998) or in the basal forebrain itself (Linville et al., 1993b).

5.4 COMPARISON BETWEEN THE ACh AND 5-HT SYSTEMS

5.4.1 Factors That Determine Vasomotor Responses to ACh or 5-HT

The underlying second messenger cascade of the M5 and the 5-HT_{1B} receptors ultimately leads to an increase in Ca^{2+} concentrations in the cell, albeit through different G proteins ($G_{q/11}$ and $G_{i/o}$ respectively). The resulting effect of the activation of these receptors in terms of blood flow, however, is highly dependent on the cellular location of the receptor. Hence an increase in Ca^{2+} in the smooth muscle cells will constrict the vessel, while a similar mechanism in endothelial cells, and possibly perivascular NOS positive neurons, could lead to Ca^{2+}/calmodulin activation of constitutive NOS, and dilation of the blood vessel.

Interestingly, for both mAChRs and 5-HT receptors, it appears that the mobilization of NO production in endothelial cells occurs more readily than the concurrent activation of the contractile mechanisms in the smooth muscle cells. This could explain why at low concentrations both ACh and sumatriptan tend to dilate vessels via the endothelial M5 or 5-HT_{1B} receptor, while at higher concentrations, constriction, via smooth muscle receptors with similar if not identical second messenger profiles and affinities, M1 (Dauphin et al.1991b; Dörje et al., 1991) and 5-HT_{1B} respectively, predominates. Indeed, it has been shown that concentrations of 5-HT and/or sumatriptan as low as 10^{-8}M can stimulate significant and almost maximal NO production in cultured endothelial cells (Ishida et al., 1998; McDuffie et al., 1999) as well as induce maximal dilation of small pial vessels (Harper and Mackenzie, 1977). The maximal constriction to either 5-HT or sumatriptan on the other hand, occurs at higher concentrations (10^{-6}M) (Hamel and Bouchard, 1991; Hamel et al., 1993; Bouchelet et

al., 2000; see Chapter 4). Similarly, high concentrations (10^{-5} to 10^{-3} M) of ACh will activate smooth muscle mAChRs to induce a vasoconstriction in some intact (Dauphin et al., 1991b) or endothelium-denuded extracerebral arteries (Van Charldorp et al., 1988; Shimizu et al., 1993), while endothelial mAChR receptor subtypes mediate the dilation of both extracerebral and peripheral vessels to low (10^{-9} to 10^{-6} M) ACh concentrations (Dauphin and Hamel, 1990; Shimizu et al., 1993).

It should be noted that numerous other conditions, in addition to agonist concentration, can also affect the vascular response to 5-HT. Our study, and others (Harper and Mackezie, 1977; Edvinsson et al.,1978; Auer et al., 1985; Lamping, 1997) have noted that vessel size can be an important factor in determining the final outcome to 5-HT and/or sumatriptan stimulation (see Chapter 4). Studies have also shown different 5-HT responses based on different receptor subtypes with opposing vascular effects. For example the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{7} have all been shown to have vasomotor effects (see section 1.4.3). In some cases the relative distribution of these receptors was shown to vary with the vessel size, vessel location, as well as the time of year (Teng et al., 1998; Vinall et al., 1991; Kaumann et al., 1994). Additionally factors such a vessel tone (MacLean et al., 1994; Sweeney et al., 1995), species (see Lincoln, 1995), agonist concentration (Kobari et al., 1993), and endothelin release (Thorin et al., 1998) have also been shown to influence the response of blood vessels to 5-HT and/or its agonists. It is therefore not surprising to find that activation of 5-HT receptors on blood vessels can, depending on conditions, induce dilation (Cocks and Angus, 1983), constriction (Hamel et al., 1993b), a dual response (see chapter 4) as well as no response at all (Toda and Okamura,1989).

The ACh response can also vary with changes in conditions, although this usually occurs only under extreme or abnormal physiological circumstances. In this regard, it has been shown that non-physiologically high concentrations of cholinergic agonist (Dauphin et al., 1991b), removal of the endothelial layer (Shimizu et al., 1993; Van Charldorp et al., 1988) or NOS inhibition (Lagaud et al., 1999), as well as pathological conditions such as hypertension, can

alter the vascular ACh response (Charpie et al., 1996; Hayashi et al.,1999). In the majority of studies, however, ACh tends to consistently dilate blood vessels in an endothelium or NO dependent manner as illustrated in the bovine or human cerebral vessels tested in this study, and by others (Tsukahara et al., 1989; Kanamaru et al., 1989; Fischer-Nakielski and Schrör, 1990). We demonstrate that changes in the tone of the vessel do not affect this dilation (see chapter 3), and in the majority of cases the final vascular response to ACh does not vary based on season or vessel size, although in the latter case the underlying dilatory mechanisms might differ, with the endothelium derived hyperpolarizing factor (EDHF) possibly playing a more prominent role in the dilation of smaller vessels. (Clark and Fuchs, 1997; Bolz et al., 1999; Lagaud et al.,1999; see below).

The relative stability of the ACh dilation across different conditions would be in line with the tonic role exerted by the basal forebrain cholinergic neurons in the control of CBF (Gomi et al., 1991; Peruzzi et al., 1996). The serotonergic neurons of the DRN on the other hand appear to affect the CBF in a phasic manner. It can therefore be suggested that since the 5-HT response is non-constitutive, it is advantageous for 5-HT to exert a variable response on the CBF that is dependent on the conditions present at the moment of activation (eg vessel tone).

5.4.2 Endothelium-Derived Factors

5.4.2.1 Nitric Oxide

Our study, and those of others have shown that ACh or 5-HT stimulation of blood vessels can result in the production and release of NO, most likely from endothelial sources. This has been shown in numerous studies on ACh in both the peripheral and cerebral circulation, and indeed is the basis by which this neurotransmitter mediates dilation. The evidence for 5-HT is less tangible. However, there are now increasing reports of this indoleamine's role in the mediation of NO-dependent dilation (Schmuck et al., 1996; Ishida et al., 1998; McDuffie et al., 1999).

In both cases, activation of NO is linked to specific receptor subtypes. In endothelial cells, the

candidates thought to be mediating dilation are the M3 (Dauphin and Hamel, 1990; Shimizu et al., 1993), and/or M5 (this study) mAChRs, and the 5-HT_{1B} (Schoeffter and Hoyer 1990; Gupta, 1992; this study) and/or 5-HT_{2B} receptors (Schmuck et al, 1996, Fozard and Kalkman 1994). They all share in common a second messenger pathway that increases intracellular Ca^{2+} levels and hence can potentially activate constitutive NOS. Evidence for their role in NO production comes from studies in which vasodilation was shown to be abolished by application of NOS inhibitors and/or removal of the endothelium. There is also direct evidence that some of these receptors, namely the M5, 5-HT_{1B} and 5-HT_{2B} can mediate the activation of NO in cell cultures or cell lines (Wang et al., 1994, 1996; Ishida et al., 1998; McDuffie et al., 1999).

In addition to endothelial sources, there is also evidence that NO release from NOS positive perivascular nerve terminals can affect vascular tone. ACh has been shown to mediate NO-dependent dilation of extracerebral blood vessels by activation of neuronal M2 (Liu and Lee, 1999) and nicotinic receptors (Toda et al., 1995, Zhang et al., 1998) on perivascular NO neurons. While there is no similar evidence for neuronal NO release by perivascular 5-HT nerve terminals on blood vessels, pre-junctional release of neuronal NO by 5-HT receptors has been shown in the peripheral nervous system in the relaxation of ileum (Kanada et al, 1993), colon (Briejer et al., 1995) and rectal smooth muscle (Prins et al., 1999). Additionally, 5-HT has also been suggested to mediate the release of NO from neuronal sources in the brain (Maura et al., 1995). Preliminary experiments for our laboratory show that serotonergic terminals innervate cortical NOS interneurons, known to closely associate with the microcirculation (Tong and Hamel, unpublished observation). However a direct effect on the blood flow has yet to be demonstrated.

