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CENTRAL SEROTONIN (5-HT) NEURONS IN THE CONTROL OF THE CEREBRAL CIRCULATION: ANATOMICAL BASIS AND FUNCTIONAL RECEPTORS

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) is known to influence cerebrovascular functions such as local cerebral blood flow (CBF) and blood brain barrier (BBB) permeability, and has been implicated in cerebrovascular diseases. The present study used a multifaceted approach to determine the distribution, density and origin of the 5-HT innervation of blood vessels at the base of the brain and overlying the cerebral cortex as well as those embedded in the cortical parenchyma. In addition, the type(s) of 5-HT receptor(s) present on intracortical blood vessels as well as their precise cellular localization within the vessel wall was investigated.

Firstly, in extracerebral blood vessels, we showed that perivascular serotonergic nerve fibers, immunocytochemically identified for the 5-HT synthesizing enzyme, tryptophan hydroxylase (TPH), are greatly reduced following removal of the superior cervical ganglia, but not after specific lesion of the ascending 5-HT fibers originating from the brainstem raphe nuclei. In addition, we demonstrated that the distribution pattern of TPH-immunolabelled perivascular fibers differed from those containing noradrenaline (identified by dopamine-β-hydroxylase). These results suggest the existence of a subset of distinct 5-HT nerve fibers in extracerebral arteries and that the serotonergic innervation, most probably, arises from the superior cervical ganglia or a structure closely related to it.

Secondly, in investigating the serotonergic input to the intraparenchymal microcirculation at the ultrastructural level, we found that central TPH-containing nerve terminals are intimately associated with intraparenchymal blood vessels and that these neurovascular associations were closer and/or more frequent in brain regions where manipulations of the brainstem raphe neurons elicited significant CBF changes, as compared to relatively unresponsive cerebral area. These associations frequently involved the perivascular astrocytes, suggesting a possible intermediary role for these non-neuronal cells in the control of microvascular functions. Furthermore, the associations between 5-HT-synthesizing nerve terminals with the microvascular bed appeared relatively selective since

neurovascular noradrenaline nerve terminals in the same cortical subdivision did not share the same characteristics in terms of frequency, intimacy or distribution around the vessel walls.

Finally, in an attempt to identify the exact site(s) of action of 5-HT on the blood vessels, we characterized, via reverse transcriptase-polymerase chain reaction and second messenger assays, the 5-HT receptor(s) present on human intracortical blood vessels as well as in cell cultures of human brain astrocytes and of endothelial and smooth muscle cells of micro-vascular origin. We reported the differential expression not only of messages but also of functional proteins for specific 5-HT receptor subtypes in the different cellular compartments of the blood vessel wall; a finding fully compatible with the ability of 5-HT to regulate microvascular perfusion and BBB permeability.

Altogether, the present thesis provides an anatomical substrate for the 5-HT-mediated responses in the microvascular bed. It demonstrates that the indoleamine can affect the microvascular bed by interacting either directly with endothelial and/or smooth muscle cells or indirectly with the perivascular astroglial cells suggesting that the neuronal-glial-vascular triad most likely constitutes the functional unit in the regulation of microvascular related responses. These studies are likely to contribute significantly to our understanding of the relationships between 5-HT and non-neuronal vascular and astroglial cells as they relate to the mechanisms involved in the regulation of CBF and BBB.

RÉSUMÉ

La sérotonine (5-hydroxytryptamine, 5-HT) joue un rôle régulateur du débit sanguin cérébral et de la perméabilité de la barrière hémato-encéphalique, et a aussi été impliquée dans certaines maladies cérébrovasculaires. La présente étude a tiré avantage de plusieurs approches méthologiques afin de déterminer la distribution, la densité et l'origine de l'innervation sérotoninergique des vaisseaux extracérébraux (polygone de Willis et leurs ramifications) et de ceux localisés dans le parenchyme cortical. De plus, le type de récepteur(s) de la 5-HT associé(s) aux vaisseaux intracorticaux ainsi que leur localisation cellulaire dans la paroi du vaisseau ont été étudiés.

En premier lieu, nous avons montré, au niveau des vaisseaux extracérébraux, que les fibres nerveuses sérotoninergiques, identifiées par voie immunocytochimique avec un anticorps dirigé contre la tryptophane hydroxylase (TPH, l'enzyme de synthèse de la 5-HT), sont réduites suite à l'ablation du ganglion supérieur cervical mais non après la destruction des fibres sérotoninergiques provenant des noyaux du raphé. De plus, la distribution des fibres périvasculaires sérotonergiques ne correspondait pas parfaitement à celle des nerfs sympathiques qui contiennent la noradrenaline (marqués par l'enzyme de synthèse de la noradrénaline, la dopamine-β-hydroxylase). Cette observation a suggéré l'existence d'une population distincte de fibres périvasculaires contenant la sérotonine.

Deuxièment, nous avons trouvé que les terminaisons sérotoninergiques centrales peuvent s'associer avec les vaisseaux intraparenchymateux. De telles associations neurovasculaires sont plus fréquentes et/ou plus intimes dans les régions cérébrales où la manipulation des neurones du raphé produit des changements notables de débit sanguin en comparaison avec les régions qui ne changent pas ou peu leur perfusion locale suite à de tels traitements. De plus, les terminaisons nerveuses périvasculaires sont fréquemment associées avec les feuillets astrocytaires, ce qui suggère un rôle possible pour ces cellules dans la régulation de fonctions microvasculaires. Les fibres noradrénergiques périvasculaires ne présentaient pas les mêmes caractéristiques de fréquence et de distribution autour du vaisseau, ce qui nous porte à croire

que les relations qui s'établissent entre un neurotransmetteur/neuromédiateur et le lit microvasculaire varient en fonction du rôle qu'il exerce localement sur la microcirculation.

Finalement, nous avons caractérisé, chez l'homme, les récepteurs de la 5-HT associés aux microvaisseaux intracorticaux, de même qu'aux cellules astrocytaires, cérébromicro-vasculaires endothéliales et musculaire lisses en culture. En utilisant une approche moléculaire et fonctionelle, nous avons montré non seulement l'expression de plusieurs récepteurs distincts de la 5-HT dans les différents compartiments vasculaires ou astrocytaires, mais aussi leur habilité à activer ou inhiber des systèmes de signalisation intracellulaire.

Cette thèse fournit une base morphologique aux effets cérébrovasculaires dans lesquels la sérotonine a été impliquée. Elle démontre que la sérotonine peut avoir des effets sur les vaisseaux intraparenchymateux, soit directement avec les cellules endothéliales et musculaires lisses ou indirectement via l'astrocytes périvasculaires, ce qui suggère que cette triade neurone-astrocyte-vaisseau joue un rôle important dans les interactions neurovasculaires fonctionelles. Cette étude a également contribué à améliorer notre compréhension des types d'interaction de la sérotonine avec les cellules non-neuronales, et plus précisement dans la régulation du débit sanguin cérébral local et la perméabilité de la barriére hémato-encéphalique.

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PREFACE OF THESIS

The structure of this thesis conforms to the manuscript based option permitted by McGill University. In accordance with the *Guidelines Concerning Thesis Preparation* of the Faculty of Graduate Studies and Research, the following excerpt is reproduced in the preface of the thesis:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory**. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include**: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent**. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

CLAIM OF ORIGINALITY AND CONTRIBUTION OF CO-AUTHORS

The work presented in this thesis constitutes an original contribution to the knowledge regarding serotonergic neurons and their possible regulation of vascular functions. The results presented herein have been either published, in press or submitted. Much of the results have also been presented orally and in poster form at the 1991, 1993, 1994, 1995, 1996 Society for Neuroscience meetings, the 1993 and 1997 International Symposium of Cerebral Blood Flow and Metabolism, the 1996 ACFAS meeting and the 1996 IBC's International Conference on Serotonin Receptors.

Chapter 1 presents an extensive review of the literature regarding serotonin and its relation with the cerebrovascular bed with a particular emphasis on the microcirculation. In Chapter 2, the specific objectives of my research project are outlined.

Chapter 3 entitled *"Cerebrovascular nerve fibers immunoreactive for tryptophan-5-hydroxylase in the rat: Distribution, putative origin and comparison with sympathetic noradrenergic nerves"* describes the immunocytochemical experiments showing that the distribution of serotonin-synthesizing cerebrovascular nerve fibers is not superimposable to that synthesizing noradrenaline, suggesting the possible existence of a subset of distinct perivascular 5-HT fibers. Furthermore, these fibers do not seem to originate directly from brainstem 5-HT raphe neurons but rather from a peripheral structure closely related to the superior cervical ganglia. This study was done in collaboration with the Cerebrovascular Group at the CNRS, Université de Paris VII (France), of which Drs J. Seylaz and E.T. MacKenzie are the group leaders. Drs P. Lacombe and G. Bonvento visited our laboratory in Montreal and started the lesion and immunocytochemical experiments, of which I completed. The manuscript was prepared and written in conjunction with Drs E. Hamel, Lacombe and Bonvento. My responsibilities in this manuscript included editing and revising it as well as all the photographic work and montage.

In Chapter 4 entitled *"Ultrastructural analysis of tryptophan hydroxylase immunoreactive nerve terminals in the rat cerebral cortex and hippocampus: Their associations with local blood vessels"* the 5-HT neurovascular associations were characterized in the frontoparietal and entorhinal cortices as well as the hippocampus. The results show that in regions where 5-HT has a more profound effect on blood flow, these associations are more frequent and/or closer. All experimental manipulations and analyses were performed exclusively by myself, under the supervision of Dr E. Hamel. I was responsible for the original version of the manuscript and worked side by side with Dr. E. Hamel is its editing and revision. The tryptophan hydroxylase antibody was kindly provided by Drs M. Ehret and M. Maitre.

Chapter 5 entitled *"Astroglial and vascular interactions of noradrenaline terminals in the rat cerebral cortex"* shows the morphological relationships of another monoamine, namely noradrenaline, and the microvasculature in the frontoparietal cortex. The results show some close noradrenaline neurovascular associations but these are less frequent and intimate than those previously characterized in the 5-HT system. Nearly all manipulations and analyses were performed by me under the supervision of Dr. E. Hamel while the rest was done by G. Molinatti, a summer student from Lyon under my supervision. The first draft of the manuscript was written by me and then revised and edited by Dr E. Hamel. The noradrenaline antiserum was a generous gift from Dr. M. Geffard.

Chapter 6 entitled *"Molecular and pharmacological characterization of functional serotonin receptors in human brain microcirculation and astrocytes"* describes the expression of various serotonin receptors on isolated human brain microvessels and capillaries as well as on endothelial, smooth muscle and astrocytic cell cultures. It also describes the possible coupling of these receptors to their expected second messenger. The majority of all experimental manipulations and analyses were performed by me under the supervision of Dr. E. Hamel. The establishment of endothelial and smooth muscle cell cultures and most second messenger assays were done by Mrs. R. Ball, a technician working in the laboratory of Dr D. Stanimirovic (National Research Council of Canada, Ottawa). The astrocyte cultures were kindly provided by Dr V.W. Yong (McGill University). Dr. J.G. Villemure provided the human tissue samples from which the cell cultures were derived while I. Bouchelet, a PhD

student in the laboratory, helped with some of the polymerase chain reaction work. The detailed first draft was written by myself and edited with the help of Drs E. Hamel and D. Stanimirovic. The final version will be submitted for publication.

In Chapter 7, a general discussion of my results and their significance is presented. In the Appendix (Chapter 9), two additional published papers entitled *"Serotonin in the regulation of brain microcirculation"* and *"In vivo synthesized radioactively labelled α-methyl serotonin as a selective tracer for visualization of the brain serotonergic system"* are also included.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5-CT:	5-carboxamidotryptamine
5-HIAA:	5-hydroxyindoleacetic acid
5-HT:	5-hydroxytryptamine, serotonin
5-HTP:	5-hydroxytryptophan
5,7-DHT:	5,7-dihydroxytryptamine
8-OH-DPAT:	8-hydroxy-2-(di-n-propylamino)tetralin
AMV:	avian myeloblastosis virus
AP:	alkaline phosphatase
BBB:	blood brain barrier
bp:	base pair
Ca^{2+}:	calcium
$[Ca^{2+}]i$:	intracellular calcium concentration
cAMP:	cyclic adenosine monophosphate
CAP:	capillaries
CBF:	cerebral blood flow
cDNA:	complementary deoxyribonucleic acid
cGMP:	cyclic guanosine monophosphate
CGU:	cerebral glucose utilization
CNS:	central nervous system
CO_2	carbon dioxide
CSF:	cerebrospinal fluid
DAG:	diacylglycerol
DBH:	dopamine-β-hydroxylase
DiI-Ac-LDL:	acetylated low-density lipoprotein
DNA:	deoxyribonucleic acid
DSP-4:	N-(2-chloroethyl-N-ethyl-bromobenzylamine)
EM:	electron microscopy
GABA:	gamma aminobutyric acid

GFAP:	glial fibrillary acidic protein
γ-GTP:	γ-glutamyltranspeptidase
HBA:	human brain astrocytes
HBEC:	human brain endothelial cells
HBSM:	human brain smooth muscle cells
IP3:	inositol-1,4,5-triphosphate
KDa:	kilodalton
LM:	light microscopy
MAO:	monoamine oxidase
MDA:	methylenedioxyamphetamine
MLC:	myosin light-chain
MLC_{20}:	myosin light-chain 20 kilodalton subunit
mRNA:	messenger ribonucleic acid
NA:	norardrenaline
NO:	nitric oxide
O_2:	oxygen
PBS:	phosphate buffered saline
PBSM:	phosphate buffered saline containing sodium metabisulphite
PCPA:	p-chlorophenylalanine
PCR:	polymerase chain reaction
pH:	hydrogen power
PKA:	protein kinase A
PKC:	protein kinase C
PKG:	protein kinase G
PLC:	phospholipase C
RT:	reverse transcriptase
SCG:	superior cervical ganglion
TPH/ TPOH:	tryptophan hydroxylase

à mon père (ז"ל)

à Muriel

CHAPTER 1

GENERAL INTRODUCTION

1.0 INTRODUCTION OVERVIEW

My PhD thesis project essentially investigated the anatomical and some functional evidence regarding the possible control by 5-HT neurons contained in the dorsal and median raphe nuclei of the cerebral circulation, with a particular emphasis on the microcirculation. Essentially, I looked at whether or not 5-HT dorsal and/or median raphe neurons directly project i) to extracerebral blood vessels, ii) to intraparenchymal blood vessels located in the hippocampus and different cortical areas and if so, iii) what is the identity of the 5-HT receptors by which serotonin can act on the microvascular bed and their specific localization within the vessel wall.

In the course of this thesis, I will first describe the anatomy of the cerebral circulation (both overlying and inside the brain) with a strong emphasis on the arterial system as it has consistently been shown to regulate cerebral blood flow. The current hypotheses of CBF regulation will also be documented. Next, several general characteristics of the central 5-HT system will be presented. The synthesis and degradation pathways of 5-HT will be discussed as well as the localization of major 5-HT cell groups and their projections. A special emphasis will be given to the distribution pattern and density of 5-HT nerve terminals in various subdivisions of the cerebral cortex and hippocampus as these regions specifically pertain to my research project.

In the following section, I will document the relevant information concerning the effects of 5-HT on the cerebral circulation. A general overview will be presented regarding the serotonergic innervation of extracerebral blood vessels and the apparent discrepancies. More emphasis was thus placed in the control of 5-HT on the local CBF as this was of major interest to my work. Finally, a description of the 5-HT receptors that mediate vasomotor responses in both central and peripheral vascular beds will be presented and the possible 5-HT effects on other vascular-related functions. A brief general description of the major features of all characterized 5-HT receptors will then be presented. Finally,

the possible mechanisms underlying vascular contraction and dilatation will be presented.

1.1 THE CEREBROVASCULAR BED

1.1.1 GENERAL ORGANIZATION

For purposes of simplicity and practicality in the present thesis, the cerebrovascular bed will be differentiated into its two main compartments, i) *the extracerebral blood vessels*, composed of the major cerebral arteries and their ramifications as small pial vessels at the base and over the convexities of the brain and ii) *the intracerebral blood vessels* that encompass primarily small arterioles, microarterioles and capillaries that are embedded in the brain parenchyma. These compartments are categorized mainly according to size and function. They differ in their respective origin and patterns of innervation as well as their response to various neuromodulators.

Blood to the brain is supplied through two pairs of arterial trunks (Fig 1.1), namely the internal carotid (anterior circulation) and the vertebral (posterior circulation) arteries. These two systems are connected to each other through the circle of Willis which is designed to maintain blood supply to both sides of the brain in the event of an interruption to one of the main arteries. The internal carotid system delivers blood to the rostral parts of the brain that includes most of the basal ganglia and interior capsule while the caudal parts of the brain including the cerebellum, the thalamus and most of the brainstem receive blood from the vertebral system. Posteriorly, the two vertebral arteries, that originate from the subclavian artery, coalesce to form the single basilar artery which in turn gives rise to the superior cerebellar and posterior cerebral arteries at its terminal end. The posterior cerebral and the posterior communicating arteries, the latter coming from the internal carotid artery, form the posterior margin of the circle of Willis. Anteriorly, the internal carotid artery bifurcates into the middle and anterior cerebral arteries, of which the former is usually the largest branch of the two. The anterior cerebral arteries on each side are connected by the anterior communicating artery at the rostral portion of the circle of Willis. This anatomical organization applies to humans,

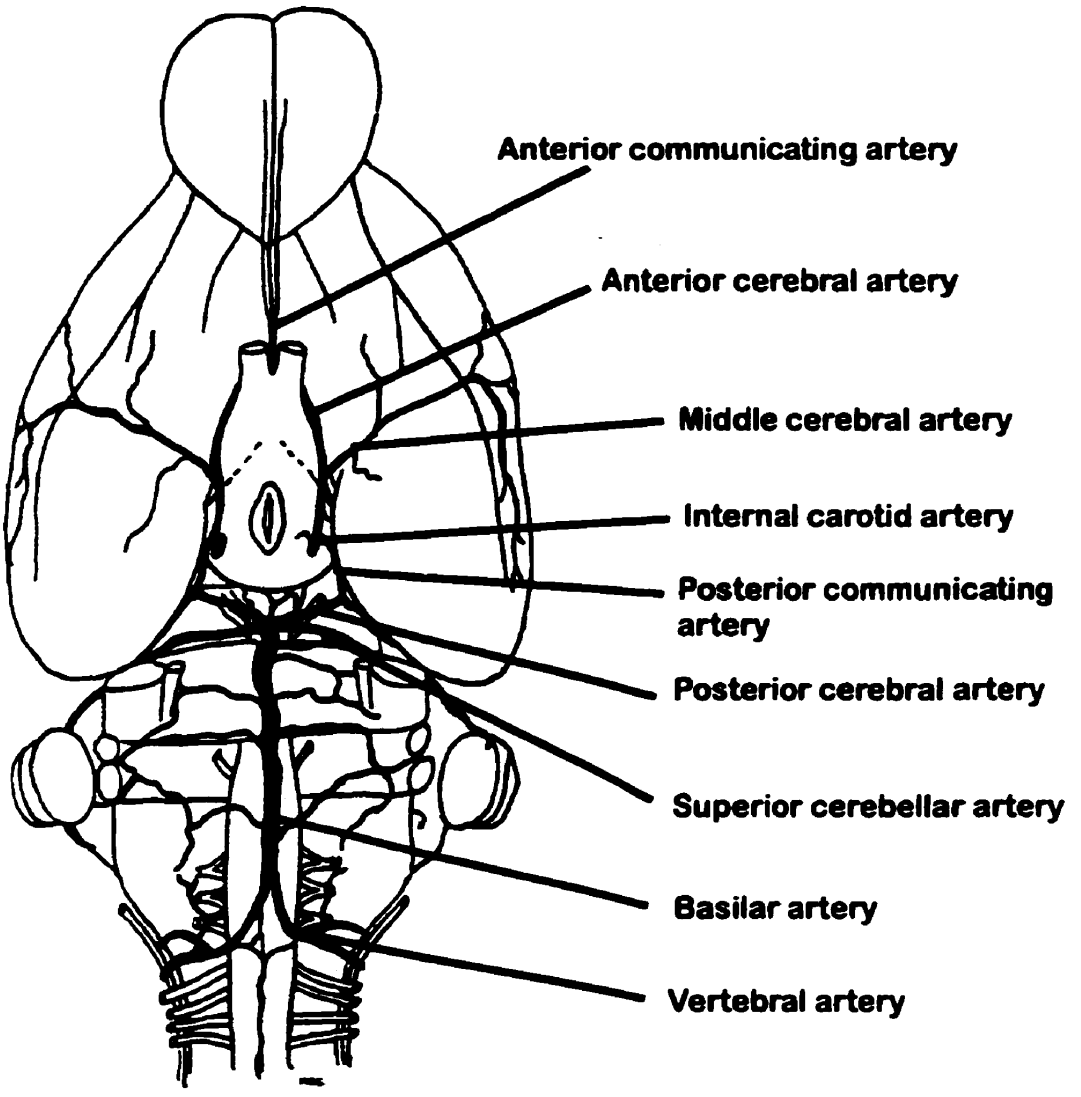

Fig 1.1: Schematic representation of the distribution of rat major cerebral arteries at the base of the brain. Note that not all branches are defined. Adapted from Zeman and Innes (1963)

primates and laboratory rodents with slight variations (Lee, 1995).

These major arteries branch out as small pial vessels (Fig 1.2) and are both located in the subarachnoid space found between the arachnoid and pial membranes. Historically, pial vessels were generally considered as the primary resistance vessels and the major arteries merely as conduit vessels. However, more recent investigations have shown that these larger arteries play an important role in blood flow resistance and consequently blood flow regulation (Faraci and Heistad, 1990). Together, the extracerebral arteries would control global cerebral blood flow (CBF) to the brain in response to physiological and systemic factors (Heistad and Kontos, 1983). At some point, small arteries further bifurcate from the pial vessels and perforate the cerebral parenchyma at right angles. As these penetrating arteries descend into the cerebral neuropil, they are initially surrounded by the Virchow-Robin's space, which is filled with cerebrospinal fluid and separates the vessel from the tissue (Fig 1.3). This perivascular space gradually disappears as the basement membrane of the vessel and the surrounding cerebral tissue fuse together (Fig 1.3). At this point, blood vessels are considered to form the intraparenchymal microcirculation and are composed of small arterioles, microarterioles and capillaries which come in direct contact with brain tissue. These vessels are thought to regulate CBF in highly localized and restricted brain regions in response to several factors (see section 1.1.4) and blood brain barrier (BBB) functions. Eventually, capillaries form numerous anastomoses which combine into venules and veins to finally drain into a number of venous sinuses.

1.1.2 MORPHOLOGY OF CEREBRAL BLOOD VESSELS

1.1.2.1 Extracerebral Blood Vessels: These vessels range in size from 700 μm in diameter for the internal carotid artery to about 50 μm for the penetrating vessels. The wall of extracerebral blood vessels consists of three layers, the tunica intima, the tunica media and, most exteriorly, the tunica adventitia. The tunica intima is composed of a single layer of endothelial cells without fenestrations that lines the lumen of the vessel

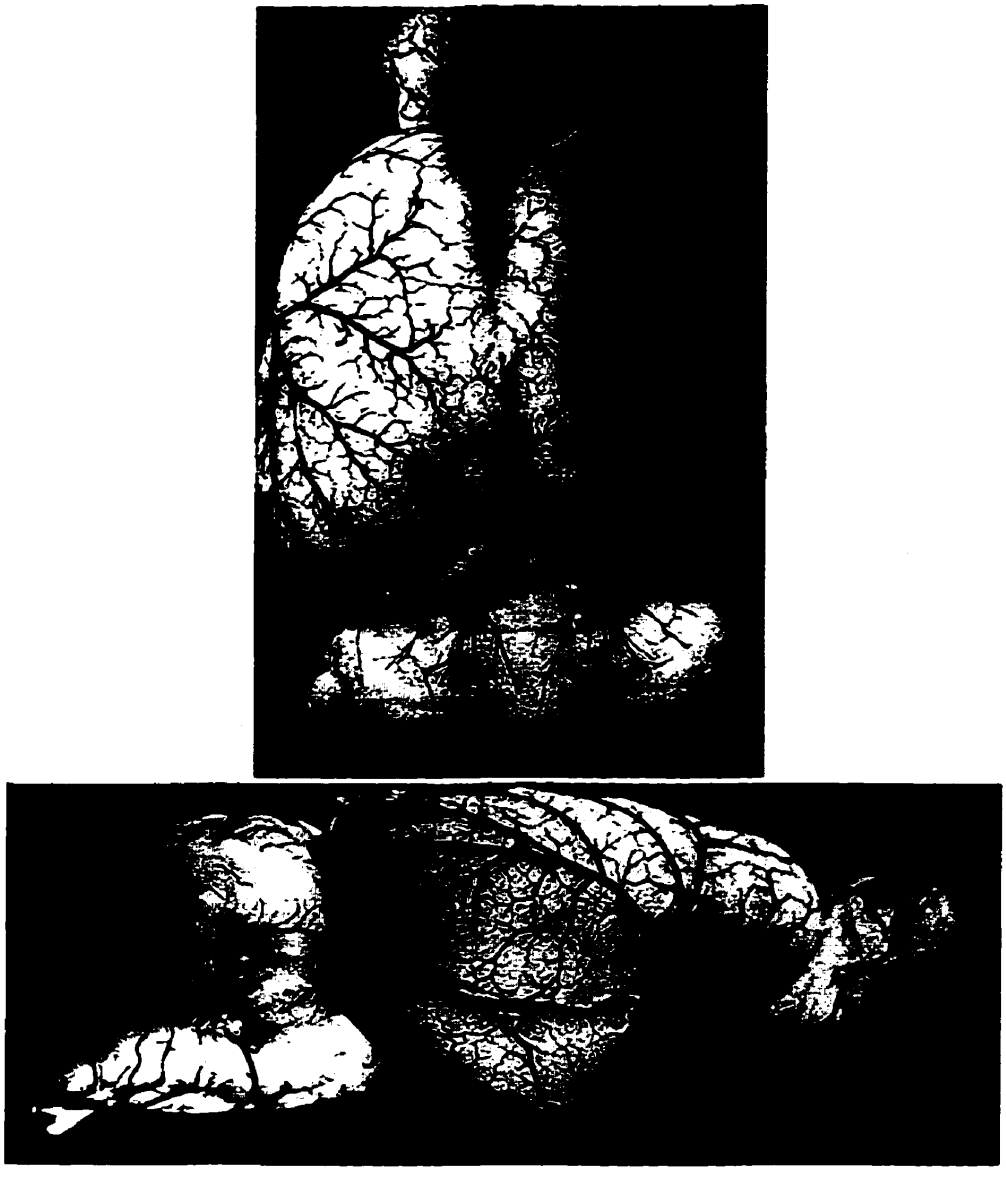

Fig 1.2: Dorsal (top) and lateral (bottom) views of the many branches of the major cerebral arteries as pial vessels in rat brain. Arteries were injected with a latex-black ink mixture. Taken from Scremin, 1995.

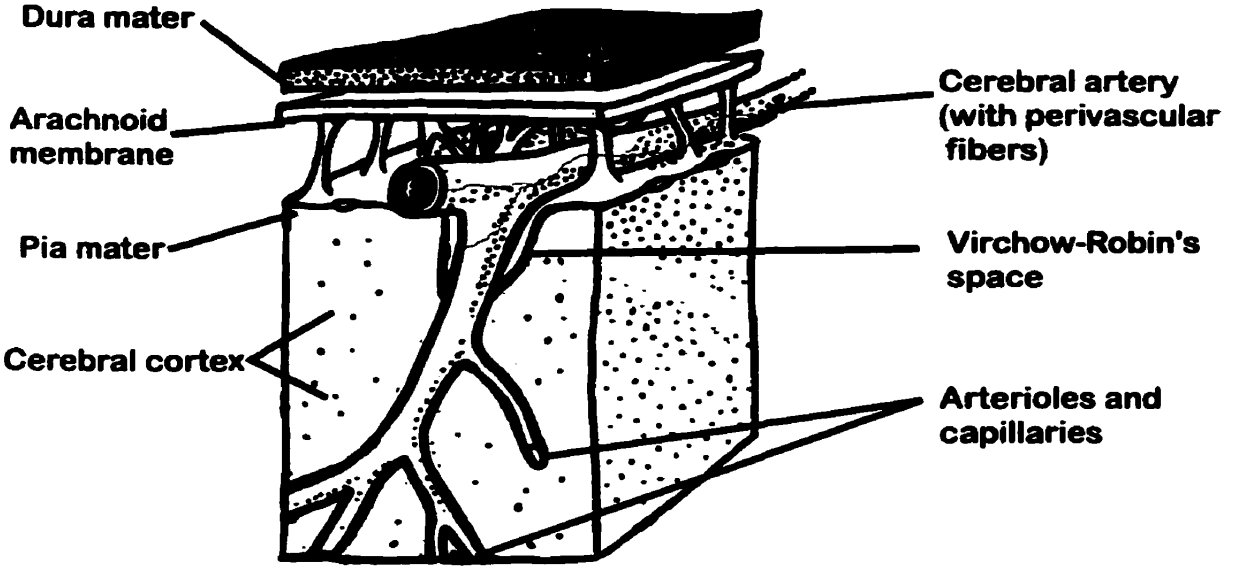

Fig 1.3: Schematic representation of the relationships between extra- and intracerebral blood vessels. An extracerebral artery (pial vessel), located in the subarachnoid space, penetrates the cortical parenchyma and is initially surrounded by the Virchow-Robins's space. As the vessel descends into the cortical tissue, the space eventually disappears giving rise to intraparenchymal arterioles and capillaries. (Adapted from Kandel and Schwartz, 1991)

and, only in larger arteries, a prominent internal elastic lamina. The tunica media is made up of collagen fibers and mostly of smooth muscle cells; the amount and number of which depend on the size of the vessel. The tunica adventitia includes bundles of collagen and fibroblasts and at the outer border this coat is made up of thin cellular processes. The adventitia in humans is separated from the media by the external elastic lamina whereas in rats it is absent. Nerve fibers of autonomic and sensory sources (see below) innervate the extracerebral blood vessels and travel in the subarachnoid space, they are localized in the adventitial layer and at the media/adventitia border.

1.1.2.2 Intraparenchymal Blood Vessels: The microcirculation is composed of small arterioles (20-50 μm luminal diameter), microarterioles (10-20 μm) and capillaries (< 10 μm). Apart from luminal diameter, intracerebral arterioles and capillaries differ from each other by the presence of one or two layers of smooth muscle cells in the former vessels. Cerebral capillaries are characterized by a single layer of flattened and elongated endothelial cells that are enclosed within a basal lamina (Fig 1.4). These endothelial cells lack fenestrations and are joined together to form *zonae occludentes*. These tight junctions are the basis of the BBB and they confer to the cerebral endothelial cells their unique characteristic in preventing certain molecules from passing freely into the brain tissue. The cerebral endothelial cells are readily permeable to oxygen, water and other vital nutrients that are important in maintaining a stable environment. Only few specialized areas lack a well-defined BBB, such as the area postrema and pineal gland, where the endothelial cells are fenestrated. Specific enzymes such as γ-glutamyltranspeptidase, alkaline phosphatase and Na^+/K^+-ATPase are present in high concentrations in cerebral endothelial cells and are useful markers to identify these cells and BBB activity (Joo, 1996). In addition, other markers have been used to characterize brain microvascular endothelial cells such as factor VIII-related antigen, angiotensin-converting enzyme, uptake of low-density lipoprotein and binding of certain specific lectins (Cancilla et al., 1993a). Other features of cerebral endothelial cells include a paucity in pinocytotic vesicles and an enrichment of mitochondria (Joo, 1996, Fig 1.4).

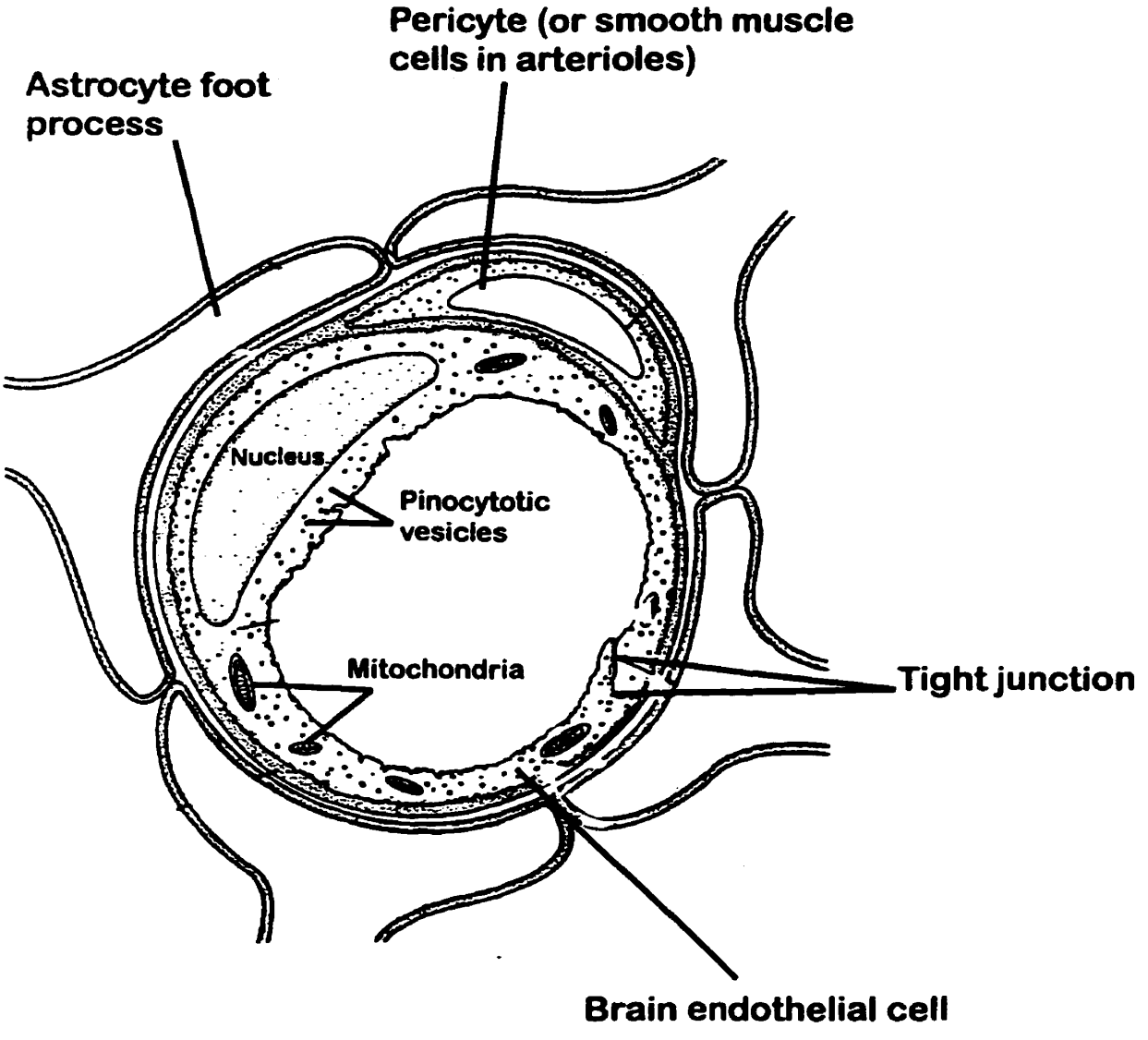

Fig 1.4: Schematic representation of a cerebral capillary. The endothelial cells that line the blood vessel lumen are characterized by the presence of tight junctions, an enrichment of mitochondria and a paucity of pinocytotic vesicles. Surrounding the endothelial cells are pericytes and their processes which in arterioles are replaced by smooth muscle cells. Invariably, the capillary and also the arteriole is surrounded by astrocytic end-feet processes. Adapted from Kandel and Schwartz, 1991

Interestingly cerebral endothelial cells have also been reported to contain contractile proteins suggesting that they could possibly mediate vasomotor responses (Owman et al., 1978).

In addition to the endothelium, pericytes and their processes are frequently contained within the capillary basal lamina. These cells are closely related to the smooth muscle cells in arterioles and have been considered their equivalent in the capillary bed. In fact, as the vessels go through the transition from arteriole to capillary, pericytes are thought to replace smooth muscle cells. They contain similar smooth muscle proteins (see Nehls and Drenckhahn, 1993; Shepro and Morel, 1993 for review) and have even been shown to develop into smooth muscle cells under certain conditions (Meyrick and Reid, 1978). By virtue of the presence of smooth muscle-related proteins, pericytes are thought to mediate microvascular contractility although direct proof is still lacking. Pericytes are also functionally related to the endothelial cells as they control, in part, their proliferation (Orlidge and D'Amore, 1987) and seem to play an active role, together with brain astrocytes (see below), in the regulation and maintenance of the BBB (Nehls and Drenckhahn, 1993).

Another important feature of intracerebral blood vessels is the astrocytic processes or end-feet that surround them and, at the capillary level, form an almost complete sheath around the basal lamina (Fig 1.4). The presence of glial fibrillary acidic protein and, at times, glycogen particles in the cytoplasm help in the identification of astrocytes. Although originally thought to play uniquely structural and supporting roles, astroglial cells have now been involved in a variety of functions within the brain. These include synthesis and release of neurotrophic factors for neuronal and vascular growth and survival (Muller et al., 1995), maintenance of the extracellular homeostasis (Hertz, 1992) and, in conjunction with neurons, regulate energy metabolism (Magistretti and Pellerin, 1996) and, possibly, CBF (Paulson and Newman, 1987; Alkayed et al., 1996). Furthermore, astrocytes have been implicated in the formation, maintenance, functional

regulation and repair of the BBB (Cancilla et al., 1993b; Montgomery, 1994).

As for extracerebral blood vessels, the intracerebral microcirculation also seems to be neurally regulated but, in this case, the neuronal input is assumed to originate from brain intrinsic neurons (Lou et al., 1987; Reis and Iadecola, 1989 for review). This aspect has been the subject of several recent studies and will be covered in more detail below (see section 1.1.3.2).

1.1.3 INNERVATION OF CEREBRAL BLOOD VESSELS

The detailed innervation of the cerebral circulation by neurotransmitters and neuropeptides has been greatly aided with the improvement of sensitive immunocytochemical techniques along with the development of specific antibodies against neurotransmitters/modulators and the enzymes involved in their synthesis. Similarly, lesion/stimulation of specific neuronal structures and tract tracing experiments have greatly helped in identifying the origin of perivascular nerves. The innervation of the extracerebral circulation has been extensively studied and several good review articles have been published over the past years (Hardebo, 1989; Bonvento and Lacombe, 1993; Dauphin and MacKenzie, 1995). In contrast, a neurogenic control of the microvascular bed is a rather novel issue and only recently did anatomical and physiological data appear in favor of such mechanism.

1.1.3.1 Extracerebral Blood Vessels The major cerebral arteries and small pial vessels are richly innervated by fibers from the peripheral nervous system, namely from sympathetic, parasympathetic and sensory ganglionic structures (Table 1.1). They are densely innervated by noradrenaline nerve fibers that originate primarily from the superior cervical ganglia with a minor component from the stellate ganglia (Edvinsson et al., 1977a; Arbab et al., 1986). In addition to noradrenaline, neuropeptide Y is present in these cerebrovascular sympathetic nerves where it is often, if not exclusively, co-localized with the monoamine (Edvinsson et al., 1984; 1987) (Table 1.1). As will be

TABLE 1.1: PERIVASCULAR INNERVATION OF CEREBRAL BLOOD VESSELS BY VARIOUS NEUROTRANSMITTERS AND MODULATORS

NEUROTRANSMITTERS MODULATORS	ORIGIN	VESSEL INNERVATED	CEREBROVASCULAR RESPONSE
Noradrenaline	Superior cervical and Stellate ganglia	A,V	Constriction
	Locus coeruleus	M	
Neuropeptide Y	Superior cervical ganglia	A,V	Constriction
	Cortical and Subcortical Neurons	M	
5-Hydroxytryptamine	Superior cervical ganglia	A	Constriction
	Raphe nucleus	A?, M	
Acetylcholine	Sphenopalatine and Otic ganglia	A,V	Dilatation
	Basal Forebrain	M	
	Cortical Interneurons	M	
Nitric Oxide	Sphenopalatine and Trigeminal ganglia	A,V	Dilatation
	Cortical Interneurons	M	
Vasoactive intestinal polypeptide	Sphenopalatine and Otic ganglia	A,V	Dilatation
	Cortical Interneurons	M	
Dopamine	Substantia nigra	M	Dilatation
Calcitonin gene-related peptide	Trigeminal ganglia	A,V	Dilatation
Substance P/ Neurokinin A	Trigeminal ganglia	A,V,M	Dilatation

A, extracerebral arteries; V, extracerebral veins; M, intraparenchymal microvessels
Adapted from Lou et al (1987) and Edvinsson et al (1993)

discussed below in section 1.3.1, there is convincing but controversial evidence that 5-HT is also present in a subset of cerebrovascular sympathetic fibers. The sympathetic innervation generally induces vasoconstriction when activated but seems to marginally affect resting CBF tone under normal conditions (Paulson et al., 1990). Rather, it influences CBF under situations of cerebrovascular stress such as hypercapnia whereby blood flow increases are attenuated by these neuromodulators (Paulson et al., 1990). The extracerebral blood vessels are also innervated by parasympathetic perivascular nerves that generally arise from the sphenopalatine and otic ganglia although some projections have been shown to originate from the microganglia of the internal carotid artery (Hara et al., 1985). These cerebrovascular nerves contain acetylcholine and vasoactive intestinal polypeptide (Hara et al., 1985; Walters et al., 1986). Moreover, the perivascular fibers that emanate from the sphenopalatine ganglia also contain nitric oxide synthase, the enzyme responsible for the synthesis of nitric oxide (Iadecola et al., 1994). Even though all these agents are potent vasodilators, their role in maintaining vascular tone under normal circumstances remains unclear. There is some evidence that they are involved in CBF regulation under stressful conditions such as cerebral hypoxia and ischemia (Kano et al., 1991; Kobetsu et al., 1992). Finally, other neuropeptides contained in sensory fibers emerging from the trigeminal ganglia have been shown to innervate the cerebral arteries (Yamamoto et al., 1983; Uddman et al., 1985; Hanko et al., 1986). These contain a variety of neuropeptides such as substance P, calcitonin-gene related peptide and neurokinin A which are all vasodilators (Table 1.1). In addition, trigeminal ganglion neurons have also been shown to contain nitric oxide synthase (Nozaki et al., 1993). Functionally, the trigeminovascular nerves are thought to relay vascular-related information pertaining to vessel diameter and metabolic conditions (McCulloch et al., 1986; Beattie et al., 1993; Branston, 1995). It has been involved with neurogenic inflammation possibly associated with the manifestation of pain during a migraine headache (Moskowitz, 1993).

1.1.3.2 Intraparenchymal Blood Vessels Microvessels as small as 40 μm in diameter

were originally suggested to be innervated by neural elements some 65 years ago (Penfield, 1932). Several decades later, ultrastructural investigations showed nerve terminals contacting the basal lamina of intracerebral blood vessels, but the type of transmitter present in these fibers could not be identified (Cervos-Navarro and Matakas, 1974; Rennels and Nelson, 1975). With the advent of more specific antibodies and improved tissue preservation techniques, morphological studies have since shown intimate contacts between the microvascular bed of various brain regions and the cell soma, dendritic processes and/or axon terminals of neurons containing various neurotransmitter and neuromodulator systems. These include noradrenergic fibers from the locus coeruleus (Swanson et al., 1977), dopamine from the substantia nigra (Felten and Crutcher, 1979), acetylcholine from the basal forebrain and cortical interneurons (Arneric et al., 1987; Chédotal et al., 1994; Vaucher and Hamel, 1995) and nitric oxide from cortical interneurons (Iadecola et al., 1993). Furthermore, intracerebral blood vessels may receive a peptidergic input from cholecystokinin (Hendry et al., 1983), substance P (Milner and Pickel, 1986), vasoactive intestinal peptidergic (Eckenstein and Baughman, 1984; Chedotal et al., 1994) and neuropeptide Y (Aoki and Pickel, 1989; Abounader and Hamel, 1996)-containing neurons of central origin.

1.1.4 MECHANISM OF CEREBRAL BLOOD FLOW AUTOREGULATION

Blood flow autoregulation is defined as the capacity of an organ to maintain blood flow fairly constant despite changes in arterial blood pressure and is particularly well developed in the brain. Under normal conditions, CBF is held relatively stable with lower and upper limits of 60 and 150 mm Hg of arterial blood pressure, respectively, although both limits can be modified by the sympathetic nervous and renin-angiotensin systems (Paulson et al., 1990; Edvinsson et al., 1993). Below 60 mm Hg, CBF decreases and above the upper limit of 150 mm Hg, a passive dilatation occurs. This regulation is thus a physiological mechanism that protects the brain from ischemia or vascular collapse when the blood pressure is low and from capillary and/or BBB damage as well as edema when it is too high (Paulson et al., 1990).

The mechanism of cerebral autoregulation is presently unknown but several hypotheses have been advanced. The first is the myogenic theory which was originally characterized in the extracerebral circulation but more recently found to apply to the intracerebral one. In this hypothesis, the cerebrovascular smooth muscle is responsive to changes in transmural pressure gradients i.e. the blood vessel constricts when transmural pressure increases and vice versa. Relaxing and contracting factors synthesized from cerebrovascular endothelial cells (Furchgott and Zawadki, 1980; Furchgott and VanHoutte, 1989) appear to be an important component of the myogenic control of CBF. As a matter of fact, a functional endothelium appears essential to detect and respond to changes in flow and pressure (Paulson et al., 1990). Indeed, nitric oxide produced by the endothelium participates in the maintenance of resting cerebrovascular tone (see Iadecola, 1993; Kontos, 1993 for reviews).

The second, the metabolic theory, was postulated quite some time ago by Roy and Sherrington (1890) and suggests that changes in local blood flow within the brain parenchyma are dependent on changes in the metabolic demand from the surrounding microenvironment. On this basis, cellular activation leads to an accumulation in vasoactive metabolites which consequently act upon the microvasculature to favor oxygen and glucose uptake while eliminating cellular metabolites that may be toxic if they accumulate at high concentrations. Thus, increases in neuronal activity lead to increases in glucose and oxygen utilization and, consequently, in turn to increases in local CBF. Based on this theory, changes in local CBF merely reflect changes in local neuronal activity, a phenomenon referred to as coupling between cerebral metabolism and CBF. Although the exact mechanism of this coupling is still not well understood, several agents have been suggested as its mediators and include K^+, H^+, Ca^{2+} as well as adenosine and adenine nucleotides (Villringer and Dirnagl, 1995). More recently, nitric oxide and epoxyeicosatrienoic acid, an arachidonic acid metabolite produced by astrocytes, have been proposed to be possible links between neuronal activity and local blood flow (Iadecola,1993; Alkayed et al., 1997). Furthermore, changes in systemic metabolism such

as the partial pressures of CO_2 and O_2 can profoundly affect CBF. Indeed, hypercapnia (elevations of PCO_2) and hypoxia (low PO_2) will increase CBF while hypocapnia (reductions in PCO_2) will have the opposite effect. Finally, blood pH may play a role in vasomotricity as well as agents that are circulating in the blood such as prostacyclins (Heistad and Kontos, 1983).

However, more recently, evidence has accumulated which suggest a partial uncoupling between neuronal activity and local CBF, that is a disproportionate change between local blood perfusion and glucose consumption (see Reis and Iadecola, 1989, for review). That these local CBF changes could be triggered independently of changes in glucose metabolic activity led to the suggestion of a third mechanism in the regulation of CBF, that has been named the neurogenic theory. In this process, the cerebrovascular bed is under the direct control of perivascular nerve fibers whose cell bodies reside in intrinsic brain structures. Nerve fibers would release neuromediators which would directly elicit vasomotor responses. The anatomical demonstration of several neurotransmitter and neuropeptidergic systems closely associated with intraparenchymal blood vessels (section 1.1.3.2) may represent the basis of this neurogenic control. *In vivo*, it is possible that these systems all work together and that the final response is an integration of all these influences.

1.2 SEROTONERGIC SYSTEM IN THE CNS

1.2.1 HISTORICAL BACKGROUND OF 5-HT

Physiologists had been aware for quite some time of the presence of a powerful vasoconstrictor agent in serum when blood was allowed to clot. The identity of this substance remained unknown for almost a century until Page and collaborators succeeded in isolating the vasoactive factor, released from platelets during blood clotting, and named it "serotonin" (Rapport et al., 1948). A year later, they identified the active compound as 5-hydroxytryptamine (5-HT, Rapport, 1949). Concurrently, Erspamer and Asero (1952), working in the enterochromaffin cells of the gastrointestinal tract, detected high concentrations of an active agent that constricted smooth muscle and called it enteramine. This substance was later purified and crystallized and shown to be identical to serotonin. The following years, serotonin was discovered in many other tissues including blood vessels (see section 3.1.1), perivascular mast cells (Rosenblum, 1973), heart (Berkowitz et al., 1974) and mammalian brain (Twarog and Page, 1953). Soon after its discovery in the CNS, it was proposed to act as a neurotransmitter.

1.2.2 BIOSYNTHESIS AND METABOLISM OF SEROTONIN

About 95% of serotonin present in the body is located in the gastrointestinal tract, mainly in the enterochromaffin cells. The remaining 5-HT is found in the CNS, blood platelets, pineal gland and mast cells of certain species such as rodent and cattle. As 5-HT cannot cross the BBB, brain cells must synthesize serotonin *de novo* from the essential amino acid, L-tryptophan. Once in serotonergic cells, L-tryptophan is hydroxylated to form 5-hydroxytryptophan (5-HTP) via the enzyme tryptophan-5-monooxygenase or tryptophan hydroxylase (TPH, E.C. 1.14.16.4), the rate-limiting step in 5-HT synthesis (Fig 1.5). 5-HTP, which is present in minor amounts, is then rapidly decarboxylated to 5-HT by the action of aromatic L-alpha amino acid decarboxylase. At this point, 5-HT is sequestered into vesicles until release (Fig 1.5). Once it is liberated into the synaptic cleft, 5-HT can interact with post-synaptic 5-HT receptors. At the synapse, the actions of 5-HT are terminated via two pathways: i) it can be rapidly metabolized to 5-hydroxy-

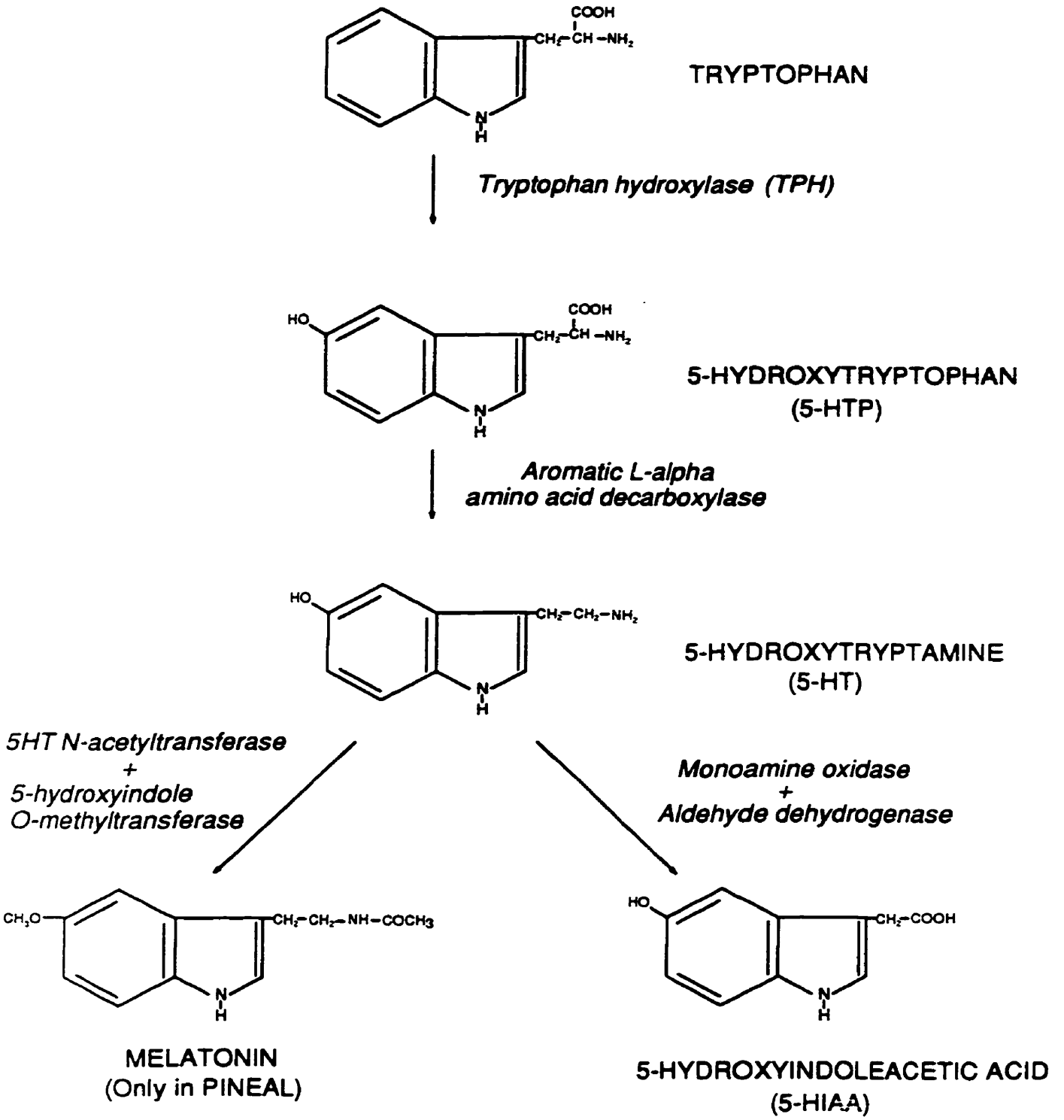

Fig 1.5: The metabolic pathways for the synthesis and degradation of 5-hydroxytryptamine

indoleacetaldehyde by the actions of monoamine oxidase type A and subsequent oxidization to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydogenase (Fig 1.5) or ii) predominantly, be taken up back into presynaptic terminals through the serotonin transporter. This transporter belongs to a large neurotransmitter transporter superfamily consisting of glycoproteins of about 600 amino acid residues and feature 12 transmembrane domains with cytoplasmic N- and C-terminals (Blakely et al., 1991; Ramamoorthy et al., 1993). These transporters represent the site of action of tricyclic and non-tricyclic antidepressants as well as drugs of abuse that include cocaine and amphetamine derivatives (Rudnick and Wall, 1992; Amara and Kuhar, 1993). This high-affinity uptake system of 5-HT neurons has greatly aided in the mapping of the distribution of 5-HT-containing cell bodies and terminals (Beaudet and Descarries, 1981; Parent et al., 1981; Descarries et al., 1982) and has recently been demonstrated that the distribution of 5-HT closely matches that of its transporter (Sur et al., 1996). Finally, in the pineal gland, 5-HT is further metabolized to N-acetylserotonin, via the enzyme serotonin N-acetyltransferase, and this product is further metabolized to melatonin (Fig 1.5).

In general, the rate of 5-HT biosynthesis is dependent on the availability of free L-tryptophan in plasma. Indeed, addition of exogenous L-tryptophan leads to an increase in 5-HT synthesis, levels and release in rats (Schaechter and Wurtman, 1989) whereas reduction of the essential amino acid in the diet has the opposite effect on 5-HT concentrations and release (Schaechter and Wurtman, 1990). Furthermore, since TPH is the rate-limiting enzyme in this process, modulating the hydroxylation step would result in a change in 5-HT synthesis and levels. Accordingly, *p*-chlorophenylalanine, a potent competitive and irreversible inhibitor of TPH, produces long-lasting reductions in 5-HT content and TPH activity (Richard et al., 1990; Weissmann et al., 1990; Park et al., 1994) although a slight increase in TPH mRNA expression is observed (Cortes et al., 1993; Park et al., 1994). In addition to L-tryptophan, the TPH enzyme requires the presence of molecular oxygen and the reduced pterin cofactor, 2-amino-4-hydroxy-6-(L-*erythro*-1',2-

dihydroxypropyl)-5,6,7,8-tetrahydrobiopterin. Optimal concentrations and levels of all three molecules are required for maximal 5-HT synthesis (Boadle-Biber, 1993, for review). Furthermore, this process seems dependent on the concentration of extracellular calcium which is thought to phosphorylate the enzyme and influence its activity (Elks et al., 1979). Finally, TPH activity and 5-HT synthesis are also reportedly influenced by activity of serotonergic neurons; that is, when 5-HT neurons are depolarized, 5-HT synthesis is increased while the synthesis is decreased when the firing rate is inhibited, as with local application of selective 5-HT_{1A} receptor agonists (Sharp et al., 1989; Bonvento et al., 1992).

1.2.3 ANATOMY OF 5-HT SYSTEM

The presence of 5-HT was discovered in the mammalian CNS close to 45 years ago (Twarog and Page, 1953) but the exact localization of 5-HT within neurons and pathways of the CNS further required the progress of histofluorescence, radioautographic, and immunocytochemical techniques before it could be established. Indeed, it was only some time later that the 5-HT neurons were first visualized in brain by histofluorescence (Dahlstrom and Fuxe, 1964). Several other anatomical approaches have since been used to establish the precise anatomical distribution of 5-HT neurons and their projections throughout the brain (Descarries et al., 1975; Beaudet and Descarries, 1981; Steinbusch, 1981; Tork, 1985; Weissmann et al., 1987). The above mentioned studies demonstrate that although their cell bodies are restricted mostly to cell clusters in the brainstem raphe nuclei, their fibers innervate nearly every brain region making the 5-HT system perhaps the most expansive and diffusively organized system in the vertebrate CNS. The number of actual 5-HT-containing cell bodies is thought to reach 20,000 and 60,000 in the rat and cat, respectively (Wiklund et al., 1981; Jacobs and Azmitia, 1992). However, the number of 5-HT nerve terminals, at least in the rat frontal cortex, is estimated at $6x10^6/mm^3$ of cortical tissue, an astounding half million 5-HT varicosities for each cell body (Audet et al., 1989). Furthermore, these cortical nerve terminals account for about 1/200 of all axon terminals present in the cerebral cortex (Audet et al., 1989). The distribution of

serotonergic cell bodies and their projections appear to be remarkably stable across phylogeny. Similarities between primates and subprimates are apparent in nuclear organization, efferent pathways, target structures and ultrastructural relationships.

1.2.3.1 5-HT Cell Bodies

Dahlstrom and Fuxe (1964) originally demonstrated 5-HT-containing cell bodies in the rat brain using the formaldehyde-induced fluorescence technique and classified them as groups of cells numbered B1-B9. These can be divided into superior and inferior groups based on their anatomical localization and projection areas. The superior group is comprised of four main nuclei which send mostly ascending projections: the dorsal raphe, the median and its laterally displaced cells called the nucleus pontis oralis and finally the nucleus caudalis linearis which is the most rostral group. The inferior group comprises cell bodies in the obscurus, pallidus and magnus raphe nuclei, in the medullary reticular formation and other regions like the solitary nucleus complex and the area postrema (Steinbusch and Nieuwenhyus, 1983) and predominantly project to the locus coeruleus and spinal cord. The 5-HT-containing neurons in the raphe nuclei form a heterogeneous population in that these are multipolar but differ greatly in size and orientation depending on the location. Of all these nuclei, the dorsal and median raphe nuclei stand out in importance as they contain the greatest number of 5-HT cell bodies but also because of their broad projection fibers.

1.2.3.1.1 Dorsal Raphe Nucleus : This nucleus is located in the ventral part of the periaqueductal gray matter of the midbrain and extends caudally well into the periventricular gray matter of the pons. It contains the largest number of 5-HT nerve cell bodies in the CNS; estimated to comprise between 40 and 60% of all 5-HT neurons in the CNS depending on the species. Within the confines of the rat dorsal raphe nucleus, the population of cells able to take up labelled 5-HT has been estimated at 11,500 cell bodies (Descarries et al., 1982) although only about 4000 TPH-containing cells have been identified in this nucleus (Weissmann et al., 1990). Furthermore, serotonergic neurons

represent only a minority, approximately one third of all cells in this nucleus (Descarries et al., 1982). A large number of dorsal raphe neurons also contain other putative transmitters and modulators such as enkephalins and other opoid peptides (Moss et al., 1983; Zamir et al., 1984; Wang and Nakai, 1993), GABA (Nanopoulos et al., 1982; Belin et al., 1983; Wang and Nakai, 1993), substance P (Moss et al., 1983; Magoul et al., 1986; Smith et al., 1994), dopamine (Descarries et al., 1986) and nitric oxide (NO, Wang et al., 1995). Original reports had shown coexistence between 5-HT and other modulators i.e. GABA, substance P and enkephalins within the same neuron (Johanssen et al., 1981; Belin et al., 1983; Leger et al., 1986) although these results have been disputed by more recent investigations (Wang et al., 1992; Tanaka et al., 1993; Stamp and Semba, 1995). Despite small differences in the distribution pattern between rat, primate and human, close similarities in the cellular organization in this nucleus are evident in these species.

1.2.3.2.2 Median Raphe Nucleus This nucleus, also called the superior centralis nucleus, contains the second largest group of 5-HT neurons in the brainstem and is mainly situated in the caudal part of the mesencephalis tegmentum (Kohler and Steinbusch, 1982). The nucleus is comprised of two distinct parts (Kohler and Steinbusch, 1982); one group found in the midline with densely packed cells and another group scattered in the periphery. As compared to its dorsal counterpart, it encompasses fewer cells which do not project as extensively. Here again this region comprises of morphologically diverse neurons which are arranged diffusely.

1.2.3.2 5-HT pathways

The ascending and descending projections from the raphe nuclei have been extensively studied using many different approaches that include anterograde degenerating techniques, radioautographic tract tracing, radioautography after injection of tritiated amino acids or 5-HT and retrograde transport of fluorescence tracers (Azmitia and Segal, 1978; Parent et al., 1981; Steinbusch and Nieuwenhyus, 1983). According to the terminology of Steinbusch and Nieuwenhyus (1983), the projections are composed of

three ascending (dorsal, medial and ventral) and two descending pathways. The ascending projections are very extensive, contain many collaterals and innervate diverse regions of the cerebral cortex, basal ganglia, limbic system and diencephalon (Fig 1.6). The dorsal component originates from the dorsal raphe and terminates mainly in the caudatoputamen complex bypassing the medial forebrain bundle. The medial pathway also arises from the dorsal raphe and travels mostly to the substantia nigra with some collaterals to the caudatoputamen complex. The ventral ascending pathway, originating from both dorsal and median raphe, courses through the ventral tegmentum area and enters the medial forebrain bundle before reaching target areas such as the hippocampus, cerebral cortex, hypothalamus and thalamus (Fig 1.6). These fibers form the transtegmental 5-HT system according to the terminolgy employed by Parent et al. (1981). The vast majority (60-90%) of raphe neurons in both rat and primates that project to the cerebral cortex and other forebrain structures have been shown to contain 5-HT (Steinbusch et al., 1980; Zhou and Azmitia, 1983; Kohler and Steinbusch, 1982; O'Hearn and Molliver, 1984; Wilson and Molliver, 1991). It is presently not known if these serotonergic neurons are colocalized with other putative neurotransmitters and neuromodulators as well as the nature of the non-serotonergic projecting neurons.

With respect to the descending pathways, the first emanates from the dorsal raphe nucleus and terminates in the locus coeruleus. The other one, and more important, the bulbospinal pathway, originates mainly from the raphe magnus, travels in the dorsolateral funiculus and innervates the substantia gelatinosa and the ventral horn of the spinal cord (Steinbusch and Nieuwenhyus, 1983). Some minor descending inputs to the cerebellum have also been described (Fig 1.6).

1.2.3.3 5-HT nerve terminals

The distribution of 5-HT nerve terminals in the brain have been described in several species such as rats, cats and monkeys (Parent et al., 1981; Steinbusch,1981; Tork,1985; Weissmann et al., 1987). According to these authors, a high density of 5-HT- or TPH-

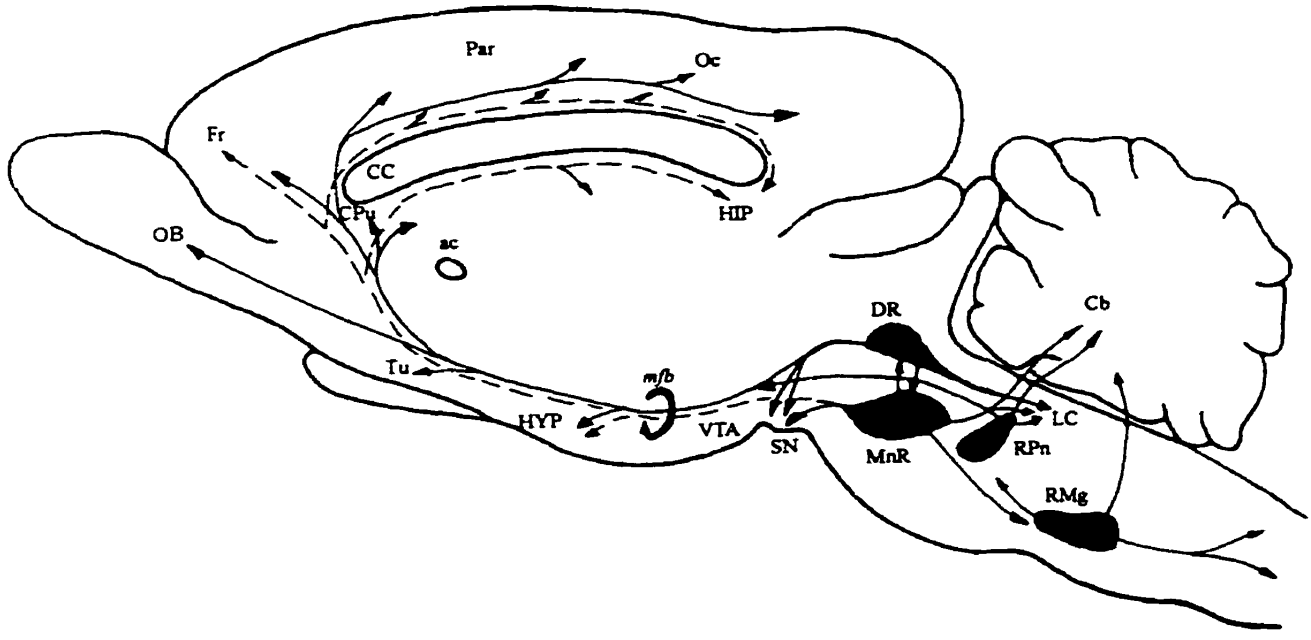

Fig 1.6: Schematic diagram of the localization of major serotonergic nuclei along with their ascending and descending projections. Taken from Cohen et al (1996).

immunoreactive nerve terminals is found in parts of the caudate nucleus and putamen, several nuclei of the amygdala, thalamic as well as the subthalamic and hypothalamic nuclei and regions of the substantia nigra. A moderate to high density is also observed in various subdivisions of the cerebral cortex and hippocampus. Lower, but detectable levels of 5-HT and TPH are found in virtually every brain area. Serotonergic nerve fibers in these areas are unmyelinated, to a major extent, although some myelinated fibers have also been shown in the medial forebrain bundle and dorsal raphe nucleus of rats and monkeys (Azmitia and Gannon, 1983).