5.4.2.2 Other Endothelium Derived Factors

It is now known that vessels tone can be altered through endothelium-derived mechanisms that are not NO-dependent such as prostacyclins, endothelin and EDHF. EDHF in particular has been shown to mediate ACh dilations in smaller arteries and microvessels by reducing Ca^{2+}

levels in smooth muscle (Bolz et al., 1999; Lagaud et al., 1999; see Garland et al., 1995), but does not appear to play a role in 5-HT-induced dilations (Frieden and Beny, 1995). The complete abolishment of the ACh and sumatriptan dilations in this study by L-NNA, indicated that they were primarily if not exclusively mediated by NO. It is nonetheless possible that under different conditions, other endothelial mechanisms might be favoured over EDRF in these arterioles.

5.4.3 Interactions between the cholinergic and serotonergic systems

In the brain, there is evidence for the interaction between cholinergic and serotonergic neurons in the mediation of several cortical functions, particularly memory and depression (Altman et al, 1987; Rada et al.,1993). In this respect, 5-HT, via pre-synaptic 5-HT_{1B} (Maura and Raiteri, 1986), 5-HT_{2A} (Muramatsu et al., 1988b) and 5-HT_3 (Maura et al., 1992) receptors can act upon cholinergic axon terminals in the cerebral cortex to modulate ACh release (Raiteri et al., 1989; Maura et al., 1992). Although there is no direct evidence on the effect of such interactions on the regulation of microvascular tone, it is conceivable that the tonic dilation effected by the basal forebrain ACh neurons could be modulated by the phasic pre-junctional activation of pre-synaptic 5-HT receptors on perivascular cholinergic terminals. Such functional interactions between these two systems would be similar to those reported in the mediation of non-vascular smooth muscle motility (Takahashi et al.,1995; Kojima and Ikeda, 1998), albeit in the peripheral nervous system.

Alternatively, ACh and 5-HT could modulate the activity of other neurons, such as the NO interneurons. These neurons, which are closely associated with microvessels (Iadecola et al., 1993; Estrada et al., 1993; Regidor et al., 1993; Tong and Hamel, 1999), have been suggested to play a role in the control of CBF, and more specifically, in the coupling of blood flow with neuronal activity (Iadecola, 1993; Estrada and DeFelipe, 1998; Lindauer et al., 1999). They are also known to be innervated by both ACh and 5-HT projection fibres (Vaucher et al., 1997b; Tong and Hamel, unpublished observation) in addition to expressing muscarinic (Moro et al., 1995a; Smiley et al., 1998) and possibly 5-HT receptors (NOS neurons being

colocalized within a subpopulation of GABA neurons which contain 5-HT receptors; Morales et al., 1996; Abi-Saab et al,. 1999). It can thus be hypothesized that the cholinergic and serotonergic innervations of these NO neurons could serve to fine tune the release of NO and therein, its effect on local perfusion. However, studies that have investigated these specific interactions have not yet been performed.

Finally, interaction between the cholinergic and serotonergic systems could also occur at an intracellular level. Cross-talk between G protein-coupled receptors, resulting in altered functions, have been demonstrated on many occasions (see Selbie and Hill, 1998) including in cerebrovascular endothelial cells (Thorin,1998). Furthermore, Sanders et al., (1995) have suggested that cross-talk between 5-HT and muscarinic receptors can inhibit serotonergic responses in human atrial vessels pre-treated with beta-blockers. While the underlying mechanisms have yet to be identified, it is quite possible for similar intracellular processes to occur in the cerebromicrocirculation considering that both 5-HT and muscarinic receptors are expressed in human microvascular cells (this study; Cohen et al., 1999).

5.5 PATHOLOGICAL IMPLICATIONS

5.5.1 The Cholinergic Pathway

Alzheimer's disease is characterized by a specific degeneration of cholinergic neurons in the basal forebrain, together with the formation of beta-amyloid deposits and a gradual decline of cognitive functions (Yankner et al., 1996). The denervation affects all cholinergic targets including NO neurons and the microvasculature (Tong and Hamel, 1999). There are also severe changes in the cerebromicrovasculature of the brain, most notably a progressive degeneration of the microvessel wall, reduced perfusion and breaches of the blood brain barrier (Kalaria 1992, 1996; Jagust et al., 1997; de la Torre, 1997, 1999). Indeed, these changes in blood vessels are so drastic that it has been hypothesized that neuronal degeneration follows cerebrovascular impairment (de la Torre, 1997, 1999).

The findings from the present study provide a direct functional link between cholinergic

neurons and brain microvasculature. They suggest that the changes in cholinergic function that occur in Alzheimer's disease could directly affect blood flow, most likely by reducing it, and hence exacerbate the pathology related to neuronal degeneration. Additionally, they provide a possible mechanism of action for compounds used in the treatment of Alzheimer's disease, which are known to increase blood flow in normal (Scremin et al., 1991) and deafferented cortex (Geany et al., 1990; Peruzzi et al., 1996) by inhibiting ACh degradation. This increase in blood flow has been speculated to be due to the vascular effect of ACh, and might account for some of the clinical improvements observed in patients treated with these drugs (Furey et al., 1997; de la Torre, 1997). Our results would provide functional arguments to support this view.

5.5.2 The Serotonergic Pathway

Current knowledge points to the involvement of 5-HT in several pathologies and diseases related to the cerebral circulation. The majority of these studies, however, have focussed on the extracerebral circulation. Nonetheless, there is no reason to believe that the intracerebral circulation is not affected as well, or that the results from this study cannot be extrapolated to the extracerebral circulation.

There is circumstantial evidence for the involvement of 5-HT in ischemia as well as in subarachnoid hemorrhage (SAH) and the resulting cerebral vasospasm. In both cases, increased 5-HT levels have been reported (Jackowski et al., 1989; Prehn et al., 1993) and viewed as possible mediators of vasoconstriction that accompanies these types of insults (Jackowski et al., 1989; Dietrich et al., 1989; Yakubu and Leffler 1997). The results from this study demonstrate that 5-HT can indeed affect brain perfusion via direct interaction with the microcirculation, and provide possible targets and/or mechanism for certain drugs known to reduce ischemic or SAH damage.

Migraine is another important cerebrovascular dysfunction in which 5-HT has been involved. According to one theory, vasodilation of extracerebral blood vessels is one of the primary

causative factor in the development of migraine headache. The antimigraine drug sumatriptan has been suggested to be effective because it eliminates this dilation by constricting cerebral blood vessels (Johnson et., 1998). Sumatriptan can in fact decrease blood flow *in vivo* in the cat and rat brain (Kobari et al., 1993; Fukuda et al., 1999), a finding which correlates well with our identification of a constrictor role for this compounds in bovine and human intracortical arterioles. However, we also showed that sumatriptan can induce a dilation of arterioles under certain conditions. If our results can be extrapolated to the extracerebral circulation, and it seems that they can (Nilsson et al., 1999a), this would imply that at low concentrations, sumatriptan could possibly dilate rather than constrict blood vessels, an effect likely to be more pronounced in the smaller vessels. This could be of great importance when prescribing sumatriptan since it could, through normal biodegradation in the body, reach a concentration with time that would dilate vessels and instigate a headache. Interestingly, sumatriptan has a high headache recurrence rate (Schoenen et al., 1997), which could be a reflection of this phenomenon.

5.6 CONCLUSIONS

This study is the first assessment of the brain intraparenchymal microcirculation in terms of cellular distribution of receptors and their vasomotor function. While previous studies have examined the properties of these vessels *in vitro* (Dacey et al., 1991; Sagher et al., 1993) or the pharmacology of the binding sites present in isolated microvascular fractions (García-Villalón et al., 1991; Linville et al., 1995), none so far has provided an evaluation of the physiological responses together with the molecular, cellular and pharmacological identification of the receptors and their signaling mechanisms. The fact that these results were obtained on human tissue samples makes them even more pertinent.

The identification of the M5 and the 5-HT_{1B} receptors in the mediation of NO-dependent dilation in intracortical arterioles are two major and novel findings of this study that deserve further investigation. A means to remove the endothelium in these arterioles would be a good first step in confirming the presence of these receptors in the endothelial layer. Additionally,

studies could be performed on vessel segments from other sources in order to establish if these receptors also play a role in the extracerebral as well as peripheral circulation. The implications of these studies could change the view of these receptors with regards to their role in control of vascular tone.

On a more global outlook, we have shown that individual resistance arterioles imbedded in the brain parenchyma are able to respond to neuronal signals in a manner that can affect the blood supply to the brain. These results are an extension of the *in vivo* physiological studies and will no doubt support the importance of the neurogenic control of CBF, not only at the level of the intraparenchymal microcirculation, but the in brain as whole. In this regard, it has been suggested that communication can occur between the intra and extracerebral circulation in order to coordinate blood perfusion between microvessels and larger surface arteries (Iadecola, 1993; Iadecola et al., 1997; Estrada and DeFelipe, 1998). The results from this study add another piece to the highly complex and intricate puzzle of the neurogenic cerebral regulation, which along of metabolic and myogenic mechanisms orchestrate the overall control of blood flow in the brain.

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APPENDIX

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Expression of Neuropeptide Y Receptors mRNA and Protein in Human Brain Vessels and Cerebromicrovascular Cells in Culture

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Summary: Neuropeptide Y (NPY) has been suggested as an important regulator of CBF. However, except for the presence of Y1 receptors in large cerebral arteries, little is known about its possible sites of action on brain vessels. In this study, we sought to identify the NPY receptors present in the human cerebrovascular bed. Specific Y1 receptor binding sites, localized on the smooth muscle of human pial vessels and potently competed by NPY, polypeptide YY (PYY), and the selective Y1 receptor antagonist BIBP 3226, were identified by quantitative radioautography of the Y1 radioligand [^{125}I]-[Leu^{31}, Pro^{34}]-PYY. In contrast, no specific binding of the Y2-([^{125}I]-PYY$_{(3-36)}$) and Y4/Y5-(^{125}I-human pancreatic polypeptide [hPP]) radioligands could be detected. By *in situ* hybridization, expression of Y1 receptor mRNA was restricted to the smooth muscle layer of pial vessels, whereas no specific signals were detected for either Y2, Y4, or Y5 receptors. Similarly, using reverse transcriptase–polymerase chain reaction (RT-PCR), mRNA for Y1 but not Y2, Y4, or Y5 receptors was consistently detected in isolated human pial vessels, intracortical microvessels, and capillaries. In human brain microvascular cells in culture, PCR products for the Y1 receptors were exclusively found in the smooth muscle cells. In cultures of human brain astrocytes, a cell type that associates intimately with brain microvessels, PCR products for Y1, Y2, and Y4 but not Y5 receptors were identified. Finally, NPY significantly inhibited the forskolin-induced cAMP production in smooth muscle but not in endothelial cell cultures. We conclude that smooth muscle Y1 receptors are the primary if not exclusive NPY receptors associated with human brain extraparenchymal and intraparenchymal blood vessels, where they most likely mediate cerebral vasoconstriction. **Key Words:** Endothelial cells—Smooth muscle cells—Astrocytes—Cerebral blood flow—Neuropeptides—Microcirculation—Ischemia.

Neuropeptide Y (NPY) is one of the most widely distributed peptides in the brain (Adrian et al., 1983), and it is known to exert a variety of functions including the regulation of the cerebrovascular tone (Edvinsson et al., 1987). In both rat and human brain, NPY neurons and projection fibers have been shown to associate with local microvessels and their perivascular astrocytes, and could therefore affect brain perfusion (see Abounader and Hamel, 1997*a*, for additional references). In fact, intracarotid or local intraparenchymal (Suzuki et al., 1989; Tuor et al., 1990) injection of NPY in the rat is known to result in a strong reduction in CBF in the ipsilateral hemisphere or locally in the ipsilateral striatum and adjacent areas. These contractile *in vivo* effects are fully compatible with the ability of NPY to induce long-lasting and sustained vasoconstriction of isolated brain extraparenchymal (Edvinsson et al., 1987) and intraparenchymal (Dacey et al., 1988) blood vessels. However, a transient NPY-mediated cerebral vasodilation, presumably mediated by nitric oxide, also has been reported after intracarotid injection of NPY in the cat (Kobari et al., 1993).

In large cerebral arteries of various species including man, the NPY-induced contractile effect has been shown

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Abbreviations used: AP, alkaline phosphatase; AST, fetal human brain astrocytes; CAP, capillaries; END, human brain microvascular endothelial cells; hPP, human pancreatic polypeptide; pIC_{50}, log molar antagonist concentration required for half maximum effect; MV, microvessels; NPY, neuropeptide Y; PCR, polymerase chain reaction; PYY, polypeptide YY; RT-PCR, reverse transcriptase–polymerase chain reaction; SMC, human brain microvascular smooth muscle cells; SSC, standard saline citrate.

to be mediated by the NPY-Y1 receptor subtype (Abounader et al., 1995; Nilsson et al., 1996). In peripheral vascular beds from some species, however, pharmacologic studies suggest that Y1 and possibly Y2 receptors are involved in this contractile response (Grundemar et al., 1992; Tessel et al., 1993). Alternatively, Y2 receptors could mediate the vasodilatory effects of NPY either directly (Neild and Lewis, 1995) or indirectly through presynaptic regulation (Hashim and Tadepalli, 1995). In the cerebral microvascular bed, little is known on the types of NPY receptors present on these nonneuronal cells. Such information is crucial to a better understanding of the role of NPY in cerebrovascular physiology and, possibly, pathophysiology, as suggested by the marked increases in NPY mRNA expression and immunoreactivity after focal ischemia in rats (Cheung et al., 1995).

Six different NPY receptors (Y1, Y2, Y3, Y4, Y5, and Y6) have been described with different affinities for NPY, polypeptide YY (PYY), the pancreatic polypeptides (PP) and their derivatives (Wahlestedt et al., 1986; Dumont et al., 1995; Gerald et al., 1996; Weinberg et al., 1996). With the exception of the Y3 subtype, all receptors have been cloned (Herzog et al., 1992; Larhammar et al., 1992; Gerald et al., 1996; Rose et al., 1995; Bard et al., 1995; Weinberg et al., 1996). In cerebral arteries, a Y1 receptor has been unequivocally identified pharmacologically (Abounader et al., 1995) and by expression of its mRNA (Nilsson et al., 1996; Abounader and Hamel, 1997*b*); message for the Y4 receptor type has been detected only in human coronary artery (Bard et al., 1995).

In the current study, we used pharmacologic and molecular approaches to investigate the expression of NPY-Y1, NPY-Y2, NPY-Y4, and NPY-Y5 receptor messages and proteins in human pial vessels, intracortical blood vessels, and cerebromicrovascular and astroglial cells in culture. The results indicate that only the Y1 receptor is consistently expressed in human cerebrovascular tissues, and that it is exclusively localized to smooth muscle cells where NPY application is coupled to inhibition of adenylyl cyclase activity. Furthermore, they show the expression of Y1, Y2, and Y4 receptor types in human brain astroglial cells in culture. Part of these data has appeared in an abstract form (Abounader and Hamel, 1997*b*).