In addition to the distribution of 5-HT-containing nerve terminals within the CNS, their ultrastructural characteristics have been well documented in the hippocampus and striatum but perhaps best in the cerebral cortex. This has been achieved mainly with 5-HT radioautography and immunocytochemically using 5-HT and more recently TPH antibodies. In general, 5-HT nerve terminals are round or ovoid and their mean diameter ranges between 0.5-0.7 μm depending on the region (Descarries et al., 1975; Takeuchi and Sano, 1984; Soghomonian et al., 1989; Séguéla et al., 1989; Oleskevich et al., 1991; Smiley and Goldman-Rakic, 1996). Within these axonal varicosities are found a heterogeneous population of vesicles. These include the small clear vesicles (25-55 nm in diameter) of varying shapes and the less frequent dense core vesicles of about 100nm in diameter region (Descarries et al., 1975; Takeuchi and Sano, 1984; Soghomonian et al., 1989; Séguéla et al., 1990; Oleskevich et al., 1991; Smiley and Goldman-Rakic, 1996). With respect to the immediate microenvironment, the 5-HT profiles were, for the most part, apposed to axon terminals and dendritic elements i.e. spines and branches. In the cerebral cortex, however, glial elements also represented an important target (Séguéla et al., 1989).

Another feature that seems to characterize this indolaminergic system is that it works primarily in a non-synaptic manner. Indeed, in the median eminence (Baumgarten and Lachenmayer, 1974), hypothalamus (Beaudet et al., 1979); striatum (Soghomonian et al.,

1989), hippocampus (Anderson et al., 1986; Oleskevich et al., 1991) and even the raphe nuclei (Descarries et al., 1982; Chazal and Ralston, 1987) which do contain some serotonergic nerve terminals (Descarries et al., 1982), very few junctional complexes are observed. In the cerebral cortex, a remarkable variation in the synaptic incidence of 5-HT axons has been reported that may differ on the basis of the species, anatomical localization, techniques used and definition of a synaptic contact. Original radioautographical results by Descarries et al (1975) in the rat frontoparietal cortex led to the conclusion that 5-HT nerve terminals were largely non-synaptic, only about 15% exhibited classical synaptic junctions. Subsequent immunocytochemical studies have suggested that as much as 80% of 5-HT nerve terminals in rat frontal, parietal and visual cortices make synaptic contacts (Papadopoulos et al., 1987). However, a more recent immunocytochemical study by Séguéla et al. (1989), using 3-dimensional reconstruction of serial sections, estimated a cortical synaptic incidence (30%) higher than previously evaluated by radioautography although the majority of terminals were still predominantly non-junctional. These observations have since been confirmed by two independent groups in the cat auditory and monkey prefrontal cortex (De Felipe et al., 1991; Smiley and Goldman-Rakic, 1996). It has also been suggested that 5-HT varicosities with the appearance of fine fibers rarely make synaptic contacts while the beaded fibers tend to do so (see below, section 1.2.3.3.1 for full description). Thus, the hypothesis originally advanced by Descarries and colleagues (see review, 1991), that 5-HT is released non-synaptically, termed volume transmission, is the most commonly acceptable one at the moment. In this scheme, 5-HT released from nerve terminals would reach distant cellular targets by diffusion through the extracellular space and activate 5-HT receptors present on neighboring neuronal and non-neuronal elements. In this mode of volume transmission, the effect of indoleamine discharge is slow, long lasting and diffuse, and is consistent with the global and widespread modulatory role of 5-HT.

I will discuss here in more detail only the distribution of 5-HT terminals and their morphological characteristics in the rat cerebral cortex and hippocampus because they are

most relevant to my work.

1.2.3.3.1 Cerebral Cortex: The distribution of serotonergic nerve terminals in the neocortex has been extensively studied mainly in rats, cats and monkeys and shown to originate from neurons in the dorsal and median raphe nucleus. This innervation is heterogeneous and more densely distributed in the insular, piriform, cingulate, frontal and parietal cortices while other cortical subdivisions such as the entorhinal, occipital and perirhinal receive a moderate input (Vertes, 1991). 5-HT nerve terminals are distributed rather uniformly in the different laminae except for layer I (molecular layer) which shows the highest density in all cortical subdivisions (Audet et al., 1989). In primates, the density and laminar distributions of 5-HT differ considerably amongst the different cortical areas (Takeuchi et al, 1983). In fact, layer I and IV contain the largest amount of 5-HT nerve terminals.

The presence of two distinct populations of 5-HT axon terminals has been suggested by some groups, following observations at the light microscopic level. These axons are reportedly different with respect to their appearance, size, origin and sensitivity to amphetamine derivatives (Kosofsky and Molliver, 1987; Tork and Hornung, 1990; Mamounas et al., 1991). The most common type of terminals, by far, is thin with small, fusiform varicosities (less than 1μm in diameter). Anterograde tracing techniques suggest that these thin varicosities arise from the dorsal raphe nucleus. They have also been called D axons and they degenerate following methylenedioxyamphetamine or p-chloro-amphetamine treatment (Kosofsky and Molliver, 1987; Mamounas et al., 1991). The second type is charaterized by large beaded oval varicosities (greater than 5 μm in diameter) and would originate from the median raphe. These axons are unaffected with administration of the amphetamine derivatives. More precise analyses at the electron microscopic level in similar cortical regions, however, have not supported these observations (Séguéla et al., 1989; Smiley and Goldman-Rakic, 1996). Indeed, irrespective of the cortical subdivisions, 5-HT axon terminals observed at the

ultrastructural level were found to average 0.66 μm in diameter and never exceed 1.5 μm (Séguéla et al., 1989). Again, these terminals were found to be predominantly non-junctional, apposed mainly to dendrites and axon terminals.

1.2.3.3.2 Hippocampus: Quantitatively, the number of 5-HT nerve terminals is about half to that observed in the cerebral cortex and about the same as in the striatum (Oleskevich and Descarries, 1990). They are heterogeneously distributed with a high density in the molecular layer of the subiculum, molecular-lacunosum layer of the CA1 region and layer oriens of CA3 while being absent in the alveus and fimbria (Oleskevich and Descarries, 1990). Electron microscopic analyses of these terminals showed that they did not greatly differ from one another with respect to size, synaptic specialization and immediate microenvironment. The hippocampal varicosities had an average diameter of about 0.57 μm, were predominantly non-synaptic (70-80%) and mostly juxtaposed to unlabelled nerve terminals (Oleskevich et al., 1991).

1.3 SEROTONIN AND THE CEREBRAL CIRCULATION

1.3.1 ANATOMICAL AND BIOCHEMICAL EVIDENCE OF 5-HT INNERVATION

1.3.1.1 Extracerebral Blood Vessels: The initial evidence that mammalian cerebral arteries are innervated by 5-HT was demonstrated by Chan-Palay (1976), who showed a plexus of nerve fibers around the rat internal carotid artery following intraventricular infusion of ^{3}[H]-5-HT, an observation later confirmed with radiolabeled 5-hydroxytryptophan (Napoleone et al., 1982). Subsequent immunocytochemical investigations with well-characterized 5-HT and/or TPH antibodies demonstrated the presence of 5-HT-containing perivascular fibers in different vascular segments of the major cerebral arteries and small pial vessels in various species such as rabbit, guinea pig, gerbil, cat, rat, and human (Griffith et al., 1982; Griffith and Burnstock, 1983; Edvinsson et al., 1983; Cowen et al., 1986; 1987; Dhall and Burnstock, 1989; Chédotal and Hamel, 1990; Mathiau et al., 1993a). In the major arteries, these studies reported 5-HT immunopositive nerve fibers forming plexuses with fibers running in all directions and describe a greater density of 5-HT fibers in vessels of the anterior circulation (i.e. internal carotid, middle and anterior cerebral arteries) than those of the posterior circulation (i.e. vertebral and basilar arteries). In pial vessels, few nerve fibers, running in all directions, could be detected.

Biochemical studies which quantified the amount of 5-HT and 5-HIAA (Table 1.2) present in these vascular compartments have shown unnegligeable levels, comparable to those found in brain tissues such as the cerebral cortex and hippocampus. There is a large degree of variability on the concentrations that appears to be species specific and these levels are higher in rat blood vessels as compared to cats, rabbits and humans (Edvinsson et al., 1983; Scatton et al., 1985; Duverger et al., 1987).

1.3.1.2 Intraparenchymal vessels: The earliest evidence that intraparenchymal blood vessels could receive a serotonergic input was described by Scheibel et al. (1975) who showed neuronal cell bodies and dendrites in the raphe nucleus, presumably serotonergic,

TABLE 1.2: CONCENTRATIONS OF 5-HT AND ITS MAJOR METABOLITE, 5-HIAA, IN CEREBRAL AND VASCULAR TISSUES

SPECIES	*TISSUE*	*5-HT (ng/g tissue)*	*5-HIAA (ng/g tissue)*	*REFERENCES*
HUMAN		57	216	i
RAT	CEREBRAL	343-521	104-323	i,ii,iii
CAT	CORTEX	138	52	i
RABBIT		138	52	i
RAT	HIPPOCAMPUS	310-535	262-565	ii,iii
RAT	CIRCLE	97	-	iv
CAT	OF	19-352	106	iv,v
RABBIT	WILLIS	61	-	iv
RAT	MAJOR CEREBRAL ARTERIES	240-310	930-1500	iii,vi
HUMAN		13	44	i
RAT	SMALL	162- 426	224- 305	i,ii,iv
	PIAL VESSELS	190-350	530-560	iii,vi
CAT		42-78	40-74	i,ii,iv
RABBIT		86-133	98-103	i,ii,iv
RAT	MICROVESSELS	176	-	vii

REFERENCES: i)Edvinsson et al (1984) ii) Scatton et al (1985) iii) Bonvento et al (1991) iv) Duverger et al (1987) v) Marco et al (1985) vi) Bonvento et al (1990) vii) Reinhard et al (1979)

in close opposition to blood vessels. A perivascular serotonergic plexus was later confirmed in this nucleus following intraventicular perfusions of radiolabelled 5-HT (Chan-Palay, 1976). Ensuing immunocytochemical investigations within the brainstem raphe nuclei supported these findings and documented contacts and intimate associations between 5-HT-containing cell bodies and dendrites and the blood vessel basement membrane at both light and electron microscopic levels (Di Carlo, 1984; Kapadia and de Lanerolle 1984). In other regions like the cerebral cortex, 5-HT-immunolabeled nerve terminals were seen to associate with penetrating arteries and even capillaries (Itakura et al., 1985). To date, the only study to measure the concentration of 5-HT in these vessels was conducted close to 20 years ago by Reinhard and colleagues (1979), in which they report relatively high amounts of the amine (176 ng/g tissue) (Table 1.2). Furthermore, the authors showed that the 5-HT levels are significantly reduced following electrolytic raphe lesions and injection of p-chlorophenylalanine, a competitive inhibitor of the TPH enzyme, and is suggestive of a neuronal source of 5-HT.

1.3.2 ORIGIN OF 5-HT INNERVATION

1.3.2.1 Extracerebral Blood Vessels: In the major cerebral arteries, many attempts have been made to determine the precise origin of 5-HT-containing nerve fibers around cerebral blood vessels. Early studies claimed the exclusive involvement of the raphe nucleus. These radioautographical experiments showed either an increase in tritiated 5-HT into the internal carotid artery following activation of the raphe nucleus (Chan-Palay, 1976) whereas no change in [^{3}H]5-HT or [^{3}H]5-hydroxytryptophan uptake was observed after bilateral removal of the superior cervical ganglia (Napoleone et al., 1982; Amenta et al., 1985). Similarly, the demonstration of retrogradely transported horseradish peroxidase in neurons of the dorsal raphe nucleus following its application to the walls of the middle cerebral artery (Tsai et al., 1985) and the increased sensitivity to 5-HT in this artery following destruction of the dorsal raphe (Moreno et al., 1991) have further supported a role for dorsal raphe neurons in the control of extracerebral blood vessels. With the demonstration of 5-HT cell bodies in the sympathetic superior cervical ganglion

(Verhofstad et al., 1981), it was also hypothesized that the perivascular 5-HT innervation originated from this structure. As expected, bilateral removal of the superior cervical ganglia resulted in an almost complete loss or major reduction in 5-HT immunoreactivity and/or 5-HT levels (Cowen et al., 1986; Chang et al., 1988a; 1989). More recently, other studies have implied that fibers around major arteries originate from both central and peripheral structures as 5-HT concentrations and TPOH activity are significantly reduced following lesions of the pathways from the dorsal raphe and the superior cervical ganglion (Marco et al., 1985; Bonvento et al., 1991; Moreno et al., 1995).

In small pial vessels, the experimental evidence suggest that perivascular 5-HT nerve fibers were derived entirely from the raphe nucleus. Accordingly, electrolytic or chemical lesioning of the dorsal and median raphe nuclei with 5,7-DHT markedly decreased the levels of 5-HT and 5-HIAA in these vascular segments (Edvinsson et al., 1983; Scatton et al., 1985; Bonvento et al., 1991) whereas their electrical stimulation resulted in a large reduction in 5-HT levels with moderate 5-HIAA increases. On the other hand, bilateral extirpation of the superior cervical ganglia failed to elicit significant changes in 5-HT and 5-HIAA concentrations (Edvinsson et al., 1983).

1.3.2.2 Intraparenchymal vessels: The origin of the 5-HT input to the intraparenchymal blood vessels is considerably less a matter of debate. The evidence unequivocally points to the raphe nucleus as the origin. Electrolytic lesion of this nucleus results in a dramatic decrease (~ 70%) in 5-HT content of blood vessels while bilateral superior cervical ganglionectomy failed to alter these concentrations (Reinhard et al., 1979). Isolated intracerebral vessels, like their extracerebral counterparts, have the capacity not only to take up 5-HT but also synthesize it (Reinhard et al., 1979; Maruki et al., 1984).

1.3.3 AUTHENTICITY OF PERIVASCULAR 5-HT FIBERS

The authenticity of 5-HT nerve fibers around major and pial arteries has been challenged by several investigators (Saito and Lee, 1987, Chang et al., 1988a; Jackowski et al.,

1988;1989). They claim that the presence of 5-HT immunoreactivity is exclusively due to the indoleamine being actively taken up from the cerebrospinal fluid (CSF), perivascular mast cells and/or platelets circulating in the blood into sympathetic noradrenaline nerve fibers. In their experiments, no 5-HT perivascular nerve fibers could be observed around cerebral blood vessels from animals perfused intracardially before sacrifice. It is only when the animals are killed by exsanguination and the vessels fixed by immersion that 5-HT-immunoreactive perivascular fibers could be evidenced.

Nevertheless, several lines of evidence have suggested that 5-HT present in both vascular segments can not be accounted entirely by uptake into noradrenergic nerve fibers. Rather these experiments suggest the presence of a subset of distinct or authentic 5-HT nerve fibers and that perivascular 5-HT is likely to be of a neuronal nature. Firstly, a difference in 5-HT and noradrenaline nerve fibers distribution is evident under normal conditions but also as a response to hormonal treatment (Cowen et al., 1986; Dhall et al., 1988). Secondly, cerebrovascular fibers have been shown biochemically and anatomically to contain the necessary machinery to synthesize, store and release 5-HT (Scatton et al., 1985). In these studies, the selective 5-HT neurotoxins, 5,7-dihydroxytryptamine and p-chloroamphetamine, along with p-chlorophenylalanine dramatically reduced the levels of 5-HT and its metabolite in small pial vessels while administration of pargyline, a monoamine oxidase inhibitor, increased 5-HT levels and decreased those of 5-HIAA. Furthermore, the TPH enzyme has been visualized immunocytochemically in nerve fibers surrounding extracerebral blood vessels (Chédotal and Hamel, 1990; Mathiau et al., 1993a), suggesting that they have the possibility to synthesize 5-HT *de novo*. Although some authors (Mathiau et al., 1993b) could not measure significant TPOH activity in brain arteries and pial vessels, many other and particularly recent reports have observed significant activity provided the tetrahydrobiopterin cofactor is included in the assay (Scatton et al., 1985; Bonvento et al., 1991; Moreno et al., 1994: 1995: Lopez de Pablo et al., 1996). Finally, the description by Chang et al. (1990) that the re-uptake systems for 5-HT and noradrenaline are different adds credence to the suggestion that 5-HT around

blood vessels can not be totally accounted for by uptake mechanisms into noradrenergic fibers.

As well, several other reports have argued against a central innervation of the extracerebral circulation. These studies failed to detect labeled perivascular fibers in animals injected in dorsal raphe neurons with the anterograde tracer, *Phaseolus vulgaris* leucoagglutinin (Mathiau et al., 1993a). Furthermore, cerebrovascular nerves in extracerebral blood vessels contain monoamine oxidase (MAO) type A which is lost following ganglionectomy (Shigematsu et al., 1989; Mathiau et al., 1993c) and raphe neurons possess MAO type B (Levitt et al., 1982; Mathiau et al., 1993c). In addition, perivascular nerve fibers to extracerebral blood vessels are not immunoreactive to the low molecular mass neurofilament (Mathiau et al., 1993c) which is typical of neurons whose cell bodies and axons are located entirely in the CNS such as the raphe nucleus (Leonard et al., 1988). Likewise, perivascular fibers contain the peripheral neuron marker, peripherin, but raphe neurons do not (Mathiau et al., 1993c).

As can be seen, considerable confusion and conflicting data exists with respect to the origin of the 5-HT perivascular nerve fibers to extracerebral blood vessels. On this basis, as part of my thesis work, I attempted to alleviate some of this confusion by investigating this issue via immunocytochemistry for the serotonin synthesizing enzyme, TPH, and lesion of either the superior cervical ganglion or the ascending pathway from the dorsal raphe nucleus, as will be discussed in greater detail in Chapter 3.

1.3.4 VASOMOTOR PROPERTIES OF 5-HT

1.3.4.1 Exogenous 5-HT: The vasomotor effects of 5-HT has been extensively studied in cerebral arteries of several species using a variety of approaches. Altogether, these studies show that cerebral blood vessels are particularly sensitive to 5-HT and that the indoleamine is one of the most potent vasoconstrictor agent of brain vessels. In isolated cerebral arteries, serotonin predominantly exerts a strong concentration-dependent

contraction, reaching up to 40-100% of the maximal contraction induced by exposure to a depolarizing solution (124 mM) of potassium (Young et al., 1986). This response to 5-HT appears to depend on various factors such as the species, the anatomical segment of the cerebral circulation studied and even the different areas within a same artery (McCulloch and Edvinsson, 1984; Young et al., 1986). Another factor apparently involved in 5-HT-mediated vasomotor response is the initial tone of the blood vessel. Although 5-HT generally induces a contractile response, some studies have demonstrated that it can exert vasodilatation in cerebral arteries under high tone. In human and feline arteries precontracted with prostaglandin $F_{2\alpha}$, 5-HT elicited a concentration dependent vasodilatation (Edvinsson et al., 1978). Furthermore, *in situ* microapplication of mock cerebrospinal fluid solutions containing 5-HT was shown to relax pial arterioles (diameter less than 70 μm) and constrict large cerebral arteries (diameter greater than 200 μm) (Harper and MacKenzie, 1977a).

Following infusion of 5-HT through the internal carotid artery, initial experiments failed to detect any change in cerebral perfusion even though blood flow in the carotid system was reduced (Grimson et al., 1969); an effect explained by the fact that 5-HT does not cross the BBB. Subsequent experiments whereby the BBB was deliberately disrupted prior to infusion of 5-HT confirmed this contention (Harper and MacKenzie, 1977b). In these experiments, 5-HT significantly decreased cerebral perfusion, oxygen consumption as well as electrical cortical activity in baboons. Indeed, Grome and Harper (1983), using the ^{14}C-iodoantipyrine quantitative radioautographic technique which directly measures CBF changes in well-delineated regions, observed decreases in blood flow in several brain areas following infusion of 5-HT with prior disruption of the BBB. Furthermore, inhibiting the activity of MAO, the major catabolic enzyme for 5-HT, also resulted in decreases in CBF (Eidelman et al., 1978). These studies agree with those where 5-HT was administered directly to the blood vessels and confirm that 5-HT induces predominantly a vasoconstractile response when the indoleamine is allowed to gain access to the cerebral circulation. Taken together, these studies support a primary

vasoconstrictive role for endogenous 5-HT.

1.3.4.2 Endogenous 5-HT

1.3.4.2.1 Raphe nuclei stimulation: Initial electrical stimulation of the dorsal raphe nucleus in cats and monkeys undertaken by Goadsby et al. (1985a,b) were found to induce vasodilatory responses, as demonstrated by a decrease in common carotid resistance. These studies, however, do not provide a direct assessment of CBF changes since the electromagnetic probes used to detect perfusion changes were placed around the common and external carotid arteries. Subsequent investigations using the ^{14}C-radioautographic technique have yielded somewhat conflicting results. In the α-chloralose anesthetized rat, electrical activation of the dorsal raphe produced significant decreases (13 to 26%) in CBF in a majority of cerebral regions including specific subdivisions of the cerebral cortex (Bonvento et al., 1989; Table 1.3). In the conscious rat, however, similar manipulations resulted in CBF elevations (15-71%) in 17 of 63 brain structures studied such as the fronto-parietal cortex while reductions were seen in a limited number of regions (Cudennec et al., 1993; Table 1.3). Other anatomical regions such as the molecular layer of the posterior hippocampus and entorhinal cortex exhibited very minor or no changes in CBF following dorsal raphe stimulation (Bonvento et al., 1989; Cudennec et al., 1993). These authors suggested that these apparent differences may be due to the use of anesthesia which could potentially affect the vasomotor responses, as described by Edvinsson and colleagues (1993). In addition, these inconsistencies have been partly explained by the ability of different subregions of the dorsal raphe nucleus to elicit opposite vasomotor responses. In fact, Underwood et al. (1992; Table 1.3), using laser Doppler flowmetry, elegantly demonstrated that depending on the precise site of stimulation within the dorsal raphe nucleus, opposite CBF responses can be observed. If caudal regions are stimulated, increases in parietal CBF are evident while activation of rostroventral regions decrease perfusion within this cortical subdivision (Underwood et al., 1992). Interestingly, the observed CBF changes (either increases or decreases) in this study are in the magnitude of those described by both

TABLE 1.3: EFFECTS OF SEROTONERGIC STIMULATION, LESION AND PHARMACOLOGICAL MANIPULATIONS ON CEREBRAL BLOOD FLOW (CBF)

RAPHE STIMULATION

EXPERIMENTAL PARADIGM	*CBF EFFECT*	*REFERENCES*
Electrical, chemical/ dorsal	⇑	Goadsby et al (1985a,b)
Electrical/ dorsal	⇓	Bonvento et al (1989)
Electrical/dorsal	⇑/⇓	Underwood et al (1992)
Chemical/dorsal	⇓	Cao et al (1992)
Electrical/dorsal	⇑/⇓	Cudennec et al (1993)
Chemical/dorsal	⇑	Underwood et al (1995)

SEROTONERGIC LESION

EXPERIMENTAL PARADIGM	*CBF EFFECT*	*REFERENCES*
5,7-DHT treatment	-	Dahlgren et al (1981)
5,7-DHT treatment	-	Itakura et al (1985)
5-HT lesion/MDA	- / ⇑	McBean et al (1990)
5,7-DHT treatment	-	Underwood et al (1992)
5-HT lesion/MDA	- / ⇑	Kelly et al (1995)

PHARMACOLOGICAL MANIPULATION

EXPERIMENTAL PARADIGM	*CBF EFFECT*	*REFERENCES*
8-OH-DPAT/5-HT_{1A} agonist	⇑	McBean et al (1991)
Sumatriptan/ 5-$HT_{1B/1D}$ agonist	-	Friberg et al (1991)
Sumatriptan/ 5-$HT_{1B/1D}$ agonist	-	Scott et al (1992)
Sumatriptan/ 5-$HT_{1B/1D}$ agonist	⇓	Kobari et al (1993)
Sumatriptan/ 5-$HT_{1B/1D}$ agonist	-	Ferrari et al (1995)
Ketanserin/5-HT_2 antagonist	⇑	Dietrich et al (1989)
Ketanserin/5-HT_2 antagonist	-	Olsen et al (1992)

Bonvento et al. (1989) and Cudennec et al. (1993). Finally, the possibility also exists that the dual and opposing vasomotor responses elicited by 5-HT may be due to the initial tone of the blood vessel. Thus, the final integrated response is likely to depend on some or all of these factors.

1.3.4.2.2 Central 5-HT lesions: In contrast to the significant blood flow alterations elicited by activation of the 5-HT pathways, destruction of this system is characterized by very limited, if any, changes in this parameter, as summarized in Table 1.3. Chemical destruction of the serotonergic system with selective 5-HT neurotoxins, namely 5,7-dihydroxytryptamine and methylenedioxyamphetamine failed to exert changes in blood perfusion in all studies (Table 1.3; Dahlgren et al., 1981; Itakura et al., 1985;; Underwood et al., 1992; Kelly et al., 1995) except one (McBean et al., 1990). In this study, only moderate but generalized increases in CBF were observed after methylene-dioxyamphetamine administration with only a few regions (7 of 32 regions) exhibiting a small but statistically significant increase in perfusion (McBean et al. 1990). The failure of lesion experiments to alter CBF locally, is indicative that 5-HT exerts a phasic, or perhaps a minor tonic, influence on the microvascular bed.

1.3.4.3 Pharmacological Manipulations: Using ^{14}C-iodoantipyrine quantitative radioautography, McBean et al. (1991) showed that the selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), reduces neuronal firing and thereby results in a decrease in 5-HT release, produced significant increases (up to 226%) in CBF in the majority of regions studied while in a minority of them very limited, if any, blood flow changes were evident (i.e. molecular layer of the hippocampus, 15% increase). Similarly, administration of the 5-HT_{2A} receptor antagonist, ketanserin, was found to significantly increase local CBF in the rat cerebral cortex (136-166%) with only small, not significant CBF increases in subcortical regions (Dietrich et al., 1989; Table 1.3). This observation was later confirmed by Cao et al. (1992) who showed that the decrease in parietal blood flow elicited by stimulation of the dorsal raphe nucleus could

be blocked by pretreatment with ketanserin (Table 1.3).

In cat and human, other pharmacological investigations have involved the infusion of sumatriptan, a non-selective 5-HT_1 receptor agonist with high affinity at the 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptors (Connor and Beattie, 1996). This agent can effectively alleviate migraine headache, possibly because of its potent contractile properties at the cerebrovascular level (Humphrey and Goadsby, 1994). In healthy individuals or in migraine patients, local CBF was unaffected with intravenous injections of sumatriptan at clinically effective doses (2-6 mg), although the middle cerebral artery on the headache side constricted following such treatment (Friberg et al., 1991; Scott et al., 1992; Ferrari et al., 1995; Table 1.3). In the cat, a similar response was observed but when higher sumatriptan concentrations (500mg/kg) are administered, CBF was significantly reduced in the parietal cortex (Connor et al., 1992; Kobari et al., 1993; Table 1.3).

1.3.4.4 Effects of 5-HT and related compounds on glucose metabolism: With the introduction by Sokoloff et al. (1977) of the quantitative radioautographic ^{14}C-2-deoxyglucose (^{14}C-2-DG) method, it became possible to measure the rate of glucose consumption within localized cerebral regions under normal and experimental conditions. As mentioned in section 1.1.4, cerebral glucose utilization (CGU) is an indication of neuronal activity. With respect to the 5-HT system, several experimental paradigms have been tested to determine the effect of 5-HT on CGU, as summarized in Table 1.4.

Activation of both dorsal and median raphe nuclei in anesthetized or conscious animals resulted in marked and widespread increases of glucose use, most notably in neocortical areas and extrapyramidal regions (Cudennec et al., 1988a) as well as in a number of subcortical nuclei (Bonvento et al., 1991). These changes are most likely induced by 5-HT as prior administration of the serotonergic neurotoxin, 5,7-dihydroxytryptamine, totally abolished the CGU changes. On the other hand, central lesions of raphe nuclei, via many different approaches, have very limited, if any, effect on CGU. Only with

TABLE 1.4: EFFECTS OF SEROTONERGIC STIMULATION, LESION AND PHARMACOLOGICAL MANIPULATIONS ON CEREBRAL GLUCOSE UTILIZATION (CGU)

RAPHE STIMULATION

EXPERIMENTAL PARADIGM	*CGU EFFECTS*	*REFERENCES*
Electrical/ dorsal, median	⇑	Cudennec et al (1988a)
Electrical/ dorsal	⇑	Bonvento et al (1991)

SEROTONERGIC LESION

EXPERIMENTAL PARADIGM	*CGU EFFECTS*	*REFERENCES*
5,7-DHT	-	Cudennec et al (1988b)
Electrolytic raphe lesion	-	Cudennec et al (1988b)
5-HT synthesis inhibition/PCPA	-	Pappius et al (1988)
5-HT lesion/ MDA	-/⇑	McBean et al (1990)
5-HT lesion/ MDA	-/⇑	Kelly et al (1995)

PHARMACOLOGICAL MANIPULATION

EXPERIMENTAL PARADIGM	*CGU EFFECTS*	*REFERENCES*
Clomipramine/ 5-HT uptake inhibitor	⇓	Freo et al (1993a)
Fluoxetine/ 5-HT uptake inhibitor	⇑/⇓	Cook et al (1994)
Fenfluramine/ 5-HT uptake inhibitor	⇑/⇓	Kapur et al (1994)
LSD, 5-MeODMT/ 5-HT agonist	⇓	Grome and Harper (1986)
Quizapine, 6-CPP/ 5-HT agonist	⇑/⇓	Grome and Harper (1986)
Quizapine/ 5-HT agonist	⇑	Freo et al (1993b)
8-OH-DPAT/ 5-HT_{1A} agonist	⇑/⇓	Kelly et al (1988)
8-OH-DPAT/ 5-HT_{1A} agonist	⇑/⇓	McBean et al (1990)
8-OH-DPAT/ 5-HT_{1A} agonist	⇑/⇓	Freo et al (1995)
Ipsapirone/ 5-HT_{1A} agonist	⇓	Wree et al (1987)
Gepirone, ipsapirone, buspirone/ 5-HT_{1A} agonists	⇑/⇓	Grasby et al (1992)
Buspirone/ 5-HT_{1A} agonist	⇓	Freo et al (1995)
RU-24969/ 5-HT_{1B} agonist	⇑/⇓	Kelly et al (1988)
m-CPP/ 5-$HT_{2B/2C}$ agonist	⇓	Freo et al (1990)
DOI/ 5-$HT_{2A/2C}$ agonist	⇓	Freo et al (1991)
Methiothepine/ 5-HT_1 antagonist	⇓	Ricchieri et al (1987)
Ondasetrone/ 5-HT_3 antagonist	⇓	Mitchell and Pratt (1991)

methylenedioxyamphetamine treatment could small but significant increases in CGU be evidenced in a limited number of cerebral regions (McBean et al., 1990; Kelly et al., 1995).

The metabolic consequences of pharmacologically manipulating 5-HT neural pathways have not been as conclusive since administration of a given drug can elicit both increase and decrease in CGU depending on the cerebral region. The differences in the metabolic responses following infusion of various 5-HT-related agents seem more related to the concentration of the compound, the brain areas that it targets and the receptors that it activates. In general, a reduction in glucose consumption is observed following pharmacological activation of 5-HT pathways (Table 1.4; see Freo, 1996 for review).

1.3.4.5 Cerebral blood flow and metabolism coupling: effects of 5-HT: As mentioned in section 1.1.4, the prevailing view of CBF regulation is that of a tight coupling of flow to metabolic activity, i.e. the changes in flow are a direct consequence of metabolic demand. Presently, the only method to distinguish between a 'primary' or 'secondary' vascular phenomenon is by statistical analyses of the relationships between local blood perfusion and metabolic activity under normal and experimental conditions, as described by McCulloch et al. (1982). However, as the authors pointed out, the results have to be interpreted with caution because of statistical limitations. In these analyses, mean CBF values under both conditions are plotted against the mean CGU values for each brain region. Comparisons between both parameters could identify if there is a general resetting of the flow-metabolism relationships as well as the structures where the CBF-CGU ratio is disturbed. An "uncoupling" phenomenon whereby metabolic and cerebrovascular changes are not superimposable would suggest a vascular effect not directly dependent on metabolic activity.

With respect to the effect of 5-HT on local perfusion and metabolic activity, four different studies in the rat have investigated the relationships between local CBF and

CGU in conditions where 5-HT release was either increased (dorsal raphe stimulation) or decreased (MDA or 8-OH-DPAT administration) experimentally (Bonvento et al., 1989; 1991; McBean et al., 1990; 1991; Cudennec et al., 1993). All four reports showed that, in some brain regions, alterations in CGU are not accompanied by a parallel change in CBF and all described a resetting of the flow-metabolism relationship (Fig 1.7). These studies demonstrate that when 5-HT release is increased, the slope of the linear relationship of CBF and CGU is decreased suggestive of a reduction in perfusion without the corresponding metabolic change. Reciprocally, if 5-HT release is decreased, then the slope shifts upwards indicating that blood flow is in excess to CGU (Fig 1.7). Taken together, these studies point to a primary vasoconstrictive effect of 5-HT and indicate that intracerebrally released 5-HT could act directly on local blood vessels to modify their diameter. On the basis of these suggestions and implications, the second part of my research project was to investigate anatomically the putative relationships between central serotonin-containing nerve terminals and the brain microvascular bed (Chapter 4).

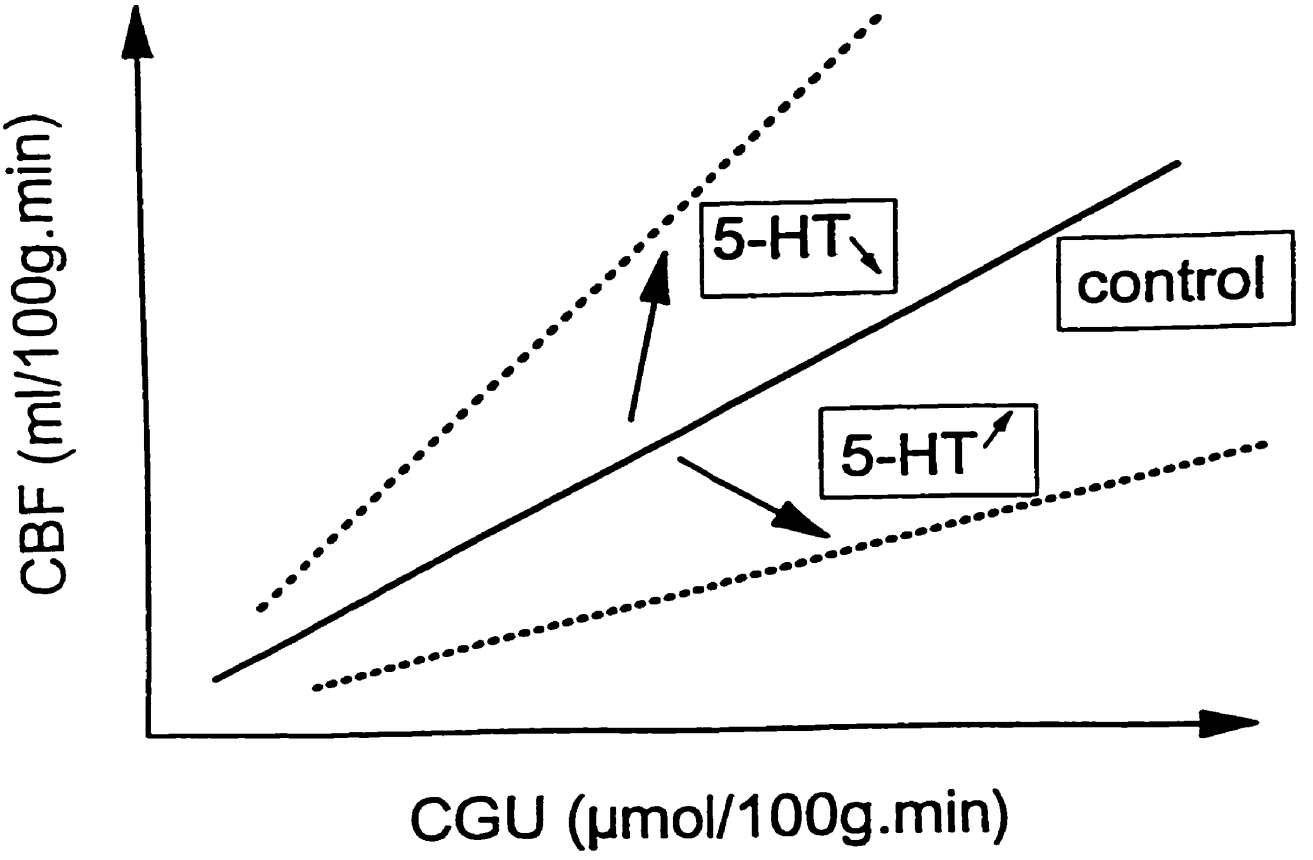

Fig 1.7: Comparison of the realtionship between cerebral blood flow (CBF) and cerebral glucose utilization (CGU) in control conditions and following changes in 5-HT neurotransmission. Note that when 5-HT release is increased, the slope is reduced while a decrease in 5-HT neurotransmission has the opposite effect. Taken from Cohen et al (1996).

1.4 SEROTONIN RECEPTORS: DISTRIBUTION IN BRAIN AND BLOOD VESSELS

The last part of my thesis work will be dedicated to the identification of 5-HT receptors present in the microvascular bed and its cellular constituents (Chapter 6). As a background to this section, I will present the evidence suggesting the presence of specific 5-HT receptors able to mediate vasomotor responses in several vascular beds of central and/or peripheral origins. However, in order to proceed logically, a brief description of the various 5-HT receptors identified so far will first be provided. Some characteristics for each receptor are summarized in Table 1.5.

1.4.1 HETEROGENEITY OF 5-HT RECEPTORS

The presence of different types of 5-HT receptors was initially suggested by Gaddum and Picarelli (1957) in the guinea pig isolated ileum. They described two categories of receptors, 5-HTD and 5-HTM, depending on the abilites of dibenzyline and morphine to block smooth muscle contraction, respectively. Subsequent experiments in the 1970's furthered our understanding and knowledge of the different 5-HT receptor types and subtypes. The introduction of radioligand binding techniques, the development of selective agents acting on specific 5-HT receptor subtypes and more recently, the advance of molecular biology techniques have all contributed significantly to our modern understanding of the multiplicity of 5-HT receptors.

5-HT receptors can be classified on the basis of their operational (i.e. drug related characteristics), transductional (i.e. receptor-effect coupling events) and structural (i.e. amino acid sequence) properties as well as their anatomical distribution. To date, five distinct receptor families (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7) have been defined. In addition, three recombinant receptors (5-ht_{5A}, 5-ht_{5B} and 5-ht_6) have been identified which provides strong evidence of two more families (Table 1.5). Since the latter receptors still await full operational and transductional characterizations in intact tissues, they must retain the lower 5-ht appellation as recommended by the Nomenclature

TABLE 1.5: OPERATIONAL, TRANSDUCTIONAL AND STRUCTURAL INFORMATION OF CHARACTERIZED 5-HT RECEPTORS

RECEPTOR	PREVIOUS NAME	AGONIST	ANTAGONIST	RADIOLIGAND	EFFECTOR PATHWAY	PRIMARY A.A. SEQUENCE
5-HT_{1A}	-	8-OH-DPAT 5-CT	WAY100135	[^{3}H]-8-OH-DPAT	⇓ cAMP	Human (Kobilak et al., 1987) Rat (Albert et al., 1990)
5-HT_{1B}	5-$HT_{1D\beta}$	Sumatriptan RU24969	GR127935 GR55562	[^{3}H]-Sumatriptan [^{125}I]-GTI	⇓ cAMP	Human (Jin et al., 1992; Adham et al., 1992) Rat (Voigt et al., 1991)
5-HT_{1D}	5-$HT_{1D\alpha}$	Sumatriptan L694247	GR127935 GR55562	[^{3}H]-Sumatriptan [^{125}I]-GTI	⇓ cAMP	Human (Hamblin et al., 1991b) Rat (Hamblin et al., 1991b)
5-ht_{1E}	-	5-HT	-	[^{3}H]-5-HT	⇓ cAMP	Human (McAllister et al., 1992)
5-ht_{1F}	5$ht_{1E\beta}$, 5-HT_6	LY334370 5-HT	-	[^{3}H]-LY334370	⇓ cAMP	Human (Adham et al., 1993; Lovenberg et al., 1993a) Rat (Lovenberg et al., 1993a)
5-HT_{2A}	D, 5-HT_2	α-Me-5-HT DOI	Ketanserin Ritanserin	[^{3}H]-Ketanserin	IP_3/DAG	Human (Yang et al., 1991) Rat (Pritchett et al., 1988)
5-HT_{2B}	5-HT_{2F}	α-Me-5-HT, DOI BW723C86	SB204741 SB200646	[^{3}H]-5-HT	IP_3/DAG	Human (Kursar et al., 1994; Schmuck et al., 1992) Rat (Kursar et al., 1992)
5-HT_{2C}	5-HT_{1C}	α-Me-5-HT DOI	Mesulergine SB200646	[^{3}H]-Mesulergine	IP_3/DAG	Human (Saltzman et al., 1991) Rat (Julius et al., 1988)

TABLE 1.5 (CONTINUED): OPERATIONAL, TRANSDUCTIONAL AND STRUCTURAL INFORMATION OF CHARACTERIZED 5-HT RECEPTORS

RECEPTOR	PREVIOUS NAME	AGONIST	ANTAGONIST	RADIOLIGAND	EFFECTOR PATHWAY	PRIMARY A.A. SEQUENCE
5-HT$_3$	M	2-Me-5-HT Phenylbiguanide	Tropisetron Granisetrone Zacopride	[^{3}H]-Zacopride	Ligand gated channel	Rat (Johnson and Heinemann, 1992) Mouse (Maricq et al., 1991)
5-HT$_4$	-	5-MeOT Renzapride	SB204070 GR113808	[^{3}H]-GR113808	⇑ cAMP	Rat (Gerald et al., 1994)
5-ht$_{5A}$	5-ht$_{5\alpha}$	5-HT, 5-CT	LSD	[^{125}I]-LSD	⇓ cAMP??	Rat (Erlander et al., 1993)
5-ht$_{5B}$	5-ht$_{5\beta}$	5-HT, 5-CT	LSD	[^{125}I]-LSD	???	Rat (Wisden et al., 1993; Erlander et al., 1993)
5-ht$_6$	-	5-HT, 5-MeOT	LSD, Methiothepine	[^{125}I]-LSD [^{3}H]-5-CT	⇑ cAMP	Rat (Ruat et al., 1993)
5-HT$_7$	-	5-HT, 5-MeOT, 5-CT	Methiothepine Mesulergine	[^{125}I]-LSD [^{3}H]-5-CT	⇑ cAMP	Human (Bard et al., 1993) Rat (Lovenberg et al., 1993b; Shen et al., 1993)

Based on review articles by Martin and Humphrey (1994) and Hoyer et al (1994)

Committee (Hoyer et al., 1994). All 5-HT receptors, with the exception of the 5-HT_3 which is a ligand-gated ion channel, are G-protein coupled receptors with their characteristic seven hydrophobic transmembrane-spanning domains and three intracellular loops (Hoyer et al., 1994; Saxena, 1995).

1.4.1.1 5-HT_1 receptor family: This receptor family was originally identified as high-affinity sites for 5-HT and could be labelled with tritiated 5-HT. Five different 5-HT_1 receptor subtypes have been recognized so far, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E} and 5-ht_{1F}. The latter two receptors are lower-cased because they have yet to be functionally characterized in intact tissues. The receptors contained in this family are characterized by a high affinity (nanomolar range) for 5-HT and all are linked to the inhibition of adenylyl cyclase whether in intact tissues or in cells transfected with the corresponding receptor gene. All five receptor genes have been cloned and display an overall sequence homology of about 40% and all 5-HT_1 receptors are encoded by intronless genes (see Hoyer et al., 1994; Saxena, 1995).

1.4.1.1.1 5-HT_{1A} Receptors: Radioautographic studies have shown 5-HT_{1A} binding sites to be widely distributed throughout the brain of various species including man. These sites are particularly enriched in regions that are components of the limbic system such as the hippocampus, entorhinal cortex and septum (Pazos and Palacios, 1985; Hoyer et al., 1986). Such distribution in binding sites is in excellent agreement with that of 5-HT_{1A} receptor mRNA (Chalmers and Watson, 1991; Pompeiano et al., 1992) and immunoreactivity (Kia et al., 1996). In addition, the above studies also found that 5-HT_{1A} receptors are abundant in the dorsal and median raphe neurons where they have been suggested to function as somatodendritic autoreceptors inhibiting neuronal firing and modulating raphe neurons activity (Sprouse and Aghajanian, 1987). Stimulation of presynaptic 5-HT_{1A} receptors results in a decrease in 5-HT synthesis, release and electrical activity (de Montigny and Blier, 1992). Although predominantly coupled to inhibition of adenylyl cyclase via the pertussis-toxin sensitive protein, Gi (de

Vivo and Maayani, 1986), some studies have also shown that, at high concentrations of 5-HT, they can also be linked to inositol phosphate production and protein kinase C activity (Fargin et al., 1989).

1.4.1.1.2 5-HT_{1B} Receptors: 5-HT_{1B} receptors were first thought to be present exclusively in rodents but have since been found in guinea pig, bovine, non-human primates and humans where they were initially called 5-$HT_{1D\beta}$ (Weinshank et al., 1992). The rat amino acid sequence is very similar to its human counterpart (93% homology) but the pharmacological profiles of the two receptors are quite different (Hamblin et al., 1992). Receptor radioautography, immuno- and *in situ* histochemical techniques have shown an abundance of 5-HT_{1B} receptors and mRNA in the basal ganglia, most notably in the globus pallidus and in the pars reticularis of the sustantia nigra (Bruinvels et al., 1993; 1994; Langlois et al., 1995). Some binding sites and mRNA, albeit at low levels, have been localized in the raphe nuclei, hippocampus and cerebellum. The 5-HT_{1B} receptors have been classically described as the presynaptic inhibitory autoreceptor in the regulation of 5-HT release (Middlemiss, 1985; Pineyro et al., 1995). They may also function as heteroreceptors modulating the release of other neurotransmitters such as glutamate and acetylcholine (Maura and Raiteri, 1986; Gothert et al., 1996). As will be considered in more detail below (sections 1.4.2.1.1 and 1.4.2.2.2), 5-HT_{1B} receptors have also been identified as mediators of vasocontractile and possibly vasodilatory responses in cerebral and peripheral blood vessels.

1.4.1.1.3 5-HT_{1D} Receptors: 5-HT_{1D} receptors are mostly found in non-rodent species although they also exist in rodents albeit in very low amounts (Bruinvels et al., 1993; 1994). With respect to their regional distribution, 5-HT_{1D} (originally called 5-$HT_{1D\alpha}$ in human, Weinshank et al., 1992), receptor and message closely mirror that of 5-HT_{1B} with which they are frequently codistributed, although there are fewer 5-HT_{1D} sites and lower levels of 5-HT_{1D} mRNA (Bruinvels et al., 1993; 1994). The 5-HT_{1B} and 5-HT_{1D} receptor subtypes are structurally related (60 and 65% amino acid homology in rat

and human, respectively) and possess very comparable pharmacological profiles (Zgombick et al., 1995). It has been suggested that 5-HT_{1D} receptors present on raphe neurons serve as inhibitory autoreceptors for somatodendritic release of 5-HT that is independent of 5-HT_{1A} receptors (Pineyro et al., 1996) while at the terminal level they would be inhibitory receptors similar to what has been reported for 5-HT_{1B} receptors. They may also possess some vasomotor properties (see sections 1.4.2.2.1 and 1.4.2.2.2).

1.4.1.1.4 5-ht_{1E} and 5-ht_{1F} Receptors: These two receptors have been cloned and classified in the 5-HT_1 family on the basis of their amino acid homology (60-70%) with other 5-HT_1 receptors and their negative coupling to adenylyl cyclase in transfected cell lines (McAllister et al., 1992; Adham et al., 1993; Lovenberg et al., 1993a). In human and monkey brains, 5-ht_{1E} mRNA is expressed at the highest level in the parietal cortex (layer IV), caudate nucleus and putamen while lower signals were observed in hypothalamic nuclei and other cortical regions (Bruinvels et al., 1994). For 5-ht_{1F} receptors, mRNA expression has been localised in the dorsal raphe nucleus, striatum, hippocampus, and in layers V and VI of the cerebral cortex (Adham et al., 1993). The precise function of both receptors are unknown but the 5-ht_{1F} subtype is thought to act as another 5-HT autoreceptor (Adham et al., 1993) and reportedly does not mediate any vasomotor responses either in cerebral (Hamel et al., 1993) or peripheral (Phebus et al., 1996) blood vessels.

1.4.1.2 5-HT_2 receptor family: Three subtypes (i.e. 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) are currently recognized as members of the 5-HT_2 receptor family. All 5-HT_2 receptors are characterized by micromolar affinities to 5-HT and activate phosphoinositide metabolism. Their coding sequences are all interrupted by introns and display approximately 60% homology between themselves.

1.4.1.2.1 5-HT_{2A} Receptors: In the CNS, 5-HT_{2A} (the original 5-HT_2) binding sites, are localized in high densities in many areas of the cerebral cortex (layers III and V

in humans), in the claustrum and in many components of the limbic system, as determined by quantitative radioautography (Pazos et al., 1985; Hoyer et al., 1986). This distribution has been confirmed and extended by the use of 5-HT_{2A} selective antibodies and by *in situ* hybridization (Mengod et al., 1990; Morilak et al., 1993). The precise role of 5-HT_{2A} receptors in the CNS is not known but they have been involved in the regulation of motor behaviors, sleep and food intake. They have been found on GABA-, acetylcholine- and somatostatin-containing neurons in various cortical regions (Morilak et al., 1993), where they presumably control neurotransmitter release. (Hirano et al., 1995). In peripheral and cerebral blood vessels of some species, this receptor is known to mediate contractile responses (see sections 1.4.2.1.1 and 1.4.2.2.1).

1.4.1.2.2 5-HT_{2B} Receptors: The 5-HT_{2B} receptor was initially discovered in the rat stomach fundus, hence its original classification as the 5-HT_{2F}. It has yet to be demonstrated in rat brain although low levels of 5-HT_{2B} mRNA have been detected in human brains (Kursar et al., 1994). Its overall distribution in the CNS is still not known. Functionally, it may be involved in fundal contractions (Wainscott et al., 1993) and, possibly in endothelial-dependent vascular relaxations (see section 4.2.2.2) but virtually nothing is known about the effects it mediates centrally.

1.4.1.2.3 5-HT_{2C} Receptors: Radioligand binding, radioautography, *in situ* hybridization and immunocytochemical studies have shown that these receptors, originally classified as the 5-HT_{1C}, are enriched on the epithelial cells in the choroid plexus (Pazos et al., 1984; Hoyer et al., 1986; Pompeiano et al., 1994; Abramowski et al., 1995). These receptors are also found, albeit in lower densities, in limbic structures such as hippocampus, septum and amygdala as well as in the basal ganglia, hypothalamus and cerebral cortex). In the choroid plexus, they are thought to regulate the production and composition of cerebrospinal fluid while in limbic structures they are suggested to influence mood, behavior and hallucinations (Hartig et al., 1990).

1.4.1.3 5-HT_3 Receptors: These receptors corresponded to the M receptor type originally defined by Gaddum and Picarelli (1957). High levels of 5-HT_3 receptor mRNA and binding sites have been found in the hippocampus, amygdala, discrete regions of the brainstem such as the area postrema, the nucleus tractus solitarius and the dorsal nucleus of the vagus nerve along with the substantia gelatinosa of the spinal trigeminal nucleus and spinal cord (Kilpatrick et al., 1987; Tecott et al., 1993). Behavioral studies have suggested that these receptors have antipsychotic, antidepressant and anxiolytic properties (Glennon, 1990).

1.4.1.4 5-HT_4 Receptors: These receptors are positively coupled to adenylate cyclase activity and two different clones, 5-HT_{4S} and 5-HT_{4L}, have been identified (Gerald et al., 1995). Radioligand binding studies have documented high densities of these receptors in the caudate nucleus, globus pallidus and putamen while moderate densities are found in the cerebral cortex and areas of the limbic system (Reynolds et al., 1995). Their mRNA distribution has been recently documented and was found to correspond well to that of binding sites (Vilaro et al., 1996; Ullmer et al., 1996). They are thought to promote neurotransmitter release and enhance synaptic transmission. Furthermore, through their presence in septo-hippocampal and nigro-striatal pathways, 5-HT_4 receptors may be involved in cognitive, emotional and motor control (Reynolds et al., 1995).

1.4.1.5 Other 5-HT Receptors: Three other receptor families, namely 5-ht_5, 5-ht_6 and 5-HT_7, have been proposed to exist. Since these receptors have only been cloned fairly recently and because they still lack selective pharmacological agents, little information is known concerning their physiological effects.

1.4.1.5.1 5ht_5 Receptors: Two related 5-HT receptors have been cloned and found not to possess a similar amino acid sequence or drug binding profiles to other previously defined 5-HT receptors. They have been referred to 5-ht_{5A} and 5-ht_{5B} and little is known about their distribution in mammalian brain. *In situ* hybridization and

immunocytochemical studies have shown transcripts and proteins for the 5-ht_{5A} throughout the rat CNS; predominantly in astrocytes (Erlander et al., 1993; Carson et al., 1996). For the 5-ht_{5B} subtype, message was highest in the medial habenula and in the CA1 region of the hippocampus (Wisden et al., 1993; Erlander et al., 1993). Originally, the signal transduction system for the 5-ht_5 receptors was unknown but they have recently been proposed to inhibit cAMP production (Carson et al., 1996). No known function has of yet been attributed to these receptors but based on their distribution, a role in learning and memory processes has been suggested (Wisden et al., 1993).

1.4.1.5.2 5-ht_6 Receptors: The highest mRNA expression, as detected by Northern blot analyses and *in situ* hybridization, of 5-ht_6 receptors was found in the striatum, nucleus accumbens, regions of the hippocampus and olfactory tubercle while moderate levels were observed in the cerebellum, several layers of the cerebral cortex and amygdala (Ruat et al., 1993; Ward et al., 1995). Very recently, antibodies against this receptor have been developed and distribution of the protein was found to match well that of the mRNA (Gerard et al., 1997). Although not yet tested on intact tissue, 5-ht_6 receptors are positively linked to cAMP production in transfected cells (Ruat et al., 1993). Based on their anatomical distribution, these receptors may play a role in antipsychotic actions (Ward et al., 1995).

1.4.1.5.3 5-HT_7 Receptors: Expression of mRNA for the 5-HT_7 receptor has been detected both by Northern blot analysis and *in situ* hybridization in both human and rat brains and shown to predominante in the thalamus, hippocampus and hypothalamus (Lovenberg et al., 1993b; Bard et al., 1993; Gustafson et al., 1996). These receptors, like the 5-ht_6, stimulate cAMP production (Bard et al., 1993). Due to the presence of 5-HT_7 message in the suprachiasmatic nucleus, these receptors have been proposed to be involved in the regulation of circadian rhythms (Inouye and Shibata, 1994). However, they have also been suggested to be involved in the therapeutical effects of some antidepressant drugs (Sleight et al., 1995). Recently, 5-HT_7 receptors have been

implicated in the 5-HT-mediated endothelium-independent relaxation (see section 1.4.2.2.2) in several vascular beds.

1.4.2 CEREBROVASCULAR 5-HT RECEPTORS

From the above description, 14 different 5-HT receptor subtypes have been identified to date and some of them have been shown to mediate vasomotor (contraction, relaxation; see below) or vascular (blood brain barrier, vascular mitogenesis, section 1.6) responses. To determine the receptor involved in specific vasomotor responses, pharmacological *in vitro* experiments on isolated blood vessels have compared the rank order of agonists and antagonists potencies at the cerebrovascular receptor and at cloned 5-HT receptors and/or have evaluated and compared the correlation between the pharmacological profile of vascular and cloned receptors (Hoyer et al., 1994). More recently, a variety of molecular biology techniques (i.e. *in situ* and Northern blot hybridization, polymerase chain reaction) have been used to determine which 5-HT receptors are expressed in cerebral and peripheral blood vessels.

1.4.2.1 Cerebral Arteries

1.4.2.1.1 Contraction: Overall, the prevailing vasomotor consequence of 5-HT administration on blood vessels throughout the cerebrovascular bed is vasoconstriction. Its potency is dependent on the species and vascular segments studied (Young et al., 1986) and the precise receptor that mediates this response also appears to be highly species specific. The 5-HT receptor mediating cerebral vasocontraction has been studied extensively in multiple species and the literature is quite vast. Here, I will attempt to summarize the most current knowledge in order to provide a general overview. Recent extensive reviews on this subject have been published (Martin, 1994; Lincoln, 1995). Cerebral vasoconstriction is mediated by 5-HT_{2A} receptors in rats (Chang and Owman, 1987; Chang et al., 1988b), 5-$HT_{1\text{-like}}$ receptors in guinea pig (Chang et al., 1988b), 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors in cats (Auer et al., 1985; Hamel et al., 1988; 1989; Connor et al., 1992), and 5-HT_{2A} and possibly 5-HT_{1A} receptors in dogs (Muller-

Schweinitzer and Engel, 1983; Peroutka et al., 1986). In human basilar and pial arteries, the receptor mediating vasoconstriction has consistently been described as a 5-$HT_{1\text{-like}}$ with no involvement of the 5-HT_{2A} (Parsons et al., 1989; Hamel and Bouchard, 1991). More detailed analyses on these receptors have concluded that the human cerebrovascular 5-$HT_{1\text{-like}}$ receptor mediating contraction is a functional correlate of the cloned human 5-HT_{1B} receptor (Hamel and Bouchard, 1991; Hamel et al., 1993a; Kalkmann et al., 1993; 1994; Bouchelet et al., 1996a). The same appears to hold true for bovine cerebral blood vessels (Hamel et al., 1993a,b).

In intraparenchymal blood vessels, very limited information is available with regards to the receptors mediating local contractile responses. In rats, administration of ketanserin effectively increases cortical CBF (Dietrich et al., 1989) and reverses cortical blood flow decreases subsequent to dorsal raphe stimulations, suggesting the presence of 5-HT_{2A} receptors in microvascular contraction in this species (Cao et al., 1992). In the cat, 5-$HT_{1\text{-like}}$ receptors (either 5-HT_{1B} and/or 5-HT_{1D}) have been proposed to mediate the contractile response in intracortical blood vessels, as infusion of sumatriptan, albeit at high concentration, elicit CBF reductions (Kobari et al., 1993).

1.4.2.1.2 Relaxation: 5-HT-induced relaxations have been described although infrequently and under certain situations, generally when the vascular tone is raised. In precontracted human and feline arteries, Edvinsson et al. (1978) suggested the involvement of β-adrenoceptors in this response as antagonists to these receptors, like propranolol and 1-N-isopropyl-p-nitrophenyl-ethanolamine, inhibited the 5-HT-mediated vasorelaxation. Subsequent investigations by Auer et al. (1985) suggested that feline cerebrovascular dilatations are elicited by 5-HT_{2A} receptors, as the blood flow increases in small pial arteries (less than 200 μm in diameter) were blocked by ketanserin. Furthermore, there has been some speculation over the past years that 5-HT_{2B} receptors could possibly mediate an endothelium-dependent relaxation in brain vessels; a mechanism that has been suggested as a possible triggering event of migraine headache

(for review, see Fozard and Kalkman, 1994; Fozard, 1996). However, direct evidence of 5-HT_{2B}-mediated vasodilation in human blood vessels is still lacking although only 5-HT_{2B}, and not 5-HT_{2C} mRNA has been shown in porcine meningeal arteries (Schmuck et al., 1996). Finally, 5-HT has been shown to dilate cerebral veins, possibly with the interaction of 5-HT_7 receptors (Ueno et al., 1995).

1.4.2.2 Peripheral blood vessels:

1.4.2.2.1 Contraction: The list of peripheral vascular beds exhibiting contraction in response to 5-HT is long and has been extensively reviewed (Martin, 1994). Like the cerebral circulation, two possible receptors, 5-$HT_{1\text{-like}}$ and 5-HT_{2A} have attracted attention. Of importance to the present discussion is the demonstration in man of the involvement of different types of 5-HT receptors-in this response depending on the vascular segment under study. Indeed, in the umbilical and pulmonary arteries, 5-$HT_{1\text{-like}}$ receptors have been implicated (MacLellan et al., 1989; Templeton et al., 1993) while in the temporal artery this vasomotor response is exerted by 5-HT_{2A} receptors (Tfelt-Hansen and Pedersen, 1992). In the coronary artery and saphenous vein, however, both receptor types appear to make a contribution in this response (Bax et al., 1992; Kaumann et al., 1994; Connor ; Bouchelet et al., 1996b).

1.4.2.2.2 Relaxation: Two types of dilatory responses have to be considered; relaxations that are endothelium-independent (mediated by vascular smooth muscle cells) and those that are endothelium-dependent and require the release of an endothelial factor to act on smooth muscle cells. These receptors have been characterized to a large extent in non-human species.

Endothelium-independent relaxation: Originally, the receptor mediating this effect, almost exclusively seen in veins (i.e. saphenous, jugular and vena cava), was suggested to be a 5-$HT_{1\text{-like}}$ (Feniuk et al., 1983; Martin et al., 1987; Sumner et al., 1989). Later, studies established the rank order of agonist/antagonist potency at this vascular

receptor and showed its positive coupling to cAMP formation, thus convincingly establishing its similarity with the 5-HT_7 receptors, as subsequently also concluded in some arteries (Leung et al., 1996; Cushing et al., 1996; Terron, 1996). In support of these pharmacological data, mRNA for the 5-HT_7 receptor has been found in human peripheral vascular smooth muscle cell cultures (Ullmer et al., 1995; Schoeffter et al., 1996). The possibility that 5-HT_4 receptors may be able to elicit vascular relaxations that are independent of endothelial cells has been advanced in sheep pulmonary arteries (Cocks and Arnold, 1992; Zhang et al., 1995).

Endothelium-dependent relaxation Two receptor subtypes have been associated with this response and they have been identified in venous (jugular, vena cava) as well as arterial (coronary) vascular segments. Both receptor subtypes involve the release of an endothelium-derived relaxing factor, most probably nitric oxide. The first receptor described shared an overall pharmacological profile similar to the 5-HT_{2B} and/or 5-HT_{2C} receptors (Glusa, 1992; Bodelson et al., 1993). The lack of selective pharmacological compounds does not allow to clearly discriminate between these two receptors thus it is impossible to determine which of two receptors is involved in the endothelium-dependent dilatation. However, as mentioned in section 1.4.2.1.2, recent molecular biological data has reported 5-HT_{2B} but not 5-HT_{2C} mRNA in porcine cerebral arteries (Ullmer et al., 1995; Schmuck et al., 1996) thus strongly supporting the 5-HT_{2B} receptor in this response.

Finally, in some isolated blood vessels such as the coronary artery and jugular vein, the receptor mediating relaxation has been shown to possess a pharmacological profile similar to that reported for the 5-HT receptor responsible for the vascular contraction (determined to be 5-HT_{1B}, see section 1.4.2.1.1); a profile totally different from that of the 5-$HT_{2B/2C}$ receptors (Schoeffter and Hoyer, 1990; Gupta, 1992).

1.5 INTRACELLULAR MECHANISMS UNDERLYING VASCULAR CONTRACTION AND RELAXATION

The rise and fall of free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) are the primary mechanisms by which vascular smooth muscles constrict or relax, respectively. The basic mechanisms involved in these vasomotor responses have been well reviewed elsewhere (Somlyo and Somlyo, 1994; Orallo, 1996) and only a brief summary will be provided here (Fig 1.8). At rest, $[Ca^{2+}]i$ is considerably lower within the smooth muscle cells (0.1 μM) than in the extracellular fluid (1-2 mM). To activate the contractile machinery, the level of Ca^{2+} inside the cell has to exceed 1 μM. When this level is reached by various mechanisms (see below), the abundant cytosolic Ca^{2+} binds to the Ca^{2+} binding protein, calmodulin. This calcium-calmodulin complex binds to an inactive myosin light-chain (MLC) kinase and phosphorylates a specific serine residue of the MLC 20 kDa subunit (MLC_{20}). Phosphorylation of this subunit allows the myosin ATPase to be activated by actin and the muscle to contract. When the cytosolic $[Ca^{2+}]$ drops, the calcium-calmodulin complex dissociates and the MLC kinase is inactive, thus allowing dephosphorylation of MLC_{20} by MLC phosphatase which results in relaxation (Fig 1.8, Somlyo and Somlyo, 1994; Orallo, 1996).

1.5.1 CONTRACTION

Two separate and almost independent intracellular pathways have been described as possible mediators of the vascular smooth muscle contraction, namely phosphoinositide (PI) turnover (Fig 1.9) and a reduction in cyclic AMP (cAMP) levels (Fig 1.10), both of which would increase $[Ca^{2+}]i$.

1.5.1.1 PI hydrolysis: Following activation of the Gq protein, phospholipase C (PLC) is activated and PI's are hydrolysed, which results in the generation of two second messengers: inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 then diffuses into the cytosol, binds to an IP_3 receptor located on the sarcoplasmic reticulum which releases Ca^{2+} stores available for binding to calmodulin, as part of the mechanisms

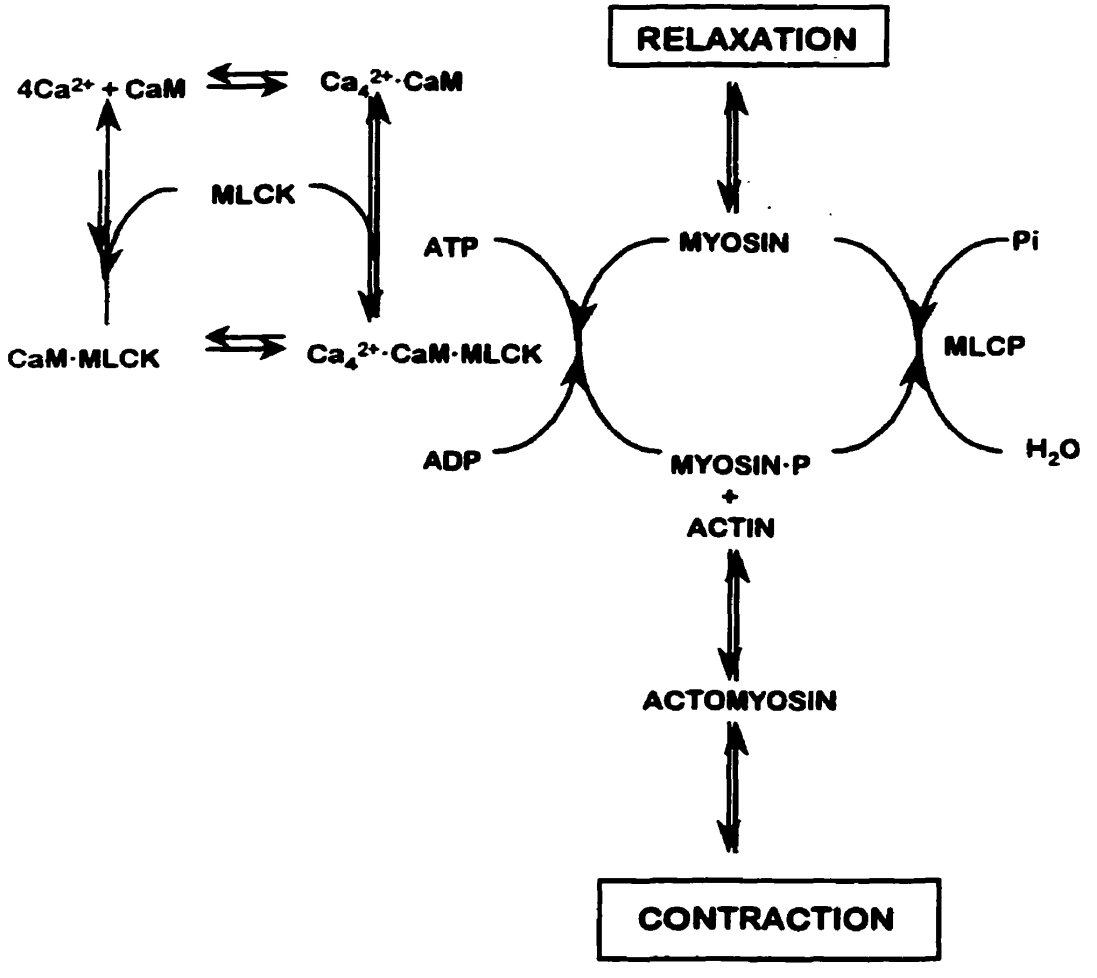

Fig 1.8: Overview of the regulation of smooth muscle contraction-relaxation by myosin phosphorylation-dephosphorylation. Elevations in intracellular Ca^{2+} levels allow the binding of calcium to calmodulin (CaM) forming a complex which can activate myosin light chain kinase (MLCK). The active kinase then phosphorylates a serine residue on myosin light chain (MYOSIN·P) which can subsequently bind to actin filaments and produce a contraction. When the Ca^{2+} levels drop, myosin light chain phosphatase (MLCP) dephosphorylates the MYOSIN·P and results in relaxation. (Adapted from Walsh, 1993)

underlying vascular contraction (see Walsh, 1994, for review). In addition, the DAG molecule activates protein kinase C (PKC) which can phosphorylate proteins and induce contractions independent of $[Ca^{2+}]i$ influx, although the exact mechanisms is presently unknown (Fig 1.9; see Walsh, 1994, for review).

1.5.1.2 cAMP reduction: Under certain circumstances, the increase in $[Ca^{2+}]i$ and subsequent contraction can not be attributed to stimulation of PI turnover but rather to inhibition of adenylate cyclase activity (Motulsky and Michel, 1988; Olivera et al., 1992). The precise mechanism by which the reduction in cAMP produces contraction has not yet been elucidated but may involve membrane depolarization which could lead to activation of voltage-operated Ca^{2+} channels (Fig 1.10; Ebersole et al., 1993). In fact, activation of 5-HT_1 receptors, which are negatively linked to adenylate cyclase activity, increase $[Ca^{2+}]i$ (Ebersole et al., 1993; Sweeney et al., 1995) in bovine pulmonary arteries or vascular smooth muscle cells derived from basilar arteries.

1.5.2 RELAXATION:

Relaxation of vascular smooth muscle essentially results from a decrease in $[Ca^{2+}]i$, this being achieved primarily by two pathways which produce an elevation of intracellular cAMP levels (Fig 1.11). In the first, increases in cAMP levels allow phosphorylation of K^+ channels which leads to hyperpolarization and decreased cytosolic Ca^{2+} influx via a voltage operated calcium channel (Nelson et al., 1990). In the second, cAMP elevations activate not only protein kinase A (PKA) but also protein kinase G (PKG), resulting in phosphorylation of phospholamban, a small protein which regulates the Ca^{2+} pump on the sarcoplasmic reticulum (Fig 1.11; Cornwell et al., 1991). Normally, phospholamban inhibits the entry of Ca^{2+} into the reticulum but when phosphorylated it activates the pump therein causing a decrease in cytosolic Ca^{2+}. It seems that it is PKG that is the physiologically important kinase in this mechanism (Lincoln and Cornwell, 1991). There is also some evidence that PKG can inhibit phospholipase C activation and Ca^{2+} release from IP_3-sensitive storage sites (Walsh et al., 1995; Abdel-Latif, 1996). Furthermore, an

endothelial-derived relaxing factor, thought to be nitric oxide, can also elicit smooth muscle relaxation via a mechanism that involves guanylate cyclase, cGMP elevations and PKG phosphorylation (Fig 1.11; Faraci and Brian, 1994).

A. Phospholipase C Dependent

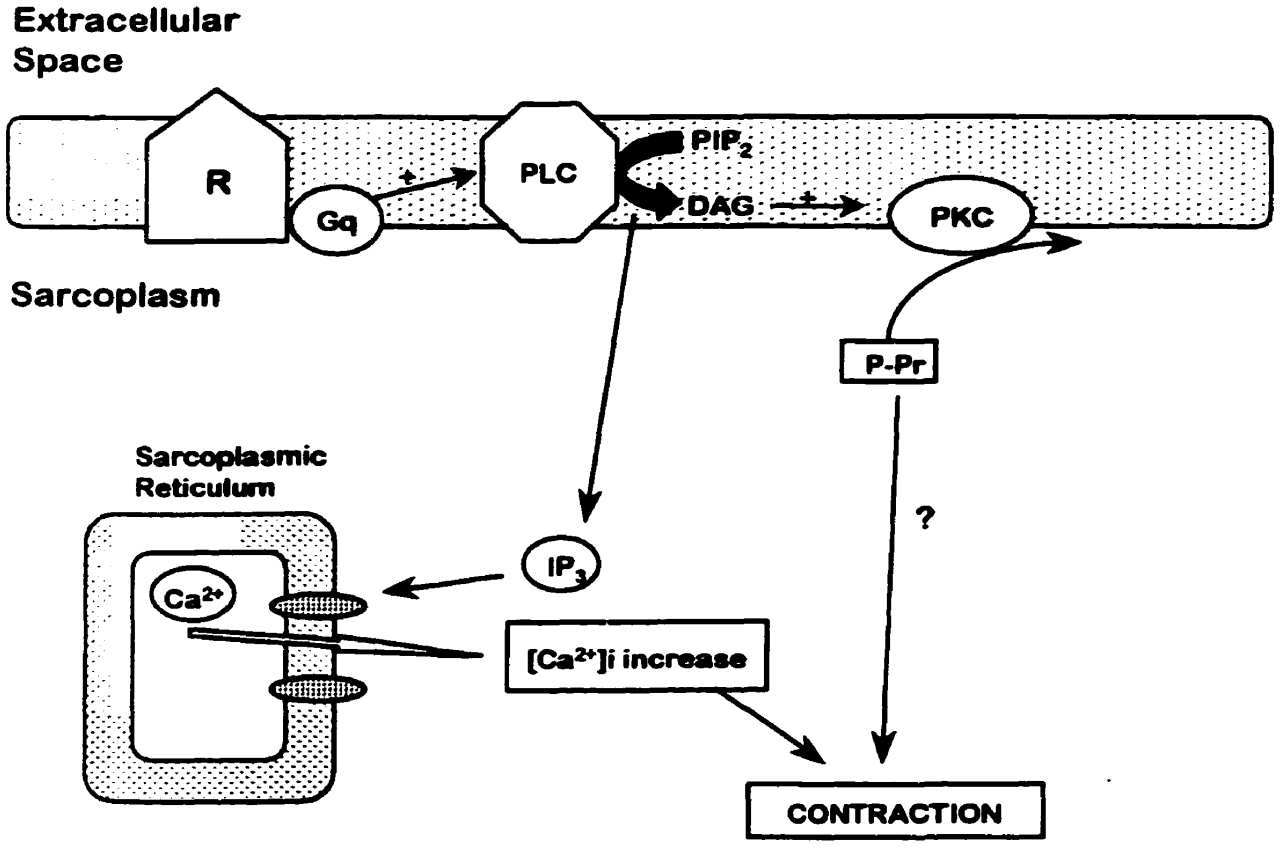

Fig 1.9: Schematic representation of phospholipase C (PLC)-dependent smooth muscle contraction. Binding of an agonist in the extracellular space to a receptor (R) activates a G-protein (perhaps Gq) which can activate PLC. The activated PLC can split phosphatidylinositol 4,5-biphosphate (PIP_2) into two second messengers: inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 diffuses into the sarcoplasm and binds to an IP_3 receptor found on the sarcoplasmic membrane. This triggers an immediate surge in sarcoplasmic Ca^{2+} levels and results in a contraction. The other second messenger, DAG, can activate protein kinase C (PKC) which is thought to enhance Ca^{2+} intracellular levels by a presently unknown mechanism. P-Pr, phosphorylation of a substrate protein.

B. Adenylate Cyclase Dependent

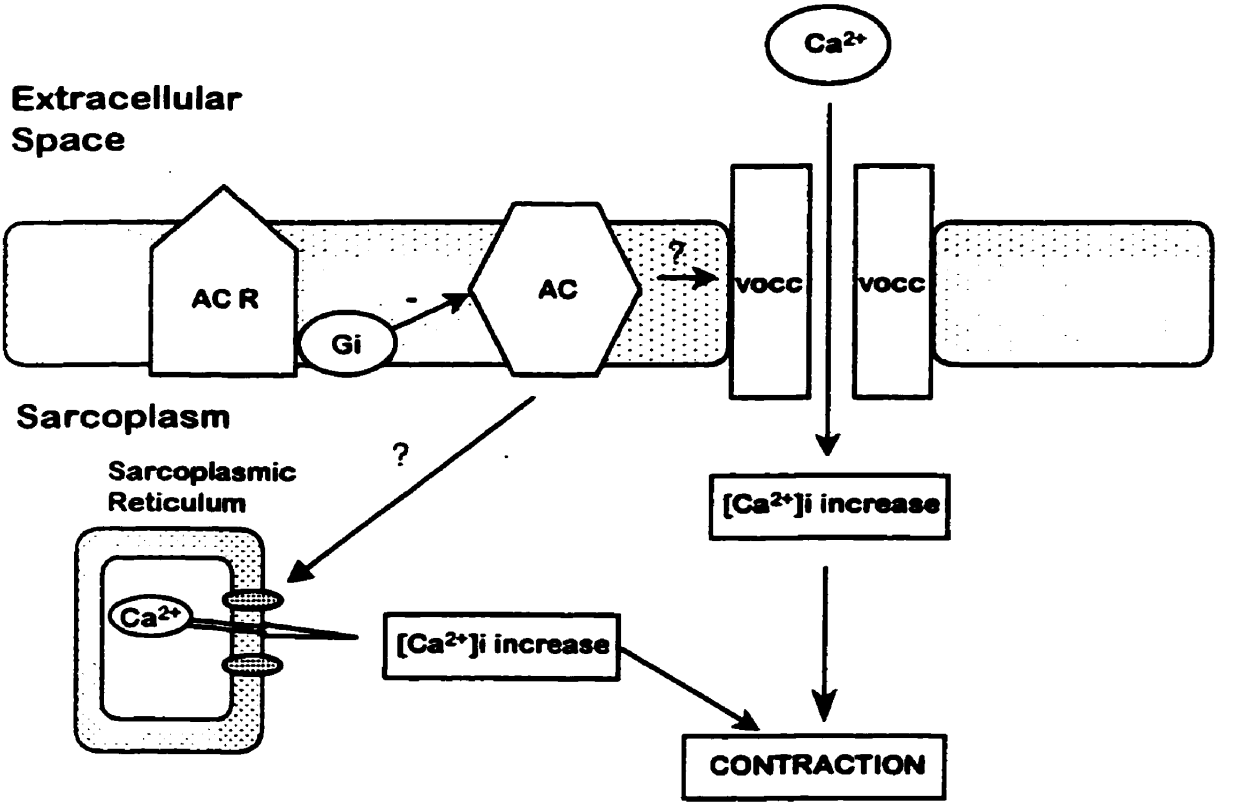

Fig 1.10: Agonists bind to a negatively linked adenylate cyclase (AC) receptor (R) by way of Gi, an inhibitory G-protein, that result in an increase in intracellular Ca^{2+} levels and subsequent contraction. The exact mechanisms by which cAMP reductions induce increases in $[Ca^{2+}]i$ and vascular contractions are not known but may involve the sarcoplasmic reticulum Ca^{2+} stores or voltage-operated Ca^{2+} channels (VOCC).

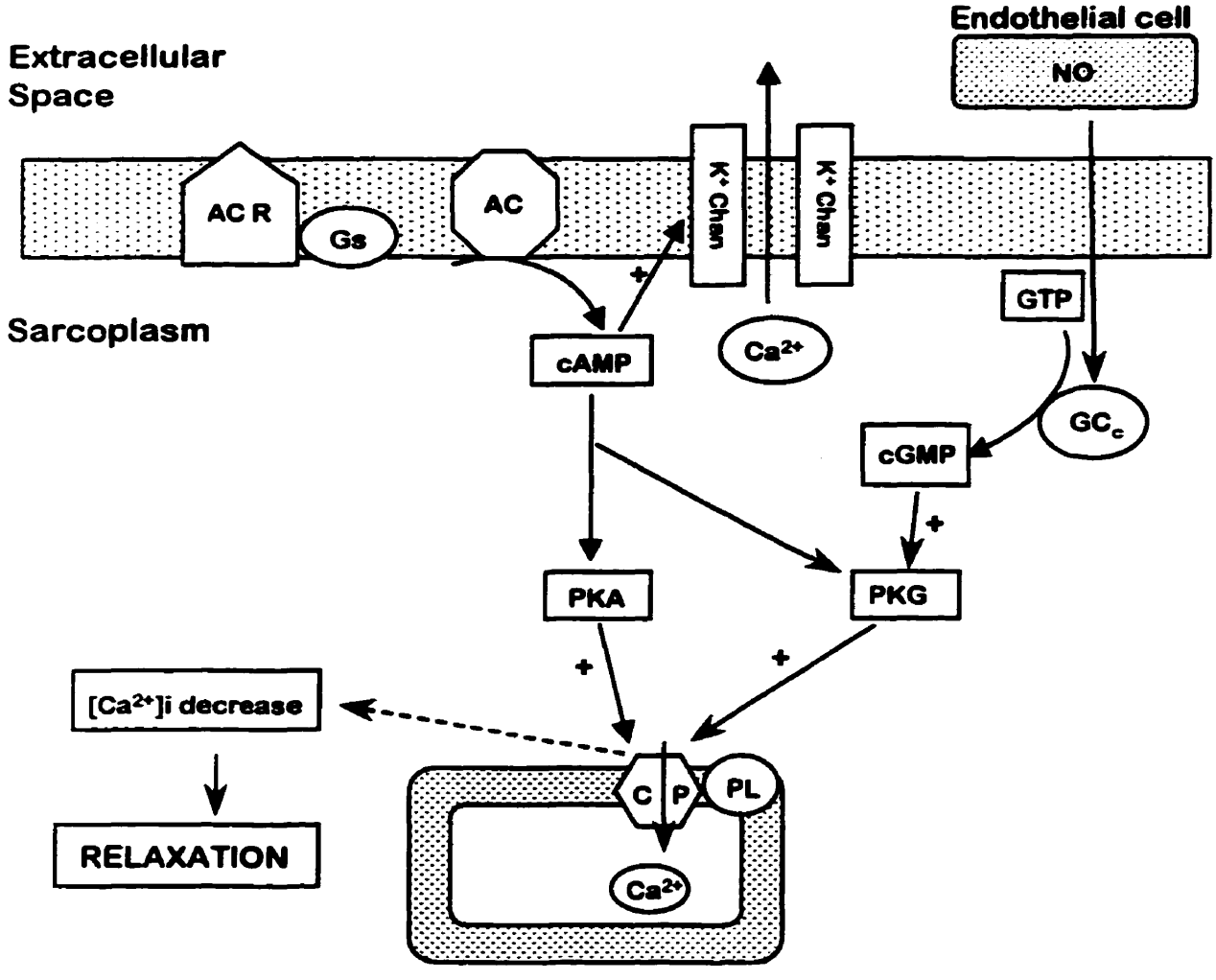

Fig 1.11: Schematic overview of the mechanisms involved in smooth muscle relaxation. Agonists bind to a positively-linked adenylate cyclase (AC) receptor (R) via the stimulatory G-protein, Gs, which increases intracellular cAMP levels. This increase can activate not only protein kinase A (PKA) but also protein kinase G (PKG) which in turn reduces intracellular Ca^{2+} levels, perhaps by phosphorylating phospholamban (PL) which activates the Ca^{2+} pump (CP). Levels of cAMP could also hyperpolarize pottasium channels (K^+ Chan) which results in the exit of Ca^{2+} from the cell. In addition, PKG is also turned on by increases in cGMP which is, most likely, a result of nitric oxide (NO) diffusion from the endothelial cells and interaction with sarcoplasmic guanylyl cyclase (GCc).

1.6 OTHER VASCULAR-RELATED EFFECTS OF 5-HT

1.6.1 BLOOD BRAIN BARRIER PERMEABILITY

As mentionned in section 1.1.2.2, cerebral endothelial cells are endowed with specialized junctions that prevent the free passage of nutrients in and out of the brain. Some agents, which include 5-HT, have been described to affect this process. It has been shown that under certain stressful conditions such as heat and forced swimming exercise in rats, 5-HT increases BBB permeability as evaluated by the passage of Evans Blue and ^{131}[I]-sodium (Sharma et al., 1990; Winkler et al., 1995). This increase in permeability is further blocked by pretreatment of a 5-HT_2 receptor antagonist (Sharma et al., 1990; Winkler et al., 1995).

However, under normal conditions the evidence for a role of 5-HT in modulating the BBB is rather contradictory. Intravenous administration of 5-HT resulted in an increase in vesicular transport of horseradish peroxidase (Westergaard, 1975) and electrical resistance (Olesen, 1985) in mice and frog cerebral microvessels; a response that is inhibited by prior infusion of a 5-HT_2 receptor antagonist. On the other hand, several other reports have shown that intravenous infusion and/or direct superfusion of 5-HT failed to elicit a change in the permeability to Evans Blue and electrical resistance (Hardebo et al., 1981; Gulati et al., 1984; Butt, 1995).

1.6.2 MITOGENESIS

There has been accumulating evidence to suggest that 5-HT functions as a growth factor. During development, high levels of 5-HT are found prior to neurite outgrowth and thus, 5-HT has been suspected to act as a trophic factor (Lauder et al., 1982). In addition, 5-HT enhances DNA synthesis in fibroblasts (Seuwen et al., 1988), promotes cell growth in glial cells (Pauwels et al., 1996) and also increases the expression of the S-100β protein in astrocytes (Haring et al., 1993). With regards to the vasculature, evidence has also been presented that shows a trophic effect of 5-HT on smooth muscle cells. In cerebral vessels, only one study has documented an effect of 5-HT on smooth muscle mitogenesis.

In this report, 5-HT potently increased DNA synthesis, as shown by an augmentation in ^{3}[H]-thymidine incorporation into DNA, in smooth muscle cell cultures harvested from the basilar artery of guinea pigs (Kent et al., 1992). A similar response was observed in smooth muscle cells of rat aorta (Nemecek et al., 1986). Furthermore, in endothelial cells derived from canine and bovine aorta, 5-HT similarly increases DNA synthesis and is thus considered a mitogen for these cells (Pakala et al., 1994). Whether or not the 5-HT mitogenic effect also applies to cells in the microcirculation is still not yet known but based on the peripheral and extracerebral data this may represent an interesting possibility.

CHAPTER 2

OBJECTIVES

OBJECTIVES

The general objective of the present thesis was to investigate the relationships between brainstem serotonergic raphe neurons and cerebral blood vessels located at the surface and over the convexities of the brain (extracerebral), but primarily, those embedded within the brain parenchyma (intracerebral). Three main topics were explored using different experimental approaches. The data obtained in this thesis will hopefully shed novel and important information concerning the anatomical basis on the regulation of the cerebral microcirculation by brain 5-HT neurons.

The **first main objective** was related to the extracerebral circulation and our analysis was twofold. Firstly, based on previous suggestions that brainstem serotonergic neurons could influence cerebral blood flow via interactions with extracerebral arteries, we investigated whether or not 5-HT-synthesizing nerve fibers originating from raphe neurons provide a direct input to major cerebral and pial arteries (Chapter 3). We also tested the possibility that this innervation originated from peripheral structures such as the superior cervical ganglia, as suggested by some investigators. For these experiments, we compared the distribution pattern and density of cerebrovascular nerve fibers immunoreactive for tryptophan hydroxylase (TPH, the synthesizing enzyme for 5-HT) around extracerebral blood vessels from control rats with rats in which the ascending projections from the raphe nucleus had been lesioned with 5,7-dihydroxytryptamine and in rats submitted to a bilateral superior cervical ganglionectomy.

Secondly, on the basis that 5-HT in perivascular nerve fibers is possibly a false neurotransmitter i.e. results from its uptake into sympathetic noradrenergic nerve fibers from the environment, we tested if distinct serotonergic nerve fibers exist. For this purpose, the distribution pattern of the TPH innervation was compared to that of perivascular fibers containing dopamine-β-hydroxylase, an enzyme selective to noradrenaline synthesizing neurons.

In the second portion of my project, we shifted our attention to the microcirculation, in which previous physiological, biochemical and pharmacological studies had indeed suggested direct influence between 5-HT brain pathways and the cerebral microvascular bed. On these assumptions, our **second main objective** was to evaluate, using TPH immunocytochemistry at the electron microscopic level, the possibility that serotonergic terminals establish privileged relationships with the intraparenchymal blood vessels, associations which could have a differential distribution depending on the area. For this purpose, we selected three brain regions known to either to exhibit significant changes (responsive area) or non-significant to no changes (unresponsive area) in local CBF following manipulations of the 5-HT system. The proximity of TPH-immunolabelled nerve terminals to local microarterioles and capillaries was assessed, their relative frequency, as well as their immediate microenvironment and synaptic frequency were established in these area. The frequency and type of associations between 5-HT nerve terminals (TPH-immunoreactive) and intraparenchymal microvessels were examined in the frontoparietal cortex (a responsive area) and compared to those in the hippocampus and entorhinal cortex (two unresponsive areas). The detailed investigations of the 5-HT neurovascular relationships were examined in an attempt to establish the anatomical basis for the reported effects of 5-HT on the microvascular bed (Chapter 4).

In an attempt to evaluate if the pattern of association with the microcirculation seen serotonergic nerve terminals is exclusive or shared by other monoamines (which are known to have widespread projections to the cerebral cortex), we studied the associations of noradrenergic nerve terminals with the microvascular bed of the cerebral cortex. To this end, noradrenaline neurovascular relationships were evaluated in the frontoparietal cortex, a region where the monoamine has minor yet significant blood flow repercussions, and compared to those described in the same area for the neurovascular 5-HT system (Chapter 5). In addition, we verified whether or not this vascular innervation was also of central origin, by means of intraperitoneal *N*-(2-chloroethyl-*N*-ethyl-2-bromobenzylamine) (DSP-4) injections, a neurotoxin which selectively destroys

projections from the locus coeruleus.

Our **third main objective** was an attempt to evaluate if the observed serotonergic neurovascular associations could eventually be functional and allow the microvascular bed to respond to neurally released 5-HT. For this purpose, we tested the hypothesis that brain microvessels harbor specific populations of 5-HT receptors that enable them to mediate the differing vasomotor responses observed following manipulations of serotonergic neurons (Chapter 6). To this end, we used reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate the expression of various 5-HT receptors in human cerebral microvessels and capillaries as well as in cell cultures of human brain microvascular endothelial and smooth muscle cells and astrocytes to pinpoint their cellular localization within the vessel wall. Moreover, using biochemical and pharmacological means, we tested the ability of these identified 5-HT receptors to induce functional responses in microvascular and astroglial cell culture preparations in assessing their coupling to the expected second messenger systems.

CHAPTER 3

CEREBROVASCULAR NERVE FIBERS IMMUNOREACTIVE FOR TRYPTOPHAN-5-HYDROXYLASE IN THE RAT: DISTRIBUTION, PUTATIVE ORIGIN AND COMPARISON WITH SYMPATHETIC NORADRENERGIC NERVES

Zvi Cohen, Gilles Bonvento, Pierre Lacombe, Jacques Seylaz, Eric T MacKenzie and Edith Hamel

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PREFACE TO CHAPTER 3

At the time that I began my thesis work, a considerable amount of information was available concerning the serotonergic innervation of extracerebral blood vessels i.e. major cerebral arteries at the base of the brain and their ramifications as small pial vessels overlying the cerebral cortex. It was well documented that brain vessels were particularly sensitive to 5-HT and the general consensus was that these vessels were richly innervated by serotonergic nerves. However, confusion existed with respect to two facets of this putative 5-HT perivascular innervation, namely whether or not previously reported 5-HT-synthesizing fibers differed from noradrenergic ones and, if so, the origin of these 'distinct' 5-HT nerve fibers.

Early experiments reported reductions in the levels of 5-HT and its major metabolite, 5-hydroxyindoleacetic acid, in major basal arteries and small pial vessels when brainstem raphe nuclei were destroyed either chemically or electrolytically (Chan-Palay, 1976; Napoleone et al., 1982; Edvinsson et al., 1983; Amenta et al., 1985; Scatton et al., 1985). This was considered as a strong evidence for a central serotonergic innervation of brain extracerebral vessels. However, the demonstration by Liuzzi et al. (1977) and Verhofstad et al. (1981) of 5-HT-immunoreactive cells and TPH activity in the superior cervical ganglia had prompted some investigators to hypothesize that this structure could be the origin of 5-HT cerebrovascular nerve fibers (Cowen et al., 1986; Alafaci et al., 1986; Chang et al., 1988a; 1989; Jackowski et al, 1989). They agreeably found that bilateral removal of the superior cervical ganglia resulted in dramatic reductions in 5-HT content and immunoreactivity in cerebral arteries. Other groups had partly reconciled these discrepancies by finding a mixed contribution to the cerebrovascular serotonergic innervation by both the raphe nuclei and the superior cervical ganglia (Marco et al., 1985; Bonvento et al., 1991; Moreno et al., 1995). Nevertheless, some investigators maintained that the presence of 5-HT in perivascular nerve fibers, as evaluated biochemically or immunocytochemically, was due to its uptake into noradrenergic sympathetic fibers where it could act as a false neurotransmitter (Saito and Lee, 1987; Jackowski et al.,

1988; 1989; Yu and Lee, 1989).

On this basis, we undertook different anatomical experimental steps in an attempt to clarify some of these issues. Previously, it had been suggested that a population of "authentic" perivascular serotonergic nerve fibers exist based on the presence of tryptophan hydroxylase (TPH), the rate limiting enzyme in the synthesis of 5-HT, in a population of cerebrovascular fibers (Chédotal and Hamel, 1990). Our first aim was to determine if the distribution of these 5-HT-synthesizing nerve fibers differed from that of sympathetic noradrenergic ones. This was achieved by comparing the distribution patterns of 5-HT-producing cerebrovascular nerve fibers with those synthesizing noradrenaline. For these experiments, animals were anesthetized, their brains fixed by intra-aortic perfusion and the major cerebral arteries and some small pial vessels were carefully dissected out and processed for light microscopic immunocytochemistry using antibodies against TPH and dopamine-β-hydroxylase (DBH, rate limiting enzyme for noradrenaline). The results suggest that the distribution pattern for both limiting enzymes are not exactly superimposable and strongly suggest the presence of a subset of "authentic" perivascular 5-HT-synthesizing nerve fibers.

In the second part of our investigation, we wanted to resolve some of the controversy concerning the origin of this cerebrovascular 5-HT innervation. In this analysis, animals were perfused and processed as above and TPH immunoreactive fibers around extracerebral blood vessels from control rats were compared to those from rats in which the ascending projections from the dorsal and median raphe nuclei were destroyed by 5,7-dihydroxytryptamine or from rats subjected to bilateral removal of the superior cervical ganglia. The results indicated that TPH immunoreactive fibers were dramatically reduced after sympathectomy while they appear unchanged following lesions of the serotonergic ascending pathway. Based on these results, we propose that the cells of origin of perivascular 5-HT fibers most likely arise from the superior cervical ganglion although these cells could not be detected by immunocytochemistry (see Chapter 3, Addendum).

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BRES 18340

Cerebrovascular nerve fibers immunoreactive for tryptophan-5-hydroxylase in the rat: distribution, putative origin and comparison with sympathetic noradrenergic nerves

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Key words: Cerebral blood vessel; Cerebrovascular innervation; Immunocytochemistry; Noradrenaline; Serotonin; Superior cervical ganglion; Raphe nucleus; Rat; Tryptophan-5-hydroxylase

The distribution of serotonergic nerves in major basal and isolated small pial arteries (diameter ≥ 50 μm) was investigated immunohistochemically using an antibody directed against tryptophan-5-hydroxylase (TPOH), the rate-limiting enzyme in the synthesis of 5-hydroxytryptamine (5-HT or serotonin), and compared to that of the noradrenergic system labeled for the selective noradrenaline (NA) synthesizing enzyme, dopamine-β-hydroxylase (DBH). In addition, the possible peripheral and/or central origins of the cerebrovascular serotonergic (TPOH-positive) nerve fibers were examined. Strongly labeled TPOH-immunoreactive (TPOH-I) fiber bundles were observed in major basal arteries and gave rise to small varicose fibers organized in a meshwork pattern. The highest density of TPOH-I fibers was found in the middle cerebral artery followed by the anterior cerebral and the anterior communicating arteries, with a moderate to low density in the internal carotid and the vertebro-basilar trunk. Of the isolated pial arteries, only the larger ones (diameter > 75 μm) were significantly endowed with TPOH-I varicose fibers. However, free floating TPOH-I nerves were observed coursing through the pia-arachnoid membranes and reaching small pial vessels. In contrast, DBH-I nerve fibers were fine and were visualized primarily as numerous varicosities distributed in a circumferential manner around the vessel wall. A very high density of DBH-I varicosities was seen in the rostral part of the circle of Willis, with the internal carotid being the most richly supplied followed by the anterior cerebral and the anterior communicating arteries; comparatively, the middle cerebral artery was moderately innervated. The differences in distribution pattern and density between TPOH-I and DBH-I cerebrovascular fibers clearly suggest that these two innervation systems are not exactly superimposable. Superior cervical ganglionectomy caused an almost complete disappearance of TPOH-I nerves in all vascular segments, with some residual fibers in selected vessels. Lesion of the central serotonergic component with the neurotoxin 5,7-dihydroxytryptamine had virtually no effect on the TPOH-I fibers in the major basal and isolated pial arteries. These results strongly suggest that the serotonergic innervation of major cerebral as well as pial arteries has a prominent peripheral origin closely related to the sympathetic system. Processing of superior cervical ganglion slices for TPOH immunocytochemistry, however, failed to unequivocally detect TPOH-I neurons.

INTRODUCTION

A role for 5-hydroxytryptamine (5-HT, serotonin) in the control of brain perfusion has been widely discussed (for review, see refs. 40, 62, 63) and, more specifically, 5-HT has been implicated in cerebrovascular dysfunctions such as vasospasm and migraine[27,28]. Anatomical studies on cerebral arteries and intraparenchymal microvessels[15] originally demonstrated a rich network of nerve fibers which contained 5-HT-like immunoreactivity[26,29,30]. However, recent evidence suggests that noradrenergic sympathetic nerves are the true site of 5-HT localization following its uptake from platelets, perivascular space, endothelial cells or cerebrospinal fluid (CSF, see refs. 2,13,64). The 5-HT uptake process appears to be distinct from that of noradrenaline (NA)[14].

On the other hand, biochemical studies have detected substantial levels of 5-HT in various segments of the cerebral vasculature[26,38,41] or microvasculature[49] and showed that cerebral vessels are endowed with all the metabolic machinery required for the synthesis,

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storage and release of 5-HT[8,53]. These biochemical data strongly argue in favor of fibers not only able to accumulate but also to synthesize the indoleamine. This hypothesis has been corroborated by our recent immunocytochemical detection of tryptophan-5-hydroxylase (TPOH), the synthesizing enzyme for 5-HT, in nerve fibers surrounding major cerebral and small pial arteries[16].

It has been proposed that 5-HT coexists with NA in cerebrovascular sympathetic nerves originating from the superior cervical ganglion (SCG)[12,13,18,35,36,52]. Alternatively, some studies suggested that 5-HT-containing cerebrovascular nerves are distributed differently from noradrenergic ones[18,23] or that only a subpopulation (approximately 25%) of NA-containing nerves also show 5-HT-like immunoreactivity[35]. Using chromatographic detection, superior cervical ganglionectomy was shown to deplete the NA content of major basal arteries and small pial vessels while selectively decreasing 5-HT levels[8,41] in major arteries. Destruction of brain ascending serotonergic pathways originating from the rostral raphe nuclei, however, resulted in substantial decreases in 5-HT concentrations in both major and small pial vessels[8,26]. It was concluded that the serotonergic innervation of small pial vessels preferentially originated from brain centers whereas that of major basal arteries had a mixed peripheral and central origin[8,41]. The observed changes in cerebral blood flow following stimulation or lesion of the raphe nuclei[6,42,43,59] would support innervation at the arteriolar and microvascular level. However, others have even reported that the serotonergic innervation of the middle cerebral artery mainly originates from the raphe nucleus[44,57].

Overall, there remains little doubt that serotonin is an endogenous component of cerebrovascular nerves and that some nerve fibers can synthesize and release the vasoactive indoleamine. Where confusion remains is regarding the peripheral and/or central origin of serotonergic nerves in major cerebral and small pial arteries and whether or not these nerves correspond to noradrenergic sympathetic ones. In an attempt to address part of these questions, we have used an immunohistochemical approach and compared the distribution of cerebrovascular nerves containing the two most selective enzymes for the synthesis of NA and 5-HT (namely dopamine-β-hydroxylase (DBH), and TPOH). Studies were performed in control rats as well as in rats subjected to superior cervical ganglionectomy or 5,7-dihydroxytryptamine (5,7-DHT) lesions of the ascending serotonergic pathways that originate in the raphe nuclei. Parts of these results have appeared in abstract forms[17,32].

MATERIALS AND METHODS

Surgical procedures

Wistar and Sprague–Dawley adult rats (weight: 300–400 g) were used in this study. All rats undergoing surgery were deeply anesthetized with sodium pentobarbital (Somnotol 65 mg/kg, i.p.).

Superior cervical ganglionectomy. The superior cervical ganglia were removed bilaterally in twelve animals but a small group ($n = 4$) had unilateral surgery. For sham-operated controls ($n = 8$), the superior cervical ganglia were exposed and left intact.

Lesion of ascending serotonergic fibers. Neurotoxic lesions with 5,7-dihydroxytryptamine (5,7-DHT, creatinine salt, Sigma Chemical Co., St. Louis, MO, USA) were carried out as previously described[8]. In short, the rats ($n = 9$) were anesthetized as above, placed in a stereotaxic frame (David Kopf) and received an injection of 2 μl of 5,7-DHT (in a solution at 2 μg/μl in 0.1% ascorbic acid in saline) into the ventral tegmental area medially (including the caudal linear nucleus of the raphe) (A +2.2; L 0; H +2.8 mm, according to the atlas of Paxinos and Watson[48]), at an infusion rate of 0.5 μl/min with a lateral angle of 4° to avoid the superior sagittal sinus. This protocol produces a selective lesion of serotonin-containing fibers without pretreatment with catecholamine uptake blockers[3,5]. Sham-operated animals ($n = 5$) were infused with 2 μl of vehicle alone.

Immunocytochemistry

Tissue preparation. Seven days after ganglionectomy or 5,7-DHT lesion, the rats were anesthetized with sodium pentobarbital (see above). They were then perfused through the ascending aorta with 100 ml of cold sodium phosphate buffer (0.1 M, pH 7.4) containing 20 mM $MgCl_2$ followed by 500 ml of 4% paraformaldehyde in the same buffer containing 15% picric acid, at 4°C over a period of 10 min. The brains were rapidly removed and post-fixed by immersion for 2 h in the same solution at room temperature. Following this period, major cerebral arteries at the base of the brain were dissected from the arachnoid membrane in one block which comprises the vertebral (VA), basilar (BA), superior cerebellar (SCeA), posterior cerebral (PA), posterior communicating (PCoA), internal carotid (ICA), middle cerebral (MCA), anterior communicating (ACoA) and anterior cerebral (ACA) arteries, as defined by Brown[10]. Isolated pial arteries (diameter ≥ 50 μm) were also carefully dissected free from the pia-arachnoid membrane (generally 20–50 small arterial segments were obtained). In some cases, the pia-arachnoid membranes were processed as such for immunocytochemistry. All vessels were washed for a period of 1-2 h in phosphate buffer.

In control animals, the pineal gland and superior cervical ganglia were removed, immersion-fixed for 2 h and washed in phosphate buffer. In those rats which underwent intracerebral injections of 5,7-DHT or vehicle, a block of neocortex was excised (a medium-high innervation by serotonergic nerves has been found at this level in layer I, with a more sparse innervation in subjacent layers[55]). These cortical tissues, superior cervical ganglia and pineal glands were stored overnight in 0.1 M phosphate-buffered saline (PBS) containing 10% sucrose followed by a second overnight storage in 30% sucrose in PBS. After this treatment, the tissues were frozen (−45°C in isopentane) and 30- to 40-μm-thick sections were cut on a freezing microtome and collected in 0.1 M sodium phosphate buffer (pH 7.4), for subsequent use in immunocytochemistry.

Antisera. The TPOH antiserum (a generous gift from Drs. D. Weissmann and M. Maître, France) was raised in a sheep against tryptophan-5-hydroxylase purified to electrophoretic homogeneity from whole rat brain[11]. The dopamine-β-hydroxylase (DBH) antibody was raised in rabbit against DBH purified from bovine adrenal (Eugene Tech International Inc., Allendale, NJ, USA). The tyrosine hydroxylase (TH) antiserum (kindly donated to us by Dr. J.F. Reinhard Jr., The Wellcome Research Laboratories, USA) was raised in a rabbit against TH purified from rat clonal pheochromocytoma (PC 12) cells[45].

Specificity of antisera. TPOH: it was previously shown that a dilution (1/500) of TPOH antiserum totally inhibited a crude preparation of TPOH enzyme over a 2 h incubation at room temperature and that electroblots carried out after gel electrophoresis of brain or raphe

dorsalis supernatants and of partially purified enzyme exhibited staining in a single band[11]. In addition, a complete immunocytochemical mapping in the rat brain using this TPOH antibody was found to selectively and specifically match the anatomical distribution of serotonin-immunoreactive nerve cell bodies, proximal dendrites and axon varicosities labeled with a 5-HT antiserum[61], thereby confirming the specificity of the antiserum in the CNS. To attest for the specificity of the TPOH antibody in the peripheral nervous system (PNS), due to the high degree of homology between the amino acid sequence of TH and TPOH[20], we performed further immunocytochemical experiments in the rat pineal gland, a structure which receives a rich sympathetic noradrenergic innervation[9,37]. As in the CNS, we found no cross-reactivity of the TPOH-antiserum with TH. Densely labeled TH-I nerves were observed in the pineal gland while adjacent sections were totally devoid of TPOH-I staining (data not shown). Therefore, under the conditions used in the present study, the TPOH antiserum has no immunocytochemically detectable cross-reactivity with TH nerve terminals in the PNS. The immunohistochemical specificity of the TPOH antiserum was also tested by replacement with normal non-immune serum, a treatment which resulted in abolition of immunostaining. Omission of the primary or secondary antibody in the respective incubation step also resulted in no staining. *DBH*: specificity of this antiserum for DBH has been shown previously in both rat CNS and perivascular systems (for references, see[58]). *TH*: specificity of this antiserum for TH has recently been described[45].

Immunostaining. Except for the vertebro-basilar trunk, one side of the cerebral arterial block of each rat was processed for TPOH-I while the other side was used for DBH-labeling. Immunohistochemical procedures were performed on free floating vessels (or tissue sections) according to protocols previously described[16,61]. Briefly, the tissues were rinsed extensively in buffer and incubated in PBS-containing bovine serum albumin (BSA, 1%, 30 min, TPOH or 2%, 2×15 min, DBH and TH) and normal goat serum (NGS, 3.3%, 30 min, DBH and TH). Then, the tissues were incubated at 4°C for 18–20 h in PBS containing the antiserum (1/1,000, TPOH and DBH; 1/2,000, TH), 0.1% Triton X-100 and 1% normal rabbit serum (NRS; TPOH) or 2% NGS (DBH, TH). Following a rinse in PBS-NRS (3×15 min, TPOH) or PBS-NGS (2×8 min, DBH, TH), the tissues were incubated with the second antibody as follows: rabbit anti-sheep (1/250, 90 min, TPOH) or goat anti-rabbit (1/50, 30 min, DBH), rinsed and incubated with goat (1/800, 45 min, TPOH) or rabbit (1/50, 30 min, DBH) peroxidase anti-peroxidase (PAP) complex in 0.1 M PBS. The peroxidase was revealed by 0.05% 3,3-(±)-diaminobenzidine (DAB) in 100 mM Tris-HCl (pH 7.4) containing 0.01% H_2O_2 for 15 min. In SCG and pineal sections, biotinylated secondary antibodies (rabbit anti-sheep (TPOH) or goat anti-rabbit (DBH, TH)) immunoglobulin G antibodies (45 min, 1/200, Vector Laboratories Inc., Burlingame, CA) and avidin–biotinylated peroxidase complex (ABC reagent, 1/100, Vector) were conjugated to the primary antibodies at room temperature (1–2 h). Immunolabeled peroxidase was visualized by incubation with DAB (see above) for 5–10 min (DBH, TH and TPOH).

Analysis of the immunostaining. All tissues were washed in 0.1 M phosphate buffer, mounted on gelatin-coated slides, dehydrated, defatted and coverslipped. The slides were examined and eventually photographed under a Leitz Aristoplan light microscope. Evaluation of the innervation pattern of TPOH- and DBH-immunoreactive fibers was performed by four observers independent of each other. The effects of the lesions were analyzed in each animal, photographed and evaluated in terms of density of nerve fibers and intensity of immunolabeling.

RESULTS

Comparative distribution of TPOH-I and DBH-I cerebrovascular nerves

In control rats, TPOH-I and DBH-I nerve fibers were both more numerous in the rostral as opposed to the caudal brain circulation. Strongly labeled TPOH-I longitudinally-oriented fiber bundles of small–medium

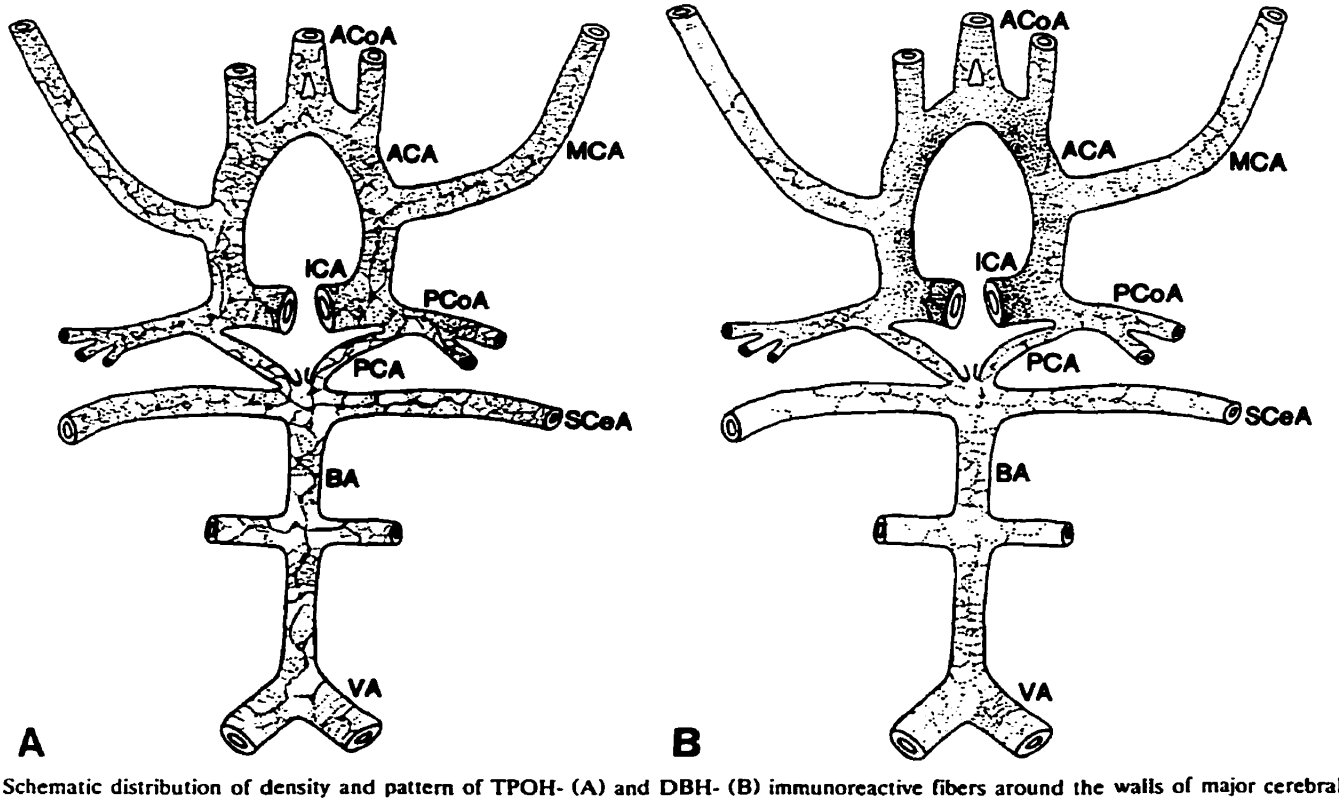

Fig. 1. Schematic distribution of density and pattern of TPOH- (A) and DBH- (B) immunoreactive fibers around the walls of major cerebral arteries. For abbreviations, see details in Materials and Methods.

caliber were evident in most vascular segments and were more numerous in vessels forming the circle of Willis. The bundles give rise to small, single and varicose TPOH-I fibers which were primarily organized in a meshwork (grid-like) pattern (Figs. 1 and 2). Few longitudinal fiber bundles were DBH-I while numerous, mostly circumferentially (spirally) oriented, bead-like varicosities were very strongly labeled by DBH antiserum (Figs. 1 and 2).

The TPOH-I cerebrovascular fibers were relatively

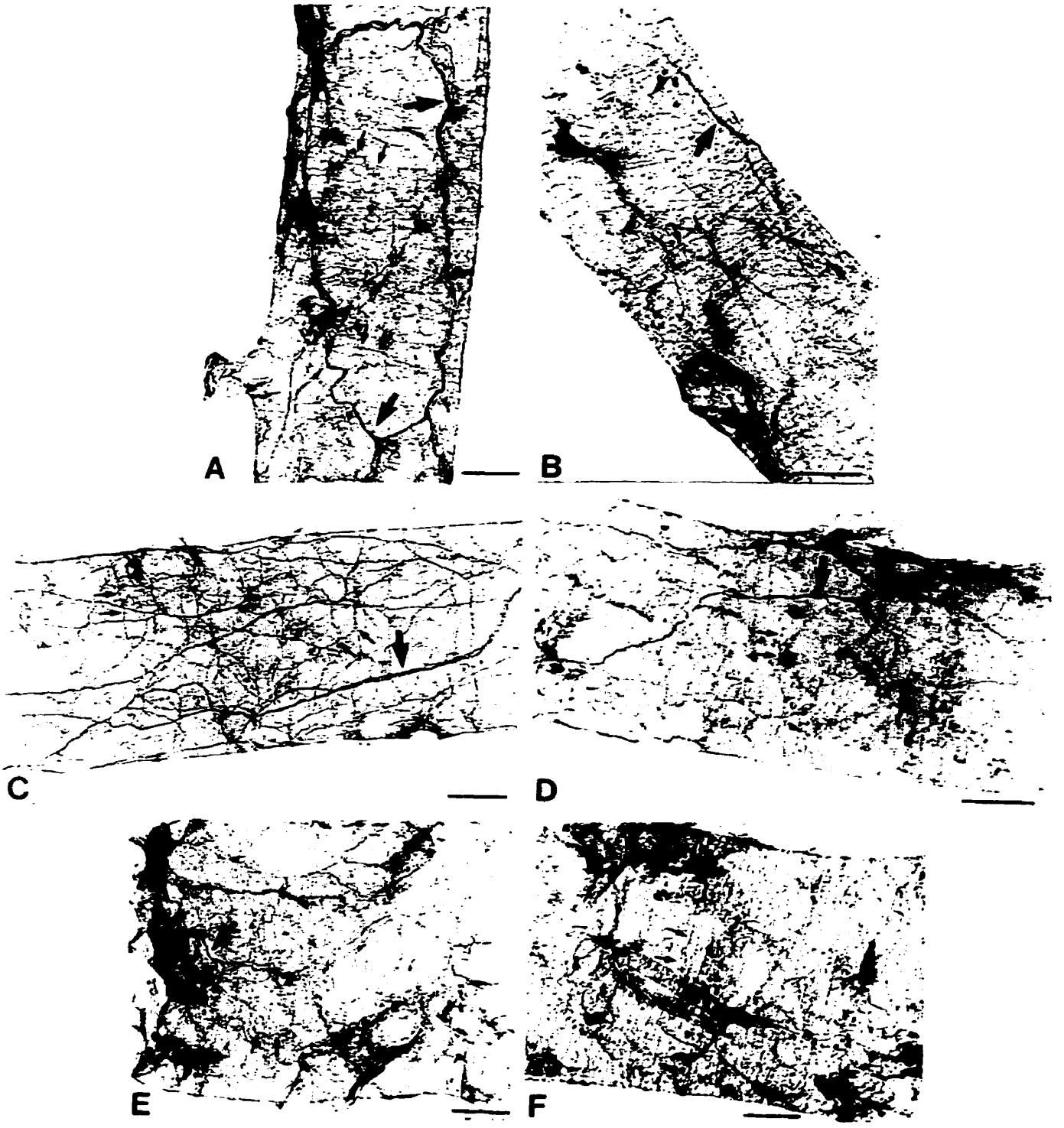

Fig. 2. Photomicrographs of TPOH- (left) and DBH-I (right) fibers in some vessels of the rostral part of the circle of Willis. The density of DBH-I fibers is highest in the ICA (F), followed by the ACA (B) and moderate in the MCA (D). Overall, DBH-I fibers have a primary circumferential orientation. In contrast, TPOH-I fibers are very abundant in the MCA (C), as compared to the ICA (E) and ACA (A), and are predominantly orientated in a meshwork pattern. Major fiber bundles (large arrows) and small varicosities (small arrows) are indicated. Bars = 100 μm.

dense in the anterior and posterior parts of the ACA and the ACoA with fewer fibers in the ICA (Fig. 2). In the ICA and, in some parts of the ACA, the distribution of TPOH-I nerves was essentially circumferential with intermingled fibers forming a grid-like pattern (Fig. 2). The MCAs were invested by numerous fine TPOH-I fibers organized in a meshwork pattern with their distal segments being particularly richly innervated (Figs. 1 and 2). A similar nerve distribution was noted in vessels such as the PCoA, PCA and SCeA (Fig. 2). It is noteworthy that the ACA occasionally exhibited a density of innervation which compared well with that of the MCA (Fig. 2) although, usually, the amount of TPOH-I fibers was substantially greater in the MCA as compared to the ACA, as illustrated in the distribution diagram (Fig. 1). In the vertebro-basilar trunk, one or two TPOH-I large fiber bundles were observed which give rise to single, small varicose fibers (Fig. 1). The BA, VA and SCeA were moderately invested with TPOH-I nerves (Fig. 1).

In pial arteries individually isolated from the pia-arachnoid membrane, numerous small, unique varicose fibers with a grid-like distribution were observed in arterioles of various caliber down to approximately 75 μm (Fig. 3). The density of TPOH innervation gradually decreased with the size of the vessels with the

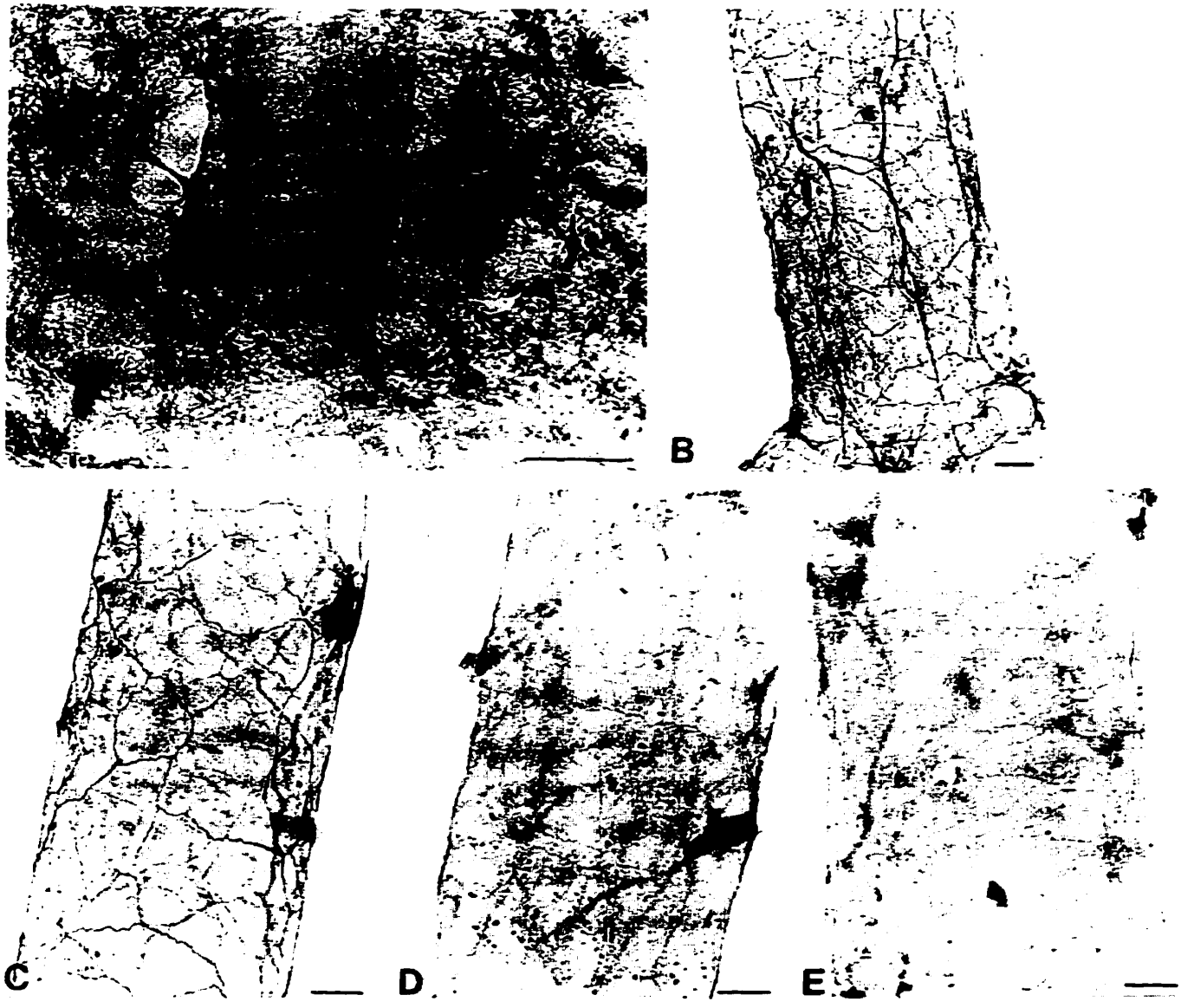

Fig. 3. Photomicrographs of TPOH-I fibers in the pia-arachnoid overlying the cerebral cortex (A), and of DBH-I (B) and TPOH-I (C) fibers around small pial arteries. A: note the varicose fibers running free in the membrane (black arrows) or above small pial vessels (open arrows). In small pial arteries, a distribution in a meshwork pattern was observed for both DBH- and TPOH-I nerves (B and C, respectively). Bilateral superior cervical ganglionectomy resulted in a near loss of TPOH- (D) as well as DBH-I (E) fibers in small pial vessels 7 days following surgery. Bars = 50 μm.

smallest ones being devoid of TPOH-I fibers. Single varicose TPOH-I fibers were occasionally seen to run in the pia-arachnoid membrane in the vicinity of small pial vessels. These fibers seem to course in the membrane and, en route, could be associated with vascular elements (Fig. 3).

The distribution of DBH-I staining in the major cerebral arteries was confined to small varicosities with a primary circumferential (or spiral) orientation. The staining was particularly dense in the ACA, ACoA and, even more so in the ICA. (Figs. 1 and 2). Overall, the DBH-I nerve terminals were more numerous and intense than those immunoreactive for TPOH. The density of innervation of DBH-I fibers in the MCA was moderate to high and resembled the meshwork distribution observed in the vertebro-basilar segment as well as that described above for TPOH-I nerves (Fig. 2). Isolated pial arteries were also endowed with DBH-I fibers and, as with TPOH-I nerves, the density of innervation decreased with the decreasing size of the

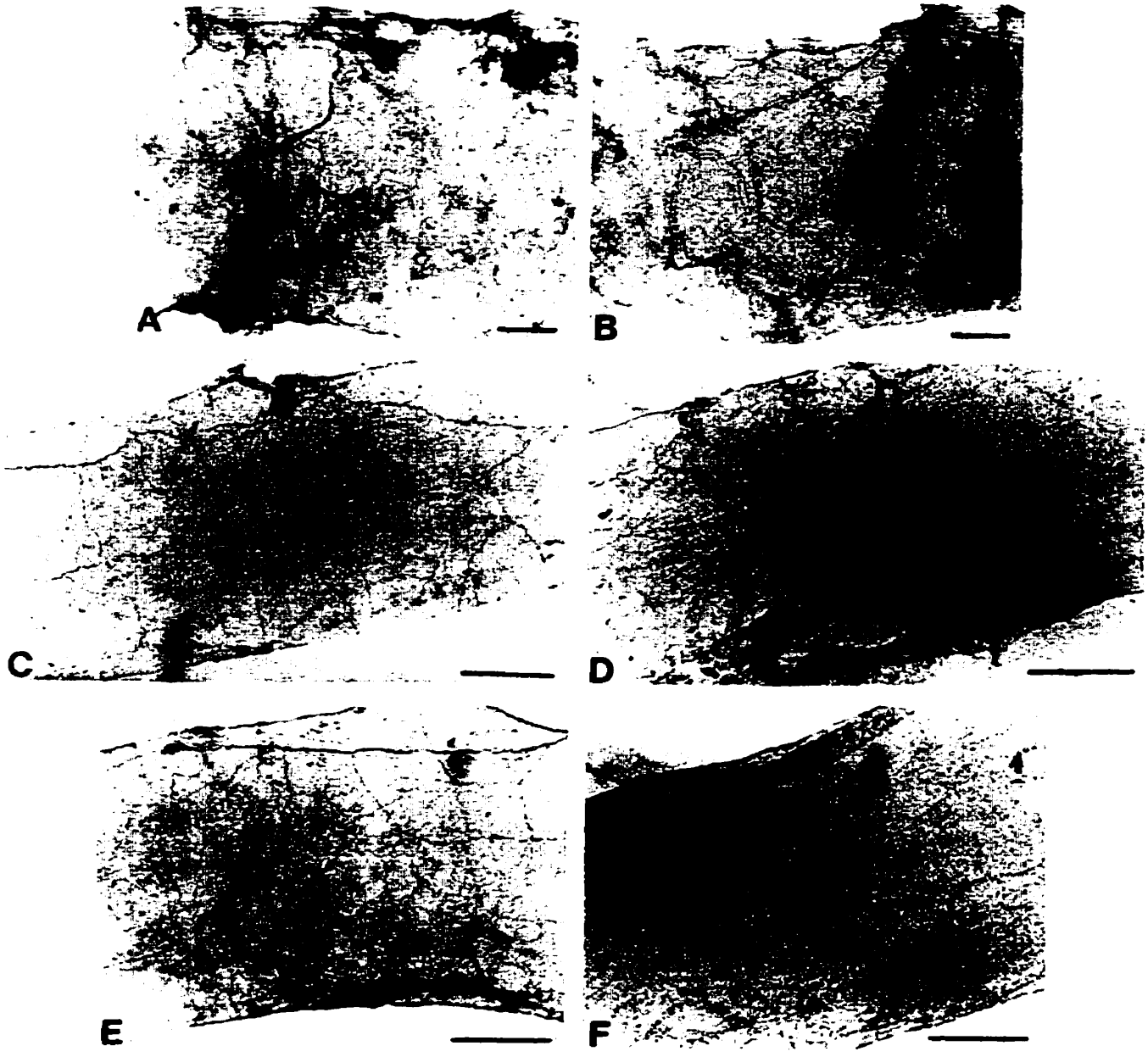

Fig. 4. Effect of superior cervical ganglionectomy on TPOH- (left) and DBH- (right) immunoreactive fibers around major cerebral arteries. Most arteries were completely devoid of immunoreactive staining as shown here in the ICA (A,B), while occasionally residual fibers remain in selective segments such as the SCeA for TPOH-I nerves (C vs. D) or in the VA for DBH-I fibers (E vs. F). Bars = 100 μm.

vessels. The distribution pattern of DBH-I fibers in ramification vessels and small pial arteries was best described as a meshwork pattern (Fig. 3).

Effects of superior cervical ganglionectomy

Unilateral superior cervical ganglionectomy resulted in an ipsilateral decrease of TPOH-I and DBH-I fibers in all segments of the cerebrovascular bed excluding somehow the vertebro-basilar segment and parts of the ACA (data not shown). Bilateral ganglionectomy caused a disappearance of almost all TPOH-I and DBH-I nerves in major basal as well as isolated pial arteries (Figs. 3 and 4). A small number of TPOH-I fibers occasionally persisted in distal segments of the MCA, PCA and SCeA (Fig. 4), although the labeling intensity was weak. Such residual fibers were not consistently observed for DBH-I. Some DBH-I fibers were still present in the VA after bilateral surgery, while TPOH-I fibers in this arterial segment were rarely observed (Fig. 4).

Effects of ascending serotonergic pathway lesion

Lesions of the ascending serotonergic pathways with 5,7-DHT failed to significantly alter the overall distribution of TPOH-I cerebrovascular fibers despite a considerable decrease in the density of cortical TPOH-I nerve terminals as compared to sham-operated controls (data not shown). The rats exhibited no apparent modification in neither the density nor the intensity of TPOH-I fibers in major basal arteries. Similarly, the amount and the labeling intensity of TPOH-I fibers were found not to be affected in isolated pial arteries

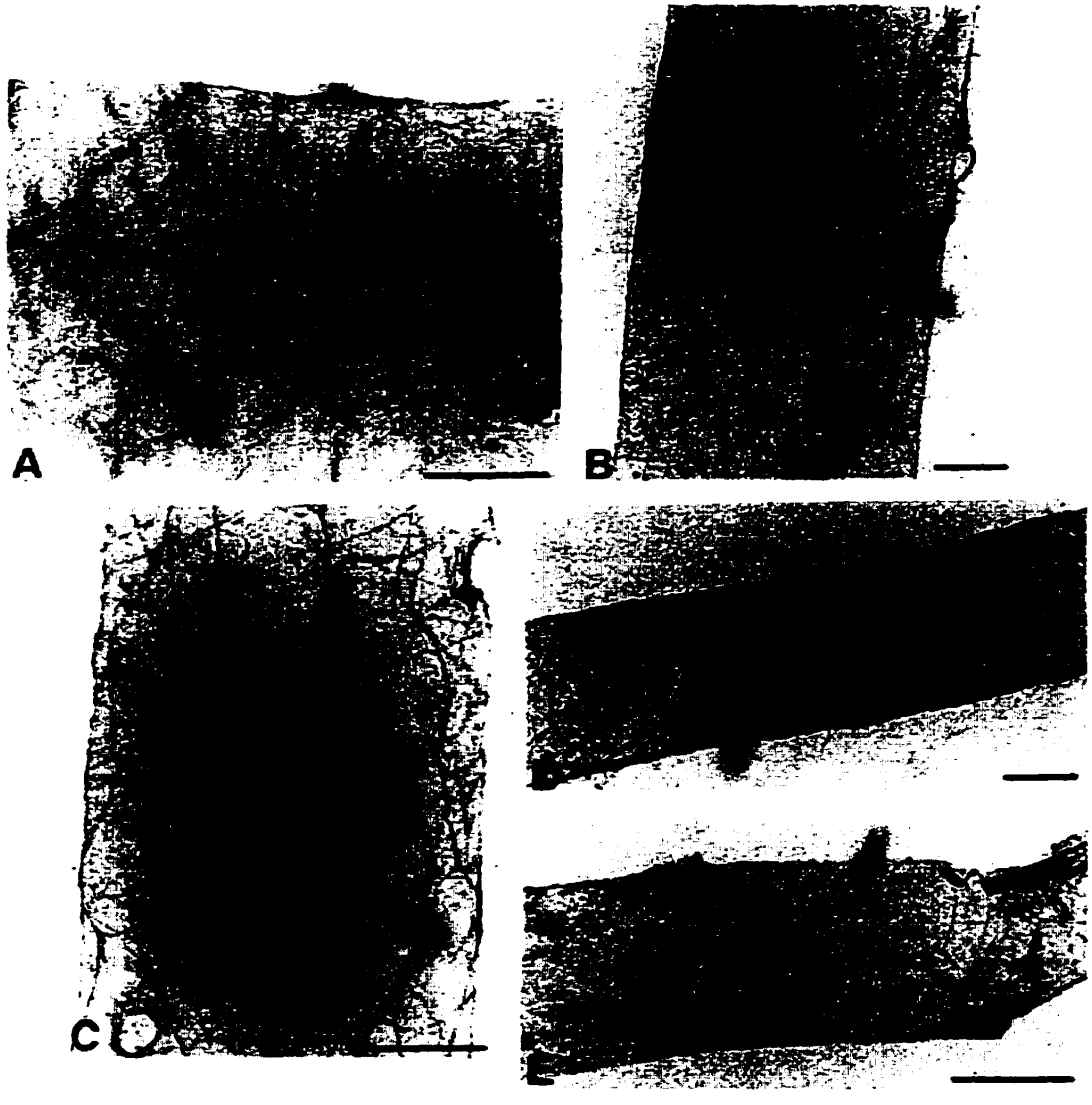

Fig. 5. Photomicrographs illustrating the effects of 5,7-DHT injection on TPOH-I nerves around major cerebral arteries such as the ICA (A), BA (B), and MCA (C) or small pial vessels (D,E). TPOH positive fiber bundles (large arrow) and small varicosities (small arrow) can still be clearly seen. Bars = 100 μm.