METHODS

Human cerebrovascular tissues

Human tissues were obtained with approval from the Institutional Research Ethics Committee from male and female individuals who either died from diseases not related to the CNS (n = 9, postmortem delay 2 to 18 hours; Brain Bank from the Douglas Hospital Research Centre, Verdun, QC, Canada) or were undergoing temporal lobe surgery at the Montreal Neurological Institute for the treatment of epilepsy (n = 6). Pial vessels (from postmortem brains) were dissected out and cleaned, and underlying cortical brain tissue then was immediately used for isolation of intraparenchymal microvessels (MV) and capillaries (CAP), as previously described (Linville and Hamel, 1995). Pial vessels, MV, and CAP were either frozen in cold isopentane (−45°C; for radioautography and *in situ* hybridization) or in liquid nitrogen (for reverse transcriptase–polymerase chain reaction [RT-PCR]) and stored at −80°C until use.

Cell cultures

Human pial vessel endothelial cells were obtained by enzymatic stripping of pial arteries. After removing intraluminal debris with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, 0.5% trypsin was injected into the lumen and kept in place for 5 minutes. The intraluminal content then was recovered into a small volume of endothelial cell culturing media (65% medium M199 containing Earle's salts, 25 mmol/L Hepes, 4.35 g/L sodium bicarbonate, and 3 mmol/L L-glutamate, 10% fetal calf serum, 5% human serum, 20% murine melanoma cell–conditioned media, 5 μg/mL insulin, 5 μg/mL transferrin and 5 ng/mL selenium, 10 μg/mL endothelial cell growth supplement, and 100 μm/mL heparin) (Stanimirovic et al., 1996), and seeded onto 0.5% gelatin-coated tissue culture dishes. Cultures reached confluence after 2 weeks, displayed cobblestone morphologic features, and contained more than 95% of factor VIII–positive cells. Endothelial cells generated from two separate segments of pial vessels removed from one brain autopsy were used in this study.

Cultures of human cerebromicrovascular smooth muscle (SMC) and endothelial (END) cells were generated from surgically removed brain tissue. The SMC cultures were derived from large intraparenchymal microvessels isolated by a sequential filtration of brain homogenate through 350- and 112-μm mesh screens. These resistance microvessels then were dislodged from the mesh screens with cold medium M199, washed, dissociated with the type IV collagenase (1 mg/mL, 15 minutes), and seeded onto 0.5% gelatin-coated culture plates in the medium containing 65% medium M199, 10% fetal calf serum, 5% human serum, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium. After 4 to 5 weeks in culture, more than 85% of cells stained positively for the smooth muscle α-actin, whereas about 10% of cells demonstrated endothelial cell morphologic features and incorporated acetylated low density lipoprotein. Six SMC-enriched cultures derived from the microvessels harvested from the 350 μm (n = 4) and 112 μm (n = 2) mesh screens were used in the current study.

To initiate brain microvessel and capillary END cell cultures, brain homogenate depleted of large microvessels was centrifuged in 20% dextran (3000 *g*, 15 minutes), the pellet was resuspended in cold medium M199, and microvessels collected on a 20-μm mesh. The vessels were dissociated with the type IV collagenase, seeded, and maintained at 37°C in endothelial cell culturing media described earlier. The END cells were purified from the culture 4 to 5 days after seeding using cloning rings (BELLCO Glass, Inc., Vineland, NJ, U.S.A.). More than 95% of cells derived by these procedures were immunocytochemically positive for factor VIII–related antigen and expressed high levels of the cerebral endothelium-specific enzymes, γ-glutamyl-transpeptidase and alkaline phosphatase (Stanimirovic et al., 1996). The morphologic, phenotypic, biochemical, and functional characteristics of END cultures used in this study have been described in detail previously (Stanimirovic et al., 1996). The END cells (passages 3 to 7) from six

different human brain biopsy specimens were used in this study.

Cultures of fetal (10 to 18 weeks of gestation) human brain astrocytes (AST) (n = 5) were provided by Drs. J. Antel and W. Yong (Montreal Neurological Institute, Montréal, QC, Canada) and prepared using established procedures (Yong et al., 1992). Brains were trypsinized (0.25%), homogenized, and filtered through a 130-μm mesh. Cell pellets were resuspended in a 95% Dulbecco's modified Eagle's medium (4500 g/L glucose) containing 5% fetal bovine serum, and plated onto poly-L-lysine–coated tissue culture dishes. Contaminating neurons disappeared after trypsinization and passaging. More than 95% of the cells in cultures used in this study (passages 2 to 5) were immunopositive for the glial fibrillary acidic protein (Yong et al., 1992).

Radioautography and *in situ* hybridization

Pial vessel cross-sections (20-μm thick) were cut on a cryostat, mounted either on gelatin-coated slides, and stored at −90°C until use (radioautography) or on clean RNAase-free slides (Superfrost plus, Fisherbrand) and stored at −20°C for no more than 24 hours (*in situ* hybridization).

Radioautography. For radioligand binding studies, sections were preincubated (60 minutes, room temperature) in 1.2 mmol/L Krebs-Ringer phosphate buffer (containing 0.12 mol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 11 mmol/L dextrose, and 25 mmol/L $NaHCO_3$) and then incubated (2 hours, room temperature) in the same buffer supplemented with 0.1% bovine serum albumin and 0.05% bacitracin, and containing about 1.10^4 cpm/mL of either the Y1-(^{125}I-[Leu^{31}, Pro^{34}]-PYY or ^{3}H-BIBP 3226), Y2-(^{125}I-PYY_{3-36}), or Y4/Y5-(^{125}I-hPP) radioligands. Incubations were carried out in the presence (nonspecific binding) or absence (total binding) of 1 μmol/L NPY. After washing (3 × 10 minutes) in 1.2 mmol/L Krebs-Ringer phosphate buffer, the sections were fixed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer (phosphate-buffered saline, 10 minutes), washed, dehydrated, and air dried before being dipped in photographic emulsion (Kodak NTB-2; 1:2 with water, Photoservice, Montreal, Quebec, Canada) and exposed at 4°C for 7 to 30 days. The cellular distribution of Y1, Y2, or Y4/Y5 binding sites within the vessel wall was evaluated under light microscopic study on 0.5% cresyl violet–counterstained sections by comparing the specificity and location of the silver grains in sections incubated under total and nonspecific (10^{-6} mol/L NPY) conditions. For competition binding experiments, sections were incubated in the presence of increasing concentrations (10^{-10} to 10^{-6} mol/L) of either NPY, PYY, or the selective Y1 receptor antagonist BIBP 3226 in the case of Y1 sites labeled with ^{125}I-[Leu^{31}, Pro^{34}]-PYY; NPY for those labeled with the selective Y1 radioligand ^{3}H-BIBP 3226; NPY, NPY_{13-36}, or [Leu^{31}, Pro^{34}]-NPY for sections incubated with the Y2 radioligand ^{125}I-PYY_{3-36}; and NPY or human pancreatic polypeptide (hPP) for displacement of the Y4/Y5 sites labeled with ^{125}I-hPP. After rinsing and dehydration, sections were directly apposed to Kodak XAR films and exposed for 2 to 15 days (4°C). Film radioautograms were used for quantitative densitometry performed on a microcomputer-based image analysis system (The Image Calculator, Soquelec, Ltd., Montréal, QC, Canada). The pIC_{50} values (mean ± SD, n = 2 or 3 different experiments) for each agonist or antagonist were determined from the competition curves, and the total binding was set at 100%.