Fig. 6. Photomicrographs showing DBH- (A), TH- (B), and TPOH-I (C) in the superior cervical ganglion. No TPOH-immunoreactive cell bodies could be detected whereas several ganglion neurons were strongly immunoreactive for DBH and TH. Bars = 100 μm.

following neurotoxic destruction of central serotonergic pathways (Fig. 5).

Distribution of TPOH-I and DBH-I cells in the superior cervical ganglion

Consecutive sections of superior cervical ganglia were processed for DBH-, TH- and TPOH-immunoreactivity. Numerous cell bodies were positive for DBH and TH with some cells being moderately and others very intensely stained (Fig. 6). In contrast, in adjacent sections processed with the TPOH antiserum we failed to clearly detect any TPOH-I cells whether they were principal or small ganglionic neurons (Fig. 6) and whether the PAP or ABC procedure was used (see Materials and Methods).

DISCUSSION

Distribution of TPOH-I nerves

Comparison of the distribution of TPOH-I cerebrovascular nerves with that of noradrenergic (DBH) sympathetic ones revealed distinct characteristics between the two systems. Firstly, TPOH-I nerves were less numerous than DBH-I ones in the arteries forming the circle of Willis and, more strikingly in the ICA. Secondly, as opposed to the noradrenergic system, arteries located beyond the circle of Willis such as the MCA, PCoA and SCeA contained as much or proportionally more TPOH-I nerves and varicosities than those contributing directly to this vascular formation. Thirdly, the TPOH-I nerves were preferentially distributed in a meshwork or grid-like pattern around the vessel wall whereas those of the sympathetic noradrenergic system had a primary spiral (or circumferential) orientation, as reported previously in several studies (for references, see ref. 58). Finally, TPOH-I and DBH-I cerebrovascular nerves have distinct patterns and densities of innervation in the major basal arteries. However, similarities were also noted, the most obvious being the higher concentration of TPOH-I and DBH-I nerves in the rostral as opposed to the caudal part of the cerebrovascular bed. In addition, the distribution patterns of TPOH-I and DBH-I nerves in arteries such as the ICA, MCA and small pial vessels were not unequivocally distinguishable.

Altogether, these differences clearly point to the existence of a network of authentic serotonin synthesizing fibers and further indicate that uptake of 5-HT into sympathetic noradrenergic nerves of the cerebral blood vessels can not exclusively account for the presence of 5-HT in this tissue. In this respect, our findings are substantiated by recent biochemical data which clearly demonstrated de novo synthesis of 5-HT in rat cerebral arteries and pial vessels[8] and specific re-uptake mechanisms for 5-HT in cerebrovascular nerves[14].

In the isolated pial arteries, both TPOH-I and DBH-I nerves exhibited a progressively decreasing gradient of innervation with the decreasing size of the vessels; the most distal vascular segments (≈ 50–70 μm) showed no innervation. A direct comparison between TPOH-I and DBH-I fibers at this level of the cerebrovascular bed was not undertaken due to the difficulty in unequivocal identification of the vessels. Thus, both the serotonergic and noradrenergic innervations appear relatively selective in directly innervating pial arterioles of large calibre. In addition, our study shows for the first time the presence of free TPOH-I fibers coursing through the leptomeninges with no obligatory association with blood vessels. This observation is reminiscent of the extensive plexus of serotonin axons in the walls of the brain ventricles[15,61] thought to be important modifiers of local CSF composition. A role of these TPOH-I leptomeningeal nerves in influencing diffusely local vasomotor activity and thus cerebral blood flow appears most likely.

Possible origin(s) of TPOH-I cerebrovascular nerves

We explored the two most probable sources for the cerebrovascular serotonergic nerve fibers, namely the rostral raphe nuclei and the cervical sympathetic trunk.

The efficacy and specificity of both types of lesions were confirmed by a large decrease in neocortical TPOH-I nerve terminals following serotonergic lesion, and by a near complete disappearance of sympathetic DBH-I nerves in most arteries following sympathectomy. Only in those arteries known to receive a sympathetic component from the stellate ganglion[1], such as the vertebro-basilar arterial system, were occasional DBH-I nerves still observed.

Examination of vascular specimen following these lesions clearly indicated that the superior cervical ganglia constitute the major pathway of cerebrovascular TPOH-I nerves in both major and isolated small pial arteries. In fact, effective lesions of the serotonergic ascending fibers provided no evidence for a contribution from the raphe nuclei, as the network of cerebrovascular TPOH-I fibers appeared unchanged following such treatment. A peripheral origin of cerebrovascular TPOH-I nerves in major cerebral vessels agree with several biochemical data and anatomical studies in which 5-HT containing nerves were studied, whether they were considered as truly serotonergic[8,18,41] or representing uptake of 5-HT into noradrenergic nerves[13,52,64]. In the present study, we are unable to ascertain whether or not the serotonin-synthesizing (TPOH-I) nerves are distinct from, or represent a subset of, noradreline-synthesizing (DBH-I) ones. Based on the differences in the pattern and density of innervation of the two systems and on the existence of sympathetic TH-I but not TPOH-I nerves in the pineal gland (see above), it appears that the two systems are not exactly superimposable. Such a conclusion is compatible with previous studies at the cerebrovascular level which showed distinct response of these two systems to hormonal treatments[22]. However, the possibility remains that there is colocalization of 5-HT in only a subpopulation of NA-containing perivascular sympathetic nerves[35].

The lack of contribution of the raphe nuclei in the serotonergic fibers contrasts with other studies[26,41,44] and, particularly, with previous biochemical data[8] which suggested a significant central component to the serotonin-synthesizing fibers in the small pial vessels. This apparent discrepancy most probably resides in the different sampling technique and respective definition of 'small pial vessels' used in the two studies. In the present anatomical investigation, only the 'major' small pial vessels ($\approx$ 50–200 μm diameter) could be isolated from the arachnoid membrane and only the larger ones ($>$ 100 μm) exhibited an important innervation network. For the biochemical determinations, the small pial vessels were sampled as a whole together with the membrane and, as such, this vascular segment included the primary ramifications of the major basal arteries (equivalent to the isolated small pial arteries) as well as the numerous, more distal small calibre vessels (20–50 μm) that could not be dissected out of the membrane for immunohistochemical staining. We are now providing evidence that these smaller pial vessels are most probably under the control of free TPOH-I fibers running in the pia-arachnoid membrane. The effect of selective lesions on these free TPOH-I nerves was not assessed due to the impossibility in getting reproducible labeling throughout the thick fragments of pia-arachnoid membrane. However, these free fibers might have a distinct origin from those innervating the major arteries and their most proximal ramifications. The possible implication of the raphe nuclei at this level of the cerebral circulation would agree with biochemical[8] as well as functional studies[6,42,43,59]. Serotonin could be released from these fibers and induce blood flow changes as already suggested[15]. Moreover, it should be emphasized that the 5-HT measured biochemically in the vascular samples can be associated with non-nervous elements such as endothelial cells[50], mast cells[25] or CSF[34]. Diffusion in CSF might enable 5-HT to reach and influence the overall cerebrovascular bed. This mechanism has been suggested before[12] and could explain the slight hypersensitivity to 5-HT noted in the MCA following raphe lesion[44].

Identification of cells of origin

Results of the sympathectomy suggested that the cells of origin of the cerebrovascular TPOH-I fibers are located in or sending fibers through the SCG. Our efforts, with the peroxidase antiperoxidase (PAP) or the more sensitive detection method[21] using the avidin–biotin complex (ABC), however, failed to clearly identify any TPOH-I cells within the SCG while DBH-I (or TH-I) ganglionic cells were readily detectable. The presence of 5-HT-containing cells in the SCG has been reported and these were identified originally as the small intensely fluorescent (SIF) cells[60]. In addition, the principal neurons of the ganglion have been shown immunocytochemically and biochemically to be able to synthesize serotonin from L-tryptophan[33,51] and, TPOH activity has been measured in extracts of the ganglion although essentially following enhancement treatments[39]. These biochemical results indicate that under normal conditions the ganglionic TPOH activity would be barely detectable and this may suggest, at least for the principal ganglion cells, that TPOH is rapidly transported toward the periphery[46]. It is thus possible that the endogeneous levels of TPOH in SCG cells are too low for immunohistochemical detection.

An alternative and most likely explanation can be drawn from molecular biology data and suggests that the TPOH located in peripheral cell bodies is not recognized by the antiserum due to differences in the post-translational modifications of the TPOH protein. Indeed, recent work has shown that the TPOH gene in the pineal gland is identical to that of the raphe nuclei[54]. However, original Northern blot analysis showed expression of TPOH in the pineal gland but failed to detect any positive reaction in the raphe nuclei[19,47]. Although TPOH expression in the raphe nuclei has now been evidenced[4,54], its expression level appears to be several orders of magnitude less (100-fold) than that of TPOH in the pineal gland[24,54], a finding which contrasts with the higher (5-fold) TPOH activity measured in the raphe nuclei as compared to the pineal gland[54]. With the TPOH antibody used in the present study, densely stained TPOH-I cells are readily observed in the raphe nuclei[16,61] but cells remain undetectable in the pineal gland (personal observations, see Materials and Methods). For reasons unknown at the present time, it appears that the TPOH-antiserum, which was produced against TPOH purified from rat brain[11] immunocytochemically reacts with a TPOH antigenic form which would be present in brain and cerebrovascular fibers but not in cell bodies of extracerebral stuctures such as the superior cervical ganglion and pineal gland. Interestingly, the presence of different forms of TPOH has been long hypothesized (for references see ref. 54) and our immunocytochemical findings reinforces such a contention. Nevertheless, it cannot be totally excluded that the cells giving rise to the cerebrovascular TPOH-I nerves are located outside the core of the ganglion itself or in neighboring structures such as the carotid body[31] or nodose ganglion[56] and their fibers interrupted by surgery.

In conclusion, we have shown that major cerebral arteries and small pial vessels of the rat are innervated by TPOH-I nerve fibers which have an almost exclusive peripheral origin closely related to the sympathetic nervous system. These results are compatible with the demonstrated uptake of 5-HT into cerebrovascular nerves of sympathetic origin, but further suggest that the uptake was, at least in part, into serotonin-synthesizing nerves. Further studies involving in situ hybridization in the SCG would help to clarify the expression of TPOH in serotonin-synthesizing cells and overcome any possible difference in protein post-translational and maturation events. As well, the exact significance and physiological role of these nerves into cerebrovascular functions, dysfunctions, hormonal or humoral regulation remains to be further elucidated.

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ADDENDUM TO CHAPTER 3

The results of this paper indicated that serotonergic neurons originating in the raphe nucleus have no direct projections to the extracerebral blood vessels. However, as mentioned in the manuscript, the results do not exclude the possibility that raphe neurons could also influence perivascular 5-HT levels and/or vasomotor properties indirectly via liberation of the indoleamine into the subarachnoid space. Our results rather suggest that the perivascular fibers containing the rate-limiting enzyme for 5-HT, tryptophan hydroxylase, arise from the superior cervical ganglia or a peripheral structure closely related to it. Using TPH immunocytochemistry, however, we could not identify the serotonin-synthesizing cells in the superior cervical ganglion, in spite of previous reports on the existence of 5-HT-immunoreactive neurons, TPH enzyme and activity in this sympathetic ganglion (Liuzzi et al., 1977; Verhofstad et al., 1981; Happola, 1988; Paivarinta et al., 1989). As hypothesized in the manuscript, we raised the possibility that two TPH isoforms exist with different antigenic properties as an explanation for the inability of our antibody, which was directed against brain TPH, to recognize the form(s) present in the pinealocytes and, possibly, in the ganglionic neurons. In fact, previous studies reported that the TPH protein present in the raphe nucleus possesses biochemical properties (i.e. molecular weight, isoelectric points) different than the protein found in the pineal gland even though the TPH mRNA coding sequence in both tissues is identical (see Kim et al., 1991 and references therein). Thus, to circumvent the possible differences in final TPH proteins and thus antigenicity, we undertook complementary experiments at the gene level to further address this issue.

Based on this information, we evaluated the expression of TPH mRNA in tissue extracts of the rat superior cervical ganglia by reverse transcriptase-polymerase chain reaction (RT-PCR). Oligonucleotide primers were designed using the NBI OLIGO 5.0 program according to the published sequence (accession number X53501) of Darmon et al (1988) derived from rat pineal gland. The primers were synthesized using an Applied Biosystems Synthesizer and purified using an OPC column (Applied Biosystems). The

primers were as follows: TPH F; 5'-TGG CTT CTC TTG GAG CTT C-3' AND TPH R; 5'-ACC TGC TGA CTC TAG CAA GG-3' and amplified a 438 bp cDNA fragment. The results, obtained from a pool (n=4) of rat superior cervical ganglia, showed the presence of PCR products of the expected size for TPH (Fig 3.1). Subsequent sequence analyses of the amplified products confirmed their homology (97%) with the expected TPH sequence cloned from the rat pineal gland (Darmon et al., 1988). This finding suggest that the TPH enzyme is expressed in superior cervical ganglionic cells but the protein levels may be too low to be detected by immunocytochemistry. It is clear that additional experiments should be devoted to the visualization of the cells within the superior cervical ganglion that express TPH mRNA by way of *in situ* hybridization.

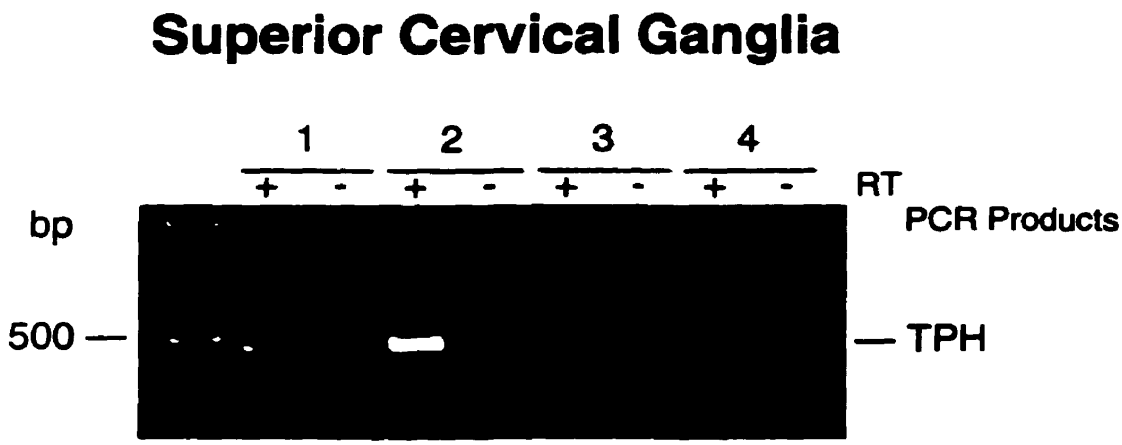

Fig 3.1: Agarose gel electrophoresis of PCR-amplified cDNA from 4 pooled superior cervical ganglia preparations using oligonucleotide-specific primers for the human tryptophan hydroxylase (TPH) gene. Samples without reverse transcriptase were included (–) to monitor for genomic and/or PCR contamination.

CHAPTER 4

ULTRASTRUCTURAL ANALYSIS OF TRYPTOPHAN HYDROXYLASE IMMUNOREACTIVE NERVE TERMINALS IN THE RAT CEREBRAL CORTEX AND HIPPOCAMPUS: THEIR ASSOCIATIONS WITH LOCAL BLOOD VESSELS

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PREFACE TO CHAPTER 4

In the previous chapter, our results indicated that brainstem serotonergic raphe neurons do not directly innervate the extracerebral circulation while it is undeniable that their stimulation can induce significant changes in local brain perfusion (Bonvento et al., 1989; McBean et al., 1990; 1991; Cudennec et al., 1993). On this basis, we sought to determine if these neurons could establish morphological relationships with the microvascular bed as several physiological, biochemical and/or anatomical studies had implied. Indeed, the levels of 5-HT around intracortical microvessels were found to be significantly reduced after neurotoxic destruction of the raphe neurons (Reinhard et al., 1979) and an uncoupling, albeit partial, is evident between CBF and metabolic demand when the raphe neurons are manipulated (Bonvento et al., 1989; 1991; McBean et al., 1990; 1991; Cudennec et al., 1993). Similarly, 5-HT neurovascular associations have been well documented in the raphe nucleus (Di Carlo, 1984; Kapadia and de Lanerolle, 1984), and occasionally reported in the cerebral cortex (Descarries et al., 1975; Itakura et al., 1985).

In order to investigate the possible serotonergic input to the brain microvascular bed, we performed an ultrastructural analysis of 5-HT neurovascular associations in the hippocampus, frontoparietal and entorhinal cortex. These cerebral regions were chosen on the basis of their differential blood flow responses to alterations in 5-HT neurotransmission (Bonvento et al., 1989; McBean et al., 1990; 1991; Cudennec et al., 1993). In these studies, significant blood flow changes were observed in the frontoparietal cortex while if present in the other two regions, these were much more modest. Therefore, our primary objective was to see if there exists an anatomical substrate that could support the local changes in cerebral perfusion.

To this end, rats were perfused, their brains processed for TPH immunocytochemistry at the ultrastructural level and TPH neurovascular associations were characterized in detail. TPH nerve terminals were considered perivascular when located within a 3 μm perimeter from the basal lamina of intraparenchymal blood vessels. This interval was chosen from

previous work (Lee, 1981; Dodge et al., 1994; Chédotal et al., 1994) and arbitrarily defined as the largest distance within which a nerve terminal could affect vascular functions either directly or indirectly via interactions with other neuronal and/or glial elements. Electron microscopic analyses included an evaluation of the proportion of perivascular terminals, their average distance from blood vessel walls, the type of blood vessels (i.e. capillaries vs microarterioles) with which they associate as well as other features such as their surface area and synaptic incidence. Furthermore, since this is the first in depth study to visualize serotonergic axonal varicosities at the electron microscopic level using a TPH antiserum, we also provided a detailed analysis of neuronal nerve terminals (i.e. non-vascular and located > 3 μm from vessel walls) taking into consideration their immediate microenvironment, size and synaptic incidence. These were compared to those available in the literature regarding serotonergic terminals identified by 5-HT radioautography or immunocytochemistry (Descarries et al., 1975; Séguéla et al., 1989; Oleskevich et al., 1991).

Overall, the morphological features of TPH nerve terminals matched very closely those previously described using 5-HT uptake or immunocytochemistry, thus confirming the reliability of the TPH antibody to label serotonergic neuronal elements at the EM level. The results showed that the serotonergic neurovascular associations in the frontoparietal cortex are more frequent and/or intimate than those in the other cerebral regions, implying a certain degree of priviledged reationships in areas that can adjust blood flow in response to manipulations of 5-HT pathways. Furthermore, a relatively high percentage of perivascular serotonergic nerve terminals were found to closely associate with astrocytic end-feet that surround the blood vessel, an observation which led us to suggest that these non-neuronal cells may play an important role in the regulation of vascular functions induced by 5-HT.

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Abstract—Physiological evidence has indicated that serotonin (5-hydroxytryptamine) could be a regulator of cerebral blood flow in various regions of the brain. In the present study, tryptophan hydroxylase immunocytochemistry was used to characterize, both at the light and electron microscopic levels, serotonergic nerve terminals and primarily their relationships with intraparenchymal microarterioles and capillaries in the rat frontoparietal cortex, entorhinal cortex and hippocampus. Irrespective of the brain area, serotonergic varicosities were primarily apposed to either dendrites or nerve terminals, were on average 0.37 μm^2 in surface area (0.69 μm calculated diameter) and 12–22% of them engaged in synaptic junctions, mostly with dendritic elements. Perivascular terminals (defined as immunolabelled varicosities located within a 3 μm perimeter around the vessel basal lamina) in the frontoparietal cortex represented 8–11% of all immunoreactive terminals counted, as determined by light and electron microscopy, respectively. In the entorhinal cortex and hippocampus, the proportion of perivascular terminals was only determined at the ultrastructural level and corresponded to 10% and 4%, respectively. In the frontoparietal cortex, serotonergic varicosities were located significantly closer ($n = 250$, 0.98 ± 0.05 μm; $P < 0.001$) to the blood vessels than those of the entorhinal cortex ($n = 116$, 1.41 ± 0.08 μm) or hippocampus ($n = 105$, 1.31 ± 0.08 μm). Of all perivascular serotonergic terminals in the frontoparietal cortex, 26% were in the immediate vicinity (0–0.25 μm) of the vessel wall, with 2.8% directly abutting on the basement membrane, while 11.6% were separated from it only by a thin astrocytic leaflet. This situation contrasts with that observed in the entorhinal cortex and hippocampus, where no immunoreactive varicosity was ever seen directly contacting the vessel basal lamina and with only 10–13% of the terminals being within 0.25 μm from the vessels. The surface area of perivascular serotonergic terminals was comparable in all regions studied and corresponded to 0.22 μm^2; these virtually never engaged in synaptic contacts with adjacent neuronal structures.

Our results indicate that tryptophan hydroxylase-immunolabelled terminals are identical to previously characterized serotonin-containing varicosities. Furthermore, the present data show intimate associations between serotonergic terminals and microvessels in the three regions examined. However, perivascular terminals in the frontoparietal cortex were more frequent and/or located much closer to local microvessels than those in the other regions, and might be more directly involved in neurogenic control of local cerebral blood flow.

The neuromediator serotonin (5-hydroxytryptamine, 5-HT) is widely distributed within the CNS and has been implicated in several brain functions, as well as in dysfunctions such as migraine[29] and cerebral vasospasm. The ascending serotonergic pathways from the midbrain dorsal and median raphe nuclei indeed appear to be involved in the processing of information pertinent to the control of food intake, anxiety, sexual behaviour, sleep[32] and, more specifically, to the regulation of cerebral blood flow (CBF) and/or blood–brain barrier permeability (for a recent review see Ref. 6). Although it was originally suggested that the raphe nuclei could alter CBF through a direct innervation of major cerebral arteries and small pial vessels,[21,39] recent evidence indicates that this is not the case and that these extracerebral blood vessels do not receive central serotonergic nerve fibres.[14,41] It is, however, undeniable that manipulations of the central raphe nuclei result in alterations in local CBF (see below). This observation is highly supportive of the concept of a central neurogenic control of brain microcirculation,[59] as first suggested for the noradrenergic system[66] and, more recently, for basal forebrain cholinergic neurons.[1,36]

Electrical and chemical stimulation of the dorsal raphe nucleus in anaesthetized rats[8,9] has been

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Abbreviations: CBF, cerebral blood flow; EM, electron microscopy; 5-HT, serotonin (5-hydroxytryptamine); LM, light microscopy; NO, nitric oxide; PB, phosphate buffer; PBS, phosphate-buffered saline; PF, paraformaldehyde; TPH, tryptophan hydroxylase; VIP, vasoactive intestinal polypeptide.

shown to result predominantly in CBF decreases in a majority of brain areas while, in awake animals, CBF increases have also been observed.[17] This apparent discrepancy was partly reconciled by Underwood and co-workers,[67] who found different responses in CBF depending on the anatomical sublocalization of the stimulation within the dorsal raphe nucleus. Additionally, pharmacological activation of the 5-HT_{1A} receptors (i.v. injection of 8-OH-DPAT[46]) or destruction of the ascending serotonergic projections (s.c. injection of methylenedioxyamphetamine[45]), both of which induce a reduction of 5-HT release, caused an increase in CBF in several cerebral areas. Altogether, these recent physiological studies strongly suggest a role for intracerebrally released 5-HT in the control of local CBF, the indoleamine eliciting primarily a vasocontractile response. Irrespective of the vasomotor effect exerted by 5-HT on brain microcirculation following manipulations of the raphe nuclei, the changes in CBF are reportedly partly independent of changes in cerebral metabolism, thus suggesting a direct effect at the level of the blood vessel itself.[7,17,45,46]

Such a hypothesis would imply close relationships between serotonergic neural elements and the microcirculation. Radioautographic[11] and immunohistochemical studies,[34] have described serotonergic cell bodies and dendrites in close apposition to the basement membrane of arterioles and capillaries in the raphe nuclei. However, there is very limited information concerning neurovascular associations in brain regions receiving serotonergic afferents, such as the neocortex,[19,31] despite the early suggestion by Reinhard and colleagues[58] that the raphe nucleus was the source of 5-HT associated with isolated intracortical microvessels.

In the present study, the associations between serotonergic nerve terminals and intraparenchymal blood vessels were investigated both at the light (LM) and electron (EM) microscopic levels. In an attempt to elucidate the morphological substrate for the functional effect of central 5-HT on CBF, the serotonergic neurovascular associations, as visualized by tryptophan-5-hydroxylase (TPH) immunocytochemistry, were characterized in the frontoparietal cortex, entorhinal cortex and hippocampus, three brain regions in which manipulations of the raphe nuclei result in distinct changes in CBF.[8,17,45,46] Parts of these results have appeared in abstract forms.[15,16]

EXPERIMENTAL PROCEDURES

Tissue preparation

Seven adult male Sprague–Dawley rats (Charles River, 250–300 g) were deeply anaesthetized with sodium pentobarbital (Somnotol, 65 mg/kg body weight, i.p.) and perfused through the ascending aorta with 500 ml of 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB; pH 7.4) containing 0.025% glutaraldehyde, followed by 1 l of 4% PF alone. For LM studies, two rats were first perfused with 30 ml of PB, followed by 1 l of 4% PF solution. Brains were removed and immersion-fixed in the PF solution for 2 h at room temperature. Following postfixation, coronal sections (40–70 μm) at the level of the frontal/parietal ($n = 5$) and entorhinal ($n = 2$) cortices, as well as in the dorsal hippocampus ($n = 2$), were cut on an Oxford Vibratome, rinsed (45 min) in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.2% gelatin (porcine skin, Sigma) and 0.001% sodium azide and then processed free-floating for TPH immunocytochemistry, with all steps performed at room temperature.

Antisera

A sheep polyclonal antibody directed against rat brain TPH,[10] the rate-limiting enzyme in the synthesis of 5-HT, was used to visualize serotonergic nerve terminals. The antiserum was diluted (1:1000–1:2000) in 0.1 M PBS containing 0.2% gelatin and 0.001% sodium azide, and for LM, 0.1% Triton X-100. The specificity of the antiserum in labelling selectively brain serotonergic cell bodies and terminals has been documented previously.[68] The secondary antibody consisted of a biotinylated rabbit anti-sheep immunoglobulin G (1:200, Vector) and was processed with the avidin–biotin–peroxidase complex (ABC kit, Vectastain, Vector), both diluted in PBS supplemented with 0.2% gelatin. Omission of the primary or secondary antiserum in tissue sections resulted in loss of raphe immunoreactive neurons and/or cortical terminals.

Immunocytochemistry

All sections were incubated overnight in a solution of anti-TPH antiserum under mild agitation. The following day, the sections were rinsed in PBS containing 0.2% gelatin (10 min) and in PBS alone (3 × 10 min), incubated with the biotinylated rabbit anti-sheep immunoglobulin G (1 h), washed as above and then incubated with the ABC kit (1–1.5 h). After rinsing, the immunocytochemical reaction product was revealed with 0.05% 3,3′-(±)-diaminobenzidine in Tris–HCl (0.1 M, pH 7.6) containing 0.005% hydrogen peroxide. Some sections were mounted on gelatin-coated slides, dehydrated, defatted and coverslipped before observation under a Leitz Aristoplan light microscope. The remaining sections, processed either for LM or EM, were extensively rinsed and then postfixed (90 min) in 2% osmium tetroxide. They were washed (0.1 M PB), stained *en bloc* with 2% uranyl acetate (45 min), dehydrated in alcohol and acetone, before being flat-embedded in Araldite 502 (Ladd). The regions of interest (frontal/parietal and entorhinal cortices or hippocampus) were trimmed from individual sections and re-embedded (48 h; 60°C) in BEEM capsules, ready to be cut (see below).

For LM studies, semi-thin (2 μm) sections of the frontoparietal cortex (including all six layers) were cut on a Reichert–Jung ultramicrotome and collected on gelatin-coated slides. For EM, ultrathin sections (straw colour, 90–120 nm) were obtained from small blocks comprising the upper layers of frontoparietal and entorhinal cortices, as well as primarily the strata lacunosum-moleculare of the CA1 region of the dorsal hippocampus. They were collected on 200-mesh copper grids, double stained with uranyl acetate and lead citrate, and then examined with a Jeol CX100II electron microscope at a working magnification of ×8000–14,000.

Tryptophan hydroxylase nerve terminals in the neuropil

The immediate microenvironment, surface area and synaptic frequency were determined in all three brain regions. In the frontoparietal cortex, TPH-immunolabelled terminals [$n = 314$, observed either directly under the EM ($n = 201$) or analysed on electron microphotographs ($n = 113$)] were chosen randomly (every fifth immunostained varicosity) and the cellular elements in contact with individual labelled terminal were identified. TPH-immunoreactive terminals in the entorhinal cortex ($n = 107$) and in

the hippocampus ($n = 108$) were selected randomly (every third varicosity in this case) and their microenvironment analysed exclusively on microphotographs.

The neuronal elements in contact with TPH terminals were classified as dendrites (dendritic trunks, shafts or spines), axon terminals, longitudinal and myelinated axons, nerve cell bodies and others (including glia and unidentifiable structures). The photographs were used to measure the surface of TPH-immunoreactive varicosities with the aid of a Bioquant II analysis program and an MTI 65 camera, and to estimate the frequency of TPH-immunopositive nerve endings engaged in synaptic contact. Only terminals in which the full contour of the membrane could be seen were included in this analysis. After measuring the average length of the synaptic contact, the synaptic incidence of the TPH terminals was calculated using the stereological formula of Beaudet and Sotelo.[5] A varicosity was considered synaptic when at least one of the apposed membranes showed a localized straightening or thickening, with some widening of intercellular space with or without a postsynaptic density (as described by Peters and colleagues[57]). For the purpose of clarity throughout this paper, TPH terminals located within the neuropil and not associated with intraparenchymal blood vessels (see below) will be referred to as neuronal terminals.

Tryptophan hydroxylase nerve terminals associated with blood vessels

A first analysis performed at the LM level was undertaken to evaluate the proportion of TPH terminals directly associated with blood vessels. Different areas of the frontoparietal cortex (taken from six different sections) were analysed with a camera lucida. All TPH-immunoreactive varicosities within a given field were counted, as well as those which were directly apposed to blood vessels. These latter terminals were expressed as a percentage of total TPH-immunolabelled varicosities. The second part of the LM analysis was to determine whether an enrichment or an impoverishment of TPH innervation could be evidenced around vessels and was performed as described previously.[13] In short, all TPH-immunolabelled varicosities on microphotographs that were found to directly touch any blood vessel wall were counted. The blood vessel contours were subsequently drawn on transparencies which were then overlaid onto adjacent cortical regions. These contours were treated as hypothetical blood vessels and all TPH varicosities touching these hypothetical vessels were counted. The numbers of TPH terminals counted over real and hypothetical vessel walls were expressed as a ratio.

At the EM level, a terminal was considered to be perivascular if located within a 3 μm perimeter around the vessel basal lamina, a distance corresponding to that reported previously for functional nerve fibres at the level of extracerebral blood vessels.[37] The percentage of perivascular TPH-immunoreactive terminals was established by calculating the number of varicosities associated with blood vessels as compared to the amount of TPH terminals within a given number of ultrathin sections. As the results of perivascular percentages in the frontoparietal cortex were comparable at the LM or the EM level (see Results), only the EM analysis was undertaken for the two other brain regions. In addition, the amounts of immunolabelled TPH terminals and blood vessel walls were quantified at the EM level in a given area corresponding to $\sim 9000\ \mu m^2$ in each of the 12 squares of EM grid examined in the respective brain regions.

The surface area and distance from the vessel basal lamina of perivascular TPH terminals were determined with the Bioquant program directly on electron microphotographs from 105 to 250 immunolabelled varicosities in the three brain regions. The type of microvessels (capillary or arteriole) associated with perivascular TPH nerve terminals was assessed directly on the electron micrographs and was based on the criteria defined by Peters and colleagues.[57] Capillaries were characterized as blood vessels (4–10 μm luminal width) containing a single layer of endothelial cells surrounded by a basal lamina which may contain pericytes and their processes. Arterioles consisted of larger vessels (> 10 μm) with one or two layers of smooth muscle.

Statistical analysis

Student t-tests were used to compare the size of the neuronal and perivascular TPH terminals in each of the three brain regions studied. A one-way analysis of variance (ANOVA, followed by a Tukey test) was used to compare the distances of the perivascular terminals from the blood vessel walls, as well as their surface area between the three brain areas examined. A P value ≤ 0.05 was considered significant.

RESULTS

Characteristics of neuronal nerve terminals

General features. At the LM level, the distribution of serotonergic fibres immunolabelled with the TPH antiserum corresponded to that reported previously by others in the three brain areas studied, whether 5-HT radioautography[2,19,52] or immunocytochemistry[61,65] was used. A high density of TPH nerve fibres was observed throughout the frontoparietal cortex, with a particularly dense labelling in the superficial layers (Fig. 1A). In the entorhinal cortex, TPH nerve fibres were predominantly distributed in layer I (Fig. 1B). In the CA1 region of the dorsal hippocampus, TPH innervation was highest in the stratum lacunosum-moleculare, with some fibres in the stratum moleculare (Fig. 1C). Sparse labelling was observed in the adjacent strata radiatum and pyramidalis. The labelling in these three terminal areas was confined to fine varicose fibres (Fig. 1).

Morphometric analysis at the electron microscopic level. Neuronal TPH terminals contained numerous small as well as some dense-core vesicles and, at times, one or two mitochondria (Fig. 2). The labelled terminals were usually round or ovoid in shape. In the three regions studied, the vast majority of TPH terminals was apposed to unstained dendritic and axonal elements, almost in equal proportions (Table 1, Fig. 2). Occasionally, the labelled terminals were in contact with small longitudinal axons or nerve cell bodies, as shown in the frontoparietal cortex (Fig. 2B). TPH terminals in the three areas were strictly similar in size (Table 1), with an overall surface area of the order of 0.37 μm^2 (ranging from 0.022 to 1.791 μm^2) and a mean diameter corresponding to approximately 0.69 μm.

TPH nerve terminals rarely exhibited synaptic contacts; only 4–6% of them were found to be engaged in junctional specializations in single thin sections (Table 1). These contacts were exclusively asymmetrical and on dendritic branches (Fig. 2D), although some specializations were also seen with cell soma (Fig. 2B). When stereologically extrapolated to whole volume, the synaptic frequencies in single thin sections yielded synaptic incidences ranging from 12 to 22%, depending on the brain regions (Table 1).

Perivascular nerve terminals

Quantification of perivascular innervation. A total of 3450 TPH-immunolabelled varicosities were counted on semi-thin sections obtained in the frontoparietal cortex. These terminals were divided according to their localization in superficial ($n = 1697$) or deep ($n = 1753$) layers of the cortex. The TPH-immunolabelled terminals that touched the walls of blood vessels within the superficial layers represented 6.2% of terminals, while such perivascular terminals corresponded to 10.1% in the deeper layers. Overall, approximately 8% of TPH nerve terminals in the rat frontoparietal cortex were associated with intraparenchymal vessels (Fig. 3). The number of TPH nerve terminals that were apposed to the walls of real and hypothetical blood vessels (Fig. 3) corresponded, respectively, to 541 and 512, with a real/hypothetical ratio of 1.07 ± 0.06.

When evaluated by EM, 10.7% (142 of 1321) of the frontoparietal cortical TPH-immunostained varicosities were perivascular. The proportion of perivascular TPH-immunopositive terminals in the entorhinal cortex and hippocampus corresponded to 10.4% (99 of 952) and 4.3% (44 of 1024), respectively, of the total terminals counted at the EM level. As evaluated for a fixed area of tissue, there was, on average, 22.7 TPH-immunostained terminals and 127.59 μm of vascular wall in the frontoparietal cortex, with a density of TPH terminals corresponding to 1.76 terminals/10 μm of blood vessel wall. For the same surface area in the entorhinal cortex, 15.6 terminals and 88.81 μm of vascular walls were quantified, for a density of 1.76 TPH terminals/10 μm of blood vessel perimeter, while in the hippocampus 15.8 terminals were found for 94.75 μm of blood vessel wall, yielding an average of 1.67 TPH terminals/10 μm of vessel perimeter.

Ultrastructural analysis of perivascular terminals. Perivascular TPH nerve terminals were morphologically similar to neuronal terminals. They were primarily associated with capillaries (81, 80 and 84%, respectively, in the frontoparietal cortex, entorhinal cortex and hippocampus), as opposed to small arterioles (Fig. 4) and large penetrating arteries.

Of the 250 perivascular TPH terminals on electron microphotographs from the frontoparietal cortex, a proportion of 2.8% appeared to be directly apposed to the vessel basal lamina (Fig. 5). An additional 11.6% of the terminals were separated from the basal

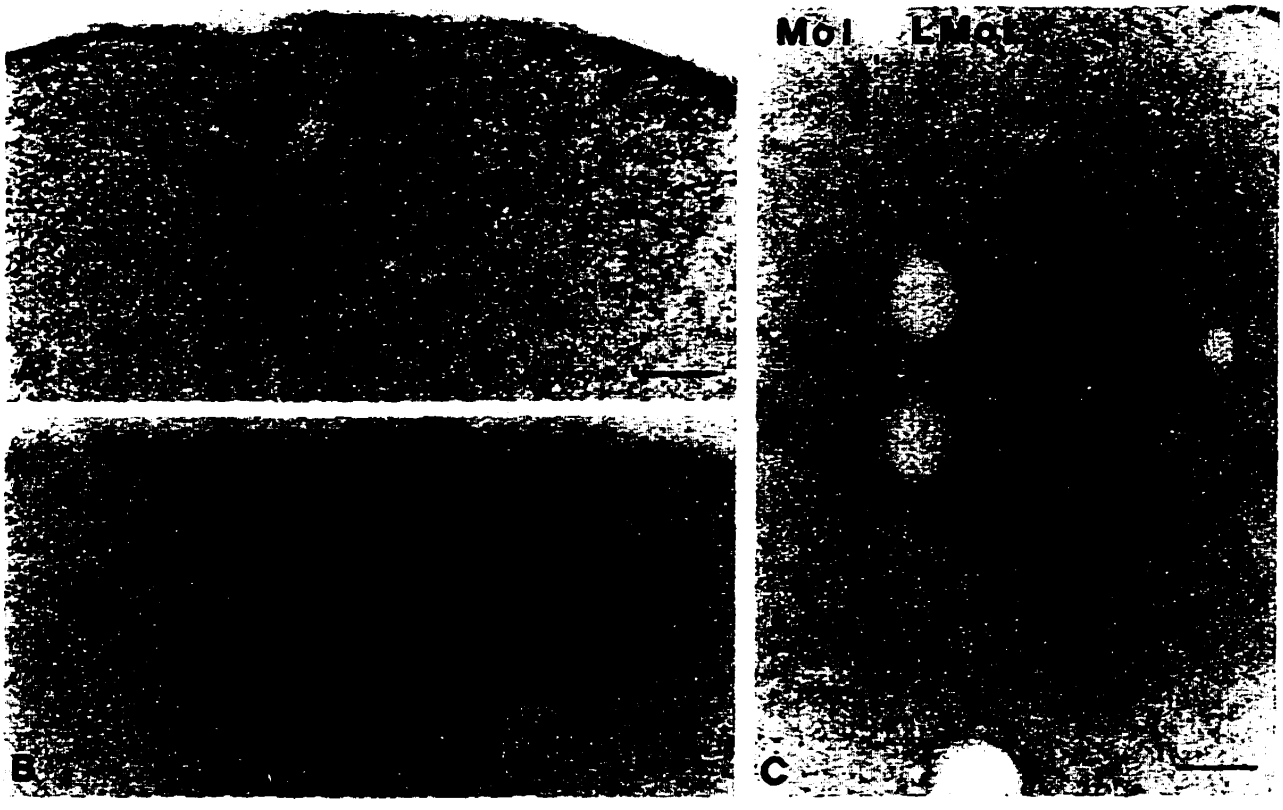

Fig. 1. Photomicrographs of 40-μm-thick coronal brain sections immunolabelled for TPH. The distribution of TPH-immunoreactive nerve terminals in the frontoparietal cortex (A), entorhinal cortex (B) and hippocampus (C) is illustrated. Note the high density of TPH varicose fibres in the superficial layers of the cerebral cortex (A, B) and in the stratum lacunosum-moleculare (LMol) of the CA1 region of the dorsal hippocampus. Mol, stratum moleculare. Scale bars = 50 μm.

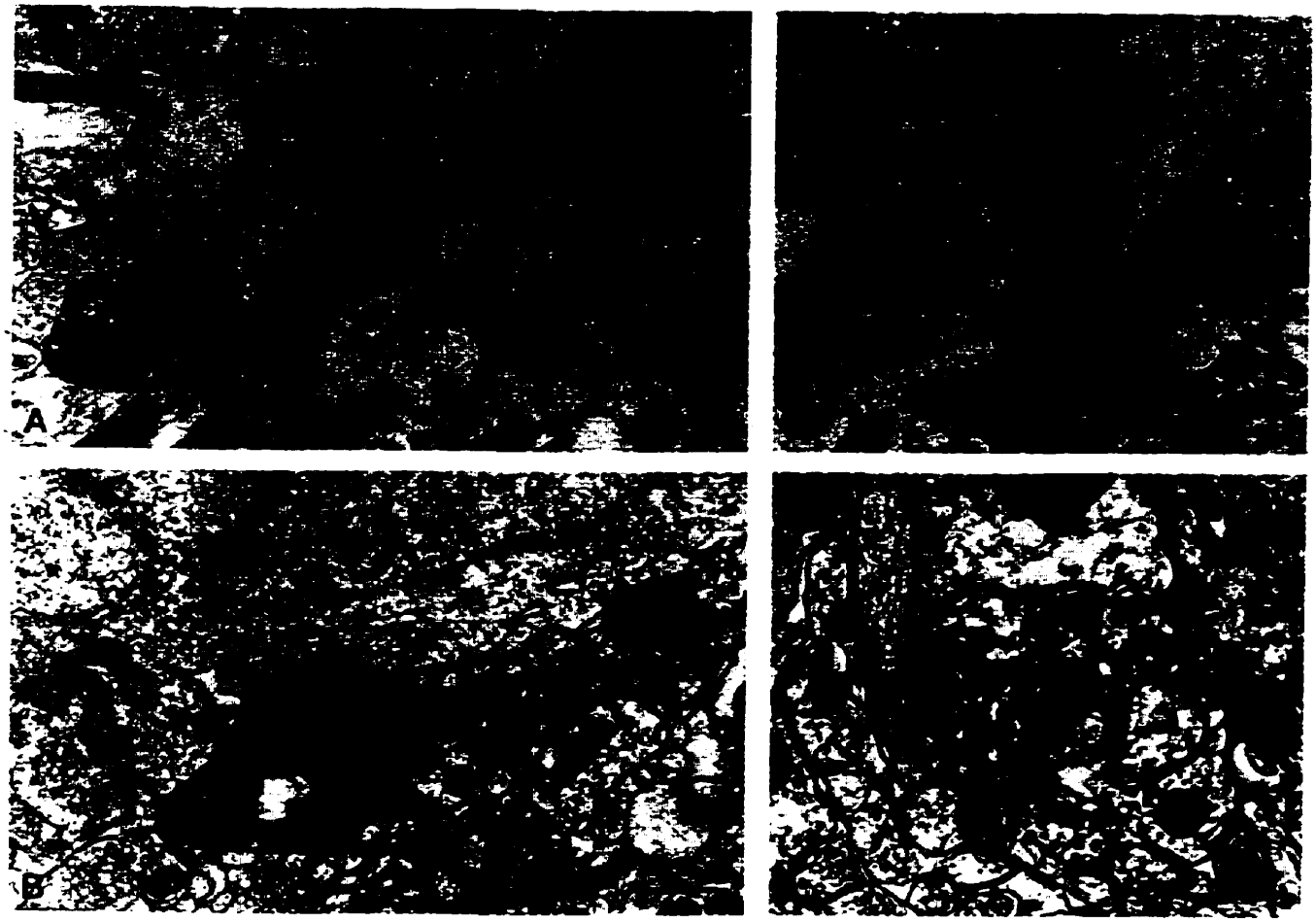

Fig. 2. Electron micrographs of TPH-immunoreactive nerve terminals in the neuropil of the frontoparietal cortex (A, B), entorhinal cortex (C) and hippocampus (D). In A, two distinct TPH-immunolabelled varicosities are found apposed either to a dendritic shaft (open arrows) or to adjacent unlabelled nerve terminals (straight arrows). In B, the cytoplasmic membrane of a nerve cell body is contacted by two immunostained varicosities, one of which is engaged in an asymmetrical synaptic contact (curved arrow). The nerve ending in C is bounded by a dendrite (open arrows) and two axon terminals (straight arrows), while the labelled terminal in the hippocampus (D) is synapsing (curved arrow) onto a dendritic shaft. Note the presence of dense-core vesicles within the varicosities (arrowheads). Scale bars = 0.5 μm.

lamina only by a thin intervening astroglial leaflet (Figs 4A, 5, 6), while another 12% were located slightly further away, up to 0.25 μm. Altogether, 26.4% of the perivascular TPH terminals located within the 3 μm perimeter around blood vessels were actually found in the immediate vicinity (0–0.25 μm) of the vessel wall (Fig. 7). Apart from this enrichment within the first 0.25 μm from the vessel wall, the frequency distribution of the perivascular TPH terminals showed an overall decrease in the number of immunolabelled varicosities as the distance from the blood vessel increased (Fig. 7).

In the entorhinal cortex and hippocampus, none of the labelled terminals was in direct contact with the vessel basal lamina and only 4.3 and 9.3%, respectively, were directly abutting onto perivascular astroglial processes (Fig. 8A, B). Similarly, only 6 (entorhinal cortex) and 4% (hippocampus) of the overall perivascular TPH terminals were located up to 0.25 μm from the basal lamina yielding respective proportions of 10.3 and 13.3% of the terminals being located within this interval. Overall, these proportions were comparable to those found across the various 0.25 μm intervals shown in Fig. 7.

None of the TPH terminals apposed to basal lamina or perivascular astrocytes was found to establish junctional specializations with these cellular elements as no gap junction, punctum adherens or synaptic junction could be observed. These perivascular TPH nerve terminals, as well as those located further away from the blood vessel, could, however, be engaged in synaptic contacts with adjacent dendrites (Figs 5, 6). These specializations were rare (stereologically extrapolated to 3.8% in frontoparietal cortex and none in the two other regions) and asymmetric.

Table 1. Ultrastructural characteristics of neuronal tryptophan hydroxylase-immunoreactive varicosities in the rat cerebral cortex and hippocampus

	Frontoparietal cortex	Entorhinal cortex	Hippocampus
No. of varicosities examined	314	107	108
Microenvironment (%)			
Dendrites	42.9	47.8	47.5
Axonal terminals	38.4	37.8	40.2
Longitudinal and myelinated axons	10.2	8.0	6.8
Cell bodies	2.6	1.5	0
Others	6.3	5.0	5.5
Topometric characteristics			
Area (μm^2)	0.37 ± 0.02	0.37 ± 0.03	0.36 ± 0.02
Average calculated diameter (μm)	0.69	0.69	0.68
Average length of synaptic contact (μm)	0.15	0.18	0.24
Synaptic frequency in single sections (%)	5.9	5.3	4.2
Extrapolated synaptic incidence (%)	22	18	12

Values indicated for the microenvironment are expressed as percentage of neuropil elements adjacent to labelled TPH neuronal terminals, as compared to the total number of appositions in each tissue. In the frontoparietal cortex, results on the microenvironment were pooled from 201 labelled terminals observed directly on the EM screen and from 113 photographed terminals, while only the latter were used for the topometric measurements. In the other two regions, results were obtained from the number of photographed terminals as indicated. The surface area of TPH terminals is given as mean $\pm$ S.E.M. Explanations for the criteria of a synaptic contact and calculation of synaptic frequency and incidence are given in Experimental Procedures.

Perivascular TPH terminals in the frontoparietal cortex were located at an average distance of $0.98 \pm 0.05\ \mu m$ from the blood vessels, which was significantly closer (ANOVA, $P < 0.001$) than perivascular terminals in the entorhinal cortex and hippocampus (Table 2). The size of the perivascular terminals was consistent from one region to another ($\sim 0.22\ \mu m^2$; Table 2) and they were significantly smaller (Student t-test, $P < 0.05$) than their neuronal congeners in the corresponding neuropil (Tables 1, 2).

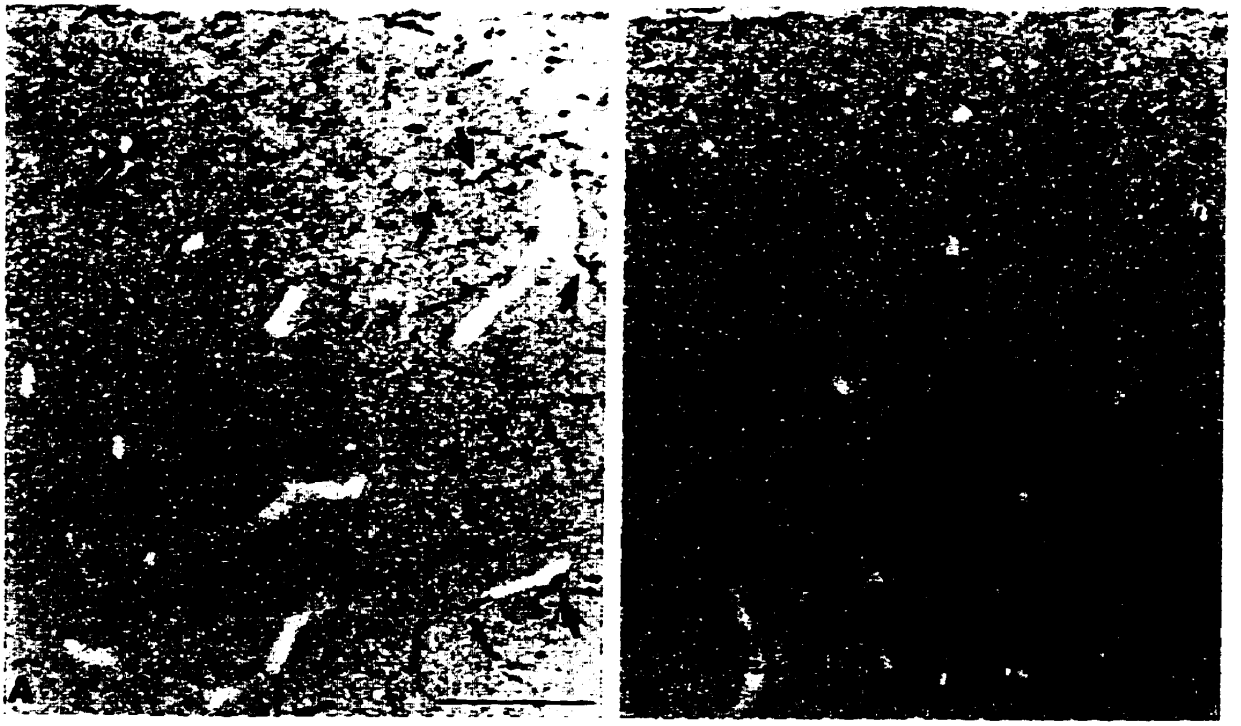

Fig. 3. Photographs of semi-thin sections ($2\ \mu m$) from the frontoparietal cortex immunolabelled for TPH. The contours of the real blood vessels in A were drawn on transparencies which were then superimposed onto an adjacent cortical region (B) and the blood vessel silhouettes were treated as hypothetical vessels (dashed lines in B). The numbers of TPH-immunoreactive terminals directly apposed to real (straight arrows) and hypothetical (arrowheads) vessels were counted and compared. Scale bars = $50\ \mu m$.

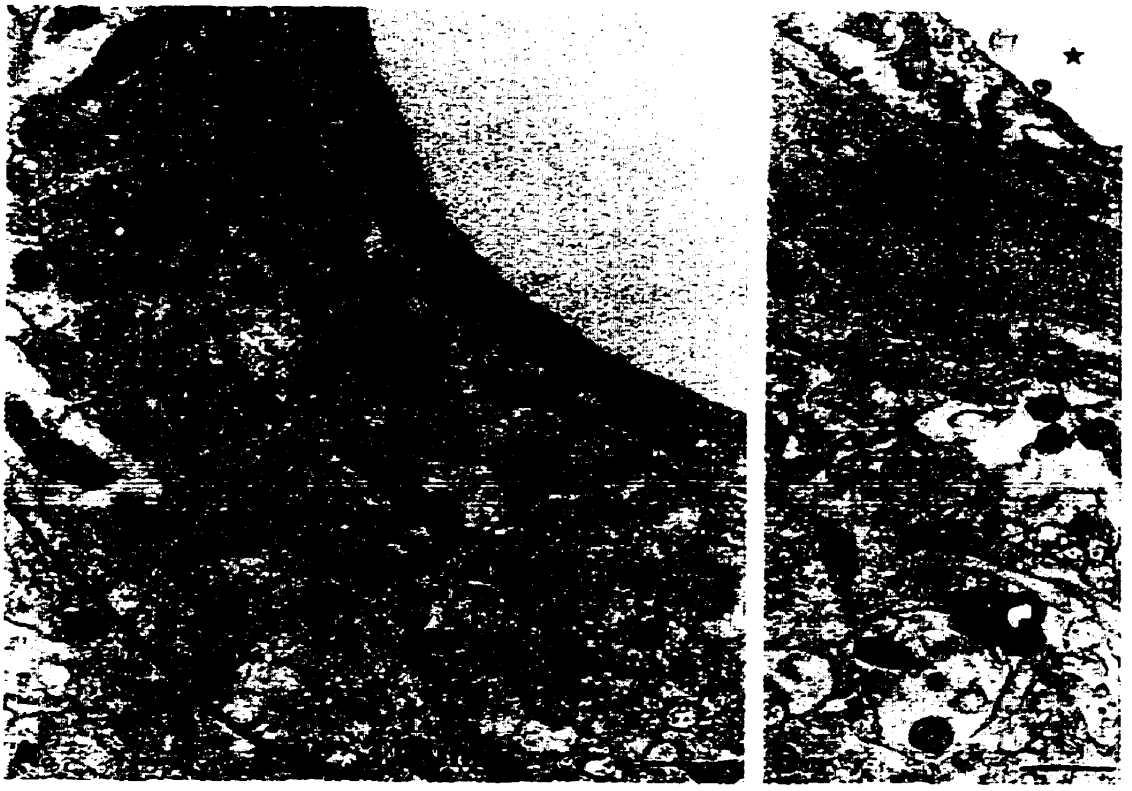

Fig. 4. Photomicrographs of perivascular TPH axon terminals associated with microarterioles in the frontoparietal cortex. The 3 μm perimeter, as well as the first 0.25 μm interval from the basal lamina (straight arrows) are illustrated in A. A perivascular TPH terminal located in the immediate vicinity (<0.25 μm) of a microvessel is separated from its basal lamina by a perivascular astrocyte (open arrow). The TPH nerve terminal in B is located approximately 1.00 μm away from the basal lamina (the average distance of perivascular terminals in this brain region). Star, blood vessel lumen; SMC, smooth muscle cell. Scale bars = 0.5 μm.

DISCUSSION

We used an antibody against TPH, the rate-limiting enzyme for the biosynthesis of 5-HT, to visualize serotonergic nerve terminals in three distinct brain regions and, more specifically, their intimate relationships with the local microcirculation. This antibody had been used previously at the LM level in brain[68] and extracerebral blood vessels,[12,14,42,64] but the present study is the first detailed ultrastructural characterization of serotonergic terminals in the rat brain using this or any other TPH antiserum. Additionally, this report is the only analysis on the fine and precise assocations of 5-HT with local cerebral microvessels in brain regions other than the raphe nucleus.

Neuronal terminals

Axon varicosities immunostained for TPH appeared, in terms of microenvironment, general morphometric features and synaptic frequency, very similar to 5-HT-containing nerve terminals identified in the rat cerebral cortex[19,55,61] and hippocampus.[52,53] TPH EM immunocytochemistry thus provides an alternative means to the ultrastructural detection of serotonergic cellular elements within the rat CNS. The possibility of visualizing the synthesizing enzyme rather than 5-HT itself could offer tremendous potential in the study of serotonergic nerve terminals following pharmacological and/or neurotoxic manipulations which result in depletion of the neurotransmitter pool from nerve endings and varicosities.

Our data further confirm the privileged association of serotonergic terminals with dendritic elements as well as other axon terminals. These axoaxonic appositions agree with a role for 5-HT in the regulation of neurotransmitter release within the cerebral cortex and hippocampus.[4,43,44] Conversely, these unlabelled axonal elements could also control the release of 5-HT from serotonergic terminals, as reported for intracortical noradrenergic terminals.[23] Overall, the non-synaptic nature of these axoaxonic and axodendritic appositions is compatible with the diffuse transmission of information to local cortical and hippocampal neurons, some of which are reportedly cholinergic or GABAergic[28,48] and endowed with a subset of 5-HT receptors.[4,48]

Although the surface area of TPH terminals varied within each cerebral area examined, the mean diameter of 0.69 μm fully agrees with the previously

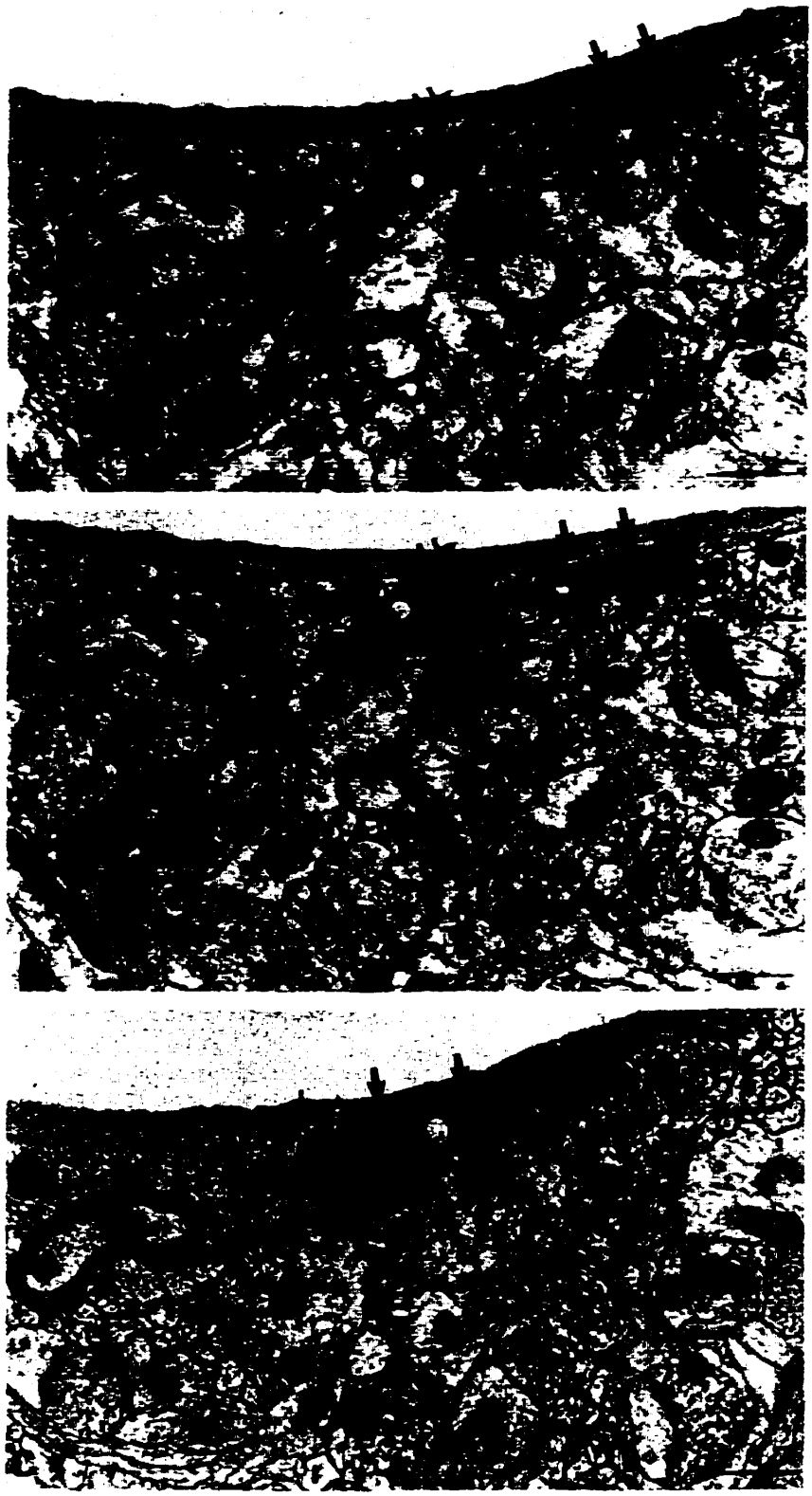

Fig. 5. Perivascular TPH varicosities in the frontoparietal cortex examined in three serial thin sections. The terminal on the right appears to directly contact the basal lamina of the capillary (A, B, straight arrows). The middle terminal, which is separated by an intervening astrocyte in A and B (open arrow), approaches the capillary to finally abut on its basal lamina, as shown in C (straight arrows). In B, this varicosity is seen forming a junctional contact (small curved arrow) with an adjacent dendrite. Labelling of the intervaricose segment is clearly visible in B and can be seen to give rise to a third varicosity (large curved arrows in B). Scale bars = 0.5 μm.

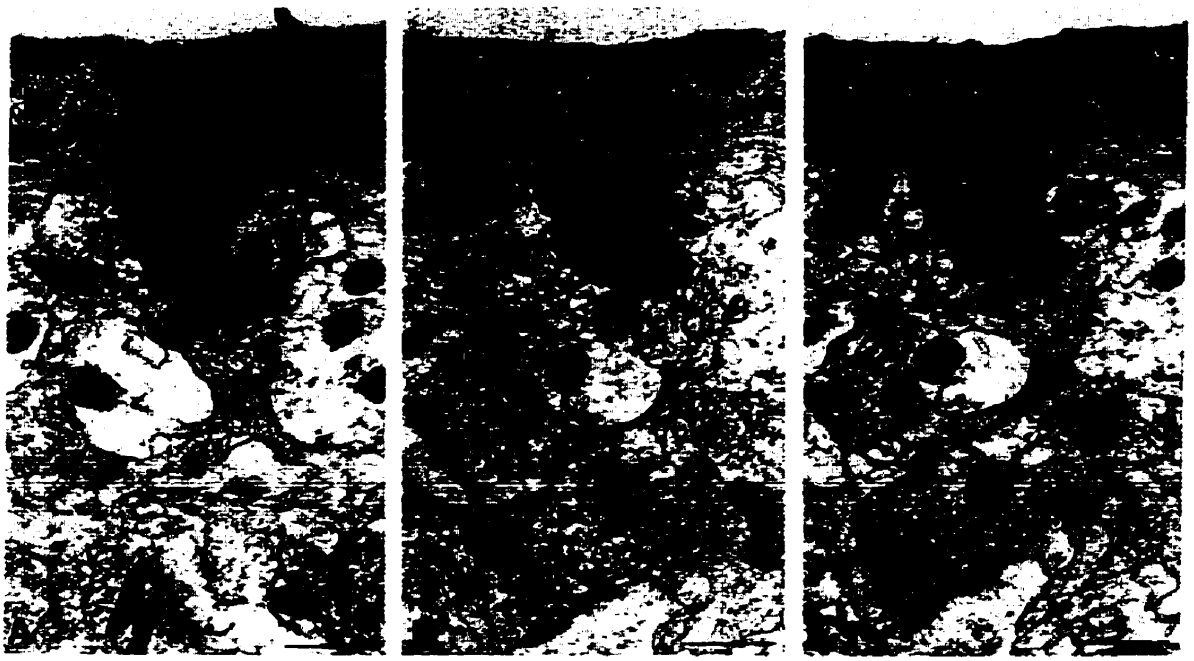

Fig. 6. Electron micrographs of serial sections of a perivascular terminal separated from the blood vessel by a thin astrocytic membrane. Notice the presence of an intervening glial process (open arrows) between the terminal and the basal lamina. The labelled terminal in B is engaged in a double asymmetrical contact with the adjacent dendrite (curved arrows). Scale bars = 0.5 μm.

reported size for 5-HT terminals.[19,53,55,61] No terminal with a diameter greater than 1.46 μm (frontoparietal cortex), 1.51 μm (entorhinal cortex) and 1.08 μm (hippocampus) was ever encountered. This observation is in contradistinction with the work of Kosofsky and Molliver,[35] who identified, at the LM level, two classes of cortical 5-HT nerve endings, one corresponding to large spherical varicosities averaging up to 5 μm in diameter. Our finding is, however, supported by other ultrastructural measurements of 5-HT terminals in the rat cerebral cortex and hippocampus,[53,61] and altogether strongly argue against the existence of a subpopulation of very large serotonergic nerve terminals. Furthermore, the low extrapolated synaptic frequency (12–22%) of TPH-immunolabelled nerve terminals documented here is fully compatible with that of 5-HT-containing terminals in the frontoparietal cortex[61] and hippocampus,[53] although divergent findings have also been reported.[55] To the best of our knowledge, there is no study available on the fine morphological and topometric features of serotonergic terminals within the rat entorhinal cortex. Our results would suggest that they resemble very closely serotonergic terminals in the frontoparietal as well as in other cortical areas.[61]

Perivascular nerve terminals

Association with the local microcirculation. A fair proportion (8–11%) of TPH-immunoreactive nerves in the frontoparietal cortex either touched a vessel wall or were located within 3 μm from the basal lamina. This analysis being performed on single sections, it is likely that on its full length a given blood vessel is approached and/or contacted by a significant amount of serotonergic varicosities. In the entorhinal cortex, a similar population (10%) of TPH terminals was identified as perivascular, while this proportion was only 4% in the hippocampus. This considerably lower population of perivascular terminals cannot be due to the fact that there were less vascular profiles for a given surface area in the hippocampus as compared to the cerebral cortex. Indeed, our results indicated that equivalent proportions of vascular walls and TPH nerve terminals were present in the three areas examined. These results thus indicate a relatively poor perivascular serotonergic innervation in the hippocampus and suggest that this local microcirculation is less likely to be influenced by 5-HT. The reciprocal statement would be that local blood vessels in the frontoparietal and entorhinal cortices may represent a putative target for neighbouring serotonergic terminals. A similar conclusion was obtained from the LM analysis of real and hypothetical blood vessels in the frontoparietal cortex, which clearly showed that TPH nerve terminals were not impoverished around blood vessel walls.

Irrespective of the area examined, capillaries were more frequently associated with serotonergic terminals than microarterioles. It is not clear if this observation is consequent to the greater amount of capillaries in brain parenchyma[47] or if, indeed, 5-HT would preferentially participate in capillary functions.[62] Interestingly, a similar association with local capillaries has been reported previously for central

neurovascular systems containing acetylcholine, vasoactive intestinal polypeptide (VIP) and nitric oxide (NO).[1,13,30]

A unique characteristic of the perivascular TPH terminals in the frontoparietal cortex was their striking enrichment (more than 25% of all perivascular terminals) in the immediate vicinity of the vessel wall. Such an intimate association with the local microcirculation was not found in the entorhinal cortex nor in the hippocampus, as these terminals were located significantly further away from blood vessels. Whether growth factors or other neutrophic factors specific to endothelial, smooth muscle or perivascular astrocytes[3] in the frontparietal cortex could attract and maintain serotonergic terminals in close proximity to blood vessels remains unclear.

Perivascular astrocytes and neurovascular innervation. In all brain regions but more frequently so in the frontoparietal cortex, TPH nerve endings located very close to blood vessels were, for the most part, abutting on perivascular astrocytic processes. Only rarely were interruptions present in the astrocyte cytoplasmic membrane, thus permitting direct contact between a TPH nerve terminal and a blood

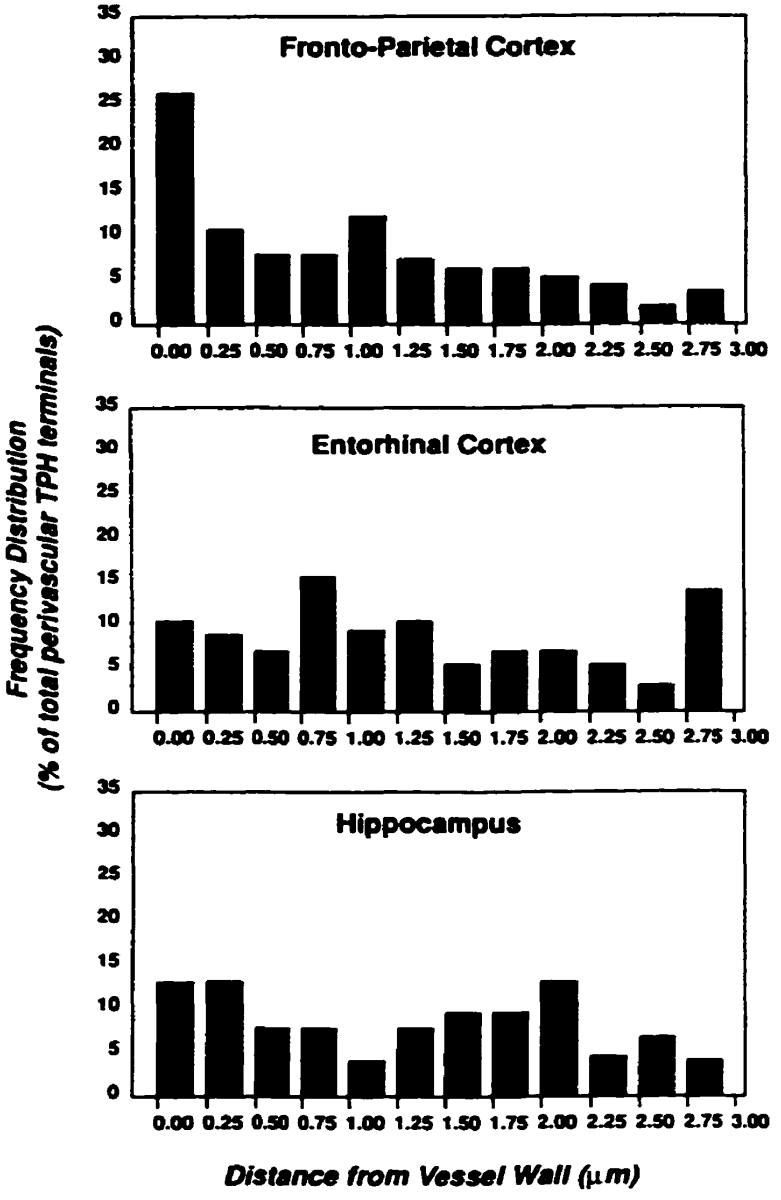

Fig. 7. Histograms of the distribution of perivascular TPH-immunoreactive axon terminals within the 3 μm perimeter around intracortical microvessels. The abscissa is divided in intervals of 0.25 μm at increasing distance from the vessel basal lamina. Bars represent the number of varicosities found in each 0.25 μm interval and are expressed as percentage of total TPH perivascular terminals counted in the brain region.

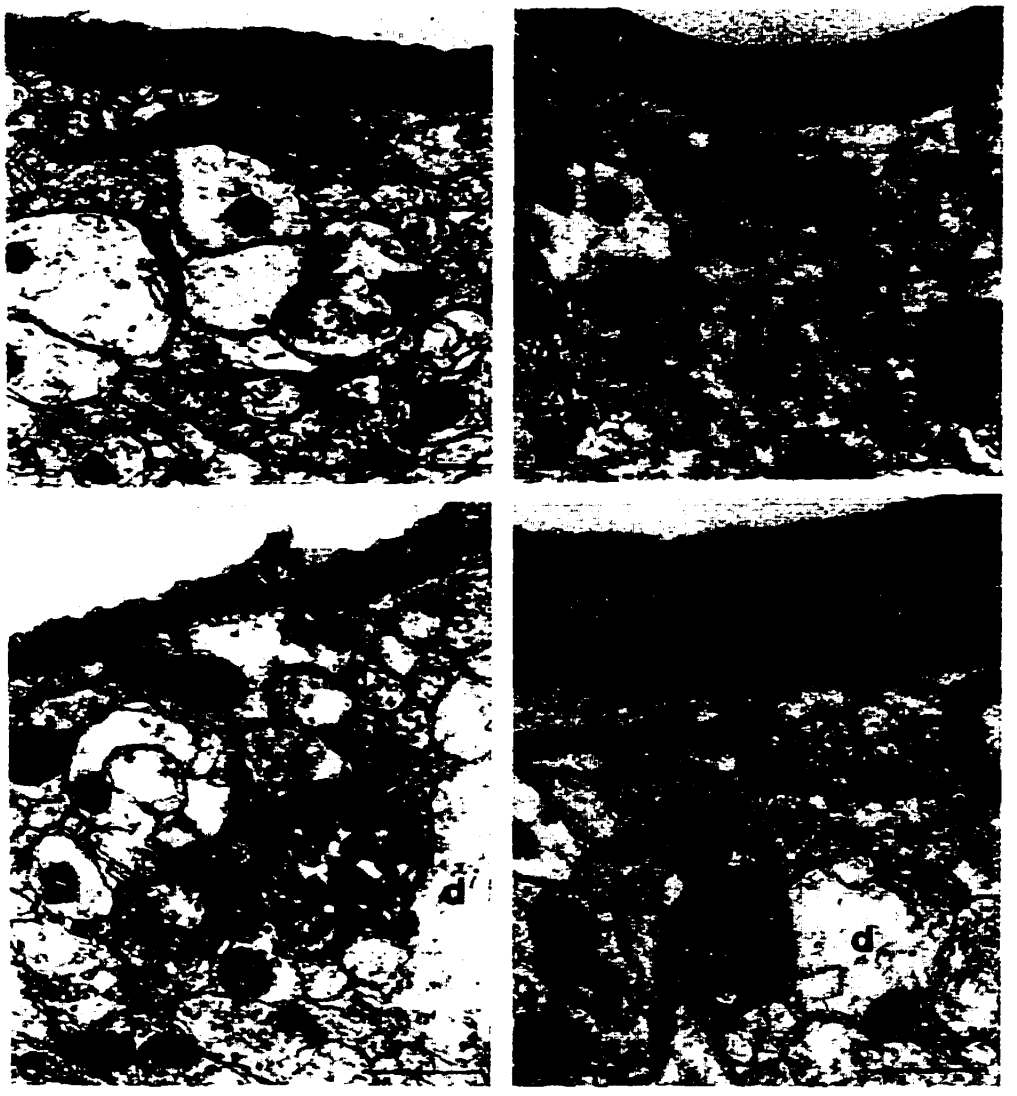

Fig. 8. Perivascular TPH terminals in the entorhinal cortex (A, C) and hippocampus (B, D). The perivascular terminals were occasionally found close to the basal lamina of the microvessel, but always separated from it by an astroglial leaflet (A, B), as shown by the presence of distinct membranes (open arrows in A and B). The perivascular terminals in C and D are located at a distance of approximately 1.00 μm from the capillaries and are surrounded by unlabelled axonal terminals (a) and dendrites (d). Scale bars = 0.5 μm.

vessel, a phenomenon that was observed only in the frontoparietal cortex. The presence of intervening astrocytic leaflets between nerve terminals and blood vessels has been documented before.[33,47] Recently, similar neuronal–glial associations have been observed for the cortical cholinergic and VIPergic neurovascular systems,[13] and it was suggested that perivascular astrocytes could be an essential intermediate cellular link in the neurogenic control of CBF. A similar role for astrocytes in the serotonergic control of CBF deserves consideration. For instance, the 5-HT_{1A} and/or 5-HT_{2A} receptor subtypes have been identified in brain astrocytes.[18,69] These cells have been implicated in the regulation of local metabolic activity, extracellular K^+ concentrations and, more generally, brain homeostasis.[27] Notable is the previous proposition that the release of K^+ from astrocytic endfeet could be a component of local CBF regulation.[56] It is also conceivable that through their gap junction[40] and ability to synthesize and release a series of potent vasoactive substances such as eicosanoids,[25,49] endothelin[38] and NO,[24,50] brain astrocytes could be important players in controlling CBF and blood–brain barrier permeability. Although direct neurovascular effects are possible, serotonergic terminals could also affect the microcirculation through neuronal–glial–vascular interactions.

Interaction of perivascular serotonergic terminals with other neurotransmitter systems. A major fraction of perivascular TPH terminals was separated from

the blood vessels by various neuronal elements present within the 3 μm perimeter. The possibility that 5-HT released from a serotonergic terminal interacts with specific 5-HT receptors located on these intermingled non-serotonergic neuronal structures is probable. Such mechanism could affect the release of neurotransmitter substances located within cortical afferents, some of them possibly releasing the effective vasoactive modulators (e.g., acetylcholine, VIP, NO, neuropeptide Y). The fact that stimulation of the raphe nucleus generally results in vasoconstriction, but occasionally in vasodilatation,[8,17,67] could be explained by the involvement of such intermediate neuronal systems for which either glial and/or microvascular cells possess receptors. Alternatively, it cannot be totally excluded that distinct microvascular 5-HT receptor subtypes could partly mediate the two opposite vasomotor effects. These various interactions could possibly lead to the equilibrium between contractile and dilatory responses and explain the inability to detect significant CBF changes in the entorhinal cortex and hippocampus[17] following manipulation of the raphe nucleus.

Non-junctional serotonergic vascular innervation. Perivascular TPH terminals, although occasionally seen to synapse onto an adjacent dendrite, never established any junctional specialization with the glial or vascular basal lamina. This observation is consistent with similar findings regarding other intrinsic neurovascular systems in various regions of the CNS[1,13,26] and implies diffusion of 5-HT through the extracellular space in order to reach its receptors on neighbouring neuronal, glial and/or vascular targets. This volume transmission mode is fully compatible with the functional innervation of major cerebral arteries and pial vessels,[22] as well as of 5-HT neurotransmission within the CNS.[20]

Interestingly, perivascular TPH nerve terminals appeared to be less synaptic and smaller than their neuronal congeners in the three brain regions examined. Although we do not have a clear explanation for this observation, it might be that blood vessel walls are the final, non-synaptic targets of these serotonergic nerve endings.

Functional correlates. Our data suggest that a subset of TPH nerve terminals are associated with the local microcirculation in various brain areas. Differences were found in terms of frequency of association with local microvessels and/or proximity of the nerve terminals with the vascular elements between the three regions examined and they may partly explain why 5-HT has differential effects on local CBF in these areas. However, other elements not considered in the present study, such as the distribution of microvascular and/or astrocytic 5-HT receptors, their subtypes and density, could represent alternative contributors to the ability of 5-HT to exert a vasomotor effect in a given region.

A consistent observation was that capillaries represented the main target for perivascular TPH nerve endings. Although the exact role of 5-HT at the level of brain capillary has not been elucidated, 5-HT receptors have been identified in cerebral endothelial cells[63] and, reportedly, 5-HT has the ability to alter permeability and transport mechanisms[62] at the level of the blood–brain barrier. Although it might be difficult to reconcile the capillary bed with vasomotor functions, this possibility cannot be excluded. Indeed, as suggested in the hamster cheek pouch,[60] it is conceivable that an endothelial-generated signal spreads upstream to induce a vasomotor effect in larger smooth muscle blood vessels. The possibility that endothelial cells themselves, through their actin- and myosin-like filaments,[54] pericytes and related enzymes involved in the control of smooth muscle cell contractility,[51] are directly contributing to vasomotion of the microcirculation also cannot be ignored.

It is much easier to conceptualize that TPH nerve terminals associated with local microarterioles could, provided that the cerebrovascular smooth muscle cells contain a given population of vasomotor 5-HT receptors, lead to the reported changes in CBF observed following serotonergic activation. Interestingly, microvascular 5-HT$_2$ receptors have been suggested as being implicated in the CBF changes in the parietal cortex.[9] It thus appears that every component of the proposed neuronal–glial–vascular

Table 2. Morphological features of perivascular tryptophan hydroxylase terminals in frontoparietal and entorhinal cortices and hippocampus

	Frontoparietal cortex	Entorhinal cortex	Hippocampus
n	250	116	105
Distance from vessel (μm)	0.98 ± 0.05	1.41 ± 0.08**	1.31 ± 0.08**
Area (μm^2)	0.22 ± 0.01*	0.20 ± 0.01*	0.23 ± 0.02*
Average calculated diameter (μm)	0.53	0.51	0.54

Except for the diameter, which was evaluated from the measured surface area, the values are expressed as mean ± S.E.M. of the number (n) of perivascular TPH terminals assessed in individual brain regions.

*Significant difference between the mean surface area of TPH perivascular terminals as compared to TPH neuronal terminals in the corresponding area (Student t-test, $P < 0.05$, Table 1).

**Statistically significant difference as compared to the frontoparietal cortex (ANOVA, $P < 0.001$; $F = 12.47$).

pathway that may govern the actions of 5-HT on the cerebral microcirculation is endowed with appropriate receptors. It is possible that such functional interactions require specific relationships with the local blood vessels, as demonstrated here in the frontoparietal cortex, as well as the presence of the appropriate receptors on glial and/or vascular elements.

CONCLUSIONS

These present morphological data show that nerve terminals immunolabelled for TPH are identical to serotonergic varicosities described previously by [^{3}H]5-HT uptake or 5-HT immunocytochemistry. In addition, these observations suggest that the microvascular bed in the frontoparietal cortex is more frequently and/or more closely innervated by serotonergic terminals (albeit in a paracrine manner) than that in the other brain regions studied. These neurovascular associations could possibly account for the differential CBF changes observed in these brain areas following stimulation of the raphe nucleus. Our results further emphasize that neuronal–glial–vascular interactions appear to be the basic substrate for neurogenic regulation of CBF.

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CHAPTER 5

ASTROGLIAL AND VASCULAR INTERACTIONS OF NORADRENALINE TERMINALS IN THE RAT CEREBRAL CORTEX

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PREFACE TO CHAPTER 5

Our morphometric analysis of perivascular 5-HT nerve terminals indicated significant differences in frequency and/or intimacy of associations with the local microvascular bed between the different regions. Although our study does not allow definite conclusions, it appeared that the brain regions which exhibited the most pronounced changes in CBF following manipulation of brainstem 5-HT neurons were also those with the best defined neurovascular associations. However, such regional selectivity could also reflect a structural property of the individual brain area. In order to evaluate if the neurovascular associations were a characteristic of the region and/or the neurotransmitter system, we investigated the morphological features of the neurovascular associations between noradrenaline terminals and intraparenchymal blood vessels in the frontoparietal cortex.

Direct contacts between catecholaminergic nerve terminals and intracerebral blood vessels were originally reported by Swanson and colleagues (1977) in the richly vascularized periventricular nucleus of the hypothalamus. Similar NA neurovascular associations have also been documented, although much less frequently, in other brain areas such as the cerebral cortex, hippocampal formation and striatum (Papadopoulos et al., 1987; Milner et al., 1989; Aoki, 1992; Paspalas and Papadopoulos, 1996). Physiologically, the available evidence suggest that central NA neurons have a rather limited, although consistent, effect on blood flow regulation under normal conditions. Stimulation and/or lesion of the locus coeruleus, the seat of intracerebral NA neurons, exert rather moderate blood flow changes (Raichle et al., 1975; De la Torre , 1977; Yokote et al., 1986; Goadsby and Duckworth, 1989; Adachi et al., 1991; Kobayashi et al., 1991); corresponding to about a 15% change in flow. In addition, it has been well documented that NA participates in BBB permeability processes. Locus coeruleus stimulation and lesion have shown to result in changes in the transport of Na^+ and K^+ ions across the BBB and an increase in permeability to water, albumin and sodium fluorescein (an impermeate molecule) and reportedly involve both of α- and β-adrenoceptors (Raichle et al., 1975; Harik and McGuingal, 1984; Harik, 1986; Sarmento et al., 1994). The presence of these adrenoceptors in whole microvessels and in their endothelial and smooth muscle cell compartments (Nathanson and Glaser, 1979;

Wroblewska et al., 1984; Kalaria and Harik 1989; Bacic et al., 1992) is fully compatible with the possibility that intracerebrally released NA can mediate functional vascular responses.

We, thus, investigated the associations between NA and intracerebral blood vessels in the frontoparietal cortex, a region with privileged 5-HT neurovascular associations as compared to the hippocampus and entorhinal cortex. In these experiments, an antibody directed against NA and not its synthesizing enzymes, i.e. tyrosine hydroxylase and dopamine-ß-hydroxylase, was used to assure selectivity and feasability at the ultrastructural level. We established the morphological features of these NA neurovascular associations (i.e. frequency, average distance from blood vessel walls, surface area and synaptic incidence) in order to be able to compare them to serotonergic neurovascular associations in the same area.

Finally, in order to establish the central origin of these NA neurovascular associations in the cerebral cortex, similar to what had been reported in the hypothalamus, we further examined these associations in rats treated with DSP-4, a neurotoxin which selectively and specifically destroys NA neurons that originate from the locus coeruleus (Berger et al., 1988), which provide innervation to the cerebral cortex as well as most other brain areas.

The results of this study show that NA neurovascular associations are less frequent than those previously characterized in the 5-HT system in the same cortical subdivision. More detailed analyses demonstrated that these NA nerve terminals are more associated with astrocyte processes and suggest that they are probably more involved in astrocytic functions. Furthermore, the findings show that the microvascular NA innervation arises from the locus coeruleus and that it does not appear to cross the glia limitans and provide fibers to extracerebral blood vessels.

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Astroglial and Vascular Interactions of Noradrenaline Terminals in the Rat Cerebral Cortex

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Summary: Noradrenaline (NA) has been shown to influence astrocytic and vascular functions related to brain homeostasis, metabolism, local blood flow, and blood-brain barrier permeability. In the current study, we investigate the possible associations that exist between NA-immunoreactive nerve terminals and astrocytes and intraparenchymal blood vessels in the rat frontoparietal cortex, both at the light and electron microscopic levels. As a second step, we sought to determine whether the NA innervation around intracortical microvessels arises from peripheral or central structures by means of injections of *N*-(2-chloroethyl-*N*-ethyl-2-bromobenzylamine) (DSP-4), a neurotoxin that specifically destroys NA neurons from the locus ceruleus. At the light microscopic level, 6.8% of all NA-immunoreactive nerve terminals in the frontoparietal cortex were associated with vascular walls, and this perivascular noradrenergic input, together with that of the cerebral cortex, almost completely disappeared after DSP-4 administration. When analyzed at the ultrastructural level in control rats, NA terminals in the neuropil had a mean surface area of 0.53 ± 0.03 μm^2 and were rarely junctional (synaptic incidence close to 7%). Perivascular terminals (located within a 3-μm perimeter from the vessel basal lamina) counted at the electron microscopic level represented 8.8% of the total NA terminals in the cortical tissue. They were smaller (0.29 ± 0.01 μm^2, $P < 0.05$) than their neuronal counterparts and were located, on average, 1.34 ± 0.08 μm away from intracortical blood vessels, which consisted mostly of capillaries (65%). None of the perivascular NA terminals engaged in junctional contacts with surrounding neuronal or vascular elements. The primary targets of both neuronal and perivascular NA nerve terminals consisted of dendrites, nerve terminals, astrocytes, and axons, whereas in the immediate vicinity (0.25 μm or less) of the microvessels, astrocytic processes represented the major target. The results of the current study show that penetrating arteries and intracortical microvessels receive a central NA input, albeit parasynaptic in its interaction, originating from the locus ceruleus. Particularly, they point to frequent appositions between both neuronal and perivascular NA terminals and astroglial cells and their processes. Such NA neuronal–glial and neuronal–glial–vascular associations could be of significance in the regulation of local metabolic and vascular functions under normal and pathologic situations. **Key Words:** Astrocytes—Cerebral blood vessels—Glycogenolysis—Locus ceruleus—Microcirculation—Ischemia.

The cerebral cortex receives a rich input from brainstem noradrenaline (NA) neurons located in the locus ceruleus (LC) (Mason and Fibigier, 1979). This cortical afferent pathway has been associated with multiple functions such as sleep–wake cycle and motor activity while in the hypothalamus, NA is involved in the regulation of temperature control as well as eating and drinking behaviors (Kramarcy et al., 1984; Lin et al., 1984; Hilakivi, 1987; Towell et al., 1989). Evidence also shows that NA and astrocytes work in tandem to modulate several cellular functions that include neurotransmission (Kimelberg, 1986), synthesis and release of neurotrophic factors (Schwartz and Mishler, 1990), and neuroprotection against ischemic insults (Blomqvist et al., 1985). Recently, NA has been shown to influence astrocytic properties such as intercellular communication, glycogenolysis, and glucose uptake (Giaume et al., 1991; Tsacopoulos and Magistretti, 1996).

A role for NA in vascular-related functions such as regulation of blood-brain barrier (BBB) permeability and CBF also has been suggested. Stimulation of the LC can increase or decrease BBB permeability, depending on the experimental paradigm (Raichle et al., 1975; Harik,

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Abbreviations used: BBB, blood-brain barrier; DSP-4, *N*-(2-chloroethyl-*N*-ethyl-2-bromobenzylamine); EM, electron microscopic; LC, locus ceruleus; LM, light microscopic; NA, noradrenaline; PBSM, phosphate-buffered saline containing sodium metabisulphite.

1986; Borges et al., 1994), and it also induces small decreases in local CBF (Raichle et al., 1975, De la Torre, 1977; Goadsby and Duckworth, 1989; Adachi et al., 1991) that can be blocked by α_2-adrenoceptor antagonists (Goadsby et al., 1985). Conversely, pharmacologic manipulations that selectively destroy central noradrenergic neurotransmission (Yokote et al., 1986; Kobayashi et al., 1991) increase CBF—a response that can be reversed by addition of NA.

Overall, these observations suggest that NA might directly interact with brain astrocytes and microvasculature. In this respect, NA fibers coursing along or approaching small blood vessels have been observed in various deep brain nuclei and in the cerebral cortex (Edvinsson et al., 1973; Jones, 1982), a region where NA neuronal–astroglial interactions have been documented (Séguéla et al., 1990). Nerve endings (presumably noradrenergic) were found to be intimately associated with intraparenchymal blood vessels in the richly vascularized paraventricular nucleus of the hypothalamus (Swanson et al., 1977), and NA terminals in the vicinity of local microvessels have been incidentally reported in the medulla oblongata and cerebral cortex (Milner et al., 1989, Papadopoulos et al., 1989).

To establish a morphologic substrate for the effects of NA on brain astrocytes and microvessels, we performed a quantitative and morphometric analysis of the neuronal–glial and/or vascular associations of NA nerve terminals by immunocytochemistry at the light microscopic (LM) and electron microscopic (EM) levels. The central origin of the NA fibers associated with cortical microvessels was assessed with the neurotoxin *N*-(2-chloroethyl-*N*-ethyl-2-bromobenzylamine) (DSP-4), which is highly selective for NA terminals originating from the LC (Grzanna et al., 1989; Fritschy et al., 1990). Parts of these results have been presented as an abstract (Cohen et al., 1994).

MATERIALS AND METHODS

Tissue preparation

Adult male Sprague Dawley rats (Charles River, 250 g, n = 7) were deeply anesthetized with sodium pentobarbital (Somnotol, 65 mg/kg body weight intraperitoneally). They were perfused intracardially first at low speed (100 mL/minute) with 50 mL phosphate-buffered saline (0.1 mol/L, pH 7.4) and then rapidly (300 mL/minute) with 600 mL of 5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer containing 0.2% of sodium metabisulfite (PBSM). After removal, the brains were immersion-fixed in the glutaraldehyde solution for 2 hours at room temperature. Brains processed for LM study were cryoprotected with 30% sucrose overnight, frozen in isopentane, and then coronal sections (30 or 60 μm) at the level of the frontoparietal cortex were obtained on a freezing microtome and collected in PBSM. Brains processed for EM analysis were immediately cut (thick sections of 60 μm) on an Oxford vibratome after the postfixation period and collected in PBSM. Before incubation with primary antibodies, the sections were reacted with 0.5% sodium borohydride and rinsed in PBSM, as previously documented (Séguéla et al., 1990). All experiments were approved by the Animal Ethics Committee based on the guidelines of the Canadian Council on Animal Care.

DSP-4 lesion of NA terminals

For noradrenergic denervation, adult rats (n = 4) received a first intraperitoneal injection (60 mg/kg; 7 to 10 days before perfusion) followed 3 to 4 days later by a second injection (50 mg/kg) of DSP-4 in sterile saline, whereas control rats (n = 5) received injections of saline alone (Berger et al., 1988). Both DSP-4–injected and control rats were perfused as just described and processed simultaneously for immunocytochemical examination at the LM level (see later).

Immunocytochemical study of NA

Free-floating sections were incubated overnight with an antiserum directed against a NA–glutaraldehyde–protein conjugate, diluted 1/5000 in PBSM and 1% normal goat serum. The production and specificity of this antibody has been previously described (Geffard et al., 1986; Mons and Geffard, 1987). The sections then were thoroughly rinsed in PBSM, incubated sequentially with a biotinylated goat anti-rabbit immunoglobulin G (1/100, Vector Labs, Burlingame, CA, U.S.A.), and the avidin–biotin–peroxidase complex (1/50, ABC, Vectastain Elite kit, Vector Labs). Rinses of 0.1 mol/L phosphate-buffered saline were carried out before and after each antibody incubation. The immunocytochemical product was revealed with 0.05% 3.3-(±) diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) containing 0.01% hydrogen peroxide in 0.1 mol/L Tris-HCl (6 minutes).

Only sections prepared for LM analysis were incubated in solutions supplemented with 0.1% Triton X-100. The 30-μm thick immunostained sections were mounted on gelatin-coated slides, dehydrated, and defatted before observation and photography under a Leitz Aristoplan light microscope. The 60-μm thick sections were used either for high-resolution LM (sections from cryoprotected brains) or ultrastructural (vibratome-cut sections) analysis. All were postfixed with 2% osmium tetroxide in 0.4 mol/L sodium phosphate buffer containing 7% dextrose and then processed for flat embedding in Araldite 502 resin (for details, see Cohen et al., 1995). After polymerization, small blocks comprising the region of interest were trimmed and reembedded, and semithin (2-μm) sections comprising all six layers of the frontoparietal cortex or thin (90 to 120 nm, straw color) sections of the cortical upper layers were obtained using a Reichert ultramicrotome for LM and EM study. Semithin sections were observed and photographed under a Leitz Aristoplan light microscope. Thin sections were recovered on copper grids, double stained with uranyl acetate and lead citrate, and examined with a JEOL CX100II electron microscope at a working magnification of ×8000 to 14,000.

Analysis at the light microscopic level

Analysis of NA-immunoreactive nerve fibers associated with local microvessels within the frontoparietal cortex was done on photomicrographs of semithin sections. For this purpose, all NA-immunopositive nerve endings in 10 different cortical slices (n = 3 rats) were counted, including those (hereafter referred to as perivascular) that were directly apposed to blood vessel walls. The perivascular terminals then were expressed as a percentage of total NA terminals. The efficacy of the DSP-4 lesion also was verified on thick and semithin sections.

Ultrastructural analysis

Neuronal terminals. All cortical NA terminals that were not perivascular (see later) were treated as neuronal. Their imme-

diate microenvironment, surface area, and synaptic frequency were determined in single thin sections. The NA immunopositive axonal varicosities (n = 130) were randomly chosen (every fifth terminal encountered) and photographed. The cellular elements (n = 712) apposed to these NA nerve endings were identified (an average of six appositions were observed for each terminal) and classified as dendrites (dendritic trunks, shafts, or spines), nerve terminals, axons, myelinated axons, astrocytic cells, and nerve cell bodies. The prints also were used to measure the surface area of each varicosity using a Bioquant II analysis program and a MTI 65 camera, and the proportion of NA terminals engaged in synaptic contacts. Varicosities were considered to be synaptic when at least one of the juxtaposed membranes exhibited a straightening or thickening with an expanded intercellular space with or without a postsynaptic density (Peters et al., 1991). The synaptic incidence, which yields a precise estimate of the overall proportion of cortical NA terminals engaged in synaptic junction, was calculated using the extrapolated stereologic formula of Beaudet and Sotelo (1981).

Perivascular terminals. The NA-immunoreactive terminals were defined as perivascular when located within a 3-μm perimeter from the basal lamina of a blood vessel, a distance corresponding to that previously reported for functional perivascular nerve fibers (Lee, 1981; Dodge et al., 1994; Chédotal et al., 1994). This interval was defined as the largest distance within which axon terminals might affect vascular functions either directly or indirectly through interactions with other neuronal or nonneuronal elements within the perivascular perimeter. The percentage of perivascular NA terminals in a given area of frontoparietal cortex was determined. For this purpose, from a total of 458 NA immunolabeled terminals observed directly on the EM screen, the population of perivascular terminals was identified with the help of scale bars to determine whether the terminal was within 3 μm from the vessel. They were then expressed as a percentage of the total amount of NA terminals counted within a given area. In addition, the first 125 perivascular NA terminals encountered were photographed and their distance from the vessel wall, surface area, synaptic incidence, and immediate microenvironment determined. The cellular elements (n = 643) contacted by NA perivascular terminals were divided as described earlier for the population of neuronal terminals. Particular attention was given to the microenvironment of nerve terminals located within the first 0.25 μm from the vessel basal lamina. The type of vessel associated with each terminal was identified directly on the EM screen and recorded for analysis. Capillaries were defined as small blood vessels (<10 μm) comprising a layer of endothelial cells within a basal lamina with or without associated pericytes. Arterioles were larger vessels (>10 μm) consisting of one or two layers of smooth muscle cells enclosed by the basal lamina (Peters et al., 1991)

RESULTS

Light Microscopy

The NA immunostaining in the frontoparietal cortex, as observed in 30-μm thick sections, corresponded to that previously described by ^{3}H-NA radioautography, dopamine-β-hydroxylase– and NA–immunocytochemistry (Audet et al., 1988; Olschowska et al., 1981; Séguéla et al., 1990). Long varicose fibers, running vertically and some sideways, were scattered throughout the neocortex with an increased density in the most superficial layer (Fig. 1). Some fibers were seen to approach and run closely to intraparenchymal blood vessels. In semithin sections (Fig. 1B and C), the quantitative analysis showed that 412 of the 6074 immunoreactive NA terminals in the cerebral cortex were directly apposed to blood vessel walls, corresponding to 6.8% of all cortical NA nerve terminals.

DSP-4 treatment. When compared with saline-injected rats, DSP-4–treated rats exhibited a marked reduction in the density of cortical NA nerve fibers, including those associated with intracortical vessels (Fig. 2). Despite this massive cortical denervation, NA fibers running in the pia-arachnoid membrane and associated with pial blood vessels at the surface of the brain parenchyma were still present (Fig. 2).

Electron Microscopy

Neuronal terminals. At the ultrastructural level, the immunostained NA profiles were found to correspond primarily to round or ovoid varicosities as well as to longitudinal axon fibers. They contained numerous small synaptic and a few dense core vesicles, with one or more mitochondria (Fig. 3). The immunolabeled nerve endings were juxtaposed mostly on dendritic processes, unlabeled axonal varicosities, astroglial processes, less so on axons, and only rarely on cell soma and myelinated axons (Fig. 3; Table 1). Overall, neuronal NA terminals had a mean surface area of 0.53 ± 0.03 μm^2 (calculated diameter of about 0.82 μm) and rarely exhibited synaptic junctions in single thin sections, exclusively asymmetrical and with dendritic elements (Table 1). When extrapolated to whole volume, a synaptic incidence of about 7% was calculated.

Perivascular terminals. A total of 458 NA-immunoreactive terminals were counted in the frontoparietal cortex, and 8.8% (n = 44) of them were found to be located within 3 μm from microvessels (Fig. 4). When studied in more detail on a population of 125 perivascular NA terminals, 23 terminals were located in the immediate vicinity (≤0.25 μm) of the vessel basement membrane (Figs. 4 through 6). The remaining terminals were distributed uniformly throughout the 3-μm perimeter around vessel walls (Fig. 6). Perivascular terminals were similar in appearance and shape to their neuronal congeners, except that they corresponded almost exclusively to axonal varicosities (Figs. 4 and 5)

FIG. 1. Photomicrographs depicting the distribution of NA-immunoreactive nerve fibers in the frontoparietal cortex in 40-μm thick sections (A) and 2-μm semithin sections (**B and C**). **A:** Long varicose fibers coursing through the cerebral cortex are observed with a slight increase in density in the upper layers. **B and C:** In semithin sections, some NA-immunopositive nerve terminals are directly apposed to intracortical blood vessels (arrows), corresponding to capillaries and small arterioles. Scale bars = 50 μm.


A

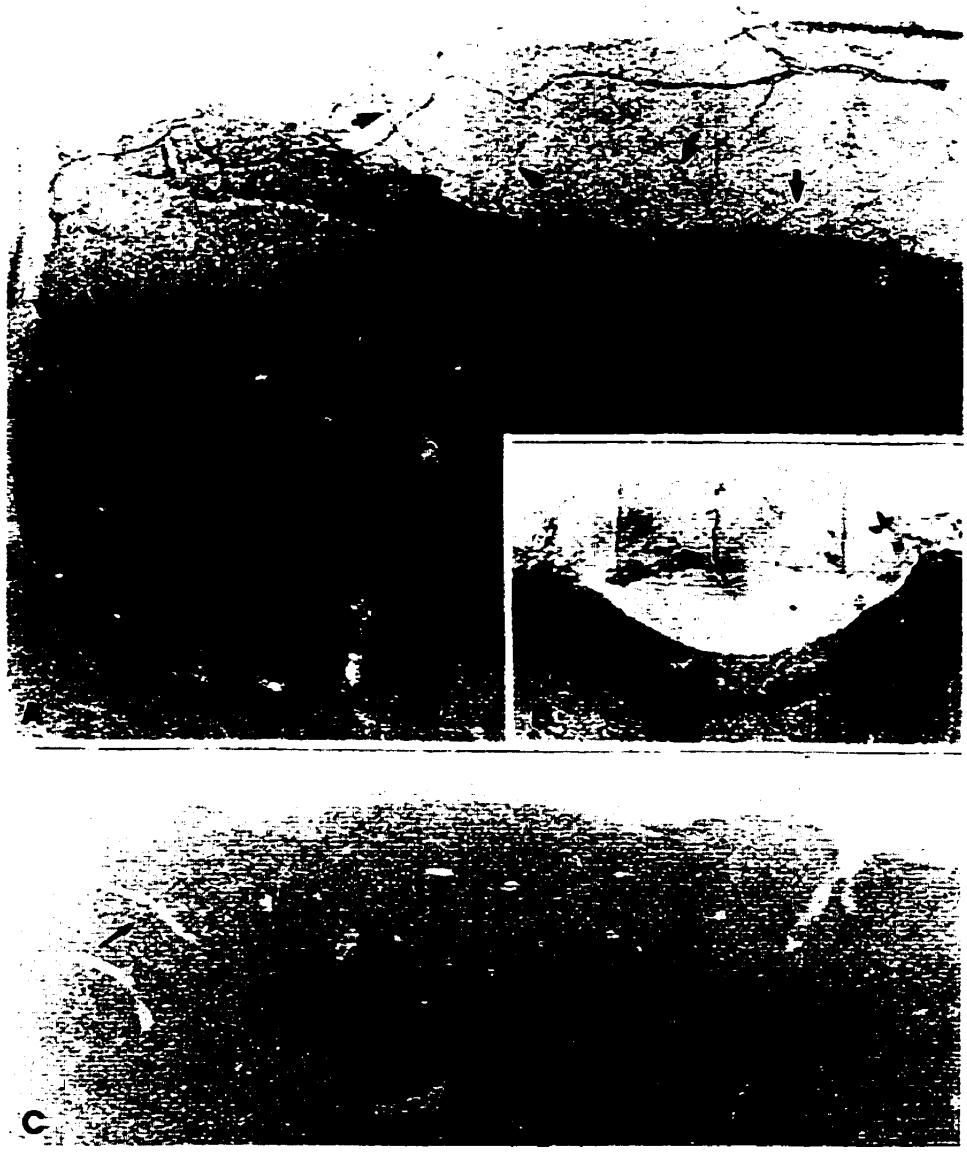

FIG. 2. Photomicrographs of thick **(A and B)** and semithin **(C)** sections immunolabeled for NA in the rat frontoparietal cortex after treatment with DSP-4. The NA varicose fibers (thick arrows) are still present in the pia-arachnoid membrane **(A)** and small pial vessels **(B)** after DSP-4, whereas in the cerebral cortex **(A and C)**, they almost completely disappear, with only residual fibers (thin arrows) being found in the neuropil and in association with intraparenchymal blood vessels. Scale bars = 50 μm.

and seldom to axon fibers. The microenvironment of the perivascular terminals, like that of the neuronal population, was composed mostly of dendrites, nerve terminals, and astrocytes (Table 1). In the closest interval (0.25 μm or less) around the vessel wall, however, 75% of the terminals (17 of 23) abutted on a perivascular astrocyte, whereas of all targets, the astrocytes (either perivascular or in the neuropil) represented the primary cellular elements of apposition (32.7%) followed by nerve terminals (25.2%), axons (21.5%), and dendrites (19.6%). Most of perivascular associations were on capillaries (~65%) compared with small arterioles (~35%). Interruptions in the perivascular astrocytic leaflets allowing NA terminals to directly touch the vascular basal lamina were rarely observed. None of the perivascular terminals were seen to engage in synaptic contact with any of the ap-

FIG. 3. Electron micrographs illustrating the general morphologic features of neuronal NA-immunostained axon terminals in the frontoparietal cortex and their immediate microenvironment. Terminals occasionally contain dense-core vesicles and are primarily apposed to dendrites (dt), axon terminals (at), axons (ax), and astrocytes (arrows). Scale bars = 0.5 μm.

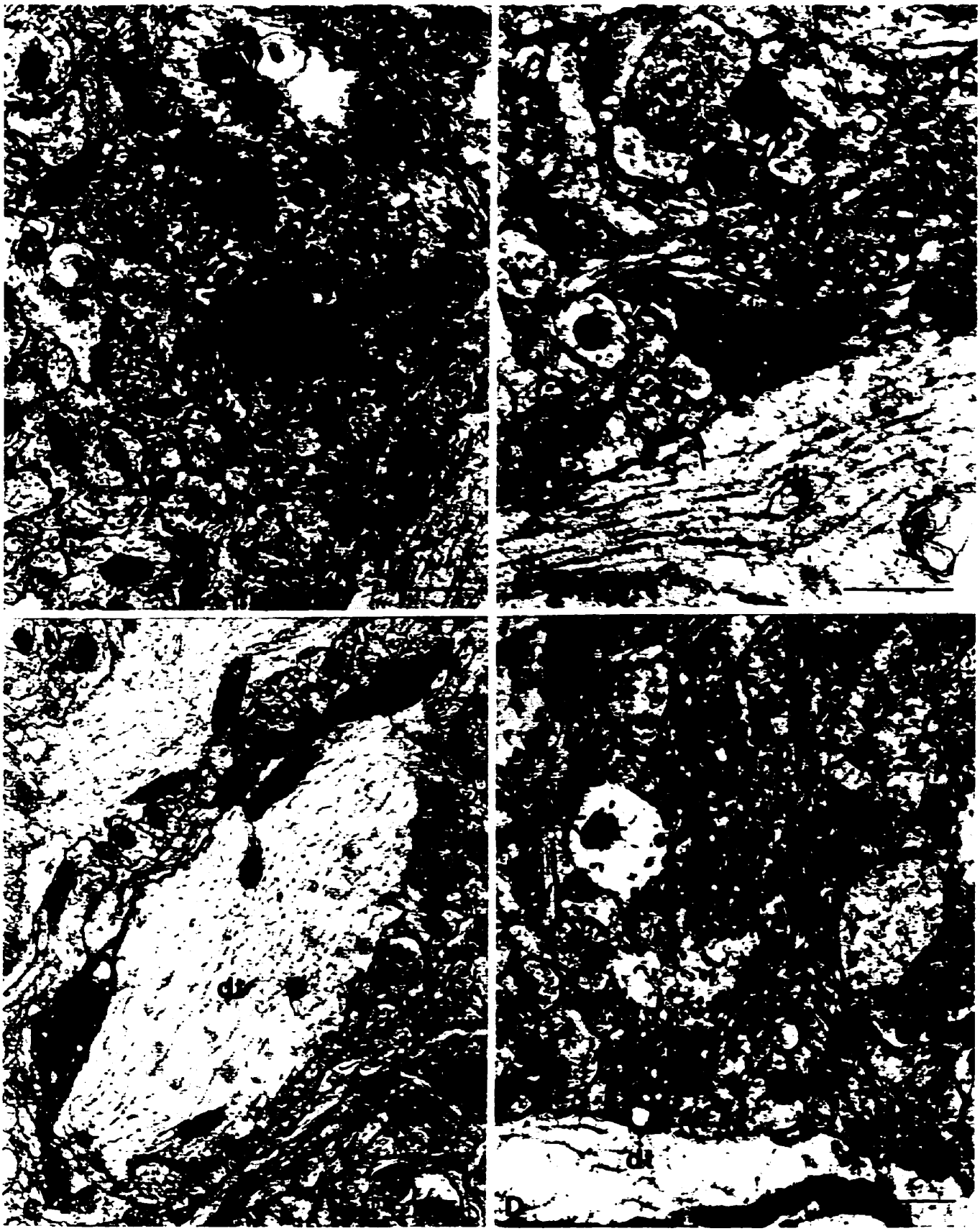
dt
dt

TABLE 1. *Morphological characteristics and microenvironment of neuronal and perivascular NA-immunoreactive nerve terminals in the rat frontoparietal cortex*

	Neuronal terminals	Perivascular terminals
Morphological features		
n	130	125
Area (μm^2)	0.53 ± 0.03	0.29 ± 0.01*
Diameter (μm)	0.82	0.61
Synaptic frequency (%)	2.4	0
Synaptic incidence (%)	7.1	0
Distance from vessel (μm)	—	1.34 ± 0.08
Microenvironment† (%)		
Dendritic elements	31.9	27.1
Nerve terminals	27.1	25.5
Astrocytic processes	21.8	25.2
Axons	18.4	21.4
Cell bodies/myelinated axons	0.8	0.8

* Perivascular population is statistically smaller ($P < 0.05$) from neuronal population.

† Immediate microenvironment juxtaposed to NA-immunolabeled nerve terminals. See Materials and Methods for detailed definition of surrounding neuronal elements.

posed cellular elements within the perivascular perimeter. The perivascular terminals were located on the average 1.34 ± 0.08 μm from intracortical blood vessel walls and were significantly smaller (mean surface area of 0.29 ± 0.01 μm^2, $P < 0.05$) than their neuronal counterparts (Table 1).

DISCUSSION

The current study examines the relations between NA-containing neurons and intracortical astrocytes and microvessels. Our results show that the LC is the major, if not exclusive, source of NA fibers to the cortical neuropil but also to the local microvascular bed. Furthermore, the results show that NA terminals in the cerebral cortex frequently target glial processes and, more frequently so, the perivascular astroglial leaflets.

General features

The LM and ultrastructural analyses of NA immunostained elements in the frontoparietal cortex yielded results consistent with those of previous radioautographic and immunocytochemical studies on cortical NA innervation (Olschowka et al., 1981; Audet et al., 1988; Papadopoulos et al., 1989; Séguéla et al., 1990). The NA innervation of the cerebral cortex was predominantly nonjunctional, and perivascular terminals never established synaptic junctions with vascular, glial, or other neuronal elements. They were smaller compared with neuronal terminals, perhaps because of the fact that the perivascular elements or blood vessels themselves were their final targets. The frequent incidence of axo-axonic appositions for both neuronal and perivascular cortical terminals is fully compatible with the capacity of NA to modulate other neurotransmitters release within the cerebral cortex (Beani et al., 1986; Pittaluga et al., 1990; Raiteri et al., 1990), an aspect that also may be highly relevant to local regulation of brain perfusion (Raszkiewicz et al., 1992).

Perivascular NA terminals

Selective destruction of NA neurons from the LC with DSP-4 (Grzanna et al., 1989; Fritschy et al., 1990) resulted in a massive denervation of not only the cortical neuropil but also the penetrating arteries and intracortical microvessels. This observation suggests that the noradrenergic input to the cortical microcirculation, like that of the hypothalamus (Swanson et al., 1977), is of central origin. This conclusion concurs with the reported "denervation supersensitivity" of cortical microvascular adrenoceptors in rats after chemical lesions of the LC (Kalaria et al., 1989) and in patients with Alzheimer's disease, a condition known to be accompanied by degeneration of the NA system (Kalaria and Harik, 1989). Together with the presence of functional high-affinity α- and β-adrenoceptors in brain microvessels, smooth muscle, and endothelial cells in culture (Nathanson et al., 1979; Wroblewska et al., 1984; Bacic et al., 1992), these observations strongly suggest that intracerebral vessels can respond to neurally released NA. Interestingly, DSP-4 treatment did not affect the NA innervation of the pial vessels, which is consistent with the superior cervical ganglion origin of their noradrenergic innervation (Duverger et al., 1987). These results suggest that distinct neurogenic mechanisms with sympathetic and central origins provide a NA input to extracerebral and intracerebral blood vessels, respectively.

However, the neurovascular associations between NA terminals and the cortical microvessels were not as frequent and/or as intimate as those reported previously for basalocortical acetylcholine and brain stem serotonin (5-hydroxytryptamine) cortical projections (Chédotal et al., 1994; Vaucher and Hamel, 1995; Cohen et al., 1995), two systems believed to affect local CBF partly through a direct action on the microvascular bed (Sato and Sato, 1992; Cohen et al., 1996). Cortical neurovascular NA associations compared much better with those of acetylcholine (Vaucher and Hamel, 1995) and 5-hydroxytryptamine (Cohen et al., 1995) in brain areas where only weak or no changes in CBF have been found. This may indicate that NA regulation of CBF, at least in the cerebral cortex, is not the primary role of these neurovascular or neuronal–glial–vascular interactions. Such contention is supported by the relatively minor changes that have consistently been observed in cortical perfusion after manipulations of the NA system (Adachi et al., 1991; Kobayashi et al., 1991) and may point to a role of perivascular NA terminals in BBB and astrocytic functions.

The NA terminals preferentially targeted capillaries

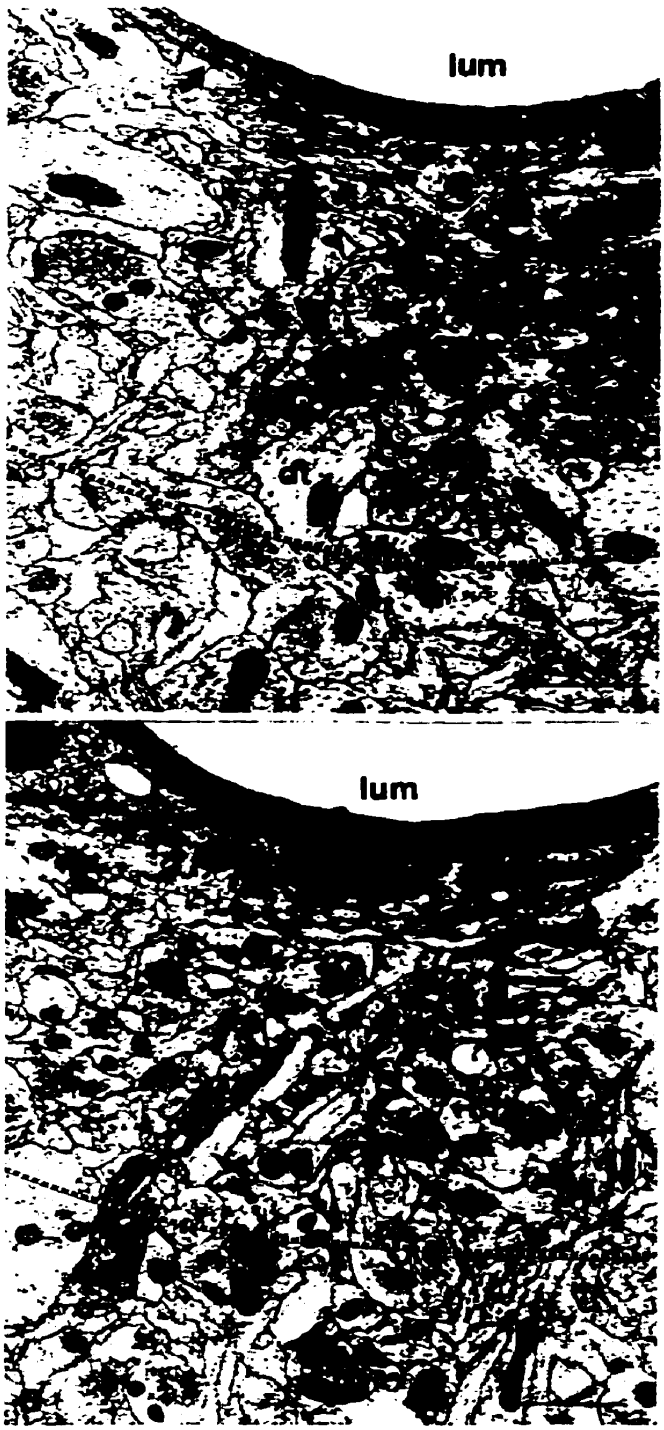

FIG. 4. Electron micrographs of NA perivascular terminals in the rat frontoparietal cortex. The 3-μm perimeter around the vessel basal lamina is delineated by the dotted line. **(A)** An NA immunoreactive nerve terminal is located close to an arteriole containing a smooth muscle profile (curved arrow) and is apposed to various neuronal elements in the neuropil including an astrocytic leaflet (straight arrow). **(B)** The NA-labeled fiber (thin arrows) is seen coursing within the perivascular area, where it seems to abut the basement membrane of the vessel because of the thin astrocytic leaflet (open arrows). Notice that part of the axon fiber is outside of the 3-μm perimeter (dotted line). at, axon terminal; ax, axon; dt, dendrite; lum, lumen. Scale bars = 1 μm.

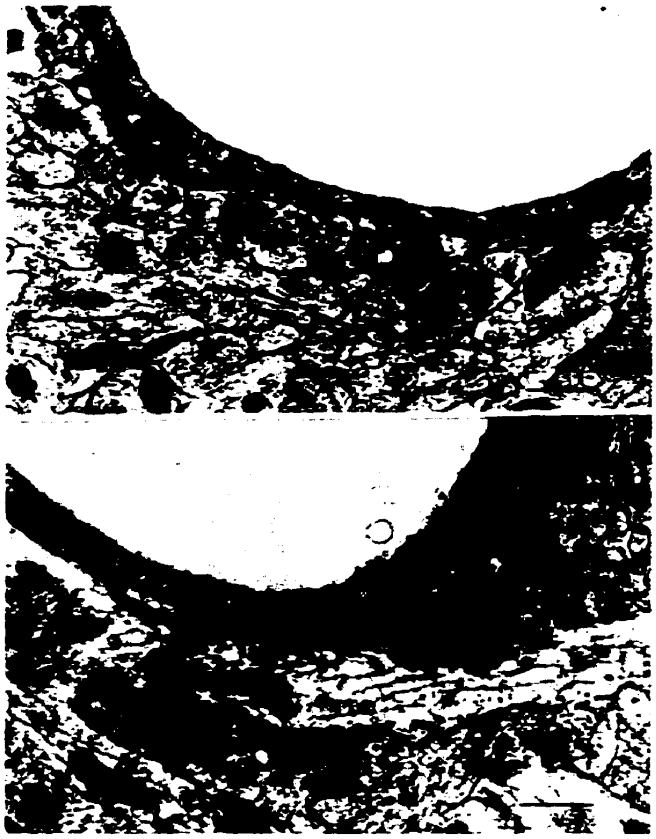

FIG. 5. Electron micrographs of NA terminals associated with cortical microvessels. NA-labeled terminals are separated from the basal lamina of a capillary (A) or microarteriole (B) only by the perivascular astrocytic leaflet (open arrows). The curved arrow in (A) points to a small pericyte embedded within the basal lamina. at, axon terminal; dt, dendrite; solid arrows, astrocyte. Scale Bar = 0.5 μm.

compared with microarterioles. In this respect, LC stimulation has been shown to produce significant changes in the transport of water and solutes across the BBB (Raichle et al., 1975; Sarmento et al., 1994; Borges et al., 1994), an effect consistent with the presence of functional adrenoceptors on brain endothelial cells (Bacic et al., 1992). The possibility that the primary role of the perivascular NA terminals is related to regulation of the BBB permeability is of interest since dysfunctions at this level, including alterations in microvascular adrenoceptors (Kalaria and Harik, 1989), are thought to contribute to the overall pathology of Alzheimer's disease (see Kalaria, 1992; De la Torre and Mussivand, 1993).

Association of NA terminals with astrocytes

The astroglial cells constituted the third most abundant targets for cortical NA-immunolabeled terminals (Table 1; Séguéla et al., 1990), a percentage that we found to be slightly higher for the perivascular NA nerve terminals and even more so for those located close to vessels. Indeed, for NA terminals located in the immediate vi-

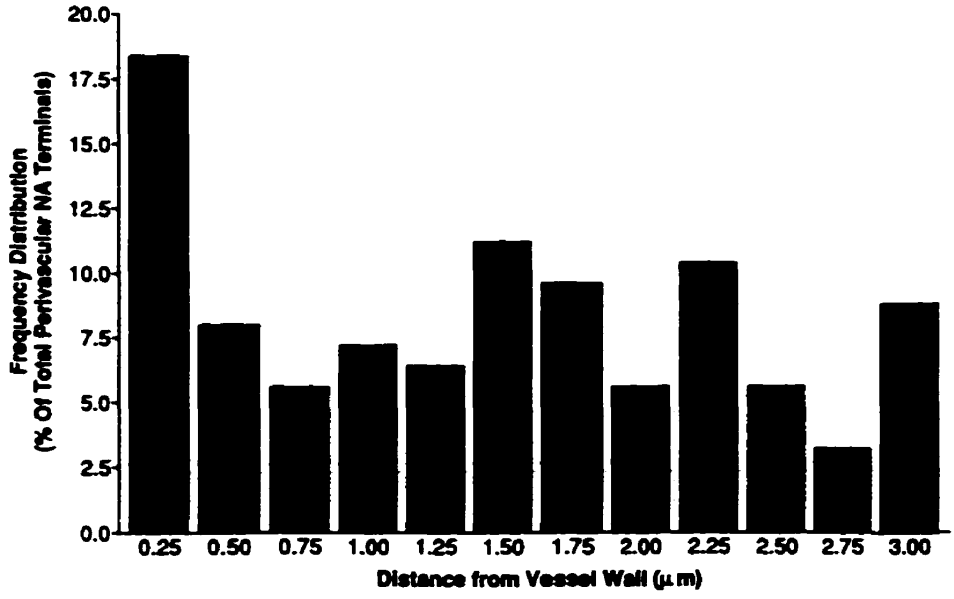

FIG. 6. Histogram of the distribution of NA-immunoreactive terminals within the 3-μm perimeter around intracortical microvessels. The distance from the blood vessels is separated into 0.25-μm intervals, and percentages correspond to the number of terminals found within each interval, compared with the total number (n = 125) of perivascular terminals.

cinity (0.25 μm or less) of blood vessels, the astrocytic processes, whether perivascular or in the surrounding neuropil, represented their major cellular target (Table 1; see Results). These observations strongly suggest that the astrocytic processes are likely to be the direct cellular target of perivascular NA terminals. Such a statement is supported by a recent, although nonquantitative, ultrastructural study on the relations of NA terminals with nonneuronal elements in the rat visual cortex (Paspalas and Papadopoulos, 1996). Together, our studies and theirs strongly underline the postulated contribution of brain astrocytes as integral components of brain neuronal–glial or vascular interactions within the cerebral cortex (Barres, 1991; Hertz, 1992; Cancilla et al., 1993). The reported ability of NA to modulate production and secretion of nerve growth factor from astroglial cells (Furukawa et al., 1987; Schwartz and Mishler, 1990) and its possible intrinsic growth properties on brain microvessels, similar to those reported on smooth muscle cells of peripheral origin (Blaes and Boissel, 1983; Chen et al., 1995), also are in line with a localization of NA terminals in the vicinity of cortical microvessels and astrocytes.

Functional adrenergic receptors of various types are widely expressed in brain astrocytes (Salm and McCarty, 1992; Hösli and Hösli, 1993), and, in the rat visual cortex, astrocytic processes exhibiting immunoreactivity to the β-adrenergic receptor subtype often were interposed between NA nerve endings and the vascular basal lamina (Aoki, 1992). It is thus likely that NA, through parasynaptic interactions with astroglial cellular elements, could regulate astrocytic functions whether related to BBB properties, intercellular communication along the astrocytic syncytium, and metabolic or ionic homeostasis (Giaume et al., 1991; Hertz, 1992; Laterra et al., 1994; Montgomery, 1994; Tsacopoulos and Magistretti, 1996). More specifically, the frequent associations between NA terminals and astrocytes may provide the morphologic basis for both the β-adrenoceptor–mediated, rapid, and long-term control of glycogen levels induced by NA in cerebral cortical astrocytes (Sorg and Magistretti, 1991, 1992). However, astrocytes also have been shown to reorganize their syncytial network, a mechanism regulated by NA (Giaume et al., 1991) to promote neuronal survival after ischemic injury (Hossain et al., 1994). In this regard, lesions of the LC increase neuronal necrosis after ischemia (Blomqvist et al., 1985), whereas stimulation of NA neurons decreases the detrimental effects of ischemia (Gustafson et al., 1990). It is thus tempting to speculate that NA neurotransmission would not only regulate astrocytic functions under normal conditions, but that NA neuronal–glial interactions could be of primary importance in protecting the brain from damage caused by ischemic insults. In conclusion, these results emphasize the current view that neurotransmitters are important mediators of astrocytic functions, whether related to their actions in neurotransmission, homeostasis, or vascular regulation.

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CHAPTER 6

MOLECULAR AND PHARMACOLOGICAL CHARACTERIZATION OF FUNCTIONAL SEROTONIN RECEPTORS IN HUMAN BRAIN MICROCIRCULATION AND ASTROCYTES

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PREFACE TO CHAPTER 6

Based on the physiological, pharmacological and our anatomical evidence, it would appear that intraparenchymal blood vessels can respond to changes in 5-HT neurotransmission (Bonvento et al., 1989; McBean et al., 1990; 1991; Cudennec et al., 1993; Chapter 4). However, limited information is available on the presence and identity of the receptor(s) that could possibly mediate local vasomotor and/or permeability 5-HT-mediated functions within the microvascular bed. In human intracortical microvessels, early radioligand binding studies suggested the presence of a 5-HT_1 receptor subtype but its exact nature could not be identified (Peroutka et al., 1981; O'Neill et al., 1988). More recent work on human cerebromicrovascular endothelial cells showed that application of 5-HT increased cAMP levels (Spatz et al., 1989; Bacic et al., 1991), suggesting the presence of either 5-HT_4, 5-ht_6 and/or 5-HT_7 receptors, all of which stimulate adenylate cyclase. However, due to the lack of pharmacological tools, the precise receptor mediating this response could not be established. In other species, pharmacological manipulations have suggested the presence of cerebromicrovascular $5\text{-HT}_{1B/1D}$ and 5-HT_{2A} receptors (Dietrich et al., 1989; Sharma et al., 1990; Cao et al., 1992; Kobari et al., 1993). This paucity of information contrasts with the relative abundance of pharmacological and molecular information on 5-HT receptor types and subtypes that mediate vasomotor responses in extracerebral and peripheral vessels from human and non-human species (see sections 1.4.2.1 and 1.4.2.2).

In the last part of my thesis, taking advantage of the recent cloning of several 5-HT receptor subtypes, we thus attempted to identify the nature of the 5-HT receptor(s) present on human isolated intracortical blood vessels. More specifically to establish their distribution within the various cellular compartments (endothelial, smooth muscle and astroglial cells) that may be relevant to the functional microvascular unit (Chédotal et al., 1994; Cohen et al., 1995; Harder et al., 1995). Whole microvessels (MVs) and capillaries (CAPs) were obtained from human post-mortem brains while endothelial and smooth muscle cell cultures were grown, in collaboration with Dr. D. Stanimirovic (National

Research Council of Canada, Ottawa), from MVs and CAPs harvested from human temporal cortex biopsies obtained from patients undergoing treatment of idiopathic epilepsy. The astrocytic cultures were derived from human fetal brains and obtained from Dr W.V. Yong (Montreal Neurological Institute).

Due to the large number of identified 5-HT receptors, our analysis was restricted to those previously suggested to mediate vasomotor responses in peripheral and/or cerebral blood vessels i.e. 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_7 receptors (see Section 1.4.2.1 and 1.4.2.2). Although there is no evidence that 5-ht_{1F} receptors mediate vasomotor effects (Hamel et al., 1993; Phebus et al., 1996), these were included in our investigation largely because they have been shown to be expressed in human extracerebral arteries (Bouchelet et al., 1996a). Based on this information, we examined the expression of mRNA of all these receptors by way of reverse transcriptase-polymerase chain reaction (RT-PCR) in the isolated MVs and CAPs as well as the three cell cultures. Further, in an attempt to provide a first assessment that the receptor messages were also translated into functional proteins, we evaluated the ability of the identified receptors to couple to their expected second messenger systems. This was examined in the three cell culture preparations by measuring their ability to decrease (5-HT_1 family) or increase (5-HT_7 family) the production of cAMP or to stimulate IP_3 formation (5-HT_2 family).

The results show a differential expression of functional 5-HT receptors in the various compartments of the microvascular wall; 5-HT_{1D} and 5-HT_7 receptors in the endothelial cells, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors in the smooth muscle and 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F}, 5-HT_{2A}, and 5-HT_7 receptors in astrocytes. The possibility of 5-HT_{2B} and 5-HT_{2C} receptors in these tissues was also tested but will be presented separately (see Chapter 6, Addendum).

ABSTRACT

Physiological and anatomical evidence suggest that 5-hydroxytryptamine (5-HT) neurons regulate local cerebral blood flow and blood brain barrier permeability. To elucidate the mechanisms via which these effects could be mediated, we used molecular and pharmacological approaches to assess the presence of functional 5-HT receptors in human brain microvascular fractions, endothelial and smooth muscle cell cultures, as well as astroglial cells known to associate with intraparenchymal blood vessels. Isolated cortical microvessels and capillaries expressed h5-HT_{1B}, h5-HT_{1D}, 5-ht_{1F}, 5-HT_{2A} but not 5-HT_7 receptor messages. When studied in the different cell types of the vessel wall, endothelial cells exhibited mRNA for the h5-HT_{1D} and 5-HT_7 receptors, whereas smooth muscle cells further showed PCR products for h5-HT_{1B} receptors. Messages for all 5-HT receptors tested were detected in human brain astrocytes with a predominance of the 5-HT_{2A} and 5-HT_7 types. In all cell cultures, the 5-HT_1 receptor agonist, sumatriptan, significantly inhibited (55-80%, $p \leq 0.05$-0.01) the forskolin-stimulated production of cAMP, an effect blocked by the selective 5-$HT_{1B/1D}$ receptor antagonists, GR127935 and GR55562. Similarly, 5-HT or the non-selective 5-HT receptor agonist, 5-carboxamidotryptamine, induced general increases (55-106%, $p \leq 0.05$) in basal cAMP levels which were abolished by mesulergine, a non-selective 5-HT_7 receptor antagonist. In contrast, only astroglial cells showed a ketanserin-sensitive increase (77%, $p \leq 0.01$) in IP_3 formation when exposed to 5-HT. Taken together, these results demonstrate that functional 5-HT receptors are differentially distributed within the various cellular compartments of the microvascular bed: h5-HT_{1B} receptors in smooth muscle cells and astrocytes , h5-HT_{1D} receptors in endothelial and astroglial cells, 5-ht_{1F} and 5-HT_{2A} receptors exclusively in astrocytes while 5-HT_7 receptors were ubiquitous to all cell types. These receptors could be involved in permeability changes, vasomotor and/or astroglial responses elicited by circulating and/or intracerebrally released 5-HT. These findings further emphasize the complexity of interactions between brain serotonergic pathways and non-neuronal cells within the CNS.