In situ hybridization. Pial vessel sections were thawed, fixed (5 minutes in 2% paraformaldehyde in 4 × standard saline citrate [SSC]) and washed (3 × 5 minutes in 4 × SSC) before immersion (10 minutes) in 0.1 mol/L triethanolamine containing 0.25% acetic anhydride. The dried sections were incubated (overnight in a humidified chamber at 56°C) in hybridization buffer (4 × SSC, 50% formamide, 10% dextran sulfate, 1 mol/L dithiothreitol, 1 × Denhardt's solution, 500 μg/mL denatured salmon sperm DNA, and 250 μg/mL yeast tRNA) containing approximately 5.10^6 cpm/mL of [^{35}S]-labeled cRNA probes (200 μL of probe per section). Adjacent sections were used for incubation with sense and antisense cRNA probes transcribed from cDNA complementary to the full coding region of the human Y1, Y2, Y4, and Y5 receptors (Larhammar et al., 1992; Rose et al., 1995; Bard et al., 1995; Gerald et al., 1996). The cDNA was subcloned in PGEM plasmids (constructed and kindly provided by Dr. Y. Tong at the Douglas Hospital Research Centre, Verdun, QC, Canada). The plasmids were linearized, antisense and sense cRNA probes transcribed (60 minutes at 37°C) in 20 μL of 1 × transcription buffer (40 mmol/L Tris-HCl, 6 mmol/L $MgCl_2$, 2 mmol/L spermidine, and 10 mmol/L NaCl; pH = 7.9) containing 20 mmol/L dithiothreitol; 10 units of RNAsin (Promega); 2 mmol/L ATP, γ-glutamyl-transpeptidase, and CTP; 2.5 mmol/L [^{35}S]-UTP; 1 μg linearized plasmid template; and 10 units of SP6, T7, or T3 RNA polymerase, depending on the cloning orientation in the respective plasmid. The cDNA template was removed by incubation (15 minutes, 37°C) with 10 units of RNAase-free RQ1-DNAase (Promega). The probes were purified by phenol/chloroform extraction and precipitated (−90°C, 60 minutes) in ethanol before resuspension of the pellet in 50 μL of RNAase-free deionized water for addition in hybridization buffer. After hybridization, the sections were subjected to high-stringency washes (4 × SSC/50% formamide, 30 minutes, 65°C and then 4 × SSC, 2 × 30 minutes at 65°C) and the remaining unhybridized probes removed with RNAase A (10 μg/mL) in 2 × SSC (30 minutes, 37°C). The sections then were washed (2 hours, 65°C in 0.5 × SSC), cleared in 70% ethanol, dried, and dipped into Kodak NTB-2 emulsion and exposed (4°C, 4 to 30 days). After development in Kodak D19, the sections were stained with 0.5% cresyl violet and observed under a Leitz Aristoplan microscope. Expression of NPY receptor mRNA was evaluated by comparing the density and location of silver grains between antisense- and sense-treated sections.

Reverse transcriptase–polymerase chain reaction

Frozen pial vessels, MV, and CAP were first powdered on dry ice whereas cell cultures were used directly for total RNA extraction, after homogenization in TRIzol Reagent (Gibco BRL, Gaithersburg, MD, U.S.A.). The suspension was treated with RQ1-DNAase (3U, Gibco BRL) and cDNA synthesized from 5 to 30 μg of total RNA (5- to 10-μg cells, 20- to 30-μg tissues) using random primers (200 ng/μg RNA, Gibco BRL) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, U.S.A.) (1 hour at 42°C). cDNA prepared from human cerebral cortex obtained at surgery was included in all polymerase chain reaction (PCR) studies as positive controls. The PCR amplification was performed in an MJ Research minicycler (Fisher Scientific, Montréal, Quebec, Canada) under the following conditions: 2 minutes at 95°C, 5 minutes at 72°C, 40 seconds at 95°C, 40 seconds at 56° to 58°C (depending on the primer used), and 40 seconds at 72°C for 37 cycles, followed by a 5-minute final extension at 72°C. The reaction mixture contained 3 to 5 μL of cDNA, 2 U of Taq DNA polymerase, and 0.5 μmol/L of each primer in a total volume of 50 μL. Specific oligonucleotide primers for the human Y1, Y2, Y4, and Y5 receptor genes were designed (NBI Oligo 5.0) according to published sequences (Larhammar et al., 1992; Rose et al., 1995; Bard et al., 1995; Gerald et al., 1996). They were synthesized in an Applied Biosystems synthesizer and purified

using an OPC column (Applied Biosystems). Some oligonucleotide primers were flanked with the restriction enzyme EcoR1 (GAATTC) and BamH1 (GGATCC) sequences. The primers were as follows: Y1 (Genbank accession #M88461), Y1-Forward: 5'-TCAGGATCCAACATCCTGATTGTGAAAC-3' and Y1-Reverse: 5'-TACTGCAGCACCAAGAGGAG-3', yielding a PCR product of 435 base pairs; Y2: (Genbank accession #36269), Y2-Forward: 5'-TGAATTCATATTGGCCTACTGCTCC-3' and Y2-Reverse: 5'-CTGGATCCTGCCATAGATGCTCTTCTCC-3' . PCR product of 492 base pairs; Y4 (Genbank accession #U35232), Y4-Forward: 5'-TGAATTCTTCATCGTCACTTCCTACAGC-3' and Y4-Reverse: CTGGATCCTAACAGACCAGGATGAAGCC-3' with PCR product of 575 base pairs; and Y5 (Genbank accession #U56079), Y5-Forward: 5'-TGAATTCGACACTAGGTTTTGCCATCTG-3' and Y5-Reverse: 5'-CTGGATCCGGACCCCTGGTATGAACTTA-3', yielding a PCR product of 510 base pairs. The PCR products were size-fractionated by electrophoresis on a 1.5% Tris/borate/ethylenediamine-tetraacetic acid agarose gel containing ethidium bromide, their size confirmed, and then photographed under ultraviolet light. Representative PCR fragments for the Y1 (SMC), Y2, and Y4 (AST) receptors were used for sequencing analysis using direct automated fluorescent sequencing (W.N. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT, U.S.A.).

Second messenger assays

The presence of functional NPY receptor proteins in microvascular cells was evaluated by measuring the ability of NPY to affect cAMP production in cultured human brain SMC (n = 4) and END (n = 3) cells, a pathway previously correlated to the vasoconstrictor activity of NPY (Lundberg et al., 1988). For comparison, one preparation of endothelial cells derived from human pial vessels also was used. Cells grown to confluence in 24-well tissue culture plates were incubated (15 minutes, 37°C) in phosphate-buffered saline containing 0.2% bovine serum albumin and 1 mmol/L 3-isobutyl-1-methyl-xanthine with NPY (0.1 and/or 1 μmol/L) either alone or in the presence of 1 μmol/L forskolin. The reaction was stopped the double extraction with 65% (v/v) ice-cold ethanol. The extracts were combined, dried (vacuum oven at 80°C) and dissolved in 200 μL of the assay buffer for measurement of cAMP levels with a commercial enzyme immunoassay kit (Biotrak, Amersham). The cell pellets were solubilized in 0.1 N NaOH and used for protein measurement by the method of Lowry and coworkers (1951). Statistical differences ($P \leq 0.05$) were evaluated by Student's t test.

RESULTS

Localization of neuropeptide Y receptor mRNA in human pial vessels

As shown in Fig. 1, sections incubated with a selective Y1 receptor [^{35}S]-labeled cRNA antisense probe exhibited a high density of silver grains, located primarily if not exclusively on the smooth muscle layer of human pial vessels (Fig. 1A). Although silver grains could accumulate at the border of the elastic lamina (Fig. 1A, arrow), endothelial cells remain unlabeled. No specific signal was observed in sections incubated with either the Y1 sense riboprobe (Fig. 1B) or the sense and antisense probes for the Y2, Y4, and Y5 receptor types (Figs. 1C through H).

Localization of neuropeptide Y receptor proteins in human pial vessels

In sections incubated with the Y1 radioligand ^{125}I-[Leu^{31},Pro^{34}]-PYY, a high level of specific binding sites (reflecting the presence of Y1 receptor protein) was observed in the smooth muscle layer, as evidenced by the difference in silver grain density in sections incubated under total and nonspecific conditions (Figs. 2A and B). In contrast, incubation with the Y2-(^{125} I-PYY$_{3\text{-}36}$) and Y4/Y5-(^{125}I-hPP) receptor ligands yielded no specific binding in any of the cellular compartments of the human pial vessels, as shown by the similar distribution and low density of silver grains in sections incubated under both total and nonspecific binding conditions (Figs. 2C through F).