INTRODUCTION

Physiological evidence suggest that brainstem serotonin (5-hydroxytryptamine, 5-HT) neurons can regulate local cerebral blood flow (CBF) (Bonvento et al. 1989, McBean et al., 1990,1991; Underwood et al., 1992, 1995; Cudennec et al., 1993) and blood-brain barrier (BBB) permeability (Sharma et al., 1990). The predominant vascular response to 5-HT is a cerebral vasoconstriction, although a vasodilatation has also been observed (see Bonvento et al., 1993 for review). This dual effect has been related to the ability of different regions within the dorsal raphe nucleus to trigger opposite vasomotor responses (Underwood et al., 1992). In support to these serotonergic-mediated changes in CBF, ultrastructural studies have documented close associations between 5-HT nerve terminals and intraparenchymal microvessels (Itakura et al., 1985). Such associations have been observed more frequently in the frontoparietal cortex in which manipulations of 5-HT neurons result in significant CBF changes (Cohen et al., 1995b). These observations, taken together with the finding that 5-HT induces partial uncoupling between local perfusion and glucose oxidative metabolism (McBean et al., 1990; 1991; Bonvento et al. 1991; Cudennec et al., 1993), suggest that neurally released 5-HT can exert direct effects on the microcirculation, possibly through interaction with specific 5-HT receptors present on blood vessels.

However, despite these proposed vascular effects in response to central changes in 5-HT neurotransmission, virtually no information is available on the nature of 5-HT receptors in the cerebral microvascular bed. Early radioligand binding studies performed on isolated intracortical microvessels from human and bovine brains suggested the presence of 5-HT_1 binding sites (Peroutka et al., 1980; O'Neill et al., 1988). The *in vivo* administration of high doses of the 5-HT_1 receptor agonist, sumatriptan, was found to induce cerebral vasoconstriction in the cat (Kobari et al., 1993) while in the rat the 5-HT_2 receptor antagonist, ketanserin, has been shown to block the decrease in cortical flow induced by dorsal raphe stimulation (Cao et al., 1992) and to induce alterations in BBB permeabililty (Sharma et al. 1990). Furthermore, stimulation of cyclic AMP (cAMP)

production (Spatz et al., 1989; Bacic et al., 1991) has been observed following 5-HT addition to human brain endothelial cells in culture. However, these studies were unable to discriminate which subtypes of 5-HT receptors are involved in these responses due to the lack of selective pharmacological agents. With the advances in molecular cloning techniques and the development of novel pharmacological tools, several new 5-HT receptors and their pharmacological profiles have been identified (for review, Hoyer et al., 1994). Some of these 5-HT receptors are likely involved in the modulation of cerebral vascular functions. For instance, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A} and 5-HT_7 receptors have been demonstrated in major cerebral arteries and/or in peripheral blood vessels of various species including human, and have been implicated in either constriction or relaxation (Chang et al., 1988; Sumner et al., 1989; Schoeffter et al., 1990; Hamel et al., 1993a,b; Kaumann et al., 1993; Leung et al., 1996, Eglen et al., 1997).

The aim of the present study was to evaluate the expression of putative vascular 5-HT receptors in the human brain microvascular bed, to identify their cellular localization within the vascular and/or astroglial compartments, and to assess their ability to mediate functional responses. The results indicate that different 5-HT receptor subtypes coupled to distinct cellular signalling pathways are expressed by the specific endothelial, smooth muscle and/or astroglial cell types of cortical microvessels and capillaries. Furthermore, the nature of 5-HT receptors identified in these cellular constituents of the cerebromicrovascular bed suggest that 5-HT is likely involved in the regulation of both the cerebral microcirculation and the astrocyte functions, as they pertain to local brain perfusion and BBB permeability. Some aspects of this study have previously been presented in abstract form (Cohen et al., 1995a; Cohen and Hamel, 1996).

MATERIALS AND METHODS

TISSUE AND CELL CULTURE PREPARATION

Isolated microvessels (MVs) and capillaries (CAPs). Human brain cortices (n=6) were obtained at autopsy from the Royal Victoria Hospital (Montreal, Canada) from men (n=4) and women (n=2) who died from diseases not affecting the central nervous system (post-mortem delay of 16.2±2.2 hr). Microvascular and capillary fractions were isolated from the cerebral cortex by centrifugation in 15% dextran, as previously described (Linville and Hamel, 1995).

The preparations were stained with cresyl violet and the purity of MVs and CAPs was confirmed by the absence of mitochondria, cell debris or contaminating neuronal elements. In some cases, activities of microvascular and endothelial cell marker enzymes, γ-glutamyltranspeptidase (γ-GTP) and alkaline phosphatase (AP), were measured and found to be highly enriched as compared to cortical tissue (Linville and Hamel, 1995).

Human brain endothelial cells (HBEC). HBEC were isolated using a modification (Stanimirovic et al., 1996) of the procedure originally described by Gerhart et al. (1988). In short, CAPs and MVs were obtained from small samples of human temporal lobe surgically removed for the treatment of idiopathic epilepsy. Cortical homogenates were sequentially filtered through 350 μm and 112 μm mesh screens, resuspended in 20% dextran and centrifuged at 3,000xg for 15 min. The pellets (CAPs fraction) were resuspended in medium M199 (Gibco BRL, Gaithersburg, MD), collected on a 20 μm mesh, dissociated with the type IV collagenase (15 min, 37°C), then seeded and maintained at 37°C in an atmosphere of 5% CO_2 in air in growth media containing 65% M199, 10% fetal calf serum, 5% human serum, 20% murine melanoma cell (mouse melanoma, Cloudman S91, clone M-3, melanin producing cells)-conditioned media, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml selenium, and 10 mg/ml endothelial cell growth supplement.

Endothelial cell colonies emerging from attached CAPs were removed using cloning rings and 2-3 of these cloned colonies were pooled and grown to confluence. Confluent cultures from six different human biopsies (passages 3-7) were used in this study. They were routinely characterized by i) the 'cobblestone' appearance, typical of microvascular endothelium, ii) the selective immunostaining for angiotensin II-converting enzyme and Factor VIII-related antibodies, as well as incorporation of fluorescently labeled acetylated-low density lipoprotein

(DiI-Ac-LDL), and iii) high activities of the BBB-specific enzymes, γ-GTP and AP (Stanimirovic et al., 1996).

Human brain smooth muscle cells (HBSM). Resistance microvessels were isolated from homogenates of human temporal lobe biopsies by sequential filtration through 350 μm and 112 μm mesh nylon nets. The blood vessels, collected on these meshes, were dislodged in cold medium M199 (containing Earle's salts, 25 mM Hepes, 4.35 g/l, sodium bicarbonate and 3 mM L-glutamine), washed 3 times in the same medium, dissociated with type IV collagenase (5 min) and seeded into 0.5% gelatin-coated tissue culture plates or multiple well dishes in the same growth media as described for HBEC. Dissociated microvessels were allowed to grow in primary culture for 4-5 weeks, by which time the slow-proliferating, spindle-shaped smooth muscle cells largely outgrew the initial sprouting of endothelial cells. Characteristic criss-crossing and palisading of several smooth muscle cell layers were observed throughout the cultures which were routinely immunostained for the smooth muscle cell protein, α-actin (Accurate Chemical and Scientific Corp, Westbury, NY). A total of 6 primary cerebromicrovascular smooth muscle cell cultures derived from either 112 μm (n=2) or 350 μm (n=4) microvascular fractions were used in the present study.

Human brain astrocytes (HBA). HBA cultures (n=5) were generously provided by Dr. Wee Yong from the Montreal Neurological Institute. The cultures, prepared as described previously (Yong et al., 1992), were generated from fetal human brains, obtained from 10-18 week old fetuses aborted for medical indications with the approval from the Institutional Ethics Review Committee. The cells pelleted after filtration of dissociated brain tissue through a 130 μm mesh were resuspended in a feeding medium containing 95% Dulbecco's modified Eagle's medium (4500g/L glucose) and 5% fetal bovine serum and plated onto poly-l-lysine coated dishes. Contaminating neuronal clumps completely disappeared after initial passaging. Passages 2-5 were used for the experiments in this study. More than 95% of cells in culture were positive for the glial fibrillary acidic protein (GFAP), as determined by immunocytochemistry.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Isolated MVs and CAPs were homogenized with a Polytron while the cell cultures were lysed either by passing the lysate through a pipette or a syringue in the TRIzol Reagent (Gibco BRL). Total RNA was isolated

using the method developed by Chomczynski (1993) and was treated with DNase I (3 U, Gibco BRL) (20°C, 15 min) to remove any residual genomic DNA. The reaction was terminated by a phenol/chloroform extraction and the RNA was ethanol precipitated. The RNA was then primed with random hexamers (200 ng/μg RNA, Gibco BRL) and reverse transcribed (15 min at room temperature and then 60 min at 42°C) in a final volume of 50 μl containing 1-3 U of avian myeloblastosis virus reverse transcriptase (AMV RT, Promega, Madison, WI). PCR amplifications were carried out for 35-40 cycles (94°C for 40 sec, 56°C to 58°C for 40 sec depending on receptors and 72°C for 60 sec) with a pre- and post-incubation of 94°C for 5 min and 72°C for 5 min, respectively. They were performed in an MJ Research thermal cycler in a final 50 μl reaction volume that included 1-5 μl of reverse-transcribed RNA template, 0.3-0.5 μM of each primer and 2 U of Taq polymerase (Promega). The PCR products were size fractionated on an 1% agarose gel in Tris-borate-EDTA containing ethidium bromide and photographed under UV light. Reactions without the RT enzyme were run in parallel as negative controls to monitor for DNA contamination in PCR. RNA extracts from each tissue were concurrently used for amplification of all receptors studied, normally repeated three times but occasionally twice. Positive and negative controls were always run in parallel. Positive controls consisted of a tissue known to express the appropriate receptor such as cerebral cortex for 5-ht_{1F}, 5-HT_{2A} and 5-HT_7 receptors (Julius et al., 1990; Lovenberg et al., 1993; Bard et al., 1993) and caudate nucleus for h5-HT_{1B} and h5-HT_{1D} receptors (Waeber et al., 1988; Jin et al., 1992). Negative controls were used to monitor for the presence of contaminating genomic DNA.

Primers. Primers for 5-HT receptors of potential interest in cerebrovascular tissue (Ullmer et al., 1995; Bouchelet et al., 1996; see also Martin, 1994 for review) were designed using the NBI OLIGO 5.0 program based on the published nucleotide sequences. Note that in accordance with the suggested nomenclature (Hartig et al., 1996), human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors will be referred to as h5-HT_{1D} and h5-HT_{1B} receptors, respectively. As well, since 5-ht_{1F} receptors still await full operational and/or transductional character-izations in intact tissues, the lower case appellation has been maintained (Hoyer et al., 1994). The primers were synthesized using an Applied Biosystems synthesizer and purified using an OPC column. All oligonucleotide 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 AGA ATA C-3') RNA polymerase promoter sequences for use in other exper-iments. **h5-HT_{1B}** (ACC # M89478), 5'-AAG CCT TCT CCT CAA GCA-3'

(upper primer, base position 75) and 5'-AGG TGA TGA GCG CCA ATA-3' (lower primer, base position 616) amplified a PCR product of 595 bp containing the region from the 5'-untranslated sequence to the 1st transmembrane domain; **h5-HT$_{1D}$** (ACC # M81589), 5'-CAC CAT CTA CTC CAC CTG TG-3' (upper primer, base position 634) and 5'-CAG AAA TCC TCT TGC GTT C-3' (lower primer, base position 920) corresponded to a PCR product of 340 bp that includes a region from the 5th transmembrane domain to the 3rd intracellular loop; **5-ht$_{1F}$** (ACC # L05597), 5'-CTT GAA GCC TTC TCT GAA CTG-3' (upper primer, base position 139) and 5'-AGA GAT GCA AGA TGG AGC AC-3' (lower primer, base position 567) matched a DNA fragment of 482 bp and contained the region from the 5'-untranslated region of the gene to the 3rd transmembrane domain; **5-HT$_{2A}$** (ACC # X57830), 5'-GAG TGT GGG TAC ATC AAG G-3' (upper primer, base position 57) and 5'-TAA GGA GAG ACA CGA CGG T-3' (lower primer, base position 349) had a PCR product of 346 bp from the 5' untranslated region of the gene to the 1st extracellular domain; **5-HT$_7$** (ACC # L21195), 5'-GGA ACA GAT CAA CTA CGG CAG AGT-3' (upper primer, base position 240) and 5'-GGT GGT GGC TGC TTT CTG TTC TCG CTT AAA-3' (lower primer, base position 1011) amplified a PCR product of 825 bp from a region of the 1st transmembrane domain to the 3rd intracellular loop.

Sequencing Analyses. Representative PCR products from HBEC (h5-HT$_{1D}$ receptor), HBSM (h5-HT$_{1B}$ and 5-HT$_7$ receptors) and HBA (5-ht$_{1F}$ and 5-HT$_{2A}$ receptors) were treated with Klenow enzyme (5 U, Gibco) and digested with the appropriate restriction enzymes. These fragments were then ligated with T4 DNA ligase (1 U, Promega) into the appropriate bacteriophage or plasmid. Competent DH5F' (Gibco BRL) cells were transformed for preparation of single-stranded or double-stranded DNA for sequencing. Nucleotide sequence analysis was performed using the Sanger dideoxynucleotide chain termination method and Sequenase (U.S. Biochemical) in an automated sequencer (ALF, Pharmacia).

SECOND MESSENGER ASSAYS

cAMP production. To measure h5-HT$_{1B/1D}$ receptor mediated responses on cAMP levels, HBEC, HBSM and HBA cells grown in 24-well tissue culture plates were incubated with forskolin (1 μM, 15 min) alone or in the presence of either the 5-HT$_1$ receptor agonist, sumatriptan (1 μM, 15 min) or sumatriptan and the selective h5-HT$_{1B/1D}$ receptor antagonists, GR127935 and GR55562 (1 μM, Skingle et al., 1993, Connor et al., 1995) in phosphate buffered saline containing 0.2%

bovine serum albumin (BSA) and 1 μM 3-isobutyl-1-methylxanthine. Sumatriptan, GR127935 and GR555-62 were generously provided by Glaxo-Wellcome Inc. The 5-HT_7 receptor-mediated responses were assessed by incubating the cells with 5-HT (1-10 μM) or the 5-HT receptor agonist, 5-carboxamidotryptamine (5-CT, 1-10 μM; Research Biochemicals Inc, Natick, MA) alone or in the presence of the 5-HT receptor antagonist, mesulergine (10-100 μM; RBI). Both 5-CT and mesulergine have been shown to exhibit high affinity at the cloned 5-HT_7 receptor (Bard et al., 1993). The cells were incubated with antagonists for 10 min (37°C) prior to the addition of agonists. The reaction was stopped 15 min after the addition of agonists by removal of the reaction mixture and adding 65% (v/v) ice-cold ethanol. The ethanol extraction was repeated twice, the extracts combined, dried (vacuum oven at 80°C) and dissolved in 200 μl of the assay buffer for determination of cAMP content with a commercial enzyme immunoassay kit (Bio-trak, Amersham). The cell pellets were dis-solved in 0.1N NaOH and protein content was measured using BSA as the standard (Lowry et al., 1951).

Inositol phosphates (IP) formation. The coupling of 5-HT_2 receptors to IP formation was determined according to the protocol originally described by Berridge et al (1982). Briefly, confluent HBEC, HBSM and HBA, grown in 24-well plates, were prelabelled with [^{3}H]myo-inositol (2.5 μCi/ml; New England Nuclear, Boston, MA) for 16-18 hours in serum- and inositol-free medium M199. Unbound [^{3}H]myo-inositol was removed by washing in M199 and the cells were then either exposed to 5-HT (100 μM) alone (15 min) or the 5-HT_{2A} receptor antagonist, ketanserin (100 μM) (10 min), followed by 5-HT (100 μM) (15 min) in the presence of 20 μM LiCl. Histamine (100 μM; Sigma Chemical Co, St Louis, MO) and endothelin-1 (100 nM; Sigma) were used as positive controls since they were previously shown to elicit pronounced IP_3 stimulation in HBEC (Stanimirovic et al., 1994a,b). The reaction was stopped by replacing the medium with cold 0.3 M trichloroacetic acid and the cells were scraped, briefly sonicated and sedimented by centrifugation. Aliquots of the supernatants were treated three times with anhydrous diethyl ether, the aqueous phases were separated by centrifugation and the remaining ether was evaporated under nitrogen. The samples were neutralized with 6.25 M sodium tetraborate and applied to a 1 ml DOWEX-AG 1X8-formate form anion exchange column. IP fractions were eluted from the columns and quantified as previously described (Stanimirovic et al., 1994b). The IP_3 fraction determined by this technique represents a mixture of IP isomers.

EXPRESSION OF 5-HT RECEPTORS

Microvessels and capillaries. A specific signal for the h5-HT_{1B} and h5-HT_{1D} receptors was consistently detected by gel electrophoresis in isolated MV and CAP fractions (Fig 6.1; Table 1). Similarly, PCR products of the expected size for the 5-HT_{2A} receptor were detected in a majority of both fractions (Fig 6.1; Table 1), although the signal was rather weak. Message for 5-ht_{1F} receptors was seen in some MV preparations and more frequently so in the CAP fraction (Fig 6.1; Table 1). No mRNA transcripts for the 5-HT_7 receptor were detected by gel electrophoresis in any of the microvascular fractions (Fig 6.1; Table 1). In all preparations, the intensity of the PCR products generated from vascular tissues was generally lower than that seen in their respective control brain tissues (not shown).

Human brain microvascular cells and astrocytes in culture. As shown in representative cultures of the microvascular and astroglial cells, virtually all HBEC were positively immunostained for the specific endothelial cell marker, Factor VIII-related antigen (Fig 6.2A) while being essentially negative for α-actin and GFAP (data not shown). Based on morphology and immunocytochemical staining, it was estimated that endothelial cells represented >95% of the total cell population in these cultures. The long-term primary cultures generated from the dissociated microvessels (>112 μm and >350 μm) were highly enriched in smooth muscle cells as demonstrated immunocytochemically by the presence of α-actin in more than 80% of all cells in culture (Fig 6.2B). Some contaminating cells incorporated dil-Ac-LDL, a specific marker for endothelium (data not shown). Essentially all cells (>95%) in HBA cell cultures stained positively for GFAP (Fig 6.2C) and were devoid of any contaminating oligodendrocytes and/or microglia (data not shown).

When expression of 5-HT receptors was studied in these cultured cells, specific PCR products corresponding in size to the h5-HT_{1D} and 5-HT_7 receptors were detected at varying intensities by gel electrophoresis in virtually all HBEC cultures examined (Fig

6.3, Table 1). Amplified fragments for the h5-HT_{1B}, 5-ht_{1F} and 5-HT_{2A} receptor were not observed in these cells (Table 1). In HBSM, strong intensity PCR products corresponding in size to the h5-HT_{1B} and 5-HT_7 receptors were evident in all HBSM cell preparations (Fig 6.4), while a specific but weak signal for the h5-HT_{1D} receptor was detected in a majority of these cell cultures (Fig 6.4). However, similar to what was observed in the HBEC, messages for the 5-ht_{1F} and 5-HT_{2A} receptors were not detected by gel electrophoresis in any of the HBSM cell cultures (Table 1). In contrast, the HBA cultures exhibited specific PCR products of moderate and strong intensity for the 5-ht_{1F} and the 5-HT_{2A} receptors, respectively. As in the vascular cells, PCR products of varying intensities for the h5-HT_{1B}, h5-HT_{1D} and 5-HT_7 receptors were detected in the astrocyte cultures (Fig 6.5, Table 1). In all PCR experiments, no products were observed if amplifications were performed in cDNA samples devoid of RT enzyme (-lanes, in Figs 6.1, 6.3-5).

Table 1: Semiquantitative expression of mRNA for various 5-HT receptors in human isolated intracortical blood vessels and cultured vascular cells and astrocytes

	h5-HT_{1B}	h5-HT_{1D}	5-ht_{1F}	5-HT_{2A}	5-HT_7
MVs	+	+	+	-/+	-
CAPs	+	++	+	+	-
HBEC	-	++	-	-	++
HBSM	++++	+	-	-	+++
HBA	++	++	+	++++	++

Relative expression for the various 5-HT receptors in all tissues based on i) number of preparations expressing these receptors and ii) abundance with respect to control amplification.

(-) denotes absence of PCR products in all or a majority (5/6) of tissue preparations. -/+: signal barely detected but present consistently, +, ++, +++, ++++: based on intensity and expression in a majority (4 to 6) of preparations studied.

Sequence analyses. The sequence of the PCR products obtained from tissue mRNA as template, matched unambiguously, i.e. 99.8% (h5-HT_{1B}), 98.8% (h5-HT_{1D}), 98.6% (5-ht_{1F}), 99.1% (5-HT_{2A}) and 99% (5-HT_7), the published sequences for their respective cloned receptors.

FUNCTIONAL AND PHARMACOLOGICAL ASSESSMENT OF 5-HT RECEPTORS

cAMP production. Stimulation of ade-nylate cyclase by 1 μM forskolin resulted in a dramatic, 6-10 fold increase in cAMP levels in HBEC, HBSM and HBA (data not shown). The non-selective 5-HT_1 receptor agonist, sumatriptan, effectively inhibited (80% in HBEC and HBSM, $p \leq 0.01$; 50% in HBA, $p \leq 0.05$) the forskolin-stimulated cAMP production in all three cell types (Fig 6.6) and this effect was completely or partially reversed ($p \leq -0.01$) in the presence of the selective h5-$HT_{1B/1D}$ receptor antagonists, GR127935 and GR55562 (Fig 6.6).

The addition of 5-HT or 5-CT alone resulted in a moderate (55-106% above basal levels, $p \leq 0.05$) stimulation of cAMP production (Fig 6.7) in all three cell types. This effect was partially but significantly ($p \leq 0.05$) inhibited by the non-selective 5-HT_7 receptor antagonist, mesulergine (Fig 6.7) in HBSM and HBA, whereas the inhibition was not significant in the HBEC cultures.

IP_3 production. In HBEC and HBSM cultures, 5-HT (1-100 μM) failed to induce any changes in IP_3 formation, even though significant increases in IP_3 levels were observed in the same cultures stimulated with histamine (100 μM) and endothelin-1 (100 nM) (287% and 1268%, respectively) (data not shown). By contrast, 100 μM 5-HT induced a 77% increase ($p \leq 0.01$) in IP_3 levels in HBA (Fig 6.8) and this stimulation was completely blocked ($p \leq 0.01$) by the 5-HT_2 receptor antagonist, ketanserin (Fig 6.8).

DISCUSSION

The present data demonstrate that specific cellular constituents of the microvascular bed of the human cerebral cortex express distinct populations of 5-HT receptors. Not only were the different messages for 5-HT receptors detected in these constituents of the blood vessel wall (i.e., HBEC, HBSM, HBA) but the proteins encoded by these mRNAs were found to functionally couple to the signalling pathways in a manner similar to that described in other tissues (Hoyer et al., 1994). The most striking example of this cell-selective localization of 5-HT receptor subtypes was evident for the h5-HT_{1B} receptor, which was consistently detected in the HBSM cell cultures derived from MVs but not in microvascular HBEC cultures derived from CAPs. As well, the 5-ht_{1F} and 5-HT_{2A} receptors were never associated with microvascular cells and their exclusive astroglial expression suggests that their detection in isolated MVs and CAPs is most likely attributed to the perivascular astroglial cells that are always present in these preparations (White et al., 1981).

EXPRESSION AND PHARMACOLOGY OF 5-HT RECEPTORS IN CEREBRAL MICRO-VESSELS AND CELLS IN CULTURE

5-HT_1 receptors. Sumatriptan, a non-selective 5-HT_1 receptor agonist which exhibits high affinity for the h5-HT_{1B}, h5-HT_{1D} and 5-ht_{1F} receptors (pKi of 8.1, 8.5 and 7.6, respectively; Connor and Beattie, 1996), effectively inhibited the forskolin-induced cAMP formation in HBEC, HSMC and HBA indicating the presence of functional 5-HT_1 receptors in all three cell types. The involvement of h5-HT_{1B} and/or h5-HT_{1D} receptor subtypes in mediating this reponse was confirmed by the ability of the selective h5-$HT_{1B/1D}$ receptor antagonists, GR127935 and GR55562 (Skingle et al., 1993; Connor et al., 1995) to block the effects of sumatriptan. Based on the RT-PCR data, it appears likely that the receptor linked to adenylate cyclase inhibition in HBEC is h5-HT_{1D} while in the HBSM cultures this inhibition is elicited primarily through a h5-HT_{1B} receptor, although a contribution from a h5-HT_{1D} receptor cannot be totally excluded (see below). Until selective 5-ht_{1F} receptor agonists or antagonists become available, some of which

are forthcoming (Overshiner et al., 1996), a possible role of 5-ht_{1F} receptor in modulating adenylate cyclase in astrocytes cannot be confirmed.

The presence of h5-HT_{1D} receptor and lack of h5-HT_{1B} receptor messages in HBEC observed in this study are in disagreement with previous reports describing the expression of h5-HT_{1B} receptors in human and porcine endothelial cells of peripheral vessels (Ullmer et al., 1995; Schoeffter et al., 1995). Our results, however, are consistent with the very low level of h5-HT_{1D} receptor message detected in some human cerebral vessels (Bouchelet et al., 1996). The HBEC used in the present study exhibit phenotypic characteristics of the BBB endothelium (Stanimirovic et al., 1996) and may harbor receptor populations different from those present in microvascular and macrovascular peripheral endothelial cells (Joo, 1996) used in other studies. In addition, 5-HT_{1D} receptors have been suggested to mediate proliferative response in aortic endothelial cell (Pakala et al., 1994) and may exert a similar role in HBEC. The expression of h5-HT_{1B} receptor in HBSM cells, however, is fully consistent with previous pharmacological (Hamel et al., 1993a,b; Kaumann et al., 1993; Beattie and Connor, 1996) and molecular studies in cerebral and peripheral arteries (Hamel et al., 1993a; Ullmer et al., 1995; Bouchelet et al., 1996). The demonstrated smooth muscle location of the h5-HT_{1B} receptor and its involvement in inhibition of adenylate cyclase in these cells are two requirements known to be necessary for eliciting a direct vasocontractile response (Zgombick et al., 1993; Ebersole et al., 1993; Sweeney et al., 1995). Functional h5-HT_{1B} receptors in brain microvascular smooth muscle cells could thus mediate an endothelium-independent vasoconstriction in cerebral microarteries similar to what has been shown in cerebral and meningeal arteries from different species including man (Bouchard and Hamel, 1991; Hamel et al., 1993a,b; Kaumann et al., 1993; Connor and Beattie, 1996). On the other hand, a weak h5-HT_{1D} signal was detected in the smooth muscle-enriched cultures. Since HBSM cultures also contained some endothelial cells, it is not possible at present to ascertain whether the h5-HT_{1D} message expressed in these primary cultures is attributable to contaminating endothelial cells or is genuinely expressed by smooth

muscle cells. Additional studies will be required to clarify this issue. Similarly, expression of h5-HT_{1B} receptors in endothelial cells of microarterioles or venules as compared to those of brain capillaries cannot be ruled out in the present study.

The multiplicity of 5-HT_1 receptor messages identified in HBA is difficult to interpret and surely points to various possibilities through which 5-HT can regulate astroglial cell functions. Functional 5-HT_{1A} receptors have been associated with astrocytes and found to stimulate the production of the serotonergic glial growth factor S-100β (Azmitia et al., 1990; Whit-aker-Azmitia et al., 1993; Hirst et al., 1994). The roles of other 5-HT_1 receptors detected in HBA in this study will need further clarification.

5-HT_{2A} receptors. Expression of 5-HT_{2A} receptors was exclusive to astroglial cells. These receptors are characterized by their coupling to IP_3 production and their sensitivity to the high affinity 5-HT_{2A} receptor antagonist, ketanserin (Hoyer et al., 1994). HBA localization of 5-HT_{2A} receptor observed in this study is in agreement with previous reports of 5-HT_{2A} receptor being expressed in brain astrocytes isolated from other species (Deecher et al., 1992; Hirst et al., 1994). Since vasomotor responses in human brain vessels do not involve 5-HT_{2A} receptors (Parsons et al., 1989; Hamel and Bouchard, 1991; Kaumann et al., 1993) as is the case in peripheral vessels (Bax et al., 1992; Kaumann et al., 1994), it appears that the role of astroglial 5-HT_{2A} receptors is restricted to mediating neuronal-glial interactions. An important function of this receptor may be related to the ability of 5-HT to stimulate glycogenolysis and release of lactate from astroglial cells in response to activation of 5-HT neurotransmission (Quach et al., 1982; Poblete et al., 1995), therefore resulting in tight coupling of brain neuronal activation and local changes in CBF (Magistretti and Pellerin, 1996). Furthermore, the inhibitory effect of 5-HT on GFAP expression in astrocytes has been proposed to be mediated by a receptor coupled to phosphoinositide turnover, possibly the 5-HT_{2A} receptor (Le Prince et al., 1990).

5-HT$_7$ receptors. 5-HT$_7$ receptors which had previously been shown to cause stimulation of adenylate cyclase in transfected cells (Lovenberg et al., 1993; Bard et al., 1993) have recently achieved their upper case status and accepted as functional receptors in biological tissues (Eglen et al., 1997). Agreeably, when tested in this study, 5-HT and 5-CT caused 55-106% increases in cAMP levels in HBEC, HBSM and HBA, most likely by activating 5-HT$_7$ receptors whose message was detected by RT-PCR in the three cell types. Expression of 5-HT$_7$ receptors, however, was not detected in intact MVs and CAPs isolated from post-mortem brains. This disparity between freshly isolated tissues and cells in culture is unlikely the artefact of cell culturing conditions since the expression of 5-HT$_7$ receptor has previously been demonstrated in peripheral blood vessels and peripheral human smooth muscle cells (Ullmer et al., 1995; Schoeffter et al., 1996). 5-HT$_7$ receptor message may possibly be expressed at lower levels *in vivo* and be more susceptible to post-mortem degradation than other 5-HT receptors, thus becoming undetectable in tissues isolated from auto-psy samples.

The 5-HT receptor antagonist, mesulergine, which exhibits high affinity to the cloned human 5-HT$_7$ receptor (pKi, 7.7) and much lower (about 100 fold) affinity to the cloned 5-ht$_6$ receptors (pKi, 5.8; Bard et al., 1993), completely inhibited the agonist-stimulated cAMP levels in all three cell types. As the mesulergine concentrations used in the present study are non-selective, they do not allow to totally exclude the possible involvement of 5-ht$_6$ receptors in this response. However, both molecular and pharmacological results strongly suggest that 5-HT$_7$ receptors are expressed and functionally coupled to adenylate cyclase stimulation in vascular and astroglial cells. Stimulation of adenylate cyclase by 5-HT has previously been reported in both cerebral endothelial cells and astrocytes (Bacic et al., 1991; Hirst et al., 1997) and expression of 5-HT$_7$ receptor message detected in smooth muscle cells derived from human peripheral vessels and porcine cerebral veins (Ullmer et al., 1995; Schoeffter et al., 1996). Functionally, 5-HT$_7$ receptors have been suggested as mediators of the endothelium-independent relaxation observed in a number of peripheral vascular segments (Feniuk et al., 1983; Sumner et al., 1989; Leung et al.,

1996; Terron, 1996), an action fully compatible with their ability to increase cAMP levels. It has also been reported that 5-HT inhibits proliferation of cultured smooth muscle cells by inducing increases in cAMP levels (Lee et al., 1991), perhaps through this receptor. Together, these observations suggest that 5-HT_7 receptors detected in the primary microvascular HBSM cultures are most likely expressed in smooth muscle cells in addition to microvascular endothelial cells. The role(s) that 5-HT_7 receptors play in cerebromicrovascular endothelial or brain astroglia cells, as also recently reported in the rat (Hirst et al., 1997) however, still remain to be further investigated.

PHYSIOLOGICAL IMPLICATIONS

The expression of functional 5-HT receptors by human cerebromicrovascular endothelial and smooth muscle cells as well as astroglia imply that these non-neuronal components of the brain parenchyma are able to respond to changes in brain serotonergic neurotransmission. It is also conceivable that changes in circulating 5-HT levels could affect cerebral endothelial 5-HT receptors even though the possible luminal localization of these receptors was not ascertained by this study. The demonstrated expression of functional h5-HT_{1B} receptor, shown to mediate cerebral vasoconstriction (Hamel and Bouchard, 1991; Hamel et al., 1993a), as well as that of 5-HT_7 receptor, identified as vasodilatory receptor in several vascular beds (Leung et al., 1996; Terron, 1996) in microvascular HBSM cells, provide strong evidence to support previous observations of both the decrease and occasional increase in cortical blood flow subsequent to stimulation of the raphe nucleus (Bonvento et al., 1989; Underwood et al., 1992, 1995; Cudennec et al., 1993). The final and integrated vasomotor response to 5-HT is likely to depend on the proportion of h5-HT_{1B} and 5-HT_7 receptors, the initial tone of the vessel (Edvinsson et al., 1978; Rosenblum and Nelson, 1990; Sweeney et al., 1995) and possibly the subdivision of the raphe nucleus being activated (Underwood et al., 1992). Other 5-HT receptors are also likely to participate in these responses, as suggested by our preliminary experiments (Cohen et al., 1996b).

Human fetal brain astrocytes were found to express several 5-HT receptors, some of which had previously been identified in neonatal and adult rat astrocytes (Deecher et al., 1992; Whitaker-Azmitia et al., 1993; Hirst et al., 1994; 1997). In this study, we report novel observations that human fetal astroglial cultures express 5-ht_{1F} and 5-HT_{2A} receptors, which were not found in either HBEC or HBSM cell cultures. Since 5-ht_{1F} and 5-HT_{2A} receptors were also detected in freshly isolated MVs and CAPs from adult brains, we conclude that perivascular astrocytes in adult brain also express these 5-HT receptor subtypes. 5-HT receptors have been implicated in the regulation of several important functions of astrocytes including stimulation of glycogenolysis (Quach et al., 1982; Poblete et al., 1995), astrocyte proliferation and maturation (Le Prince et al., 1990; Seuwen and Pouyssegur, 1990; Pauwels et al., 1996) and development of central serotonergic pathways (Azmitia et al., 1990; Whitaker-Azmitia et al., 1993). Therefore, it is possible that these astrocytic 5-HT receptors fulfil different roles related to neurotransmission, ion and pH homeostasis, induction and maintenance of the BBB as well as control of brain perfusion (Mineau-Hanschke et al., 1989; Barres, 1991; Hertz, 1992; Cohen et al., 1995b).

In conclusion, the demonstrated expression and functional coupling of various types of 5-HT receptors to intracellular signalling in vascular and astroglial cells strongly suggest that human brain intracortical microcirculation has the capacity to adjust local brain perfusion in response to changes in central 5-HT neurotrans-mission either directly or indirectly via neuronal-astroglial-vascular interactions. The results also indicate that serotonergic neurons can influence astroglial function through a variety of receptors and underline the possible interaction between serotonergic neurotransmission and non-neuronal cells within the brain.

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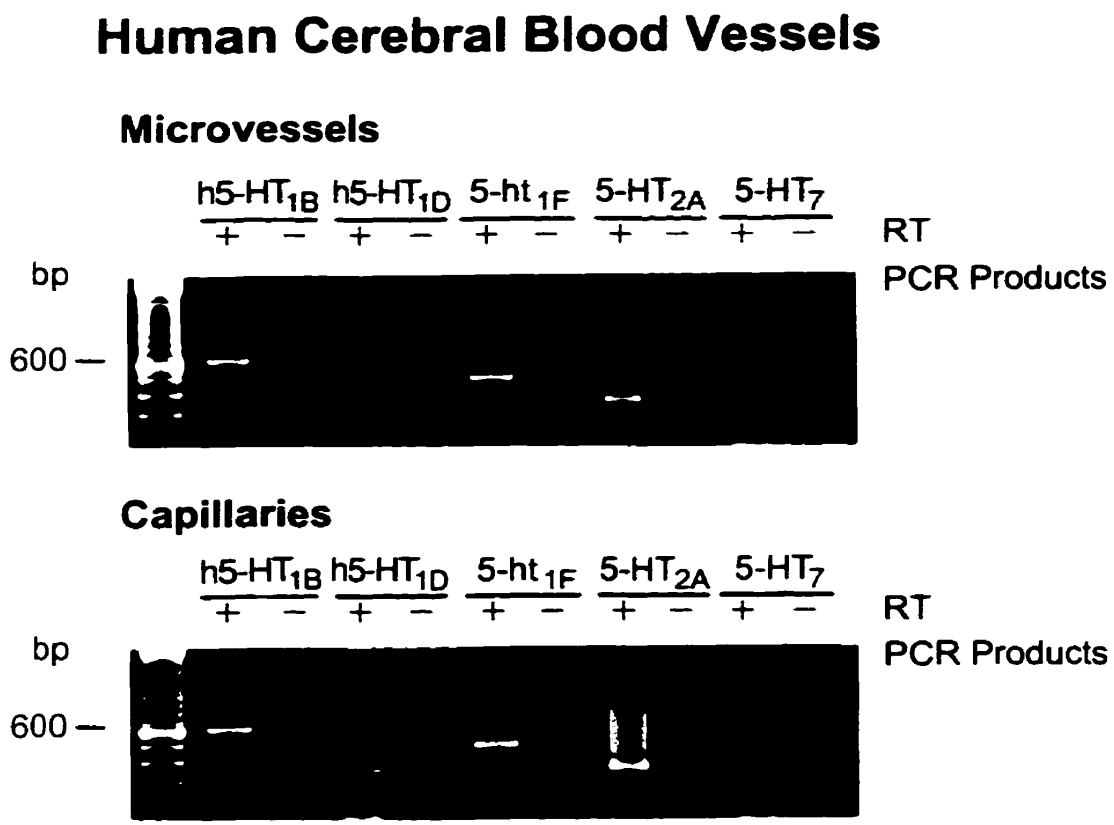
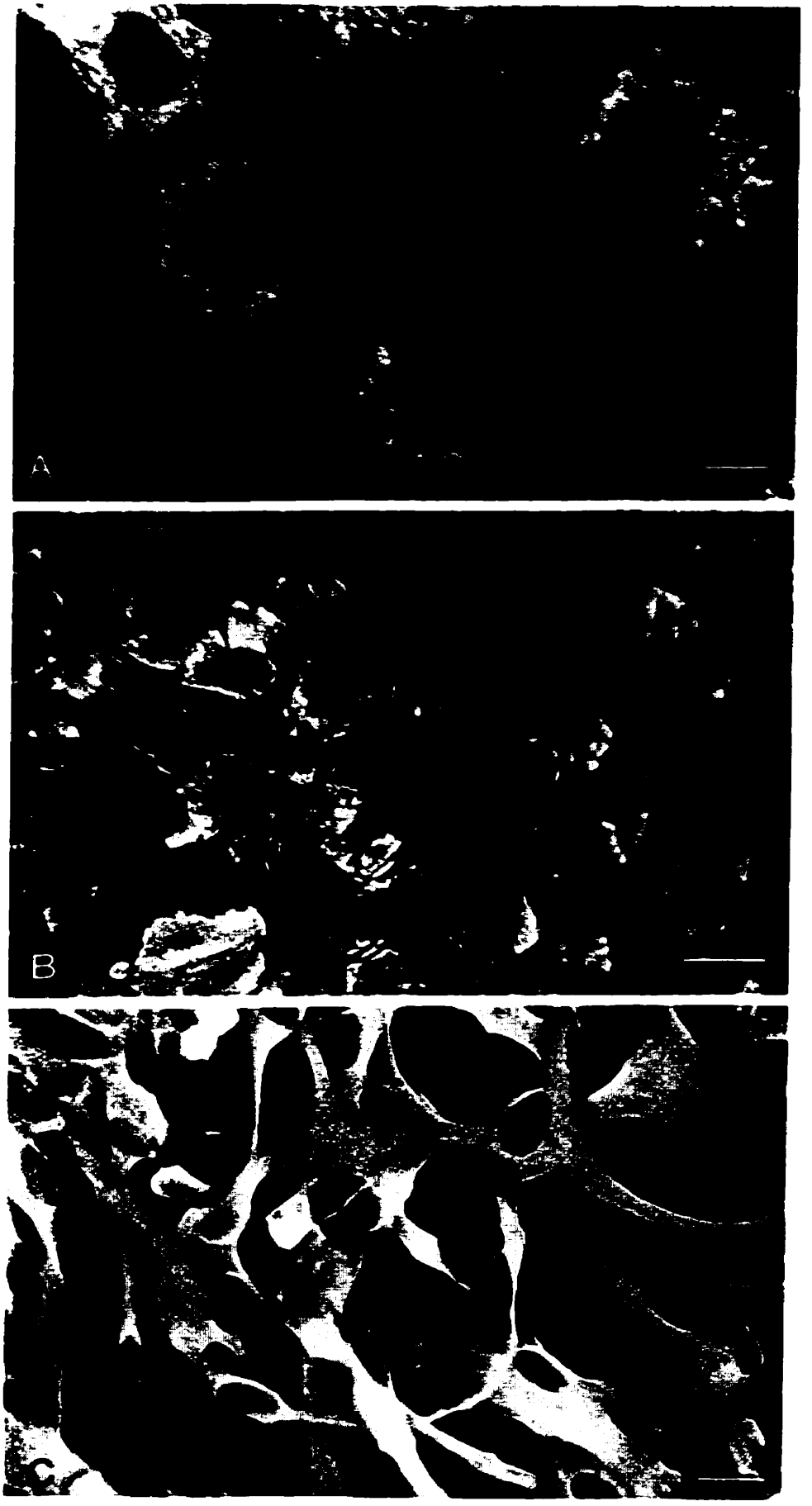


Fig 6.1: Identification of 5-HT receptors in isolated human brain MVs and CAPs by RT-PCR. Agarose gel electrophoresis of PCR-amplified cDNA from representative tissues using specific oligonucleotide primers for h5-HT_{1B}, h5-HT_{1D}, 5-ht_{1F}, 5-HT_{2A} and 5-HT_7 receptors . To control for genomic DNA and/or PCR contamination, amplification was performed with or without reverse transcriptase enzyme (+ or - RT lanes) added in cDNA synthesis reaction (see Materials and Methods). PCR products of the appropriate size (see Materials and Methods for exact size) were observed for all except the 5-HT_7 receptors.

Fig 6.2: Photomicrographs depicting the immunocytochemical characterization of human brain microvascular endothelial (HBEC) and smooth muscle cells (HBSM) as well as astrocytes (HBA). (A) HBEC cultures selectively immunostained for the Factor VIII-related antigen as shown here by immunofluorescence. (B) Primary HBSM cell cultures immunocytochemically stained for α-actin. The vast majority of cells were immuno-positive for a-actin except a few which most likely correspond to endothelium since they incorporated the endothelial specific marker DiI-Ac-LDL (not shown, see Materials and Methods). (C) All cells in HBA cultures were immunopositive for GFAP. Bar scales = 5 μm (A), 10 μm (B,C).


A
B

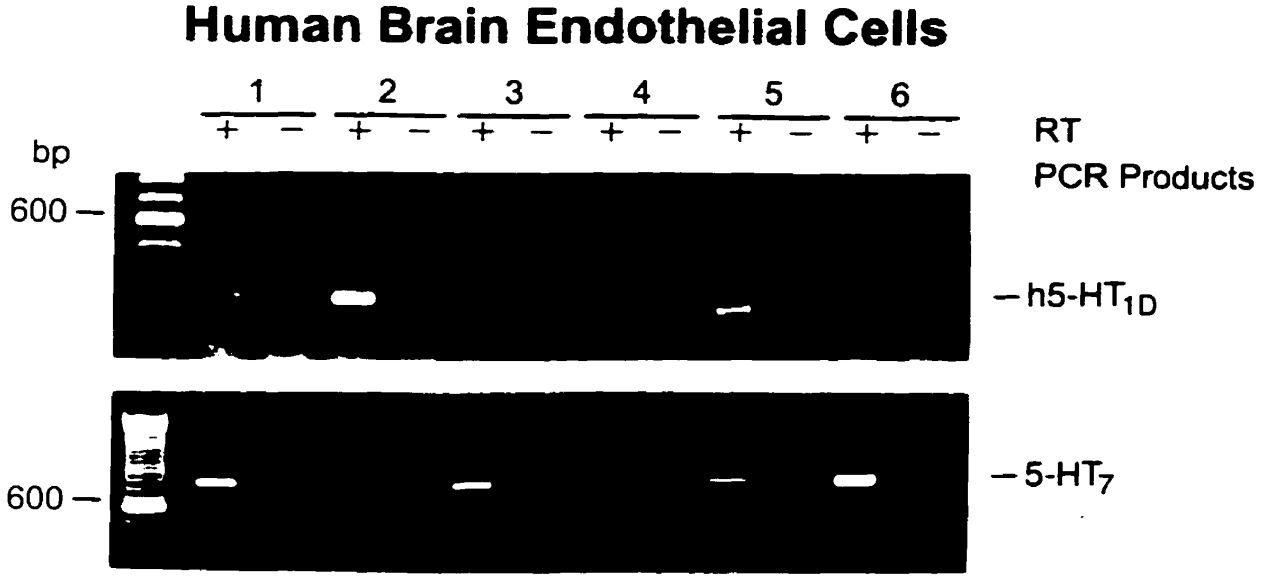


Fig 6.3: Agarose gel electrophoresis of RT- PCR products from six different cultures of HBEC. Only messages for the h5-HT_{1D} and 5-HT_7 receptors were detected by RT-PCR in these cells. The presence or absence of RT enzyme in the cDNA reaction was always used to monitor possible DNA contamination (+ and - RT on Figure).

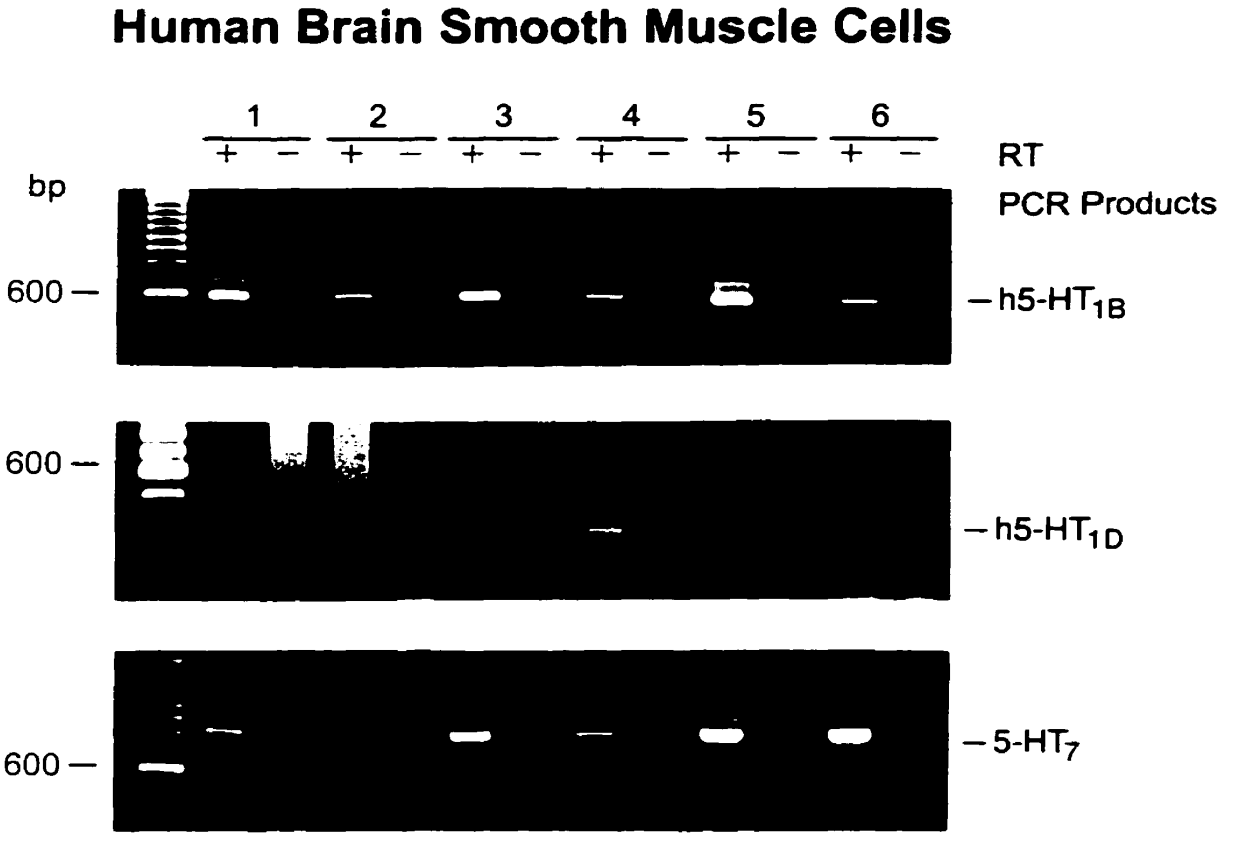


Fig 6.4: Identification of 5-HT receptors in six primary cultures of HBSM cells by RT-PCR. As seen by gel electrophoresis, h5-HT_{1B}, h5-HT_{1D} and 5-HT_7, but not 5-ht_{1F} or 5-HT_{2A} receptor messages were detected in these cells. Note the weak signal intensity for the h5-HT_{1D} receptor. Controls for genomic DNA contaminations (+ or - RT) are indicated.

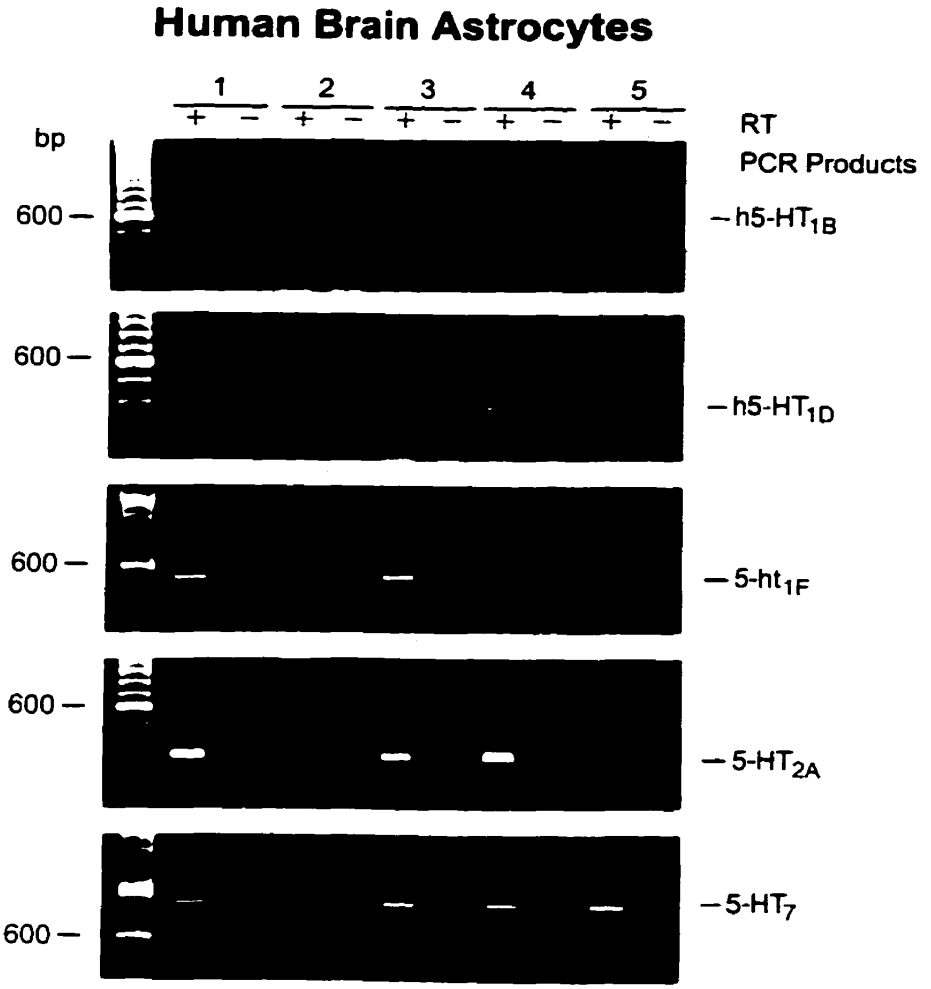
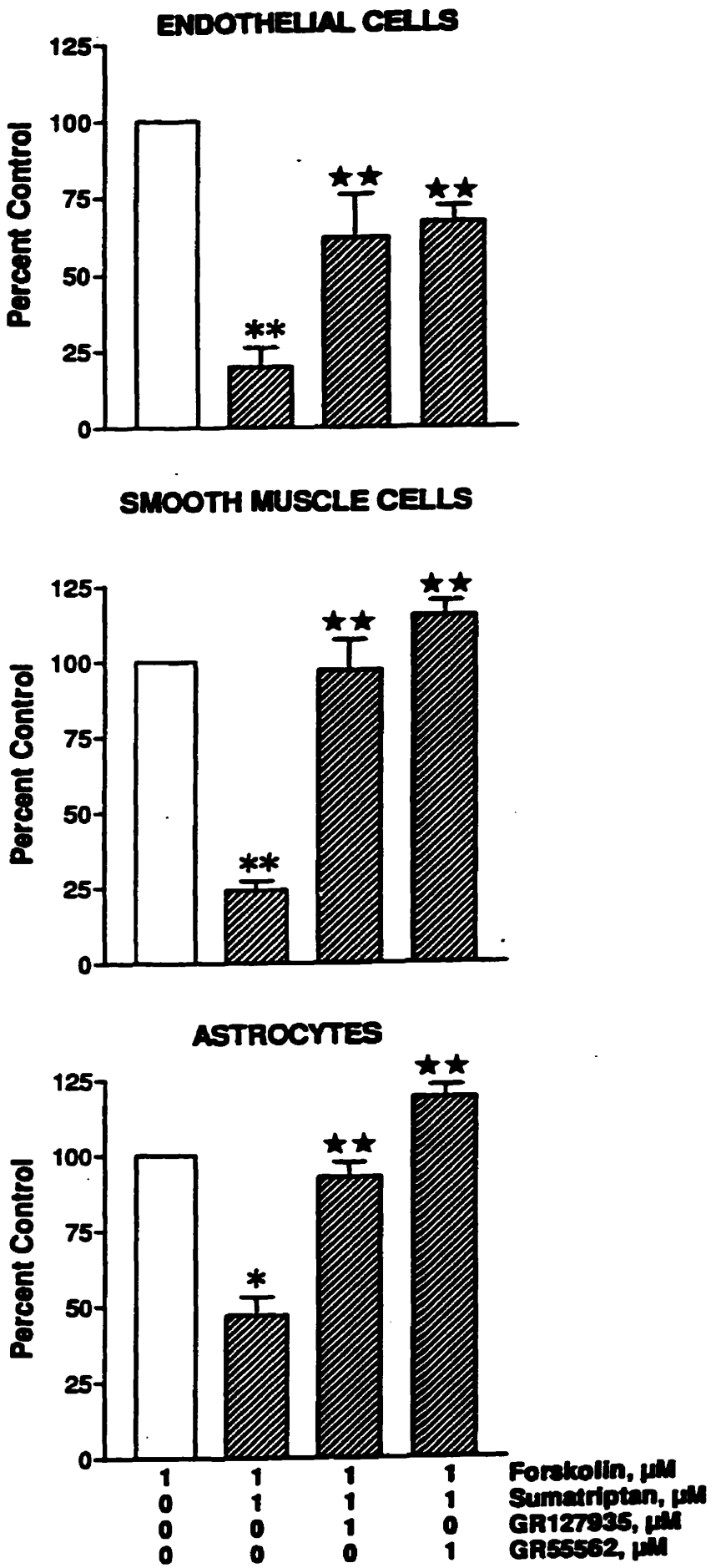


Fig 6.5: Gel electrophoresis of PCR products for the 5-HT receptors expression from five different cultures of HBA. Messages for all 5-HT receptors were detected in these cells with stronger signals for the 5-HT_{2A} and 5-HT_7 receptors. Samples with and without reverse transcriptase (+ or - RT) are shown.

Fig 6.6: Effects of the 5-HT_1 receptor agonist, sumatriptan, on the forskolin-stimulated cAMP production in HBEC, HBSM and HBA cultures. Forskolin-stimulated cAMP levels were taken as controls (100%, open box). In all three cells types, sumatriptan sigificantly inhibited the stimulatory effect of forskolin, a response partially (HBEC) or totally (HBSM and HBA) blocked by the selective 5-$HT_{1B/1D}$ receptor antagonists, GR127935 and GR5562. Note the most efficient response in the HBSM cell cultures. *:$p \leq 0.05$ and **:$p \leq 0.01$(agonist); ★★:$p: \leq 0.01$ (antagonists); Student's *t* test.


ENDOTHELIAL CELLS
Percent Control
125
100
75
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25
0
**
★★
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SMOOTH MUSCLE CELLS
Percent Control
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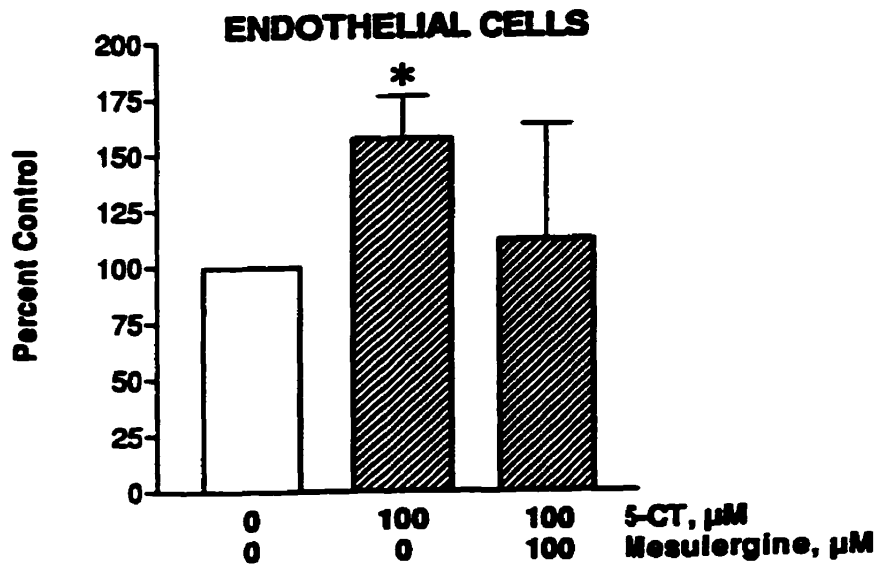
1 1 1 1 Forskolin, μM
0 1 1 1 Sumatriptan, μM
0 0 1 0 GR127935, μM
0 0 0 1 GR55562, μM

Fig 6.7: Effects of 5-HT, 5-CT and mesulergine on cAMP production in HBEC, HBSM and HBA cultures. The basal levels of cAMP in the cells were taken as controls (100%, open box) and the stimulatory effect of 5-HT or 5-CT was significant in all cultures. The non-selective 5-HT_7 receptor antagonist, mesulergine, abolished the agonist-mediated increase in cAMP levels, this effect was not significant in the HBEC cultures. *: $p \leq 0.05$ (agonist) and ★: $p \leq 0.05$ (antagonist); Student's *t* test.


ENDOTHELIAL CELLS
Percent Control
200
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*
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5-CT, µM
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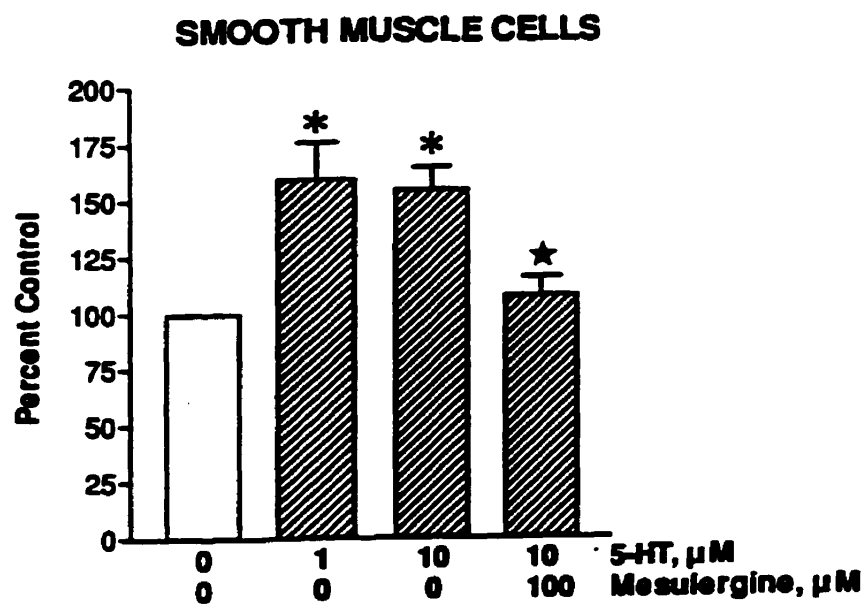
Mesulergine, µM


SMOOTH MUSCLE CELLS
Percent Control
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175
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5-HT, µM
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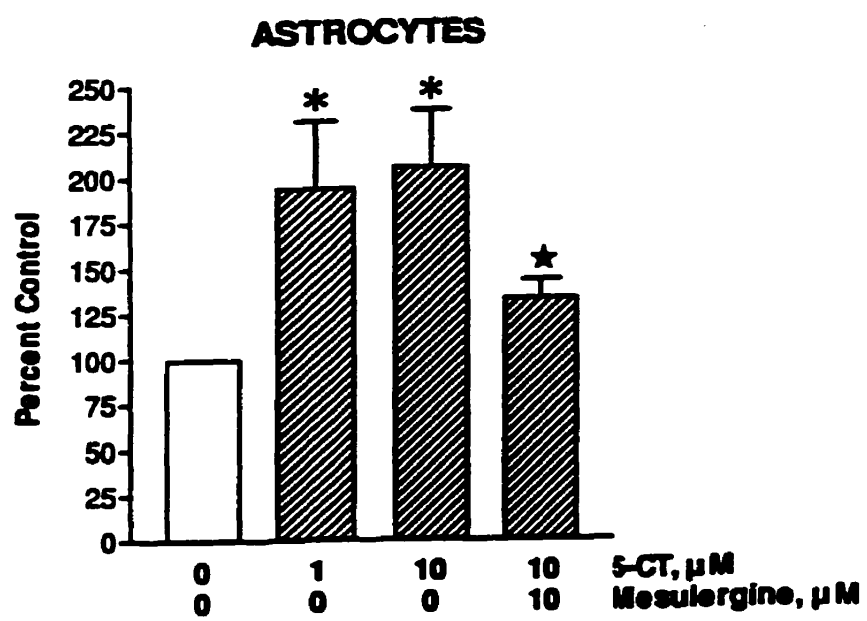
Mesulergine, µM


ASTROCYTES
Percent Control
250
225
200
175
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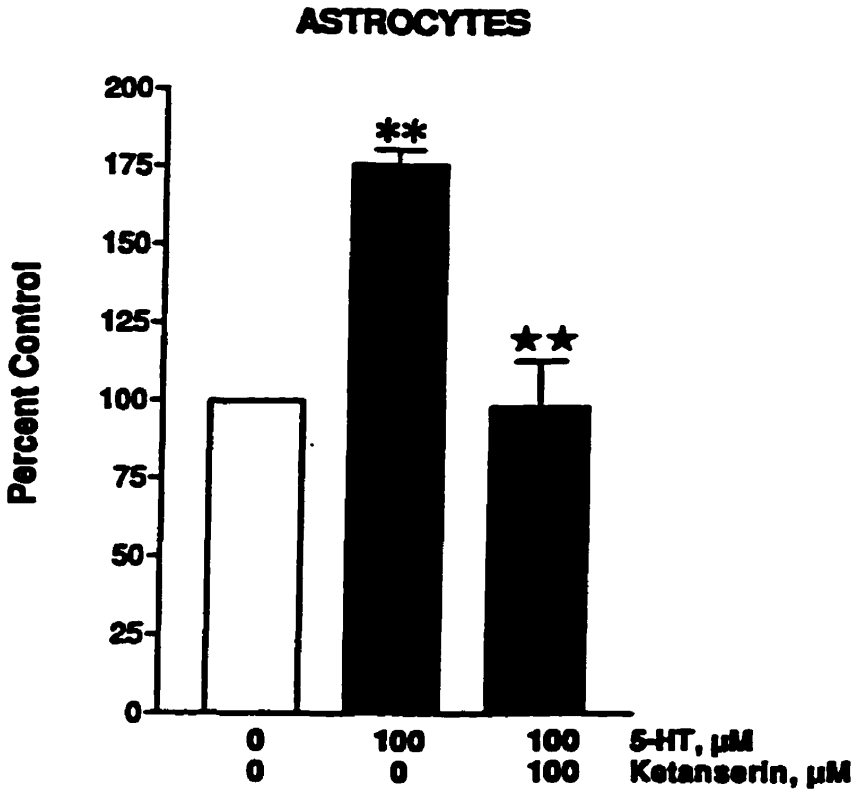

Fig 6.8: Stimulation of IP_3 formation in HBA cultures in the presence of 5-HT, and potent blockade by the selective 5-HT_{2A} receptor antagonist, ketanserin. *:p≤ 0.01 (agonist) and ★★:p≤ 0.01 (antagonist); Student's *t* test.

ADDENDUM TO CHAPTER 6

The present study provides convincing evidence for the presence of functional 5-HT receptors within respective compartments of the vessel wall with a distribution pattern that could be summarized as follows: 5-HT_{1D} and 5-HT_7 receptors in endothelial cells, 5-HT_{1B}, 5-HT_{1D} and 5-HT_7 receptors in smooth muscle cells and the latter three receptors along with the 5-ht_{1F} and 5-HT_{2A} in the astrocytes. Not included in this chapter were the 5-HT_{2B} and/or 5-HT_{2C} receptors, which have been suggested to mediate smooth muscle relaxations in peripheral blood vessels possibly via the release of nitric oxide (NO) from the endothelial cells (Leff et al., 1987; Sumner, 1991; Glusa and Richter, 1993; Bodelson et al., 1993; Ellis et al., 1995; Glusa and Roos, 1996). However, the lack of selective pharmacological agents has hampered the exact identification of this receptor. More recently, evidence has been provided in the pig strongly suggesting that the 5-HT_{2B} receptor subtype mediates relaxation in meningeal arteries (Schmuck et al., 1996).

In collaboration with Isabelle Bouchelet, a PhD student in our laboratory, we investigated this issue and further evaluated if these receptors were also present in the microvascular bed. We used RT-PCR and found the exclusive but ubiquitous expression of 5-HT_{2B} receptor in human pial vessels, microvessels, capillaries as well as in endothelial, smooth muscle and astroglial cell cultures (Fig 6.9). 5-HT_{2C} receptor mRNA transcripts could not be detected in any of the cerebrovascular tissues. Based on these findings, the results strongly suggest that endothelial 5-HT_{2B} and not 5-HT_{2C} receptors would mediate the endothelial-dependent cerebral vasorelaxation. In addition, preliminary experiments have shown that 5-HT elicited a concentration-dependent increase in NO production in the microvascular endothelial cultures (Fig 6.10). However, this elevation in NO production was not significantly inhibited by the addition of ritanserine, a 5-HT_2 receptor antagonist with high affinity at the cloned 5-HT_{2B} receptor. It was, however, enhanced by methysergide, a 5-HT_1 antagonist with some agonist properties at the cloned 5-$HT_{1B/1D}$ receptor sites. These results have to be further detailed but clearly indicate that of all 5-HT_2 receptors, only the 5-HT_{2B} has a vascular localization in human brain extracerebral

vessels and microvessels. The ability to induce Ca^{2+} influx and, possibly, NO production in vascular cells may be compatible with their suggested vasodilatory role. Further studies will be required to establish the respective role of endothelial, smooth muscle and astroglial 5-HT_{2B} receptors (Bouchelet et al., in preparation) before a clear understanding of their putative role in triggering migraine attack could be established.

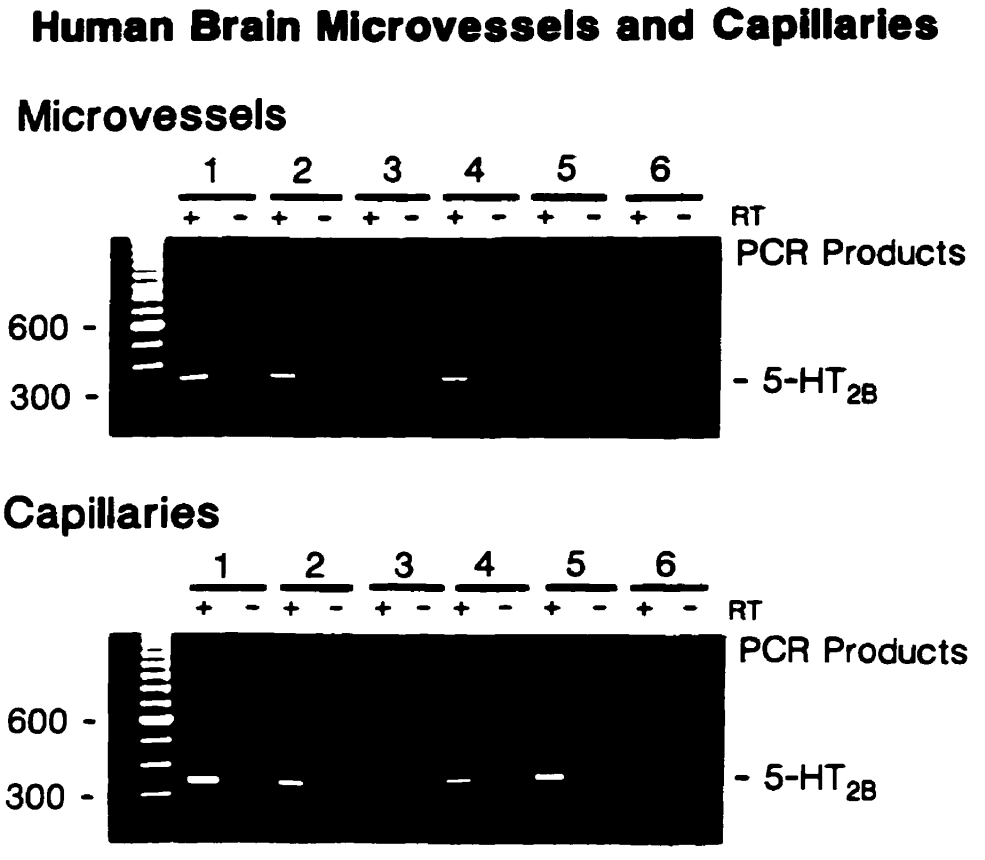

Fig 6.9A: Agarose gel electrophoresis of PCR-amplified DNA from human post-mortem microvessels and capillaries using human 5-HT_{2B} receptor specific oligonucleotide primers.

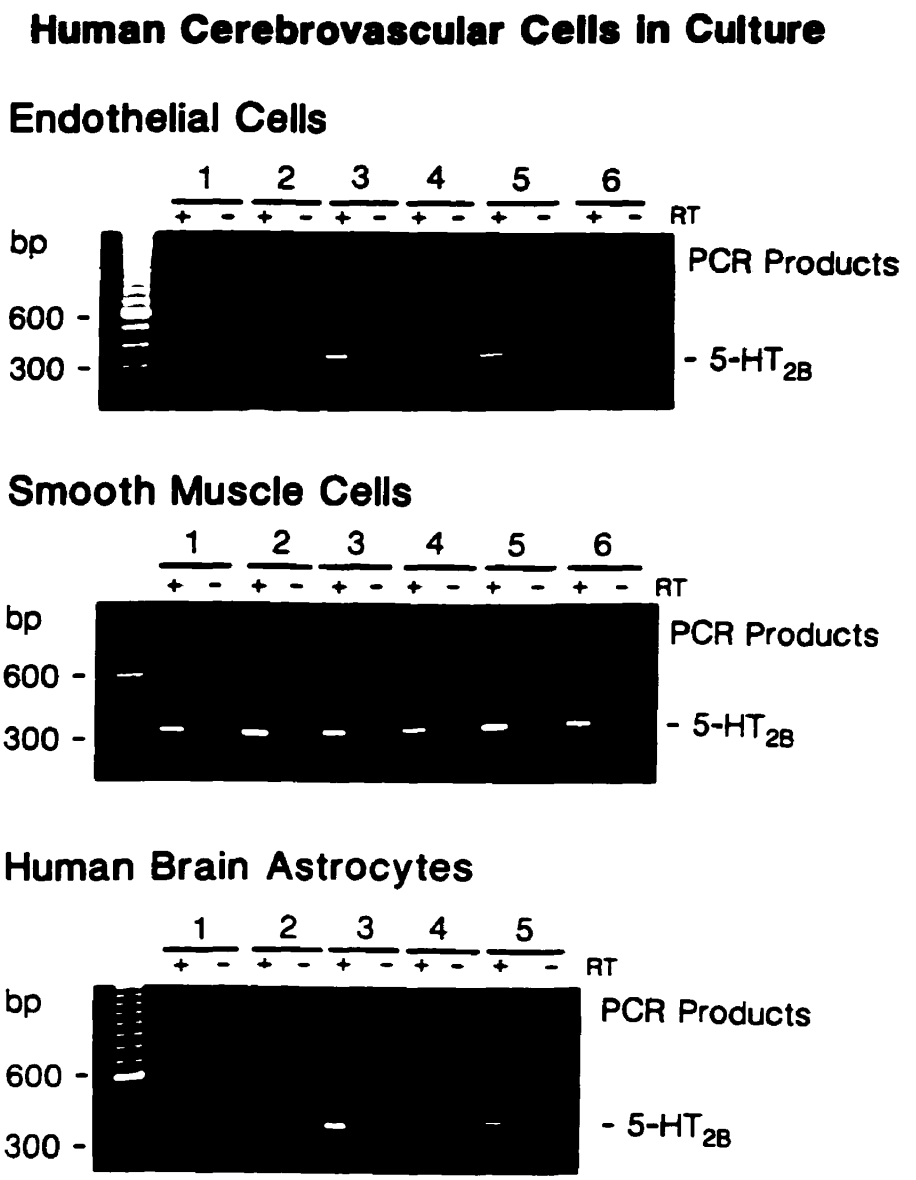

Fig 6.9B: Agarose gel electrophoresis of PCR-amplified DNA from human endothelial cells, smooth muscle cells and astrocytes using human 5-HT_{2B} receptor specific oligonucleotide primers.

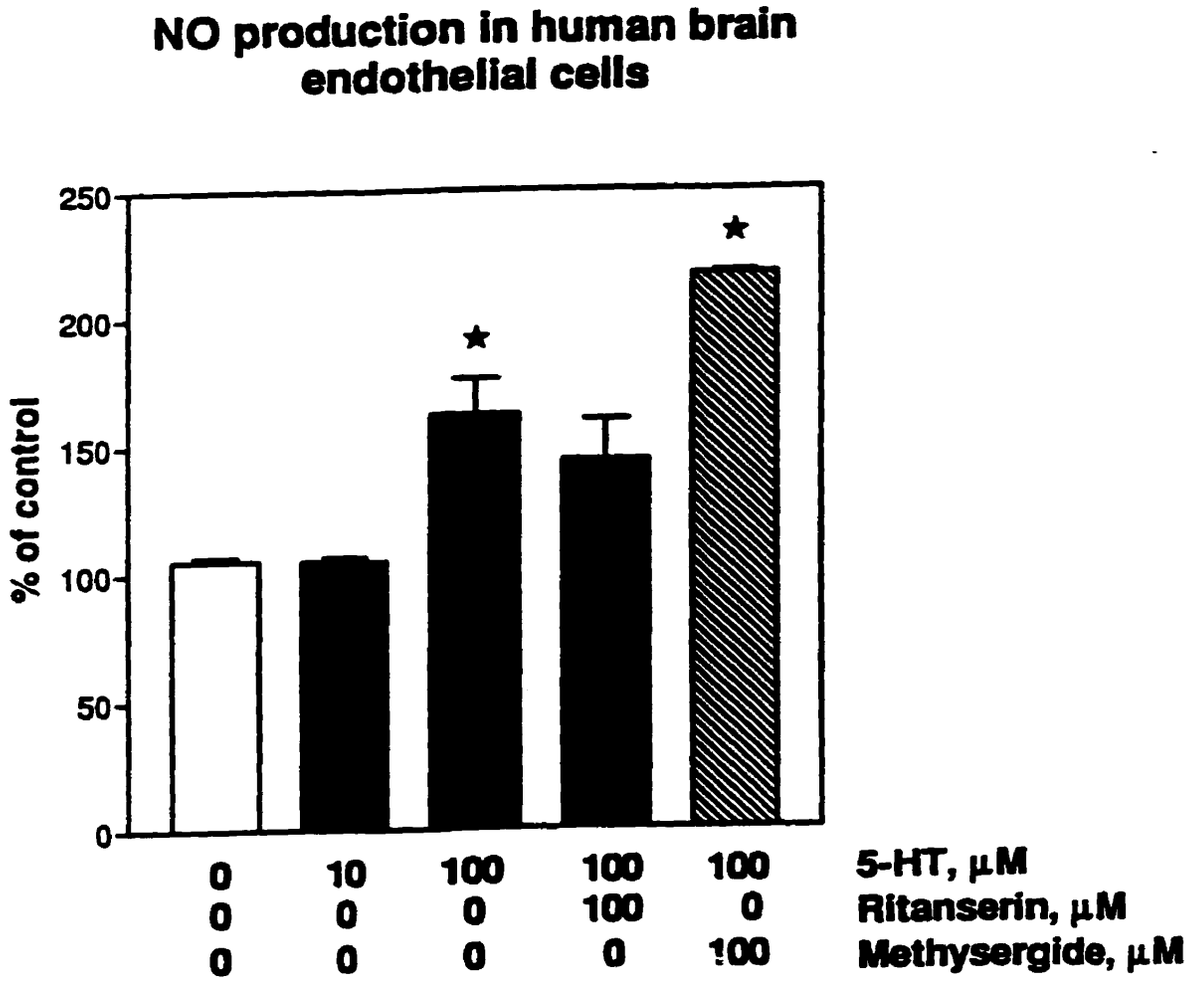

Fig 6.10: Production on nitric oxide (NO) in human cerebromicrovascular endothelial cells after incubation with 5-HT and related compounds. ★ $p \leq 0.05$, Student's *t*-test.

CHAPTER 7

GENERAL DISCUSSION

7.0 GENERAL DISCUSSION

The major findings of my thesis work can be summarized as follows:

- Major cerebral arteries and small pial vessels do not receive a major input directly from serotonergic neurons located in the dorsal and/or median raphe but rather from neurons that originate from a peripheral structure closely related to the superior cervical ganglion (Chapter 3).
- Serotonergic nerve terminals that associate with the local microvascular bed are not distributed uniformly within the different brain regions. In areas that significantly adjust their local perfusion in response to manipulations of central 5-HT neurons (e.g. frontoparietal cortex), more intimate associations are seen as compared to areas (e.g. entorhinal cortex and hippocampus), where moderate blood flow changes are observed during such treatments. These neurovascular relationships, which frequently involve the perivascular astrocytes, could form the anatomical basis for the functional effects observed (Chapter 4).
- The intimacy and frequency of the neurovascular associations appear to be a characteristic not only of the brain regoin but also of the neurotransmitter, as suggested by the differences in serotonergic and noradrenergic neurovascular relationships in the frontoparietal cortex (Chapters 4 and 5).
- Brain intraparenchymal blood vessels and their cellular compartments harbor functional 5-HT receptors (Chapter 6), most likely able to mediate the local changes in CBF and BBB permeability observed following changes in either neuronal or circulating 5-HT levels.

Taken together, these results clearly underscore the anatomical interactions between serotonergic nerve terminals and non-neuronal vascular and astroglial targets. Further, they provide strong evidence that these targets can respond to changes in central serotonergic neurotransmission through specific G-coupled receptor subtypes.

7.1 EXISTENCE AND ORIGIN OF SEROTONERGIC SYNTHESIZING PERIVASCULAR NERVE FIBERS IN EXTRA-CEREBRAL BLOOD VESSELS

7.1.1 AUTHENTICITY OF PERIVASCULAR 5-HT NERVE FIBERS

Previously, some investigators had claimed that 5-HT in perivascular nerves was due to its uptake in sympathetic noradrenergic nerve fibers (Saito and Lee, 1987; Jackowski et al., 1989) and was therefore a false neurotransmitter. In Chapter 3, we labelled serotonin-containing nerve fibers using a well characterized TPH antibody (Cash et al., 1985; Weissmann et al., 1987). This antiserum, produced from rat brain, was used because it offered two distinctive advantages to our purposes: firstly, it selectively labels the rate-limiting enzymatic step in 5-HT synthesis, TPH, an enzyme unique to serotonergic neurons and secondly and more importantly, its presence in a given population of nerve fibers cannot be ascribed to any uptake mechanisms, as has been alluded for 5-HT-containing nerve fibers visualized around cerebral blood vessels. Thus, our findings, which showed a difference in the distribution patterns of TPH-containing (serotonergic) cerebrovascular nerve fibers with those that contained dopamine-β-hydroxylase (noradrenergic), are rather suggestive of the presence of a subset of 5-HT-synthesizing perivascular fibers, in agreement with previous (Scatton et al., 1985; Cowen et al., 1986; Alafaci et al., 1986; Dhall and Burnstock, 1989) and recent (Moreno et al., 1994; 1995; Lopez de Pablo et al., 1996) reports. This would imply that 5-HT contained in perivascular nerves can not be attributed uniquely to uptake mechanisms. However, the exact percentage of 5-HT-synthesizing nerve fibers as compared to noradrenergic nerves and their precise sublocalization within the circle of Willis were not determined in the present study.

7.1.2 DIRECT RAPHE PROJECTIONS TO EXTRACEREBRAL BLOOD VESSELS ?

Following our demonstration that 5-HT-synthesizing nerve fibers exist around extracerebral blood vessels, our next objective was to determine the origin of these perivascular fibers. Two possibilities were considered: i) the dorsal and/or median raphe nuclei, which are the main source of intracerebral serotonergic neurons and ii) the superior cervical ganglia, which has been shown to contain the 5-HT synthetic machinery. Using lesion experiments

(e.g. destruction of the ascending projections from the dorsal and/or median raphe by 5,7-DHT injections) coupled to TPH immunocytochemistry, we could not observe a detectable change in the density of TPH immunoreactive nerve fibers in major and pial arteries as compared to control animals. These findings strongly imply that perivascular 5-HT-producing nerve fibers do not directly arise from brainstem raphe neurons. However, it is impossible to totally exclude a minor contribution of nerve fibers projecting from the raphe nuclei to the cerebral blood vessels. Our finding that raphe neurons have no direct input to pial and major cerebral arteries has been corroborated by other groups. Indeed, using anterograde tract tracing methods, fibers from the dorsal raphe nucleus were never found to reach extracerebral blood vessels although some cortical fibers were apparently seen to cross the glia limitans (Mathiau et al., 1993a). Furthermore, nerve fibers around pial vessels were exclusively immunoreactive for the specific peripheral marker, peripherin, which is only expressed in neurons whose processes are located outside the CNS (Portier et al., 1984; Mathiau et al., 1993c). Interestingly, we found (Chapter 5) that locus coeruleus neurons only innervate intraparenchymal but not extracerebral blood vessels, as selective destruction of locus coeruleus neurons and their projections by the neurotoxin DSP-4, completely eliminated intracortical noradrenaline fibers including those around microvessels while sparing sympathetic fibers around pial arteries. Together, these results are strongly indicative that brain neurons, although related to the intraparenchymal circulation, do not exit the brain parenchyma to directly innervate extracerebral blood vessels. Conversely, it is generally accepted that nerve fibers of peripheral sources do not descend into brain parenchyma beyond the region where the perivascular Virchow-Robin's space ends and the microcirculation begins (Jones, 1970; Marin-Padilla, 1988).

One issue that remains to be resolved is how to reconcile our findings and those of Mathiau et al. (1993a,b) of a peripheral serotonergic innervation with the considerable biochemical evidence suggesting that raphe neurons functionally innervate extracerebral blood vessels? One possibility would be the ability of the serotonergic nerve terminals located in the most superficial layer (molecular layer) of the cortical mantle, some of which reportedly float into

the subarachnoid space (Chapter 4; Mathiau et al., 1993a), to empty their contents in the cerebrospinal fluid (CSF), which bathe the blood vessels. The indoleamine could then either interact with cerebrovascular receptors to elicit vasomotor responses or be taken up by perivascular nerve fibers, hence the modifications in perivascular 5-HT levels following raphe stimulation and/or lesion (Edvinsson et al., 1983; Scatton et al., 1985; Bonvento et al., 1990; 1991). This could represent a pool of releasable 5-HT from non-serotonergic perivascular nerve terminals. Thus, it is conceivable that upon raphe stimulation or lesion, the amount of 5-HT liberated into the CSF is altered and, as a consequence, the responsiveness of blood vessels. Similarly, it has been amply documented that 5-HT fibers are present in supraependymal structures and often protrude into the ventricular system (Aghajanian and Gallager, 1975; Lorenz and Richards, 1982; Dinopoulos and Dori, 1995). Its possible release (Buma, 1989) into the CSF could influence not only CSF properties (Dinopoulos and Dori, 1995) but also extracerebral blood vessels. The demonstration of increased sensitivity to 5-HT in the middle cerebral artery following destruction of the dorsal raphe (Moreno et al., 1991) is fully compatible with such mechanisms.

7.1.3 POSSIBLE PERIPHERAL ORIGIN

Based on our results and previous ablation studies (Cowen et al., 1986; Alafaci et al., 1986; Chang et al., 1988; 1989; Bonvento et al., 1991), it seems that the superior cervical ganglion is the most likely source of serotonin-synthesizing fibers around extracerebral blood vessels. We showed that bilateral removal of the superior cervical ganglia dramatically reduced the number of TPH-positive fibers. Together with the raphe lesion experiments, our finding indicated that the vast majority of 5-HT cerebrovascular nerve fibers originate from a peripheral structure closely related to the sympathetic superior cervical ganglia. However, because of the absence of detectable TPH immunoreactive cells in the ganglia, we could not unequivocally confirm that this structure is the source of perivascular serotonergic nerves. Yet previous demonstrations of 5-HT synthesis from its precursor, L-tryptophan, *in vivo* and in cultured sympathetic neurons (Sah and Matsumoto, 1987; Happola, 1988), together with the presence of 5-HT-immunoreactive neurons mostly in small intensely fluorescent cells

(Verhofstad et al., 1981; Happola et al., 1986; Paivarinta et al., 1987; 1989; Happola, 1988), TPH message (Chapter 3, Addendum) and TPH activity (Paivarinta et al., 1989) within the superior cervical ganglion convincingly indicate that cells located within this structure can synthesize and, most likely, release 5-HT (Sah and Matsumoto, 1987). It is important to stress that in the majority of these studies, the animals were pretreated with either L-tryptophan, monoamine oxidase and 5-HT uptake inhibitors, in order to obtain detectable levels of 5-HT.

Our inability to detect TPH-containing sympathetic neurons may be explained by several factors. It can be due to the antigenic properties of the TPH antibody used. This antibody was produced from whole rat brain and it may not recognize the different TPH isoforms in peripheral structures because of differences in post-translational modifications of the proteins (Kim et al., 1991). It has been known for some years that two TPH proteins exist even though there is a unique TPH message for both proteins (Dumas et al., 1989; Kim et al., 1991; Mathiau et al., 1994). In peripheral structures such as the dura mater and pineal gland, the TPH protein has a molecular weight of 62 KDa while in central sources i.e. raphe nuclei, it corresponds to a 56 KDa product (Dumas et al., 1989; Kim et al., 1991; Mathiau et al., 1994). Accordingly, the TPH antibody used in the present study labels very well raphe neurons but not the cells of the pineal gland. This, however, could very well be explained by the paucity of TPH protein in the pineal gland as compared to the raphe nucleus (three fold less, Dumas et al., 1989), making it difficult to detect by immunocytochemistry. It is thus likely that the levels of TPH protein are a decisive factor to enable its detection by the antibody we used. Such conclusion would be compatible with the possibility that the TPH protein does not accumulate within sympathetic neurons but is rather transported along the nerve fibers to the terminals in order to provide a continuous supply of newly synthesized 5-HT into the releasable pool. Although unlikely, we cannot totally exclude that the cells of origin are located outside, but close, to the superior cervical ganglion such as in the carotid body and nodose ganglia, two structures that project to cerebral blood vessels and which are known to contain 5-HT-immunoreactive cells (Gronblad et al., 1983; Kameda, 1990;

Nosjean et al., 1990). At the time of surgical removal of the superior cervical ganglia, fibers from these other structures might have been disrupted, which could contribute to reductions in TPH immunoreactivity seen in the present study.

7.1.4 TPH: AN ACTIVE OR INACTIVE ENZYME IN CEREBROVASCULAR FIBERS

A confounding report was the immunocytochemical visualization of TPH within cerebrovascular nerve fibers but no detection of its end-product, 5-HT, raising the possibility of an inactive form of the enzyme (Mathiau et al., 1993a). Similar TPH/5-HT mismatches have also been documented in other tissues such as the dura mater (Keller and Marfurt, 1991; Stanley et al., 1993; Mathiau et al., 1994) and the dopaminergic periventriculo-hypophyseal pathway (Vanhatalo and Soinila, 1995). Their functional significance is unknown but may be related to the neurotrophic role exerted by 5-HT during embryogenesis (Lauder et al., 1982; Whitaker-Azmitia et al., 1996). Thus, conceivably, early in development 5-HT is needed as a trophic factor and consequently TPH is in its active form. However, as the animal ages, less of the indoleamine is required and the enzyme is then partially inactivated although not totally eliminated. Interestingly, similar observations have also been documented in other regions of the nervous system such as the hypothalamus and spinal cord whereby the noradrenergic and adrenergic synthetic machinery is present but not its corresponding end-product or transmitter (Grzanna and Coyle, 1978; Jaeger et al., 1983; Ross et al., 1984).

The inability of Mathiau and colleagues (Mathiau et al., 1993b) to measure any detectable TPH activity in extracerebral blood vessels was challenged by more recent and better controlled experiments in which the pterin cofactor, an essential requirement for optimal enzyme activity, was added to the assays, thus allowing TPH activity to be detected in the cerebral blood vessels (Moreno et al., 1994; 1995; de Pablo et al., 1996). This confirms earlier reports which showed, although indirectly, a functioning TPH enzyme by *de novo* 5-HT synthesis in nerve fibers around major cerebral arteries and pial vessels (Scatton et al., 1985; Bonvento et al., 1991).

Altogether, we conclude that serotonin in nerves fibers around extracerebral blood vessels can not be totally attributed to uptake into noradrenergic fibers. A certain amount of 5-HT is indeed synthesized within the nerve fibers that seem to originate, to a large extent, from the superior cervical ganglia and would accordingly require the presence of a functional TPH enzyme. Under normal conditions, the 5-HT synthetic machinery is present and functional in perivascular fibers but not synthesizing the indoleamine. When cerebrovascular stress occurs, then serotonin is required and therefore produced. The demonstration of 5-HT immunoreactivity in extracerebral blood vessels only in rats subjected to experimental embolic stroke (Ueda et al., 1994) adds support to this hypothesis.

7.2 5-HT NEUROVASCULAR ASSOCIATIONS

7.2.1 GENERAL INFORMATION

Our ultrastructural findings (Chapter 4) show that intraparenchymal blood vessels are associated with 5-HT-producing nerve terminals. Despite the lack of direct evidence from our study, it is justified to assume that these 5-HT nerve fibers arise from the brainstem raphe neurons. Over the years, evidence has accumulated suggesting that cerebral microvessels can be functionally regulated by raphe neurons (Bonvento et al., 1989; McBean et al., 1990; 1991; Underwood et al., 1992; Cudennec et al., 1993) and the results presented here provide a morphological basis for such hypothesis. A more detailed analysis of these neurovascular associations in three brain regions, which differentially adjust their blood flow response to changes in 5-HT neurotransmission, indicated that the most responsive area (e.g. frontoparietal cortex) exhibited more frequent and/or intimate neurovascular associations than those in less responsive areas such as the hippocampus and entorhinal cortex (Bonvento et al., 1989; McBean et al., 1990; 1991; Cudennec et al., 1993). This regional selectivity gained further support from other systems which either do not induce significant blood flow changes in any area (e.g. noradrenergic fibers from the locus coeruleus) or which lack of effects has been limited to well-defined areas (e.g. cholinergic basal forebrain neurons and the perirhinal cortex, Vaucher and Hamel, 1995; Vaucher et al., 1997).

In the frontoparietal cortex, the noradrenergic system was less intimately associated with blood vessels than projections arising from brainstem serotonergic neurons. The average distance of perivascular noradrenaline nerve terminals from blood vessels was significantly greater to those characterized in the 5-HT system (Student *t* test, $p \leq 0.001$) and were less frequent within 0.25 µm of the vessel basal lamina (26.5%, 5-HT; 18.4%, noradrenaline). Noradrenaline terminals were characterized by their tendency to associate with astrocytic processes, whether located around intraparenchymal blood vessels or in brain parenchyma (see also, Séguéla et al., 1989). This observation could suggest that noradrenaline terminals in the cerebral cortex are more concerned with the regulation of astrocytic rather than vascular functions. Noradrenaline has, in fact, been reported to influence astrocytic-related functions such as glycogenolysis and glucose uptake (Sorg and Magistretti, 1991; 1992) and BBB permeability (Raichle et al., 1975; Harik, 1985; Sarmento et al., 1994) while having consistent but rather limited effects on brain perfusion (Raichle et al., 1975; Goadsby and Duckworth, 1989; Adachi et al., 1991). In this respect, the noradrenaline neurovascular associations in the frontoparietal cortex (Chapter 5) resembled more closely those characterized for 5-HT in the hippocampus and entorhinal cortex as well as those for acetylcholine in the perirhinal cortex (Chapter 4; Vaucher and Hamel, 1995). Interestingly, these are brain regions where blood flow changes are rather moderate when the projecting neurons are stimulated. In the extracerebral circulation, a distance of less than 1 µm between a nerve terminal and a blood vessel has been associated with an effective functional neurogenic control of the CBF (Lee, 1981; Dodge et al., 1994). Interestingly, an average distance of about 1 µm and, more so, an enrichment of perivascular terminals within the first 0.25 µm from the vessel wall, have been found in brain regions where significant changes in perfusion are observed, namely in the frontoparietal cortex for both serotonergic and cholinergic terminals (Chapter 4; Chédotal et al., 1994; Vaucher and Hamel, 1995; Cohen et al., 1996). Conversely, in brain region where serotonergic, noradrenergic and cholinergic blood flow regulation is more moderate, the average distance between nerve terminals and blood vessel is greater than 1 µm and the terminals do not exhibit such a preferential enrichment in the immediate vicinity of the vessel wall (Chapters 4 and 5; Vaucher and

Hamel, 1995; Cohen et al., 1996). Thus, there appears to be a correlation between the intimacy of the neurovascular associations and CBF regulation in the two systems described in my thesis as noted previously for the cholinergic system. That these anatomical findings would underlie the final microvascular response, however, in purely speculative and surely depends on many other aspects such as distribution and/or relative predominance of specific receptor populations, among other integrative local mechanisms.

7.2.2 NONSYNAPTIC TRANSMISSION OF THE 5-HT SYSTEM

In the three regions studied (Chapter 4), perivascular 5-HT nerve terminals never exhibited a specialized junction at the site of contact with glial or vascular cells although a minority of synaptic junctions could be evidenced with neuronal elements. This, together with the known paucity of synaptic junctions established by 5-HT nerve terminals in various brain regions (Descarries et al., 1975; 1982; Beaudet et al, 1979; Soghomonian et al., 1989; Oleskevich et al., 1991) support the original suggestion that 5-HT neurotransmission is primarily via non-synaptic, paracrine mechanisms in the mode of volume transmission (see Descarries et al., 1991 for review). Further, the demonstration that 5-HT nerve terminals generally do not contact blood vessels directly implies that the vascular effects of 5-HT are mediated by diffusion around and through the astrocytic leaflet to reach vascular elements endowed with functional 5-HT receptors. The detection of various 5-HT receptors on endothelial, smooth muscle and astroglial cells (Chapter 6) adds further proof of this nonsynaptic mechanism. The lack of synaptic specialization between 5-HT terminals and the intraparenchymal blood vessels would be similar to the non-junctional, yet functional, innervation of extracerebral blood vessels by a variety of neuromediators (Edvinsson et al., 1993).

7.3 5-HT RECEPTORS ON MICROVASCULAR CELLS AND ASTROCYTES

In addition to the physiological evidence suggesting that the microcirculation can respond to neurally released 5-HT and with our ultrastructural demonstration of close 5-HT neurovascular associations, we also report the presence of functional 5-HT receptors within

the different cellular compartments of the microvascular unit. These receptors are likely to mediate some of the cerebrovascular effects elicited by 5-HT. In our analysis, we included not only cultures of microvascular endothelial and smooth muscle cells but also of brain astrocytes, a cell type known to intimately associate with intraparenchymal blood vessels (White et al., 1981; Peters et al., 1991). Our anatomical results, which suggested that perivascular astrocytes act as an intermediary in the serotonergic (and noradrenergic) neurovascular associations and as such to actively participate in the regulation of vascular functions (see section 7.4), further prompted us to include this cell type in the analyses.

I would like to stress that the microvascular receptor results obtained in this study are quite unique and original considering that they were generated from human brain endothelial and smooth muscle cells of microvascular origin. Very few laboratories have access to such tissues and we believe that these data provide a convincing, novel and relatively precise assessment of the mode of interaction between 5-HT neuronal systems and non-neuronal cells within the CNS.

Using RT-PCR, the messages for the 5-HT_{1D}, 5-HT_{2B} and 5-HT_7 receptors were detected in all cell types (5-HT_{1D} receptor mRNA was very faint in the endothelial fraction), 5-HT_{1B} receptor mRNA was present in the smooth muscle and astrocytes while transcripts for the 5-HT_{2A} and 5-ht_{1F} receptors were only found in astrocytes. Complementary second messenger investigations further indicated that these are functional vascular and astroglial receptor proteins. In the following section, I will highlight and discuss the most relevant findings in view of possible microvascular responses. These include 5-HT_{1D} and 5-HT_{2B} receptors of the capillary endothelium that would regulate mostly BBB permeability but perhaps vasomotor functions and 5-HT_{1B} along with 5-HT_7 receptors that could possibly mediate microvascular contraction and dilatation, respectively.

7.4 FUNCTIONAL NEURONAL-GLIAL-MICROVASCULAR INTERACTIONS

7.4.1 BLOOD-BRAIN BARRIER FUNCTIONS

In our ultrastructural study, we found that serotonergic nerve terminals were predominantly associated with capillaries (80%) as opposed to small arteries and microarterioles, an observation which would suggest that these association are likely to influence capillary functions, an important one being BBB permeability. The overwhelming evidence clearly points to the tight junctions between endothelial cells as the primary morphological locus of the physical barrier (see Cancilla et al., 1993b; Joo, 1996 for review). However, it is also well established that perivascular astrocytes play important roles in various aspects of the BBB (Cancilla et al., 1993b; Bradbury, 1994). In fact, these non-neuronal cells enhance tight junction formation in brain microvascular endothelial cells *in vitro* (Janzer and Raff, 1987; Tao-Cheng et al., 1987) and have been involved in the maintenance and/or repair of the BBB (Cancilla et al., 1993; Bradbury, 1994). The demonstration that 5-HT can increase vesicular transport, electrical resistance and permeability to Evans Blue and [^{131}I]sodium (Westergaard, 1975; Olesen, 1985; Sharma et al., 1990; Winkler et al., 1995) is in line with our findings of specific populations of 5-HT receptors in cerebromicrovascular endothelial cells as well as the high frequency of pericapillary serotonergic terminals, which could also interact with the perivascular astrocytes (see below).

The identity of the 5-HT receptor that generates these BBB changes, i.e. increased ion transport and permeability to tracers, is unknown but 5-HT_{2A} receptors appear as potential candidates, at least in the rat and frog (Olesen, 1985; Sharma et al., 1990; Winkler et al., 1995). Our results, however, clearly indicate that 5-HT_{2A} receptors are not found in human endothelial cells suggesting that alternative receptors, possibly the endothelial 5-HT_{1D} and/or 5-HT_{2B} subtypes, could play such a role. Nevertheless, a contribution of a 5-HT_{2A} receptor, located on the perivascular astrocytes, could represent a complementary mechanism. 5-HT_{1D} and 5-HT_{2B} receptors have been postulated as mediators of the endothelial-dependent vasodilatation in brain and peripheral vessels (see below). Their ability to promote NO synthesis and release could provide a means by which they could also

affect BBB properties at the capillary level. Indeed, NO is able to affect ion and nutrient transport across the BBB (Janigro et al., 1994) and the 5-HT-induced increase in vascular permeability, at least in the skin of the mouse, involves NO (Fujii et al., 1994). The preliminary data presented in this thesis regarding the slight increase in NO production and release in cerebromicrovascular capillary endothelial cells following incubation with 5-HT (Chapter 6, Addendum) would support these observations. Additional experiments are needed to confirm that 5-HT-mediated permeability changes occur in human brain, to precisely identify which 5-HT receptor subtype(s) mediates such an effect and whether or not NO is involved. The possibility also exists that other capillary endothelial receptors such as the 5-HT_7 also participate in the regulation of the BBB. These endothelial receptors are being unequivocally identified for the first time in any arterial preparations, whether derived from central and/or peripheral vessels of any species (Ullmer et al., 1995). The presence of 5-HT_7 receptors on cerebral endothelium, however, is fully compatible with previous studies which showed that 5-HT application to human microvascular endothelial cells stimulated cAMP production (Spatz et al., 1989; Bacic et al., 1991).

7.4.2 CEREBRAL BLOOD FLOW REGULATION

7.4.2.1 Direct Effects: Historically, the resistance microarterioles with their smooth muscle cells and their ability to change diameter have been considered as the ones controlling blood perfusion. Thus, the 5-HT-containing nerve terminal/arteriole interactions could participate in this regulation. In fact, we demonstrate the presence of two functional receptors, namely 5-HT_{1B} and 5-HT_7, on smooth muscle cells derived from intraparenchymal arterioles that could mediate the 5-HT-induced contraction and dilatation, respectively, in line with previous findings, albeit in different tissues.

In human pial vessels, molecular and pharmacological evidence indicate that 5-HT_{1B}, and not 5-HT_{1D} or 5-ht_{1F}, receptors mediate the 5-HT-induced cerebral vasoconstriction (Hamel et al., 1993; Beattie and Connor, 1995; Bouchelet et al., 1996; Phebus et al., 1996). The present evidence strongly points to a similar conclusion in the microcirculation. Thus, in

both extra- and intracerebral blood vessels, 5-HT_{1B} receptors would be the 5-HT receptor type responsible for the 5-HT induced contractile response. In brain parenchyma, following raphe stimulation, neurally released 5-HT would interact with the 5-HT_{1B} receptors, present on smooth muscle cells and result in vasoconstriction. The 5-HT_{1B} receptor, as part of the 5-HT_1 family, is negatively coupled to adenylate cyclase and as such its activation can increase intracellular Ca^{2+} levels via a phosphoinositide independent pathway, although the precise sequence of events is presently not known (see Fig 1.10). In fact, in bovine pulmonary arteries and vascular smooth muscle cells, activation of 5-HT_1 receptors lead to an increase in intracellular calcium. (Ebersole et al., 1993; Sweeney et al., 1995).

A large body of convincing evidence suggest that 5-HT_7 receptors are responsible for the 5-HT-mediated smooth muscle relaxation of several vascular beds, albeit of peripheral sources (Feniuk et al., 1983; Sumner et al., 1989; Cushing et al., 1996; Leung et al., 1996; Terron, 1996). The message of these receptors have been identified in human smooth muscle cultures from aorta as well as the pulmonary and uterine arteries (Ullmer et al., 1995; Schoeffter et al., 1996). Together with our findings of 5-HT_7 receptor gene expression and functional protein in microvascular smooth muscles, it is tempting to suggest that this receptor could mediate cerebral vasodilatation, at least in human microarterioles and arterioles. Its proposed vasorelaxant properties are further supported by its ability to enhance intracellular cAMP. The increase in cAMP would activate protein kinase G and phosphorylate phospholamban, the Ca^{2+} pump regulator on the sarcoplasmic reticulum, and in turn decrease cytosolic Ca^{2+} and result in vasorelaxation (Fig 1.11; Walsh, 1993).

In counterpart, it may be more difficult to explain how capillaries could influence CBF locally. However, quite a large body of evidence exists to suggest that capillaries may have the ability to control their diameter and as such participate in the contraction/dilatation of upstream microarterioles. Indeed, endothelial cells and pericytes of brain capillaries contain contractile proteins such as actin, myosin and tropomyosin (Owman et al., 1978; Nehls and Drenckhahn, 1993; Shepro and Morel, 1993) and endothelial cells from peripheral vessels

have been shown to contract in response to several vasoactive agents (Boswell et al., 1992). In addition, an interesting concept has recently attracted attention based on the demonstration by Segal and Duling (1986) that application of acetylcholine to a hamster cheek pouch arteriole elicits a bidirectional vasodilatation in feeding arteries. A similar mechanism was later described by Dietrich and Tyml (1992) in the capillary bed of rat and frog skeletal muscle using noradrenaline as the vasomotor agent. These authors showed that iontophoretic and microapplication of noradrenaline on capillaries resulted in a reduction in their endogenous blood flow by constricting the supplying arteriole, located 1 mm away from source of noradrenaline, suggesting that capillaries can function as a communicating medium. Subsequent investigations by the same group suggested that the capillary bed functions as a sensor and integrator of biological signals to transmit relevant information, possibly via electrical signals, to arterioles located upstream (Song and Tyml, 1993). The demonstration of junctions in human and bovine brains between individual endothelial cells but also between endothelial and smooth muscle cells (Larsons et al., 1987; Aydin et al., 1991) as well as the transmission of electrical information between these cells, albeit in peripheral vascular beds (Segal and Beny, 1992; Beny and Pacicca, 1994) provides strong anatomical support of such mechanism. In fact, the capillary fraction has been recently shown in the rat skeletal muscle to have as profound an effect on microvascular flow as arterioles (Mitchell et al., 1997). Capillaries would be ideal candidates to sense and relay information for the regulation of local blood flow as they are abundantly and ubiquitously found in brain parenchyma; about ten times more frequently than any other blood vessel (MacDonald and Rasmussen, 1977).

It is also possible that the endothelium can regulate local cerebrovascular perfusion via the production and release of either endothelial derived constrictor (i.e. endothelin or thromboxane A_2) or dilator (i.e. NO or prostacyclin) factors (for review see Wahl and Schilling, 1993), in part under the influence of various neurotransmitters. In this respect, 5-HT_{1D} and 5-HT_{2B} receptors are thought to mediate endothelial-dependent vasorelaxations via NO release in peripheral vascular segments (Leff et al., 1987; Schoeffter and Hoyer,

1990; Gupta, 1992; Glusa and Richter, 1993; Schmuck et al., 1996) and our demonstration of these receptor types on the endothelium provides support that 5-HT-induced relaxations may be mediated via this cell type. Furthermore, since 5-HT has been shown to be a powerful modulator of arachidonic acid turnover (Strosznadjer et al., 1994), it is tempting to speculate that metabolites such as hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids, which possess constrictor and relaxant properties respectively (Harder et al., 1995), may be produced and released as a result of 5-HT. That these mechanisms would occur *in vivo* is purely speculative at present but is theoretically plausible and could thus be under the regulation of various neurogenic factors.

7.4.2.2 Effects via perivascular astrocytes On the basis of our observations that a non-negligeable percentage (11.6%) of perivascular 5-HT nerve terminals directly target the astroglial processes that commonly surround intracerebral blood vessels, we proposed that perivascular astrocytes may play an important role, as an intermediary cell, in some aspects of the vascular functions exerted by 5-HT, thus being an integral constituent of the functional microvascular unit (Chapter 4). The demonstration of many functional 5-HT receptors on these non-neuronal cells (Chapter 6) further supports the notion that neurally released 5-HT could be involved in direct astroglial as well as astroglial-vascular functions. In fact, astrocytes have been involved in certain aspects of CBF regulation. For example, the astroglial perivascular end-feet have been shown to remove from the extracellular space K^+ ions generated as a result of increased neuronal activity, ions which produce vasodilatation of the cerebral vascular smooth muscle cells (Paulson and Newman, 1987). In addition, astrocytes produce and release compounds such as NO (Murphy et al., 1993) and endothelins (MacCumber et al., 1990), which are potent vasodilators and constrictors, respectively (Kontos, 1993; Iadecola et al., 1994, Kanaide, 1996). Recently, astrocytes have been shown to release arachidonic acid metabolites such as epoxyeicosatrienoic acids which possess potent vasodilatory properties on smooth muscle cells (Harder et al., 1995; Alkayed et al., 1996). These molecules, like NO (Iadecola, 1993) have been proposed to be the link between neuronal activation and changes in local brain perfusion (Alkayed et al., 1996).

Another interesting property of astrocytes is their interconnections by gap junctions (Giaume and McCarthy, 1996), whereby a large population of astrocytes behaves like a syncytium allowing to relay information between remote brain areas. As suggested above, capillaries could possibly sense and integrate messages from the environment and pass it on to blood vessels located upstream. Through the perivascular astrocytes, it is thus possible that, in addition, to the endothelial-endothelial and endothelial-smooth muscle dialogues, an additional level of interaction could involve the perivascular astrocytic syncytium. It is important to mention that the manner in which astrocytes contribute to the 5-HT-mediated control of the CBF, if in fact they do, is unknown. Their strategic position with brain vascular cells and their ability to release vasoactive substances together with the detection of specific populations of 5-HT receptors (Chapter 6) on these non-neuronal cells provide good evidence, however, to suggest such a role.

7.4.3 MITOGENESIS

Another possible function of 5-HT on the microvascular bed may be related to mitogenesis. In canine and bovine aortic endothelial cells, 5-HT exerts a powerful mitogenic effect via a 5-HT_{2A} and/or 5-$HT_{1B/1D}$ receptor subtypes (Pakala et al., 1994). Similarly, in smooth muscle cells derived from bovine pulmonary artery serotonin reportedly exerts a dual effect on the regulation of growth in these cells. Indeed, incubation of 5-HT stimulates smooth muscle DNA synthesis, likely mediated by internalization of 5-HT but also inhibits synthesis via a mechanism apparently associated with an elevation in cAMP levels (Lee et al., 1991; 1994). Our observation of functional microvascular endothelial and smooth muscular 5-HT_7 receptors is possibly compatible with this function.

7.5 FACTORS DETERMINING VASOMOTOR RESPONSES

Taken together with physiological and pharmacological evidence, our results allow to suggest i) that the microcirculation could be responsive to neurally released and/or circulating 5-HT, ii) that 5-HT, via specific endothelial, muscular and/or astroglial receptors, can elicit or modulate vasomotor or permeability responses in the microvascular bed and iii)

that 5-HT could also mediate specific astroglial functions as well as vascular and/or astroglial growth via a plethora of receptors with specific location in these non-neuronal cells.

Based on the available information, it seems that 5-HT exerts a relatively minor tonic influence on the microvascular bed as destruction of this system, either by lesions of the raphe nucleus and/or methylenedioxyamphetamine administration, induces negligeable, if any, changes in resting CBF (Itakura et al., 1985; McBean et al., 1991; Underwood et al., 1992; Kelly et al., 1995). It has thus been suggested that this system may be tonically active during specific physiological or pathophysiological conditions. For instance, during the sleep-wake cycle, it is well documented that the firing activity of dorsal raphe neurons varies considerably, being almost silent during rapid-eye-movement sleep where elevations in regional CBF are also observed (Masden, 1993). That the blood flow increases are corrolated to the activity of the raphe neurons still remains to be established but may be interrelated.

5-HT may also come into play during conditions of hypercapnia, in which the indoleamine could limit the accompanying large increases in CBF. Such effect would be consistent with the numerous observations suggesting that the vasomotor response of 5-HT depends on the initial tone of the blood vessels (Edvinsson et al., 1978; Rosenblum and Nelson, 1990; Sweeney et al., 1995; Kelly et al., 1995). Indeed, a differential 5-HT-induced vasomotor response has been described in extracerebral and peripheral segments depending on the preexisting tone of the vessel i.e. whether or not the vessels are in a preconstricted (high tone) or relaxed (low tone as during hypercapnia) state. 5-HT causes a vasodilation in the former and a vasoconstriction in the latter. Similar observations have also been documented *in vivo* in human subjects. In migraine patients whose middle cerebral artery was dilated unilaterally on the headache side, infusion of the antimigraine compound and 5-$HT_{1B/1D}$ receptor agonist, sumatriptan, effectively reduced blood velocity (i.e. constriction) but not in vessels on the other side (Friberg et al., 1991). From this observation, the authors concluded

that sumatriptan acts predominantly on dilated arteries. Such mechanism could explain the lack of vasomotor effects when the agent is administered to healthy individuals (Scott et al., 1992), or expectedly to migraine sufferers outside their migraine episode when the vessels are not in a dilated state. Whether or not similar conclusions can also be extended to the microcirculation warrants further investigations. However, the initial tone of the microvessel may also be a determinant of the integrated final vasomotor response to 5-HT. They suggest a subcellular interplay between endothelial-dependent and -independent factors, possibly at the level of activation and/or inactivation of G-coupled proteins such as Gi or Gs. The identity of these factors and the exact intracellular mechanisms involved in these interactions, however, is presently unknown.

Other factors may also be involved in the resulting vasomotor response. The respective amount of the different 5-HT receptor types present in the microvascular unit may be a contributing factor. A different vasomotor response may be elicited depending on the receptor that is being activated. Finally, as demonstrated experimentally in the rat, it seems that the ultimate vasomotor consequence elicited by 5-HT, at least in the microcirculation, may depend on the subregion of the dorsal raphe nucleus that is being activated. Underwood and colleagues (1992) showed that stimulation of rostral regions within this nucleus decreased local CBF while activation of caudal areas elicited opposite responses. The differences in vasomotor response in this case may merely reflect the 5-HT receptor population that is being activated following stimulation of the raphe nuclei. However, the possibility also exists that changes in blood flow (either increase or decrease) are not in fact directly mediated by 5-HT but rather by non-serotonergic neurons located in the raphe nucleus (see section 1.2.3.2.1). It is well documented that different neurotransmitters and neuromodulators, such as enkephalins, opoid peptides, substance P and NO, are present in raphe neurons and possess vasomotor properties (Kelly et al., 1989; Iadecola et al., 1993; Benyo and Wahl, 1996) and, possibly, the blood flow alterations are a result of activation of these neurons.

7.6 GENERAL CONCLUSIONS

In conclusion, the results presented in this thesis demonstrate that raphe neurons do not provide a substantial direct input to extracerebral blood vessels. They may, however, influence extracerebral perivascular 5-HT levels or 5-HT-induced vasomotor responses via the release of 5-HT into the subarachnoid space where it could either be taken up by perivascular nerves or act on 5-HT receptors in major cerebral and pial arteries. The majority of 5-HT contained in cerebrovascular nerve fibers appear to originate from the superior cervical ganglion or a structure closely related, although the visualization of TPH sympathetic neurons is still lacking. In the microcirculation, the results show that intraparenchymal blood vessels are innervated by raphe neurons and that neurovascular associations are more intimate and frequent in cerebral regions where significant blood flow changes have been previously shown and could, thus, form the anatomical basis for the functional effect observed. These neurovascular relationships frequently involve the perivascular astrocytes which suggests a neuronal-glial-vascular tripartite organization as the fundamental and functional unit in the neurogenic control of the cerebral microcirculation. This suggestion was corroborated by our findings of various 5-HT receptors not only on endothelial and smooth muscle cells of microvascular origin but also on astrocytes, supporting the possibility that 5-HT is capable of directly influencing the microvascular bed and indirectly through the perivascular astrocyte. Altogether, the physiological, morphological and molecular biological data are strongly indicative of a functional innervation of the brain microcirculation and that the vasomotor responses are not totally dependent on metabolic factors. Our findings also reflect important interactions between neurons and non-neuronal cells within the CNS to modify vasomotor functions that may depend on the state of 5-HT neurotransmission and, possibly, other neurotransmitter systems.

7.7 FUTURE DIRECTIONS

The most logical continuation of this project would be to specifically demonstrate whether or not the putative 5-HT receptors mediate the proposed vascular effects. The present results

identified 5-HT receptors on different cellular constituents of the blood vessel wall and showed their ability to interact with their G proteins to initiate the cascade of intracellular signalling. Whether or not these receptors mediate the expected effects *in vivo* remains to be established by more sophisticated approaches allowing to visualize the microcirculation either *in situ* (Fergus et al., 1995) or in isolated preparations (Dacey and Bassett, 1987) and in changes in microvascular reactivity under different conditions.

Another interesting avenue to pursue would be to identify the factors and their mechanisms at the G-protein signalling level, which determine the final integrated 5-HT vasomotor response. As discussed previously, the final vasomotor response, at least with respect to the serotonergic system, is largely dependent on the initial tone of the blood vessel. Knowledge of the subcellular mechanisms involved in this process would potentially be critical in the development of novel compounds for future therapies related to vasomotor functions and dysfunctions such as migraine, ischemia and possibly subarachnoid hemorrhage.

CHAPTER 8

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CHAPTER 9

APPENDED PAPERS

SEROTONIN IN THE REGULATION OF BRAIN MICROCIRCULATION

Z. Cohen, G. Bonvento, P. Lacombe and E. Hamel

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IN VIVO-SYNTHESIZED RADIOACTIVELY LABELLED ALPHA-METHYL SEROTONIN AS A SELECTIVE TRAVER FOR VISUALIZATION OF BRAIN SEROTONIN NEURONS

Z. Cohen, K. Tsuiki, A. Takada, A. Beaudet, M. Diksic and E. Hamel

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SEROTONIN IN THE REGULATION OF BRAIN MICROCIRCULATION

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Abstract—Manipulation of brainstem serotonin (5-HT) raphe neurons induces significant alterations in local cerebral metabolism and perfusion. The vascular consequences of intracerebrally released 5-HT point to a major vasoconstrictor role, resulting in cerebral blood flow (CBF) decreases in several brain regions such as the neocortex. However, vasodilatations, as well as changes in blood–brain barrier (BBB) permeability, which are blocked by 5-HT receptor antagonists also can be observed. A lack of relationship between the changes in flow and metabolism indicates *uncoupling* between the two variables and is suggestive of a direct neurogenic control by brain intrinsic 5-HT neurons on the microvascular bed. In line with these functional data are the close associations that exist between 5-HT neurons and the microarterioles, capillaries and perivascular astrocytes of various regions but more intimately and/or more frequently so in those where CBF is altered significantly following manipulation of 5-HT neurons. The ability of the microvascular bed to respond directly to intracerebrally released 5-HT is underscored by the expression of distinct 5-HT receptors in the various cellular compartments of the microvascular bed. Thus, it appears that while some 5-HT-mediated microvascular functions involve directly the blood vessel wall, others would be relayed through the perivascular astrocyte. The strategic localization of perivascular astrocytes and the different 5-HT receptors that they harbor strongly emphasize their putative pivotal role in transmitting information between 5-HT neurons and microvessels. It is concluded that the cerebral circulation has full capacity to adequately and locally adapt brain perfusion to changes in central 5-HT neurotransmission either directly or indirectly via the neuronal–astrocytic–vascular tripartite functional unit. Dysfunctions in these neurovascular interactions might result in perfusion deficits and might be involved in specific pathological conditions. Copyright © 1996 Elsevier Science Ltd.

CONTENTS

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CONTENTS (*Continued*)

ABBREVIATIONS

BBB	Blood–brain barrier
CBF	Cerebral blood flow
CGRP	Calcitonin gene-related peptide
CGU	Cerebral glucose utilization
CNS	Central nervous system
6-CPP	6-Chloro-2-(1-piperaxinyl)pyrazine
CSF	Cerebrospinal fluid
2-DG	2-Deoxyglucose
5,7-DHT	5,7-Dihydroxytryptamine
DL-h	DL-Homocysteic acid
DOI	1-(2,5-Dimethoxy-4-iodophenyl)2-aminopropane
DRN	Dorsal raphe nucleus
EMF	Electromagnetic flowmetry
FDG	Fluoro-deoxyglucose
glu	Glutamate
H_2	Hydrogen clearance
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine
IAP	Iodoantipyrine
KA	Kainate
LDF	Laser Doppler flowmetry
LSD	Lysergic acid diethylamide
m-CPP	Meta-chlorophenylpiperazine
MDA	Methylenedioxyamphetamine
5-MeODMT	5-Methoxy-*N*,*N*-dimethyltryptamine
NO	Nitric oxide
8-OH-DPAT	8-Hydroxy-2-(di-*N*-propylamino)tetraline
PCPA	*p*-Chlorophenylalanine
PE	Photoelectric
PET	Positron emission tomography
REM	Rapid eye movement
SPECT	Single photon emission computed tomography
RU-24969	5-Methoxy 3-(1,2,3,6-tetrahydro-4-pyridinyl)1H indole succinate
Xe	^{133}Xenon technique

1. INTRODUCTION

Shortly after the discovery of 5-hydroxytryptamine (5-HT, serotonin) as a potent vasoconstrictor agent in blood serum (Rapport *et al.*, 1948), the indoleamine was found in many other tissues including the mammalian brain (Twarog and Page, 1953) and the xexquisite sensitivity of cerebral blood vessels to 5-HT was recognized (Edvinsson *et al.*, 1977). This led to the suggestion, more than 10 years ago, that 5-HT could play a pivotal role in the coupling between cerebral blood flow (CBF) and metabolism (Edvinsson *et al.*, 1984). Since then, considerable evidence has accumulated which strongly points to the involvement of the brainstem raphe nuclei, the seat of serotonin neurons that send projections throughout the CNS, in the control of cerebral perfusion. The present review will focus on the effects of brain intrinsic 5-HT neurons on the local regulation of CBF and metabolism, but also on other possible functions related to the permeability of the blood–brain barrier (BBB) and vascular growth. The morphological relationships that exist between brainstem serotonergic neurons and intraparenchymal blood vessels will be presented as an anatomical basis for the functional changes observed in CBF following manipulation of serotonergic neurotransmission. Novel data on the putative vascular 5-HT receptors, together with their distribution in the distinct cellular components of the functional microvascular unit, will be considered in light of their possible role in the local regulation of brain perfusion, metabolic demand and maintenance of the microvascular bed. Finally, the implication of 5-HT in pathophysiological conditions associated with the microcirculation will be reviewed in an attempt to evaluate the possible role of neurovascular disturbances in disease etiology, or to better understand the recent orientations of new therapeutical strategies. We have elected only to expose briefly the controversial role of brainstem 5-HT raphe neurons in the regulation of extracerebral blood vessels. However, we have considered in the discussion pathologies related to 5-HT dysfunctions at the level of extracerebral and/or intraparenchymal blood vessels, including those for which a clear relationship with the cerebral microcirculation has not yet been established.

1.1. The Vascular Compartments of the Cerebral Circulation

Before considering the interactions between brain serotonergic neurons and the microcirculation, it is important to highlight the differences between the two vascular compartments that control blood supply to the brain. The *extracerebral* vessels include the major cerebral arteries at the base and over the convexities of the brain together with their ramifications as small pial vessels that run in the subarachnoid space. These vessels ramify widely and some will bifurcate to perforate the cortical mantle. In their descent into the cortical parenchyma, the perforating vessels are surrounded initially by the Virchow–Robin's space which gradually disappears as they give rise to *intracerebral* or *intraparenchymal* microvessels. These encompass the microarterioles, small veins and capillaries embedded into brain parenchyma and are commonly referred to the

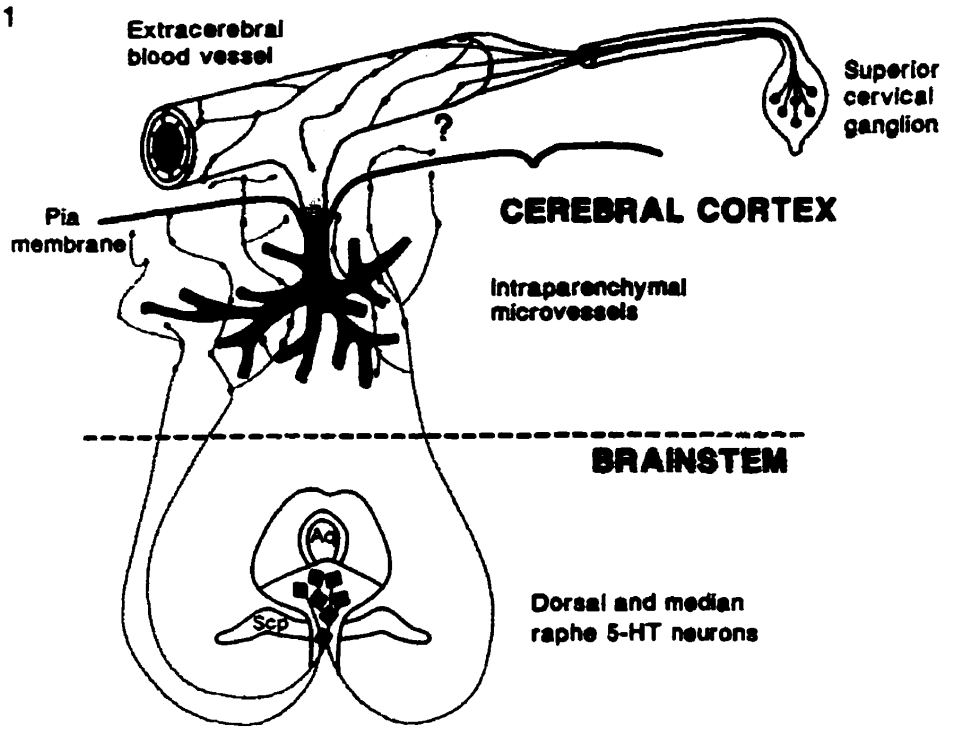

Fig. 1. Schematic representation of extracerebral and intraparenchymal blood vessels and of the origin of their respective 5-HT innervations. Extracerebral blood vessels, overlying the cerebral cortex and running in the subarachnoid space, are innervated by serotonergic nerve fibers that putatively arise from the superior cervical ganglion and/or (?) from 5-HT neurons originating in the brainstem raphe nuclei. As these vessels bifurcate and perforate the cortical mantle, they are initially surrounded by the Virchow–Robin's space. When this space vanishes, the intraparenchymal microvessels, composed of microarterioles (small veins and capillaries) develop. These vessels are innervated by serotonergic fibers originating in the raphe nuclei that project widely within the cortical parenchyma and, to a lesser extent, to the cortical microvascular bed. Some of these fibers in the most superficial cortical layer seem to float in the subarachnoid space and may functionally affect extracerebral vessels (?). Abbreviations: Aq, aqueduct; Scp, superior cerebellar peduncle.

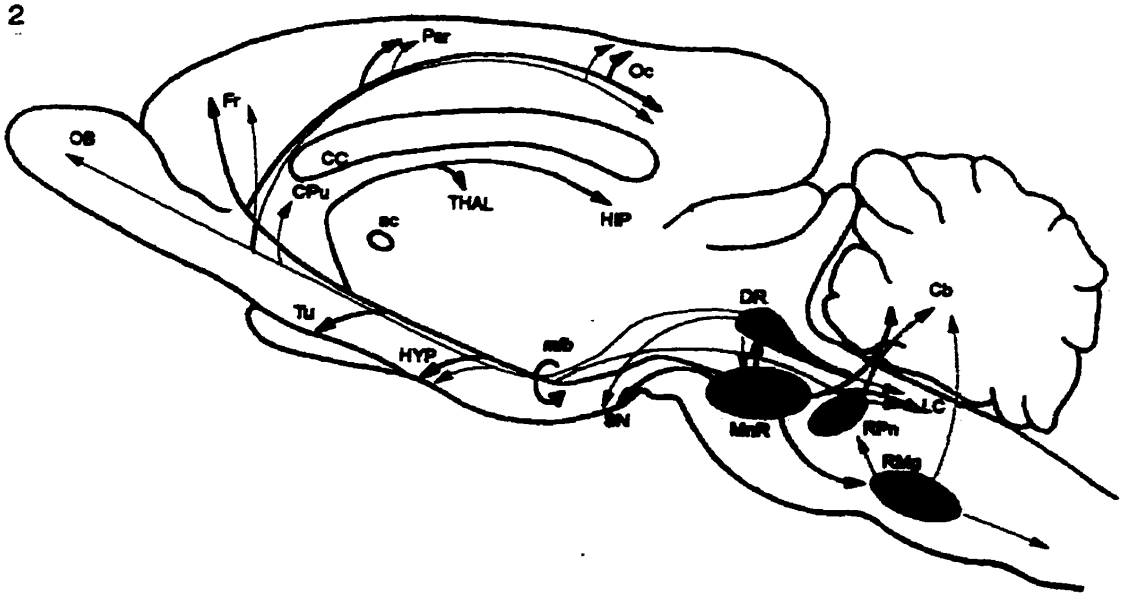

Fig. 2. Schematic representation of the serotonergic system of the rat brain. Only the main raphe nuclei and their major ascending and descending projections are outlined. Abbreviations: ac, anterior commissure; Cb, cerebellum; CC, corpus callosum; Cpu, caudate putamen; DR, dorsal raphe; HIP, hippocampus; HYP, hypothalamus; LC, locus coeruleus; mfb, median forebrain bundle; MnR, median raphe; OB, olfactory bulb; Rmg, raphe magnus; Rpn, raphe pontis nuclei; SN, substantia nigra; THAL, thalamus; Tu, olfactory tubercule; Fr, Par, Oc, frontal, parietal and occipital cortices.

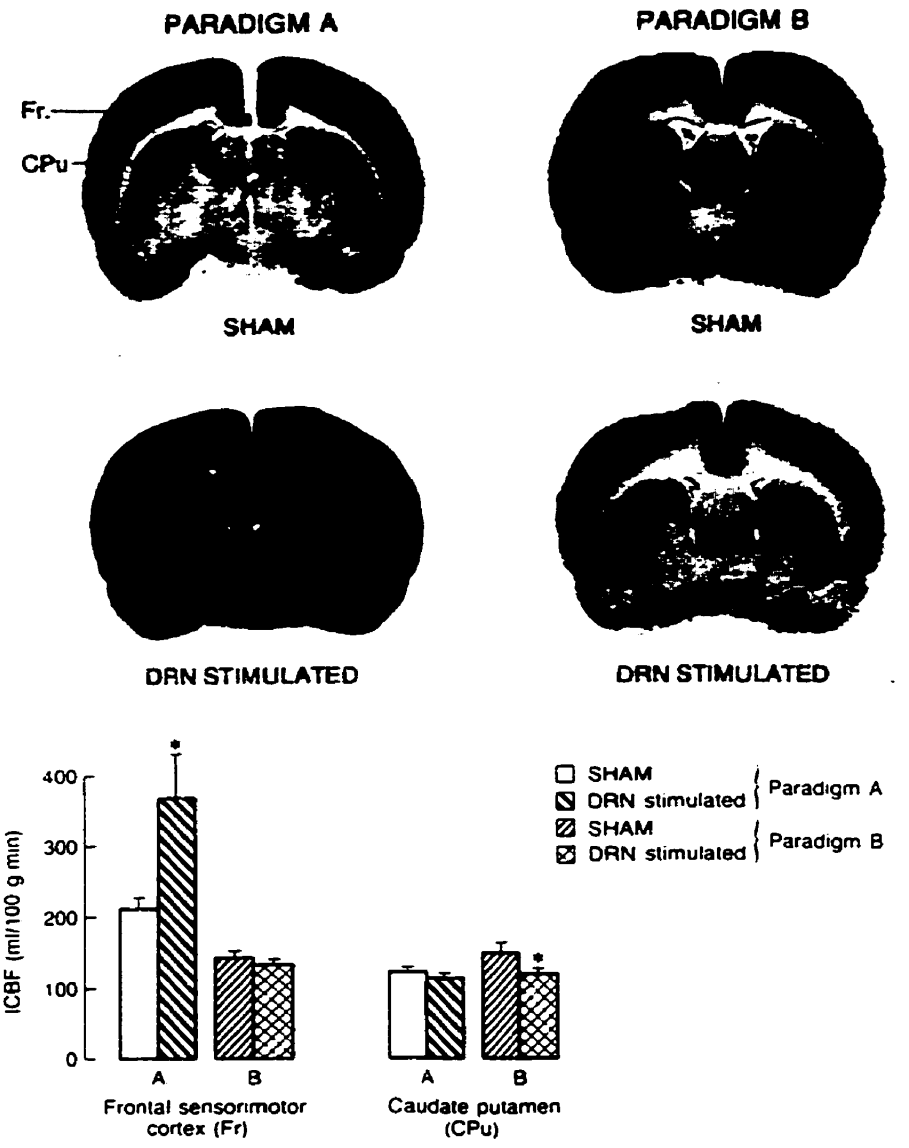

Fig. 3. Representative autoradiograms of local cerebral blood flow (lCBF, expressed in ml/100 g min) changes in sham and dorsal raphe nucleus (DRN) stimulated rats under two different experimental paradigms, conscious (paradigm A) and anesthetized (paradigm B) animals. The heterogeneous changes in lCBF following DRN stimulation under paradigms A and B are exemplified in two different brain areas, frontal sensorimotor cortex and caudate-putamen nucleus.

cerebral microcirculation. The two vascular segments are categorized according to their size and localization, and they differ in their respective origins, patterns of innervation and territory of irrigation (Fig. 1). The major arteries and, primarily, the pial vessels, are resistance vessels that control global blood supply to the brain, while the intraparenchymal vessels would be involved in the regulation of local CBF and BBB permeability.