Quantitative densitometry of film radioautograms from human pial vessel sections incubated with the Y1 radioligand ^{125}I-[Leu^{31}, Pro^{34}]-PYY showed competition of the bound ligand from the muscle layer by increasing concentrations of unlabeled NPY, PYY, and the selective Y1 receptor antagonist BIBP 3226 (Fig. 3A). Their respective pIC_{50} values were of 7.86 ± 0.06, 7.43 ± 0.04, and 8.42 ± 0.21 (mean ± SD). Nonspecific binding (evaluated in the presence of 10^{-6} mol/L of NPY, PYY, or BIBP, respectively) corresponded to about 25% to 35% of total binding (Fig. 3A). The Y1 radioligand ^{3}H-BIBP 3226 was competed from the pial vessel sections in a concentration-dependent manner by NPY with a calculated pIC_{50} value of 8.01 ± 0.01 (mean ± SD) (Fig. 3B). In contrast, film densitometry showed no significant difference in the density of binding sites between sections incubated with the radioligand alone or in the presence of various concentrations (10^{-9} to 10^{-6} mol/L) of either NPY, [Leu^{31}-Pro^{34}]-NPY, or NPY$_{13\text{-}36}$ for Y2-(^{125}I-PYY$_{3\text{-}36}$) sites, or hPP (10^{-7} to 10^{-10} mol/L) in the case of Y4/Y5-(^{125}I-hPP) binding sites.

Neuropeptide Y receptors expression in cerebrovascular tissues and microvascular and astroglial cells

In agreement with radioautographic and *in situ* hybridization experiments, specific PCR products of the expected size (435 bp) for the Y1 receptor were detected in most (67% to 100%) human pial vessels, isolated MV, and CAP, as well as in most SMC and AST cell cultures (Fig. 4, top panel). However, no amplification of Y1 receptor message could be evidenced in endothelial cells derived from pial vessels or in any of the cerebromicrovascular END cell cultures tested (Fig. 4, top panel). Message for the Y2 receptor was detected in a few (approximately 20%) of vascular preparations and a single culture of microvascular endothelial cells. Messages for the Y4 and Y5 receptors could not be detected in any of the vascular tissues, END, or SMC cultures (Fig. 4).

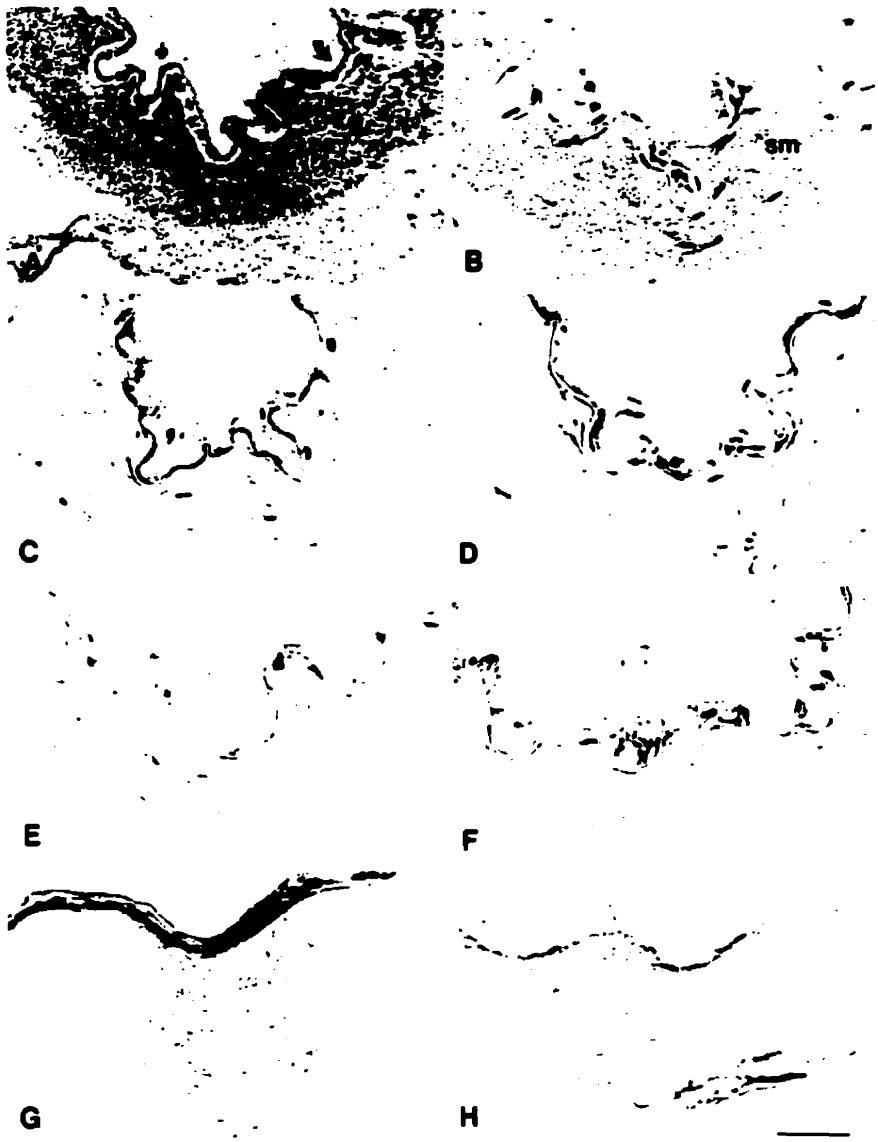

FIG. 1. Photomicrographs of emulsion-dipped sections of human pial vessels incubated with antisense (left panels) or sense (right panels) cRNA probes complementary to the coding sequence of the human neuropeptide Y (NPY)-Y1 **(A and B)**, NPY-Y2 **(C and D)**, NPY-Y4 **(E and F)**, and NPY-Y5 **(G and H)** receptor genes. Notice that only sections incubated with the Y1 antisense cRNA probe exhibited a high density of silver grains particularly visible over the smooth muscle (sm) layer. The luminal border of the elastic lamina (curved arrow) often creates a rim artifact where silver grains get trapped. Bars = 100 μm.

However, PCR products of the expected size for the Y2 (491 bp) and Y4 (578 bp) receptors were detected by gel electrophoresis in most (80% and 67%, respectively) of AST cells in culture (Fig. 4, middle and bottom panels). Sequence analysis of the selected PCR products matched the published sequences for their respective cloned human Y1 (99.8%), Y2 (99%), and Y4 (99.5%) NPY receptors.

Functional neuropeptide Y receptors in microvascular cell cultures

As shown in Fig. 5, functional NPY receptor proteins were detected in the muscular compartment of cerebral microvessels. The basal release of cAMP was slightly inhibited by NPY in SMC (about 30%, not significant) but not in END cells. Forskolin (1 μmol/L) resulted in a 10- to 20-fold increase in cAMP production in these cultures (Fig. 5). The NPY (0.1 and 1 μmol/L) significantly ($P < 0.05$) inhibited the forskolin-stimulated cAMP production in SMC (40% and 51%, respectively), whereas a 1-μmol/L NPY concentration had no effect on the evoked cAMP production in microvascular END cell cultures (Fig. 5) or in endothelial cells derived from human pial vessels (data not shown).

DISCUSSION

This study provides the first comprehensive analysis of NPY receptors message and protein distribution, as well as cellular localization in human cerebral blood vessels. The results obtained by both pharmacologic and molecular approaches suggest that the Y1 receptor subtype is the only receptor, of the known and cloned human NPY receptors, to be significantly expressed in human brain vessels, irrespective of their extraparenchymal or intraparenchymal location. These NPY receptors are exclusively located on the smooth muscle cell layer not

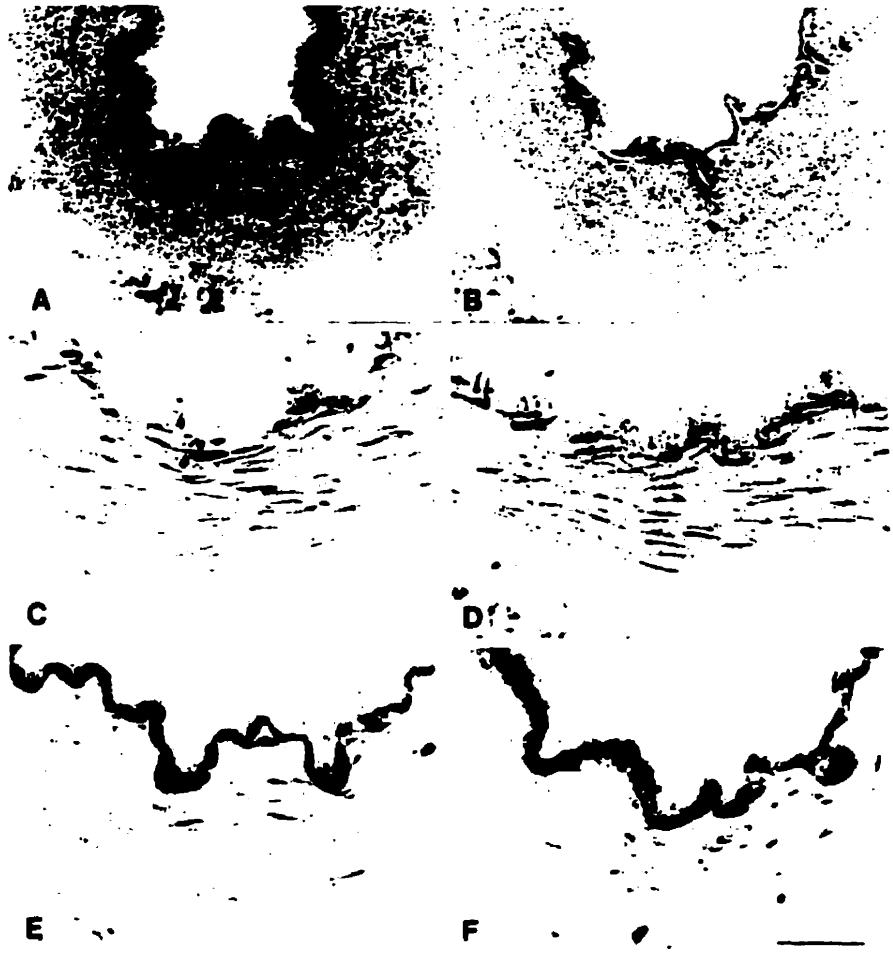

FIG. 2. Photomicrographs of emulsion-dipped human pial vessel sections incubated with radioligands for the NPY-Y1 (^{125}I-[Leu^{31}, Pro^{34}]-PYY) **(A and B)**, Y2 (^{125}I-PYY$_{3-36}$) **(C and D)**, and Y4/Y5 (^{125}I-hPP) **(E and F)** receptors under conditions for total (left panel) and nonspecific (right panel) binding. Notice the high density of silver grains over the smooth muscle layer of sections incubated with the Y1 radioligand. Diffuse labeling was observed for the other radioligands with no difference in silver grain density between total and nonspecific binding. Bar = 100 μm.

only of pial arteries, but also of intracortical microvessels, where they were shown to inhibit adenylyl cyclase activity. Moreover, the results show that brain astrocytes express multiple NPY receptor subtypes, identified as Y1, Y2, and Y4 by RT-PCR. Taken together, these data indicate that NPY has the ability to influence nonneuronal cells within the human brain by stimulating vascular Y1 and heterogeneous astroglial NPY receptors and, as such, could participate in the regulation of brain perfusion and metabolic or ionic homeostasis.

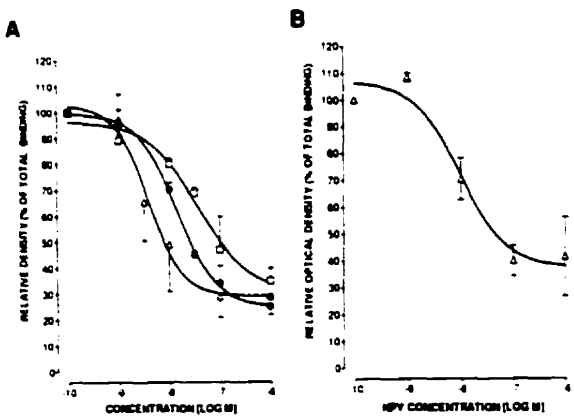

FIG. 3. Competition of ^{125}I-[Leu^{31}-Pro^{34}]-PYY–labeled smooth muscle specific binding sites by increasing concentrations (10^{-10} to 10^{-6} mol/L) of NPY (●), PYY (□), or the selective Y1 receptor antagonist BIBP 3226 (△) **(A)**, and of ^{3}H-BIBP 3226–labeled smooth muscle Y1 specific binding sites by NPY **(B)**, as determined by film radioautographic densitometry. Bars = SD.

The identification of Y1 receptors in human pial vessels by *in situ* hybridization and radioautography agrees with previous pharmacologic and RT-PCR studies (Abounader et al., 1995; Nilsson et al., 1996). However, the results further indicate that the Y1 receptor is the only NPY receptor associated with human cerebral arteries, and that it has an exclusive muscular localization. This localization concurs with a recent immunocytochemical study of cerebrovascular Y1 receptors in the rat (Bao et al., 1997). The Y1 nature of the human cerebrovascular NPY receptor is pharmacologically supported by the potency of NPY, PYY, and the Y1 receptor antagonist BIBP 3226 (respective pIC_{50} values of 7.86, 7.43, and 8.42) in competing for ^{125}I-[Leu^{31}-Pro^{34}]-PYY–labeled specific smooth muscle binding sites. These compare well with the affinities reported for these compounds at the cerebrovascular Y1 receptor mediating contraction of

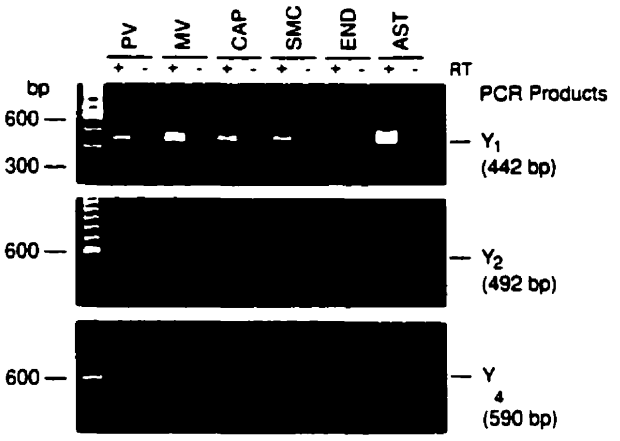

FIG. 4. Gel electrophoresis of polymerase chain reaction (PCR) products for the NPY-Y1, NPY-Y2, and NPY-Y4 receptors amplified from reverse transcribed DNA (+) from human pial vessels (PV), isolated intracortical microvessels (MV) and capillaries (CAP), as well as from human microvascular smooth muscle (SMC), endothelial (END), and astroglial (AST) cells in culture. Expression of the Y1 receptor was detected in every segment of the human cerebrovascular bed and cells in culture except for the END cells. Expression of Y5 receptor mRNA could not be detected in any vascular or astroglial preparations, despite a positive and specific amplification in human cerebral cortex (not shown). Samples without reverse transcriptase were included (-) to monitor for background amplification and possible contamination.

human cerebral arteries (Abounader and Hamel, 1995; Nilsson et al., 1996). In addition to vasoconstriction, other roles for cerebrovascular smooth muscle Y1 receptors could be considered, such as mediation of mitogenesis, as reported in peripheral vascular smooth muscle cells (Zukowska-Grojec et al., 1993).

The results suggest that human cerebral arteries differ from some peripheral (femoral and intestinal) vascular beds in animal species in which both muscular Y1 and Y2 receptors have been implicated in the control of arterial or arteriolar vascular tone (Tessel et al., 1993; Neild and Lewis, 1995). Indeed, no evidence was obtained from the current molecular or anatomical studies for the presence of smooth muscle Y2 receptors in human brain vessels. Whereas the Y3 receptor also has been pharmacologically excluded as the mediator of the NPY-induced human cerebral vasoconstriction (Abounader and Hamel, 1995), additional work is required to verify its status at the cerebrovascular level when the human clone becomes available. Based on the current molecular and radioligand binding data, it is unlikely that Y4 and Y5 receptors exist in the human cerebrovascular bed in any significant amount.

A novel finding from the current study is that of a selective expression of Y1 receptor mRNA in the human brain microvascular bed, more specifically, at the level of the smooth muscle cells where the presence of functional receptor proteins was confirmed by the ability of NPY to inhibit cAMP production. Such association of microvascular Y1 receptors with the smooth muscle cell layer of human intracortical microvessels is fully compatible with the recent immnocytochemical demonstration of Y1 receptor in rat brain microarterioles (Bao et al., 1997) and with previous reports in rodents suggesting that Y1 receptors mediate the endothelium-independent NPY vasoconstriction in the microcirculation, albeit of peripheral origin (Kim et al., 1994). The presence of smooth muscle Y1 receptors in human intracortical microvessels strongly suggests that perivascular NPY nerve terminals (Abounader and Hamel, 1997*a*) could induce local cerebral vasoconstriction, a hypothesis supported by previous observations in the rat of a local decrease in CBF after intraparenchymal application of NPY (Tuor et al., 1990) and of an NPY-induced contractile response in isolated intracortical arterioles (Dacey et al., 1988). The ability of NPY to inhibit the forskolin-stimulated increase in cAMP levels in human cerebromicrovascular

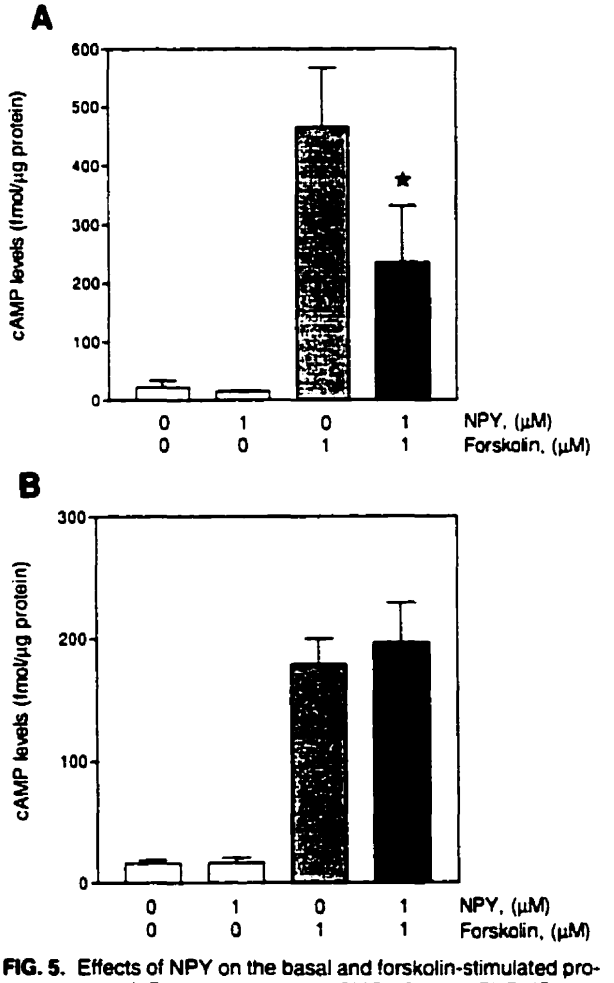

FIG. 5. Effects of NPY on the basal and forskolin-stimulated production of cAMP in microvascular SMC **(A)** and END **(B)** cell cultures. The basal levels of cAMP were slightly but not significantly decreased by NPY in SMC but not in END cell cultures. However, NPY (1 µmol/L) significantly inhibited (51%, $P < 0.05$) the forskolin-induced cAMP production in SMC while having no effect in END cells. Bars = SD; $^*P < 0.05$.

SMC cells, a signaling pathway associated with a direct vasocontractile response to NPY (Lundberg et al., 1988; Larhammar et al., 1992), further supports such a role for NPY at the microvascular level.

The failure to detect NPY receptors in a significant proportion of endothelial cells further suggests that muscular Y1 receptors are the primary if not exclusive mediators of the NPY-induced vascular responses in human brain vessels located both outside and within the cortical mantle. Interestingly, in endothelial cells from human umbilical vein, the Y1 receptor mRNA is only detectable during cell differentiation (Karwatoska-Prokopezuk et al., 1996). Therefore, although neither the NPY-Y1 receptor's message nor protein were detected in any preparation of human brain endothelial cells, it cannot be excluded that these receptors can be induced during development or by certain physiologic conditions.

In addition to being abundantly expressed in human brain cells, the current study shows that Y1 receptors appear to be an integral and functional component of brain vascular and astroglial cells. This distribution contrasts with that of the other cloned NPY receptors, which were either never present (Y4, Y5) or detected in only a few (Y2) cerebrovascular tissues or microvascular endothelial cell cultures. The detection of a Y2 message in isolated microvascular fractions is likely attributable to the associated perivascular astrocytes (see later). The apparent absence of endothelial NPY receptors is consistent with previous studies in nonhuman vessels, both in peripheral arteries (Pernow and Lunberg, 1988) and microvessels (Kim et al., 1994). However, it cannot be excluded that Y2 receptors could regulate vascular functions through receptors located presynaptically on nerve terminals, as documented in the peripheral circulation (Hashim and Tadepalli, 1995) or, alternatively, on astroglial cells, a cell type that is intimately associated with the microvascular bed.

We observed expression of multiple subtypes of NPY receptors (i.e., Y1, Y2, and Y4 receptors) in fetal human brain astrocytes. The presence of astroglial NPY receptors had been reported previously in the rat, either by identification of specific binding sites for NPY in cultures of rat brain astrocytes or by the observation of a membrane potential depolarization and an increase in $[Ca^{2+}]i$ after application of NPY (Hosli and Hosli, 1993; Gimpl et al., 1993). The possibility that these receptors are still expressed and functional in adult human brain deserves further investigation. Since astroglial cells are an integral component of isolated MV and CAP, the relative or complete absence of PCR products for the Y2 or Y4 receptor in adult human isolated MV and CAP suggests that these receptors are expressed at low levels in adult astrocytes or, alternatively, that their primary location is not the perivascular astrocytes.

In conclusion, the current study demonstrates the existence of Y1 receptors not only on smooth muscle of human cerebral arteries, but also of intracortical microvessels. In view of the potent effect of NPY on normal brain perfusion and the documented increase in brain NPY levels or expression in focal ischemia (Cheung et al., 1995), the exclusive presence of NPY-Y1 receptor on human brain myocytes may offer a unique opportunity to target the cerebrovascular system. Moreover, these data underscore the possibility that NPY released from human brain neurons can, through vascular smooth muscle Y1 receptors, directly influence cerebrovascular functions related to regulation of local cerebral perfusion. They also suggest that NPY interactions could occur through astroglial receptors, which may be involved not only in the control of microvascular functions, but also of brain metabolic and ionic homeostasis, gliosis, and recovery after injury (Zoli et al., 1997).

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