1.2. Serotonergic Innervation of Cerebral Blood Vessels

1.2.1. *Extracerebral Blood Vessels*

Original *in vitro*, *in situ* and *in vivo* studies clearly demonstrated the potent vasoconstrictor role of 5-HT on extracerebral blood vessels of various species including man (see Parsons, 1991; Bonvento and Lacombe, 1993 for review). However, the indoleamine also has been shown to elicit vasodilatation under certain conditions (Edvinsson *et al.*, 1977). While the predominant vasomotor effect is undeniably a vasoconstriction, these two opposite 5-HT-mediated vascular responses can most likely be explained, at least in part, by differences in the population(s) of 5-HT receptors and in their endothelial vs smooth muscle location, but possibly also by the original intrinsic tone of the vessels before exposure to 5-HT (Edvinsson *et al.*, 1977; see Section 3.4).

The remarkable sensitivity of brain vessels for 5-HT has been paralleled by the visualization, as evidenced by radioautographical, biochemical and immunocytochemical methods, of a rich network of nerve fibers around major cerebral arteries and pial vessels that contain 5-HT (Chan-Palay, 1976; Griffith *et al.*, 1982; Edvinsson *et al.*, 1983; Scatton *et al.*, 1985) or its synthesizing enzyme, tryptophan hydroxylase (Chédotal and Hamel, 1990; Cohen *et al.*, 1992; Mathiau *et al.*, 1993). Whether the fibers have a peripheral and/or central origin and whether the presence of the indoleamine in these nerves is due to its uptake or synthesis within authentic serotonergic perivascular nerves have remained rather controversial issues (Table 1). Clearly, a general agreement has not been reached, but compelling evidence would suggest that the serotonergic innervation of major cerebral arteries and small pial vessels emanates from the sympathetic superior cervical ganglia, although an additional component from the raphe nucleus has not been totally excluded (Edvinsson *et al.*, 1983; Marco *et al.*, 1985; Moreno *et al.*, 1991, 1995; Fig. 1 and Table 1). Similarly, despite the suggestion that 5-HT in perivascular nerve fibers is a false neurotransmitter due to its uptake into sympathetic noradrenergic nerves (Saito and Lee, 1987; Chang *et al.*, 1988, 1989, 1990; Jackowski *et al.*, 1989a; Mathiau *et al.*, 1993), biochemical and anatomical studies have proposed that a subset of these fibers could possibly synthesize the indoleamine (Scatton *et al.*, 1985; Marco *et al.*, 1985; Chédotal and Hamel, 1990; Bonvento *et al.*, 1991a; Cohen *et al.*, 1992) and, as such, be considered authentic serotonergic nerves. The details of this controversy are beyond the scope of the present review and have been thoroughly discussed previously (Cohen *et al.*, 1992; Bonvento and Lacombe, 1993).

1.2.2. *Brain Microvessels*

Since the original suggestion that the cerebral microvessels might be innervated (Penfield, 1932), ultrastructural studies have demonstrated clearly the presence of axon terminals, albeit of unknown chemical content in most cases, in direct contact with intraparenchymal blood vessels (Cervos-Navarro and Matakas, 1974; Rennels and Nelson, 1975). These perivascular terminals were thought originally to arise from peripheral structures, but it is now quite clear that nerve fibers from extrinsic sources do not enter the brain parenchyma beyond the transition zone where the Virchow–Robin's space disappears (Jones, 1970; Marin-Padilla, 1988). More recently, several intrinsic brain neurons containing various neurotransmitters and neuromodulators, including 5-HT (see Section 3), have been shown to establish intimate associations with the microcirculation in a variety of brain regions (Swanson *et al.*, 1977; Hendry *et al.*, 1983; Milner and Pickel, 1986; Arnerić *et al.*, 1988; Cohen *et al.*, 1994). In addition to their well-established role in BBB functions, there has been accumulating evidence suggesting that capillaries have the potential to modulate vasomotricity and participate in the regulation of vascular perfusion. The presence of smooth muscle contractile proteins have been detected in both endothelial cells and pericytes of brain capillaries (Owman *et al.*, 1978; Nehls and Drenckhahn, 1993; Boado and Pardridge, 1994) and,

Table 1. Summary of the Possible Origin(s) for Perivascular 5-HT Fibers Around Extra- and Intracerebral Blood Vessels

Origin	Blood vessels	References
Superior cervical ganglion	Major cerebral arteries; small pial vessels	Cowen *et al.*, 1986, 1987; Saito and Lee, 1987; Chang *et al.*, 1988, 1989; Bonvento *et al.*, 1991a; Cohen *et al.*, 1992
5-HT as a false or an authentic neurotransmitter in the above sympathetic perivascular fibers **(controversial)**		Saito and Lee, 1987; Jackowski *et al.*, 1989a; Chang *et al.*, 1988, 1989, 1990; Chédotal and Hamel, 1990; Moreno *et al.*, 1991; Cohen *et al.*, 1992; Mathiau *et al.*, 1993
Raphe nucleus	Major cerebral arteries; small pial vessels **(controversial)**	Edvinsson *et al.*, 1983; Marco *et al.*, 1985; Scatton *et al.*, 1985; Moreno *et al.*, 1991, 1995; Bonvento *et al.*, 1991a;
Raphe nucleus	Intraparenchymal vessels	Reinhard *et al.*, 1979

Table 2. Effects of Raphe or Serotonergic Lesion on Cerebral Metabolism and Cerebral Blood Flow

Experimental paradigm	Treatment	Method	Effects	References
		Cerebral metabolism		
5-HT lesion	5,7-DHT (i.c.v.)	2-DG	–	Cudennec *et al.*, 1988b
Raphe lesion	Electrolytic	2-DG	–	Cudennec *et al.*, 1988b
Inhibition of 5-HT synthesis	PCPA	2-DG	–	Pappius *et al.*, 1988
5-HT lesion	MDA	2-DG	– or ↑	McBean *et al.*, 1990
5-HT lesion	MDA	2-DG	– or ↑	Kelly *et al.*, 1995
		Cerebral blood flow		
5-HT lesion	5,7-DHT	Xe	–	Dahlgren *et al.*, 1981
5-HT lesion	5,7-DHT (cortex)	H_2	–	Itakura *et al.*, 1985
5-HT lesion	MDA	IAP	– or ↑	McBean *et al.*, 1990
Raphe lesion	5,7-DHT (raphe)	LDF	–	Underwood *et al.*, 1992
5-HT lesion	MDA	IAP	–	Kelly *et al.*, 1995

Symbols: –, no change; ↑, increase.
Refer to the list of abbreviations in the text for the different treatments and methods.

under certain conditions, these pericytes can develop into smooth muscle cells (Meyrick and Reid, 1978).

A direct role for 5-HT in the control of brain microcirculation was first suggested by the work of Reinhard and colleagues (Reinhard *et al.*, 1979), who showed that lesion of the brainstem raphe nuclei resulted in a dramatic decrease (~ 70%) in the 5-HT content of rat isolated cortical microvessels while bilateral superior cervical ganglionectomy had no effect. As will be described in detail throughout this review, these original findings provided the impetus to numerous physiological, anatomical, as well as pharmacological and molecular biology studies that all converge to demonstrate that manipulations of brainstem serotonergic neurons can result in precise and selective changes in local blood supply to the brain (Fig. 1).

2. METABOLIC AND VASCULAR CONSEQUENCES OF MANIPULATING BRAIN 5-HT NEURONS

Some general considerations about the serotonergic neurons must first be emphasized. Indeed, this system represents a widespread neurochemical network in the vertebrate CNS with the major targets being the caudate-putamen, susbtantia nigra, cerebral cortex, thalamus, hippocampus, amygdala and hypothalamus (Vertes, 1991). Notably, such an extensive fiber network originates in a very limited number of cell groupings located in the midline raphe nuclei of the brainstem (see Fig. 2). These neurons are among the first to appear during development and the distribution of their cell bodies and projection fibers has remained stable across phylogeny since the serotonergic network, as described originally in the rat, is also present in the monkey and human brain (Jacobowitz and MacLean, 1978). One major implication of this similarity is that the vast majority of the cerebrovascular studies reviewed herein, performed in the rat, can most likely be extrapolated to the human brain.

2.1. Lesion of Central 5-HT Neurons

On the basis of these anatomical considerations, and due to the fact that the serotonergic system is involved in a variety of complex and important physiological activities such as sleep, locomotion, sexual behavior, pain and mood (Jacobs and Azmitia, 1992), it was originally deemed of interest to look at the metabolic and vascular consequences of serotonergic neurons ablation. The experimental paradigms used to destroy brainstem 5-HT neurons included non-specific but localized lesions of the raphe nuclei (electrolytic) or more specific but rather global lesions of the serotonergic system (neurotoxins or amphetamine derivatives). Chronic interruption of serotonergic transmission resulted in no significant effect upon the glucose utilization in the majority of brain regions examined (Table 2), as determined using the autoradiographic 2-deoxyglucose (2-DG) technique (Sokoloff *et al.*, 1977). However, 6–9 weeks after lesion with methylenedioxyamphetamine (MDA), discrete increases in glucose use were noted in few neocortical regions, the globus pallidus and hippocampus (McBean *et al.*, 1990). These findings led to the conclusion that an intact serotonergic transmission was not a major determinant in brain cells integrated functional activity. Like the noradrenergic system, it could very well be that compensatory changes account for this lack of metabolic changes (Savaki *et al.*, 1984). However, the most valuable explanation would be that the serotonergic system exerts a phasic, rather than tonic, influence upon functional neuronal activity.

In line with this conclusion is the overall lack of vascular changes observed following lesion of serotonergic neurons and this, irrespective of the experimental paradigms or methods used to measure CBF (see Table 2). When changes occurred, these were induced, once again, only by amphetamine derivatives and resulted in minor and focal flow increases. A recent study using laser–Doppler flowmetry similarly demonstrated that basal cortical blood flow was not altered by an acute and specific lesion of dorsal raphe serotonergic neurons (Underwood *et al.*, 1992). Altogether, these observations would favor a relatively minor tonic influence of the serotonergic system on resting CBF.

2.2. Stimulation of Central 5-HT Neurons

In contrast to the virtual absence of metabolic and vascular changes following blockade or removal of serotonergic neurotransmission, electrical stimulation of either the dorsal or the median raphe nucleus in conscious as well as anesthetized animals was found to induce significant alterations in local cerebral glucose utilization (CGU) and brain perfusion (Table 3). Interestingly, the pattern of increased cortical glucose use in the conscious rat closely matched the somatotopic delineation of the rat's head and face (Cudennec *et al.*, 1988a), an observation which could be of importance when considering the neuronal events leading to a migraine attack (see Section 5). Although mediated by 5-HT, since they were prevented by prior serotonergic lesion, these increases in glucose use were neither restricted to brain regions known to receive a serotonergic innervation nor to those endowed with a high density of 5-HT receptors. Cudennec and colleagues (Cudennec *et al.*, 1988a) proposed that "the functional consequences of raphe stimulation might correlate better with the existence of a relatively important population of true serotonergic synapses in a given brain region". Nonetheless, these data add strong evidence to the aforementioned hypothesis that the physiological or pathological importance of the central 5-HT neurons may be more readily demonstrated during periods of phasic activation.

The vascular consequences of activating 5-HT neurons in conscious or anesthetized animals have been associated with both increases and decreases in CBF (Table 3). In an attempt to reconcile these apparent contradictory results, three major points should be considered. Firstly, the different methods used to measure blood flow do not provide similar indices of cerebral perfusion. The autoradiographic method whereby [^{14}C]iodoantipyrine (IAP) is used as the radioactive tracer gives a quantitative map of cerebral blood flow values, while the flowmetry techniques only provides qualitative flow values either in a restricted cortical area when using the laser–Doppler flowmetry or in a major arterial trunk when electromagnetic probes are used (for review see Lacombe and Diksic, 1996). Since this latter technique is restricted only to extracerebral blood flow (the probes are commonly placed around the common carotid artery), it is difficult to compare the results obtained with this method by Goadsby and colleagues (Goadsby *et al.*, 1985a, 1985b) with those of other studies measuring intracerebral flow changes (Bonvento *et al.*, 1989b; Underwood *et al.*, 1992; Cudennec *et al.*, 1993). Second, as previously reported (Bonvento *et al.*, 1989a), the state of vigilance of the animal should also be taken into account when dealing with serotonin (see Fig. 3). It is well known that there exists a close relationship between the sleep–wake–arousal cycle and the activity of serotonergic neurons (for review see Jacobs and Azmitia, 1992). In fact, serotonergic cells change their firing rate from 1–3 spikes/sec during quiet waking behavior to a complete cessation of activity during rapid eye movement (REM) sleep (Fig. 4). Furthermore, dorsal raphe neurons respond differently according to their prior level of activity (Fornal *et al.*, 1994). Considering these observations and the suggested phasic influence of serotonergic pathways, it can be inferred that electrical stimulation of dorsal raphe cells most likely modify differently serotonergic neurotransmission in conscious as opposed to anesthetized animals. Also, the indirect vascular consequences of the behavioral changes that occur during stimulation of the raphe nuclei in conscious animals should be considered. This behavior, known as the *serotonin syndrome*, consists mainly of tremors, hindlimb abduction, head weaving and forepaw padding (Jacobs, 1976), and is accompanied by metabolic and vascular activations of various key relay stations of the extrapyramidal system such as the red nucleus, substantia nigra and cerebellar hemispheres (Cudennec *et al.*, 1993). Finally, another component that adds complexity of the vascular changes resulting from activation of serotonergic neurotransmission relates to the observation that the dorsal raphe nucleus is apparently organized in two different cerebrovascular regulatory regions (Underwood *et al.*, 1992). Selective electrical stimulation of the rostroventral or caudal portions of this nucleus elicited decreases or increases in blood flow, respectively (Fig. 5).

Therefore, it appears undeniable that activation of the ascending serotonergic system consistently in-

Table 3. Effects of Raphe Stimulation on Cerebral Metabolism and Blood Flow

Stimulated area	Stimulation paradigm	Method	Effects	Anesthesia (+ or −)	References
			Cerebral metabolism		
Dorsal and median raphe	Electrical	2-DG	⇑	−	Cudennec *et al.*, 1988a
Dorsal raphe	Electrical	2-DG	⇑	+	Bonvento *et al.*, 1991b
			Cerebral blood flow		
Dorsal raphe	Electrical	EMF	⇑	+	Goadsby *et al.*, 1985a
Dorsal raphe	Chemical (DL-h)	EMF	⇑	+	Goadsby *et al.*, 1985b
Dorsal raphe	Electrical	IAP	⇑	+	Bonvento *et al.*, 1989b
Dorsal raphe	Electrical	LDF	⇓ or ⇑	+	Underwood *et al.*, 1992
Dorsal raphe	Chemical (glu)	LDF	⇓	+	Cao *et al.*, 1992
Dorsal raphe	Electrical	IAP	⇑	−	Cudennec *et al.*, 1993
Dorsal raphe	Chemical (glu, KA)	LDF	⇑	+	Underwood *et al.*, 1995

Symbols: ⇑, increase; ⇓, decrease; +, anesthesia; −, conscious.
Refer to the list of abbreviations in the text for the different treatments and methods.

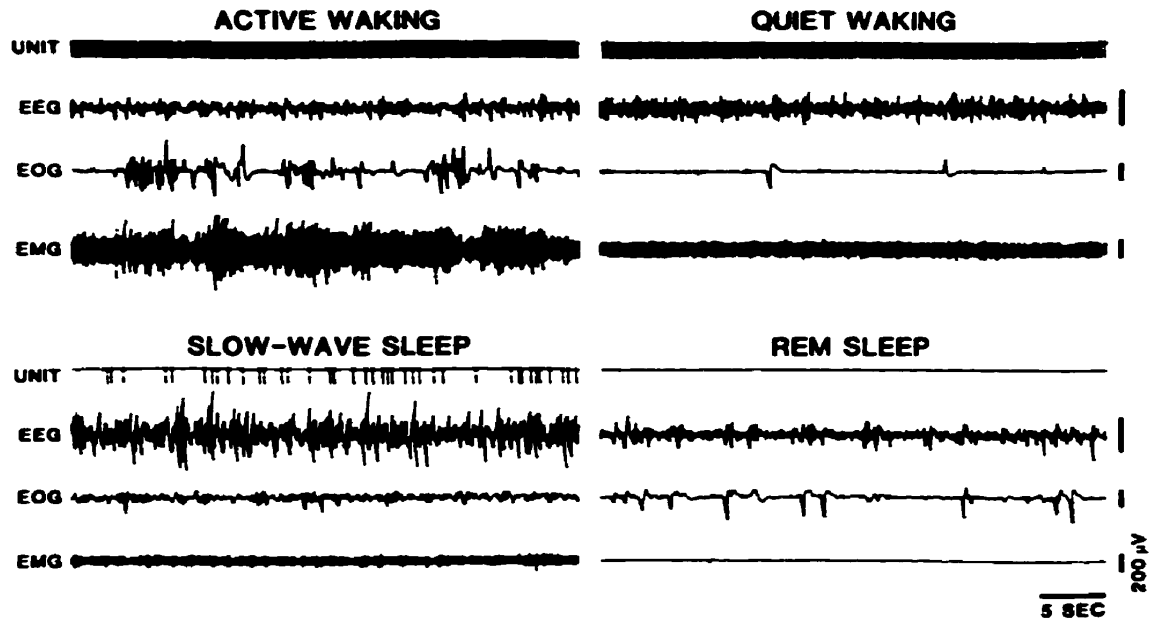

Fig. 4. Polygraph recordings of a typical activity in DRN serotonin neurons (UNIT), during the sleep/wake cycle. The electroencephalogram (EEG), electrooculogram (EOG) and electromyogram (EMG) recordings are also included. Note the positive relationship between firing rate and the level of behavioral arousal, from 4.5 spikes/sec during waking to a complete cessation of activity during rapid eye movement (REM) sleep. Reproduced with permission from Fornal *et al.* (1994).

duces vascular repercussions which can either be widespread in nature (anesthetized animals) or restricted to few regions (conscious animals) but always are confined to well-defined brain areas such as the fronto-parietal and posterior parietal cortices and some relays of the extrapyramidal system such as the red nucleus and cerebellar hemispheres. Reciprocally, blood flow changes were virtually never observed in regions such as the entorhinal cortex, globus pallidus and posterior hippocampus. By analogy to the well documented and potent vasoconstrictor effect of serotonin on the extracerebral circulation (Section 1), it can tentatively be concluded that the main cerebrovascular effect of raphe stimulation is vasoconstriction. That this effect is serotonergically mediated is substantiated by the work of Cao *et al.* (1992) who showed that the decrease in cortical blood flow induced by chemical stimulation of the dorsal raphe nucleus was abolished by administration of either 5-HT_1 or 5-HT_2 receptor antagonist, methysergide or ketanserin (see Fig. 6).

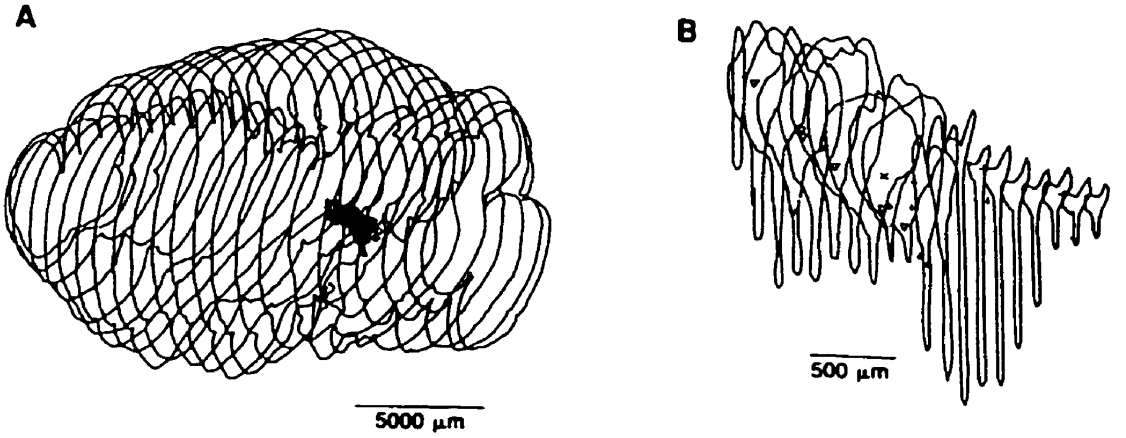

Fig. 5. Three-dimensional reconstruction (A) of the rat brain with the precise location of the DRN and (B) of the full extent of the magnified DRN. Note that stimulation sites resulting in decreased CBF are localized within the rostroventral portions of the DRN (Δ; $n = 9$), whereas those that increased CBF were within the caudal portions of the nucleus ($+$; $n = 5$). Reproduced with permission from Underwood *et al.* (1992).

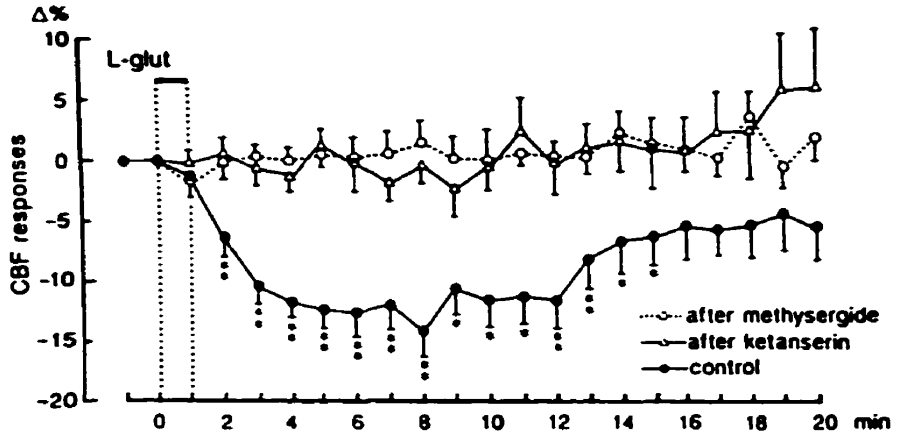

Fig. 6. The CBF changes, measured on the parietal cortex by laser-Doppler flowmetry, evoked by chemical stimulation of the DRN (50 nmol of L-glutamate in 100 nl) in control condition and following intravenous administration of methysergide (1 mg/kg) or ketanserin (0.5 mg/kg). Note that the decrease in CBF is completely abolished by these two 5-HT receptor antagonists. Reproduced with permission from Cao *et al.* (1992).

2.3. Pharmacological Manipulations of 5-HT System

When manipulating pharmacologically serotonergic neurotransmission, one is confronted with the major limitation that almost all serotonergic compounds induce marked behavioral changes when injected in the circulation. This caveat suggests that the reported variations in metabolic activity are likely to reflect an integration of the central and peripheral actions of the selected pharmacological agents. For example, 8-OH-DPAT (5-HT_{1A} receptor agonist which induces an intense *serotonin syndrome*) increased glucose utilization in brain regions related to motor functions (motor cortex, extrapyramidal and cerebellar areas), whereas buspirone (5-HT_{1A} receptor agonist which is not accompanied by any motor behavior) did not modify glucose use in these areas (see Table 4). Moreover, although the metabolic changes were localized to the site where the primary interaction between the drug and its receptors occurred, they could also be observed in regions far away but functionally related to the initial event (Kelly *et al.*, 1988). Within these limitations,

Table 4. Effects of Pharmacological Manipulation of the Serotonergic System on Cerebral Metabolism and Blood Flow

Pharmacological agents	Site of action	Method	Effects	References
		Cerebral metabolism		
Clomipramine	5-HT uptake inhibitor	2-DG	⇩	Freo *et al.*, 1993a
Fluoxetine	5-HT uptake inhibitor	FDG	⇩ or ⇧	Cook *et al.*, 1994
Fenfluramine	5-HT releaser/uptake inhibitor	FDG	⇩ or ⇧	Kapur *et al.*, 1994
LSD, 5-MeODMT	5-HT agonist	2-DG	⇩	Grome and Harper, 1986
Quipazine, 6-CPP	5-HT agonist	2-DG	⇩ or ⇧	Grome and Harper, 1986
8-OH-DPAT	5-HT_{1A} agonist	2-DG	⇩ or ⇧	Kelly *et al.*, 1988
8-OH-DPAT	5-HT_{1A} agonist	2-DG	⇩ or ⇧	McBean *et al.*, 1991
8-OH-DPAT	5-HT_{1A} agonist	2-DG	⇩ or ⇧	Freo *et al.*, 1995
Ipsapirone	5-HT_{1A} agonist	2-DG	⇩	Wree *et al.*, 1987
Gepirone, ipsapirone, buspirone	5-HT_{1A} agonist	2-DG	⇩ or ⇧	Grasby *et al.*, 1992
Buspirone	5-HT_{1A} agonist	2-DG	⇩	Freo *et al.*, 1995
RU-24969	5-HT_{1B} agonist	2-DG	⇩ or ⇧	Kelly *et al.*, 1988
m-CPP	5-$HT_{2B,2C}$ agonist	2-DG	⇩	Freo *et al.*, 1990
DOI	5-HT_{2A}/5-HT_{2C} agonist	2-DG	⇩	Freo *et al.*, 1991
Quipazine	5-HT_3 agonist	2-DG	⇧	Freo *et al.*, 1993b
Methiothepin	5-HT_1 antagonist	2-DG	⇩	Ricchieri *et al.*, 1987
Ondansetron	5-HT_3 antagonist	2-DG	⇩	Mitchell and Pratt, 1991
		Cerebral blood flow		
8-OH-DPAT	5-HT_{1A} agonist	IAP	⇧	McBean *et al.*, 1991
Buspirone	5-HT_{1A} agonist	PET	⇩ or ⇧	Grasby *et al.*, 1992
Sumatriptan	5-HT_{1D} agonist	SPECT	–	Scott *et al.*, 1992
		PE	⇩	Kobari *et al.*, 1993
Ketanserin	5-HT_2 antagonist	IAP	⇧	Dietrich *et al.*, 1989

Symbols: ⇧, increase; ⇩, decrease.
Refer to the list of abbreviations in the text for the different treatments and methods.

however, a decrease in glucose consumption has almost systematically been evidenced following pharmacological activation of central serotonergic pathways (Table 4).

More relevant to our main interest are the vascular consequences, generally associated with cerebral vasoconstriction, of pharmacological manipulations of central 5-HT pathways (Table 4). A relatively recent autoradiographic investigation by the group of Kelly supports the hypothesis of a vasoconstrictor role for 5-HT (McBean *et al.*, 1991). These authors showed that systemic administration of 8-OH-DPAT, a 5-HT_{1A} receptor agonist which decreases 5-HT release by interacting at the somatodendritic receptors (see Section 2.4.2 below), increased CBF in 16 out of 26 brain areas studied. Also in line with this study, as well as that of Cao *et al.* (1992), is the observation that administration of the 5-HT_2 receptor antagonist ketanserin elicits increase in CBF (Dietrich *et al.*, 1989).

2.4. Regulation of Raphe Nucleus 5-HT Neurons

Serotonergic neurons are characterized by their unique pattern of slow (3 spikes/sec) and clock-like intrinsic electrical activity. They have endogenous biological mechanisms for the generation of their electrophysiological signature of slow and highly regular discharge pattern, but this electrical activity is regulated tightly so that no excessive serotonergic transmission can readily occur during physiological conditions. We will highlight briefly some of the major regulatory mechanisms of 5-HT neurotransmission, as these may have significant impact on 5-HT-mediated metabolic and vascular functions within the CNS.

2.4.1. *Behavioral State*

As mentioned above, the firing rate of serotonergic neurons gradually declines as the animal becomes sleepy and completely ceases during REM sleep (see Fig. 4). Using microdialysis (Iwakiri *et al.*, 1993) or voltammetry (Imeri *et al.*, 1994), it was shown that the lowest 5-HT levels are observed during periods of desynchronized sleep. One interesting feature is their anticipated increase in activity just before the end the REM period, suggesting that serotonergic neurons could prepare the waking behavior (for review see Jacobs and Fornal, 1991). It has been suggested, at least in the domestic cat in which most electrophysiological data were performed, that the main physiological stimulus that consistently modifies the electrical activity of serotonergic neurons is the sleep–wake–arousal state.

2.4.2. *Neuronal Feedback Regulation*

The fact that an increase in brain 5-HT levels induces a decreased serotonergic activity (a phenomenon known as neuronal feedback) was described 25 years ago (Aghajanian, 1972). Electrophysiological, as well as biochemical, studies have provided evidence that this inhibition takes place at the level of 5-HT raphe neurons via a direct action at somatodendritic 5-HT_{1A} receptors (for review, see Jacobs and Fornal, 1991). This inhibition is very potent in controlling 5-HT neurotransmission, as the systemic administration of 5-HT_{1A} receptor agonists (e.g. 8-OH-DPAT) totally abolishes the firing rate of serotonergic raphe neurons (Sprouse and Aghajanian, 1987) and their local injection in the dorsal or median raphe nucleus reduces extracellular 5-HT levels in the respective projection areas, namely striatum and hippocampus (Bonvento *et al.*, 1992). Interestingly, the tonic inhibitory influence of the 5-HT_{1A} autoreceptors is observed only in conscious animals (Fornal *et al.*, 1994) and is more effective during periods of increased neuronal activity, i.e. during waking rather than during sleep, when the firing rate is close to zero (Fornal *et al.*, 1994).

2.4.3. *Afferent Control*

In addition to being able to regulate their intrinsic neuronal activity, serotonergic neurons are under the influence of a large number of afferent inputs. These include serotonergic, GABAergic, noradrenergic, histaminergic and glutamatergic projections for various brain areas (for references see Jacobs and Azmitia, 1992), of which only the noradrenergic fibers from the locus coeruleus exert an excitatory influence. Administration of drugs affecting any one of these will either engage or disengage serotonergic activity and could be of valuable interest to act specifically upon serotonergic transmission. However, local administration of noradrenergic agonists into the raphe nuclei probably would not promote serotonergic neurotransmission, as the endogenous noradrenergic input has been shown to operate at a near maximal effect under basal conditions.

In conclusion, most of the available evidence points to a phasic influence of brain serotonergic pathways on neuronal and microvascular functions and suggests that any attempt to activate 5-HT neurotransmission should only be performed in conscious animals and will, most likely, be limited in potency. Altogether, these observations imply that the local metabolic and vascular consequences of changes in 5-HT neurotransmission *in vivo* will depend strongly on intrinsic activity of the 5-HT neurons and will be more manifest during physiological activations or pathological situations than under basal conditions.

3. MORPHOLOGICAL BASIS FOR A FUNCTIONAL 5-HT INNERVATION OF BRAIN MICROVESSELS

3.1. Neurovascular Associations

The direct effects of 5-HT on cerebrovascular functions such as blood flow and BBB permeability (see Sections 2 and 4) imply close and intimate relationships between serotonergic neuronal elements and the cerebral microvascular bed (Figs 7 and 8). In the raphe nucleus, neuronal cell bodies and dendrites, presumably serotonergic in nature, had long been observed in close apposition to local blood vessels (Scheibel *et al.*, 1975). Later, a perivascular serotonergic nerve plexus was evidenced in the raphe

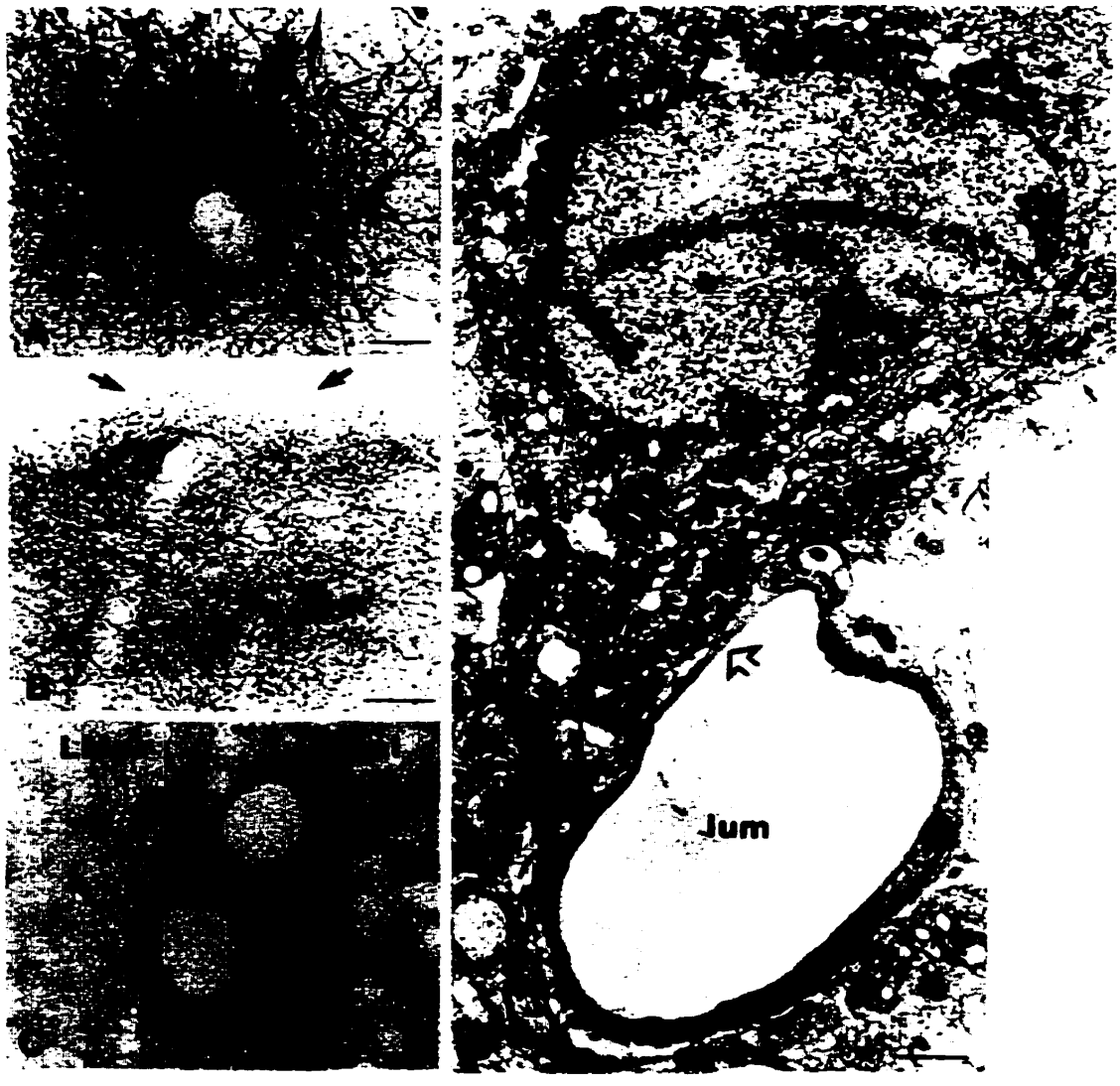

Fig. 7. Photomicrographs showing serotonergic neurons and their projection fibers (immunostained for tryptophan hydroxylase) in the raphe nucleus [(A) and (D)], fronto-parietal cortex (B) and hippocampus (C). Serotonin neurons closely associated with intraparenchymal blood vessels within the raphe nuclei, as observed at the light (A) and electron (D) microscopic levels. In (B), serotonin fibers are evenly distributed throughout the cerebral cortex with an increased density in the superficial layers. Note that some fibers overlay intracortical vessels, while others are floating in the subarachnoid space (arrows). In (C), the majority of fibers are in the stratum lacunosum-moleculare (LMol) where some can be seen to surround local blood vessels (curved arrow). In (D), an immunoreactive cell body, delineated by thin arrows and separated from the blood vessel by a thin astroglial leaflet (open arrow), and dendrites (curved arrows) are depicted. Abbreviations: Mol, stratum moleculare; lum, blood vessel lumen. Scale bars = 50 μm for [(A)–(C)] and 1.5 μm for (D).

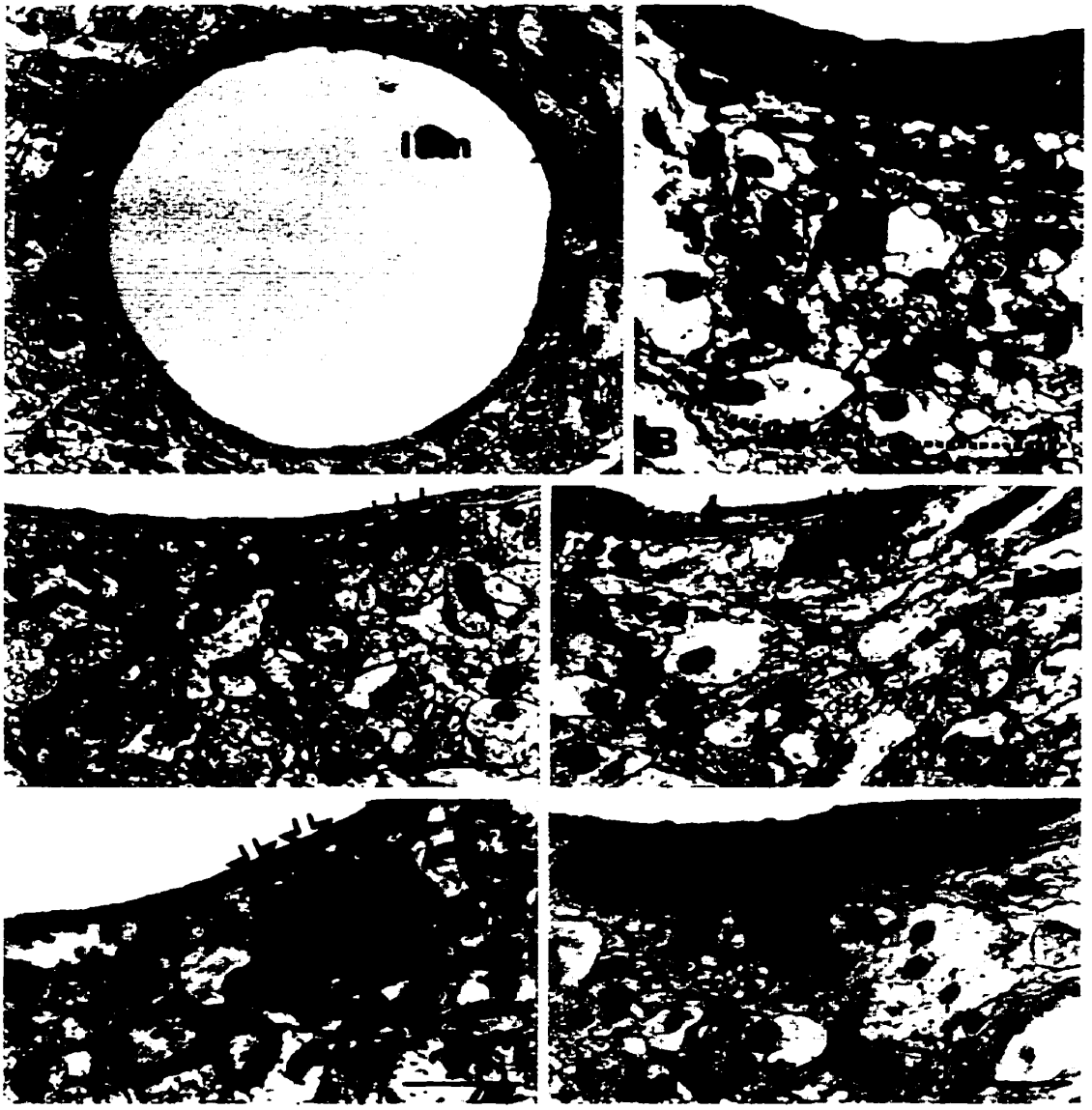

Fig. 8. Electron micrographs of serotonergic (immunostained for tryptophan hydroxylase (TPH)) nerve terminals associated with intraparenchymal blood vessels. A capillary (A) is shown in its entirety with a TPH axon terminal in its immediate vicinity (≤ 0.25 μm) while another terminal (B) is located at about 1.0 μm from the vessel wall. Also shown is the 3 μm perimeter around the blood vessel within which serotonergic nerve terminals are considered perivascular (see Cohen *et al.*, 1995a). Perivascular nerve terminals rarely abutted directly on the vessel basal lamina [small arrows in (C) and (D)] but frequently on the intervening perivascular astrocytic leaflet [open arrows in (E) and (F)]. Both terminals [(E) and (F)] are engaged in a symmetrical contact with a dendrite (curved arrows). Scale bars = 1 μm.

Table 5. Characteristics of Neuronal and Perivascular Serotonergic Nerve Terminals in Different Brain Regions

	Fronto-parietal cortex		Entorhinal cortex		Hippocampus	
	Neuronal	Perivascular	Neuronal	Perivascular	Neuronal	Perivascular
Area (μm^2)	0.37 ± 0.02	0.22 ± 0.01	0.37 ± 0.03	0.20 ± 0.01	0.36 ± 0.02	0.23 ± 0.02
Synaptic frequency (%)	5.9	1.7	5.3	0	4.2	0
Synaptic incidence (%)	22	3.8	1.8	0	12	0
Average distance from vessel (μm)	—	0.98 ± 0.05	—	1.41 ± 0.08	—	1.31 ± 0.08
Perivascular percentage (EM*)	—	10.7	—	10.4	—	4.3

Data taken from Cohen *et al.*, 1995a.
*EM, electron microscopy.

nucleus by Chan-Palay (1976), following intraventricular perfusion of radiolabeled 5-HT. Immunocytochemical investigations also documented contacts and intimate associations between serotonergic neuronal processes and the capillaries or arterioles of the raphe nucleus, both at the light and electron microscopic levels (Di Carlo, 1984; Kapadia and de Lanerolle, 1984). However, only a few studies reported on such neurovascular associations in the terminal field areas of 5-HT raphe neurons (Descarries *et al.*, 1975; Itakura *et al.*, 1985).

Recently, an analysis of the vascular associations of serotonergic neurons has become available in different brain areas (Cohen *et al.*, 1995a) selected on the basis of their blood flow changes following manipulations of central 5-HT neurons (Bonvento *et al.*, 1989b; McBean *et al.*, 1990, 1991; Cudennec *et al.*, 1993). As detailed in Sections 2.1 and 2.2 (Table 2-4), these studies overall showed significant blood flow decreases in the fronto-parietal cortex, while little or no change could be observed in the hippocampus or entorhinal cortex.

Perivascular serotonergic terminals, identified immunocytochemically for tryptophan hydroxylase, were characterized as the terminals that directly touched vessel walls at the light microscopic level and as those located within a 3 μm perimeter from the vessel basal lamina at the ultrastructural level (Fig. 8). This interval was considered as the largest distance within which a nerve terminal can affect blood vessel functions either directly or indirectly via interaction with other elements located closer to the blood vessel (for details, see Lee, 1981; Dodge *et al.*, 1994; Chédotal *et al.*, 1994). Irrespective of their location within the cerebral cortex or hippocampus (Table 5), perivascular serotonergic terminals shared several similarities such as their overall appearance, size and preferential association with capillaries ($\sim 80\%$) as opposed to microarterioles ($\sim 20\%$). Interestingly, their surface area ($0.22\ \mu m^2$) was significantly ($P \leq 0.05$) smaller than that ($0.37\ \mu m^2$) of the neuronal (not located in proximity to blood vessels) serotonergic terminals in the same area, possibly suggesting that the microvascular walls were their final target. The perivascular terminals never exhibited synaptic contacts with vascular or glial elements and only rarely with perivascular dendritic processes of unknown neurochemical content. This paucity of synaptic specialization was similar to that of serotonergic terminals in brain parenchyma and concurs well with the established mode of volume transmission for the indoleamine (Descarries *et al.*, 1991). Such a paracrine mechanism of action would rather depend on the presence of 5-HT receptors in order to exert specific and selective neuronal and/or vascular effects, as in the case of extracerebral blood vessels (Edvinsson *et al.*, 1993).

3.2. Regional Differences

Perivascular 5-HT terminals, however, were found to exhibit very distinct features depending on their localization within the brain (Table 5). For instance, in the fronto-parietal and entorhinal cortices, 10% of all 5-HT nerve terminals in the parenchyma were associated with microvessels, whereas in the hippocampus, only 4% of terminals abutted on vessel walls. Interestingly, a detailed analysis of the vascularity of these three regions showed that the difference in perivascular innervation was not due to proportional differences in the ratio of serotonergic terminals density over total vascular area between the various areas (Cohen *et al.*, 1995a). Thus, it appears that there exists a preferential relationship between 5-HT terminals and the microvascular bed of the cerebral cortex. Further, even within the limit of the cerebral cortex, significant differences were observed which pointed to the microcirculation of the fronto-parietal cortex being most closely associated with 5-HT terminals (Fig. 9). In fact, perivascular serotonergic terminals in this cortical subdivision were located closer to the blood vessels, and particularly so in their immediate vicinity ($\leq 1\ \mu m$) (Figs 8 and 9, Cohen *et al.*, 1995a). Of interest is the fact that, in peripheral and extracerebral blood vessels, a distance of less than 1 μm for perivascular nerve terminals has been correlated with functional innervation (Dodge *et al.*, 1994). Such perivascular proximity (1 μm) also was noted for the innervation of brain cortical microvessels by basal forebrain cholinergic neurons (Chédotal *et al.*, 1994; Vaucher and Hamel, 1995), a well-known functional neurovascular system which exerts a potent regulation of cortical cerebral blood flow (Sato and Sato, 1992). In addition to being more numerous in the microvascular environment, perivascular serotonergic terminals in the fronto-parietal cortex exhibited a clear enrichment towards the blood vessels while, in the other two regions, their distribution around the vessel wall was regular and appeared totally independent of the presence of the microvascular elements (Cohen *et al.*, 1995a; Table 5).

Although anatomical proximity does not allow to confer functional correlates, it suggests nevertheless that any alteration in brain serotonergic neurotransmission would be 'sensed' more closely by the

microvascular bed in the fronto-parietal cortex. Many other factors, such as cerebrovascular 5-HT receptor distribution, density, coupling efficacy, local neurogenic and metabolic factors, including the properties of the perivascular astrocytes, may, however, contribute to these terminals being able or not to induce changes in microvascular functions.

3.3. Astrocytes as Intermediates of Neurovascular Function

Astrocytic processes almost completely surround the basal lamina of intracerebral blood vessels (Peters *et al.*, 1991). It was not surprising, therefore, to find that a relatively high percentage of perivascular serotonergic elements abutted on perivascular astrocytic leaflets (Kapadia and de Lanerolle, 1984; Cohen *et al.*, 1995a; Figs 7 and 8). These neuronal–glial interactions in the proximity of microvessels occupy a privileged position that makes the astrocyte a pivotal cellular element to transfer information between the brain neurons and the microcirculation. In view of their connectivity through gap junctions (Massa and Mugnaini, 1985), the versatility of metabolic and homeostatic functions that they fulfil (see Hertz, 1992; for review, see Tsacopoulos and Magistretti, 1996), as well as the diversity of neurotransmitter receptors that they harbor (Hösli and Hösli, 1993), brain astrocytes appear perfectly suited to regulate neurovascular functions. Only circumstantial evidence exists, however, to suggest that astrocytes may have a direct influence on the microcirculation. For instance, the tight control of K^+ ion concentration by astrocytic end feet, together with the association of this ion in the regulation of local CBF (Paulson and Newman, 1987), would support an involvement of perivascular astrocytes in the control of CBF. Further, the synthesis and release of potent vasoactive substances such as eicosanoids, endothelin and nitric oxide (NO) by astrocytes (see Cohen *et al.*, 1995a for references) make it plausible that these factors are the pathway through which 5-HT acts to regulate CBF (Moncada *et al.*, 1991) or BBB permeability (Janigro *et al.*, 1994). Serotonin released from perivascular nerve terminals could activate specific populations of astrocytic receptors (see Section 3.3) which would initiate the synthesis and/or release of vasoactive substances.

Without excluding the privileged neuronal–glial–vascular interactions in the mediation of the vascular responses to changes in brain serotonergic neurotransmission, it is also quite clear that serotonergic terminals have the potential to influence the microcirculation directly. This is evidenced by a small population of terminals being directly apposed to microarterioles and capillaries (Fig. 8), as well as by the recent identification of specific population of 5-HT receptors in cerebral microvessels and capillaries (see Section 3.4; Fig. 10). Alternatively, it is possible that 5-HT affects vascular functions through the recruitment of other vasoactive neuromodulators. An important percentage of perivascular 5-HT terminals were indeed associated with nerve terminals of unknown content located closer to the blood vessel (Cohen *et al.*, 1995a). The release of 5-HT onto these nerve endings, perhaps possessing specific 5-HT receptors, could result in the subsequent liberation of vasoactive agents which, in turn, may modulate vascular functions.

The diversity of 5-HT receptors, astrocytic vasoactive substances as well as the initial tone of the microvascular bed, which is dependent on serotonergic as well as other vasoactive neurotransmitter activity, possibly could explain the dual effects

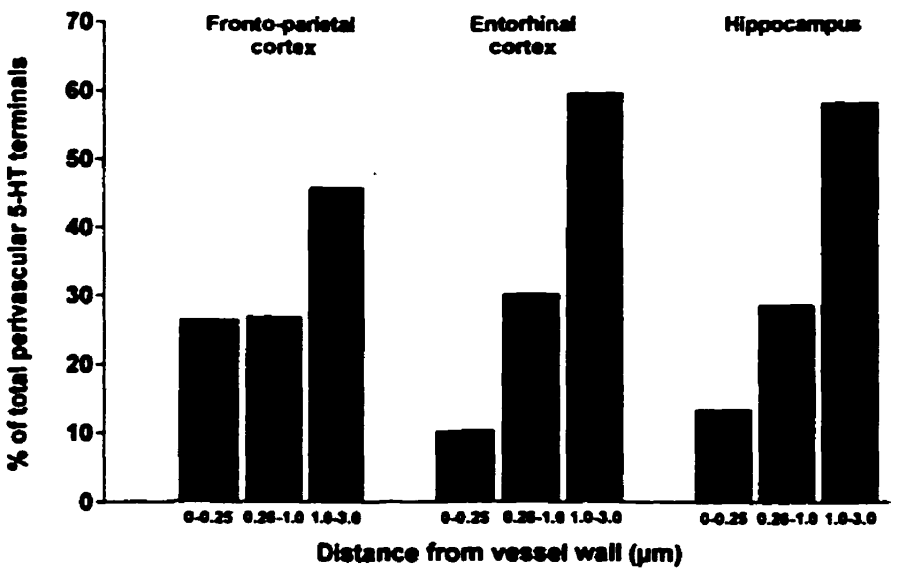

Fig. 9. Histograms depicting the distribution of serotonergic (immunostained for tryptophan hydroxylase) nerve terminals around intracerebral microvessels. Perivascular terminals in the fronto-parietal cortex are more abundant in the immediate vicinity (0–1.0 μm) of blood vessels, while in the entorhinal cortex and hippocampus, they are proportionally more numerous as the distance from the vessel increases ($\geq$ 1.0 μm).

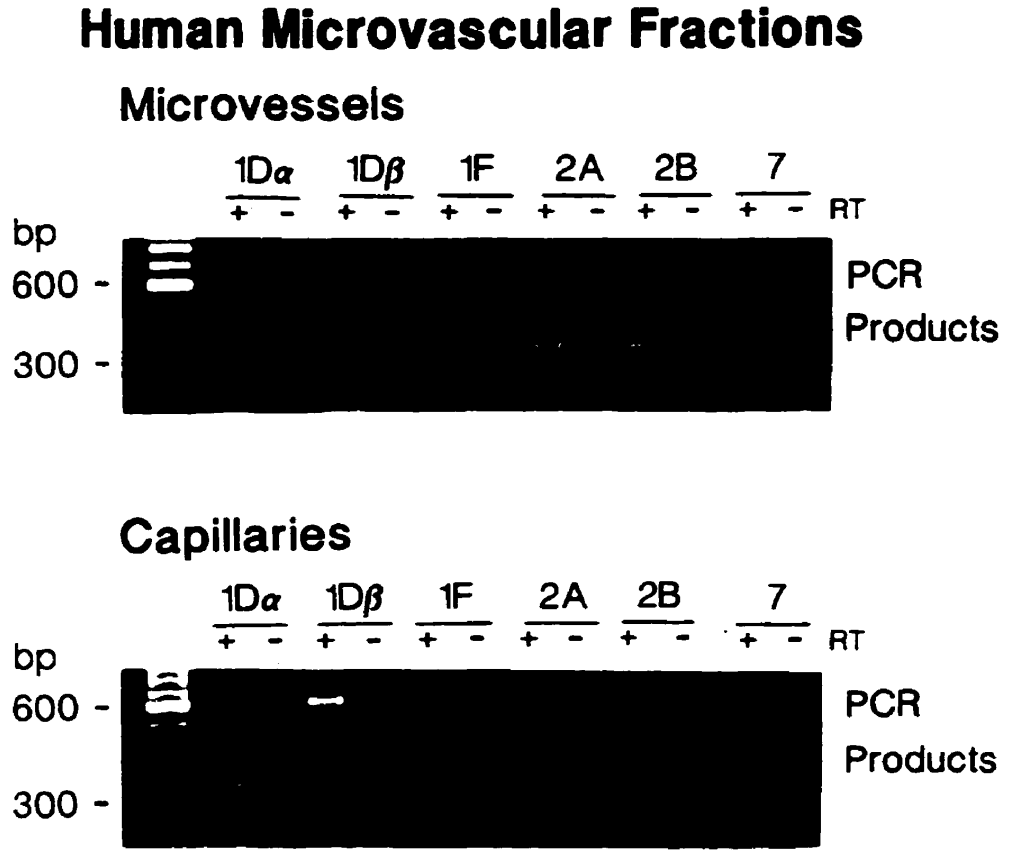

Fig. 10. Representation of 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_7 mRNA expression in human cortical microvessels and capillaries isolated from post-mortem brains. Total RNA was extracted, reverse transcribed and submitted to polymerase chain reaction (PCR) using receptor-specific oligonucleotide primers. The PCR products were separated by gel electrophoresis and visualized under UV light. No products are seen in experiments which contained no reverse transcriptase enzyme (-RT) in the PCR reaction.

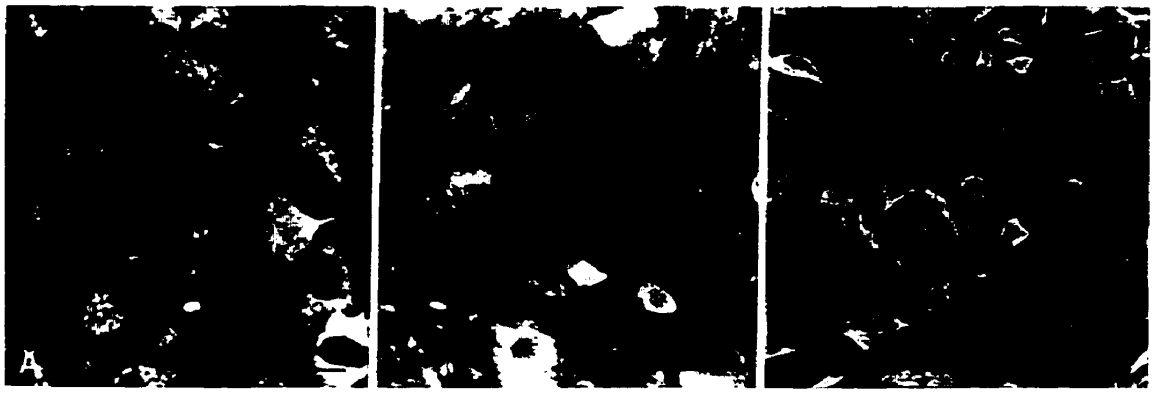

Fig. 11. Photomicrographs of cell cultures of human brain endothelium (A), smooth muscle (B) and astrocytes (C), respectively immunostained for factor VIII, α-actin, and glial fibrillary acidic protein. Capillary (≤ 20 μm) or microvessel (≥ 350 μm) fractions were isolated from temporal cortex biopsies and confluent subcloned endothelial or primary smooth muscle cell cultures were obtained (for further details, see Stanimirović *et al.*, 1996). Note that the primary cultures of smooth muscle cells contain sparse endothelial cells. Astrocytes from human fetal brains were obtained from Dr W. Yong (Yong *et al.*, 1992). Scale bars = 10 μm in (A) and 20 μm in (B) and (C).

(constriction and dilatation) that have been observed following stimulation of the raphe nucleus (for details, see Section 2).

3.4. Microvascular and Glial Sites of Action for 5-HT

3.4.1. *Multiple 5-HT Receptors and their Role in Vasomotor Functions*

The recent and rapid advances in molecular cloning techniques have allowed identification of at least 16 different 5-HT receptors, which can be organized into seven distinct classes designated 5-HT_1–5-HT_7. With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, all the others are G-linked proteins and are composed of seven putative transmembrane domains. The 5-HT_1 receptor subtype family is negatively coupled to adenylate cyclase activity, while that of 5-HT_2 is coupled to the phosphatidylinositol turnover. The newly cloned 5-HT_4, 5-HT_6, and 5-HT_7 receptors all stimulate adenylate cyclase, while no functional coupling has yet been described for the 5-$HT_{5A/5B}$ receptor subtypes (Hoyer *et al.*, 1994).

Before addressing the exact sites of action of 5-HT in the cerebral microcirculation, it appears highly relevant to discuss recent information obtained regarding the 5-HT-mediated vasomotor effects in the extracerebral blood vessels. The 5-HT receptor that mediates vasoconstriction in cerebral blood vessels has been studied for several years (for review, see Parsons, 1991) and to some extent appears to be species specific. In the rat, 5-HT_2 (probably corresponding to 5-HT_{2A}) receptors are involved primarily in cerebral vasoconstriction, while in the majority of species, including man, this effect results from activation of 5-HT_1 receptors (Connor and Beattie, 1996; Hamel, 1996). Recently, pharmacological correlates and molecular biology studies have allowed identification of the 5-$HT_{1D\beta}$ as the receptor mediating the 5-HT-induced vasoconstriction (Hamel *et al.*, 1993; Bouchelet *et al.*, 1996) in human cerebral arteries. This observation has been confirmed and extended (Ullmer *et al.*, 1995) to suggest, in a more general manner, that interaction with a 5-$HT_{1D\beta}$ receptor alone or in combination with a 5-HT_{2A} receptor appears to mediate vasoconstriction even in peripheral blood vessels of several species. However, more work is needed to identify the receptor involved in cerebral vasodilatation mediated by 5-HT (Edvinsson *et al.*, 1977), a response which has gained popularity recently due to its possible implication in initiating migraine attack (see below and Section 5). Similar mechanisms could also take place in the microvascular bed to increase CBF locally (Underwood *et al.*, 1992; Cudennec *et al.*, 1993), but clearly a better understanding of the presence and roles of 5-HT receptors in brain intraparenchymal blood vessels is needed before such conclusion can be made.

3.4.2. *5-HT Receptors in Brain Vascular Cells and Astrocytes*

In the cerebral microvascular bed, O'Neill *et al.* (1988) originally reported the presence of 5-HT_1 receptors in human isolated microvessels but the exact subtype could not be identified. On the other hand, Cao *et al.* (1992) showed that the cortical CBF decreases elicited by raphe stimulation in rats can be abolished by the 5-HT_2 receptor antagonists, methysergide and ketanserin. They suggested that 5-HT_2 receptors, most likely present on microvessels, could mediate the cerebral vasocontractile response. However, despite the fact that low doses of sumatriptan have no effect (Scott *et al.*, 1992), administration of high concentrations of this 5-HT_{1D} receptor agonist induced significant decreases in cortical CBF (Kobari *et al.*, 1993), suggesting that the 5-HT_{1D} receptor, like in extracerebral blood vessels, could trigger microvascular constriction. The 5-HT_2 receptor antagonists such as ketanserin also can reverse the increase in BBB permeability induced by 5-HT (Sharma *et al.*, 1990). Altogether, these responses indicate that brain microarterioles and capillaries can functionally respond to activation of specific receptor populations by intracerebrally released 5-HT.

Recent molecular biology data in human brain isolated microvessels and capillaries (Fig. 10), as well as in cultured human brain endothelial and smooth muscle cells (Figs 10 and 11), have indicated the presence of messages for several 5-HT receptors (Cohen *et al.*, 1995b; Cohen and Hamel, 1996). Their association with specific cellular components of the functional microvascular unit, which is composed of the microarterioles or capillaries and their associated perivascular astrocytes, has been identified (Fig. 11, Table 6). It can be deduced from these data that the 5-$HT_{1D\alpha}$ receptor localized on endothelial cells may be implicated in the changes in BBB permeability induced by 5-HT (see Section 4.2.1), while the 5-$HT_{1D\beta}$ receptor most likely represents the smooth muscle receptor responsible for local constriction of brain microarterioles. The 5-HT_{2B} receptor, in contrast, seems ubiquitously distributed in the vascular and astrocytic elements of the microvascular bed, with a possible predominance in smooth muscle. This receptor, along with the 5-HT_7 receptor, which is also present in all vascular cells, putatively may be involved in the vasodilatation occasionally elicited by 5-HT (Fozard and Kalkman, 1994; Hoyer *et al.*, 1994).

Various groups have shown the presence of 5-HT receptors, primarily of the 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C}, in brain astrocytes (Deecher *et al.*, 1992; Hirst *et al.*, 1994; Poblete and Azmitia, 1995). Recent observations have extended these findings in humans and showed that, within the microvascular functional unit, the 5-HT_{2A} receptor is associated exclusively with the astrocytes, as no expression could be detected in endothelial or smooth muscle cells (Table 6). Thus, any 5-HT_{2A}-mediated microvascular effect in human brain would be automatically secondary to signalling through the perivascular astrocytes. This is in contrast with the ability of the brain microvascular cells to respond directly to 5-HT via activation of receptors such as the 5-HT_{1D}, 5-HT_{2B}, and 5-HT_7 (Table 6). In addition to the 5-HT_{2A}, brain astrocytes harbor messages and proteins for other 5-HT receptors (Table 6, Hösli and Hösli, 1993; Cohen *et al.*, 1995b; Cohen and Hamel,

Table 6. Relative Expression of 5-HT Receptors in Human Vascular Cells and Astrocytes in Culture

Receptors	Endothelial cells	Smooth muscle cells	Astrocytes
5HT1Dα	++*	+	++
5HT1Dβ	−	++++	++
5HT1F	−	−	±
5HT2A	−	−	++++
5HT2B	+++	++++	+++
5HT7	++	++	++

*The + and − signs are used to provide an overall assessment of the presence of message for a given receptor based on the proportion of samples that show mRNA and the relative intensity of the PCR signal as determined by gel electrophoresis. From − to ++++: from no expression to high levels of expression. Data taken from Cohen *et al.*, 1995b) and Cohen and Hamel (1996).

1996) that also could be involved in metabolic and microvascular functions.

Thus, the functional microvascular unit is endowed with specific subtypes of 5-HT receptors, some of which have a selective endothelial, muscular or astrocytic distribution (Fig. 12). It is, therefore, beyond any doubt that 5-HT released from perivascular nerve terminals could result in direct, as well as astrocytic-mediated, vascular effects, either concerned with local CBF or BBB permeability. Indeed, in addition to being expressed, some of these 5-HT receptors have been shown to be functionally coupled to their expected second messenger systems in brain vascular cells (Cohen *et al.*, 1995b), indicating that the receptor proteins are fully operational.

4. FUNCTIONAL SIGNIFICANCE

Three major conclusions can be drawn from the preceding sections: (i) activation of ascending serotonergic pathways induces circulatory changes; (ii) there exist intimate associations between serotonergic nerve terminals and the cortical microvascular bed; and (iii) various 5-HT receptor subtypes are present at specific sites within the microvascular functional unit. Thus, it appears that the serotonergic system fulfils all the criteria to act upon the cerebral circulation via a neurogenic mechanism (see below, Section 4.1). Some functional data do indeed support this hypothesis.

4.1. Cerebral Blood Flow Regulation

4.1.1. *Neurogenic Control of Cerebral Blood Flow*

Coupling between CBF and brain function is a landmark characteristic in the regulation of the cerebral circulation (for review, see Lou *et al.*, 1987; Villringer and Dirnagl, 1995). The prevailing hypothesis to account for this phenomenon is that of metabolic homeostasis formulated by Roy and Sherrington (1890), whereby an increased neuronal activity would lead to accumulation of vasoactive metabolites which, in turn, increase blood flow. More recently, an alternative and attractive concept of *central neurogenic control of brain circulation* was formulated to account for the high spatial and temporal resolution of coupling. It hypothesizes that the brain can directly act upon the cerebral circulation via intrinsic neuronal pathways (for extensive review, see Reis and Iadecola, 1989). The intimate relationship between 5-HT neurons and the cerebral microcirculation, as evidenced at the ultrastructural level and presented above, now constitutes a strong anatomical substrate for such a neurogenic mechanism. On a functional point of view, the only argument that allows to discriminate the two modes of CBF regulation (e.g. metabolic vs neurogenic), relies on the comparison of the relationship that exists between blood flow and metabolism during control and test conditions (Lacombe and Diksic, 1996). The observation of an *uncoupling*, i.e. when the relationship between CBF and metabolism (reflected by CGU) is modified between the two conditions, has been used to suggest a separate control of blood flow (Fig. 13). The most appropriate statistical approach to analyze this relationship was developed originally by McCulloch and colleagues (McCulloch *et al.*, 1982) and relies on a repeated measure analysis of variance. However, it should be stressed that interpretation of these results, in terms of identifying the specific brain structures where *uncoupling* occurs, must be viewed with caution due to the high dimensionality of the data (Ford *et al.*, 1991). As reported in Table 7, study of the flow/metabolism coupling has been performed under two distinct paradigms leading either to increase or decrease in 5-HT release. A remarkable consequence of manipulating serotonergic transmission is a consistent resetting of the flow/metabolism coupling (see Fig. 14). Decreasing 5-HT neurotransmission increased the slope of the linear relationship between flow and metabolism, indicating that flow is in excess of the metabolic demand (McBean *et al.*, 1990, 1991). Conversely, increasing 5-HT release decreased the slope of the linear correlation observed under control conditions, indicating a diminution in flow without any significant corresponding metabolic changes (see Fig. 14). Altogether, these observations strongly support a vasoconstrictor role for endogenously released serotonin.

Thus, the vascular changes observed after either inhibition or activation of the serotonergic system are not exactly superimposable to the underlying changes in metabolism. Such a phenomenon could be well explained by a direct or indirect (via astrocytic or neuronal processes) action of 5-HT on the local microvascular bed in parallel and/or in anticipation to the metabolic response within the neuropil.

4.1.2. *Hypercapnic Challenge*

The classical test of cerebrovascular responsiveness to hypercapnia was used to provide an indication of the role played by central 5-HT neurons in the regulation of brain circulation. To this purpose, the CBF response to an increased CO_2 arterial tension was compared before and after a serotonergic lesion (Table 8). The results have been inconsistent, as no change or an enhancement in the hypercapnia-induced cerebrovascular dilatation was observed following serotonergic lesion. These apparent dis-

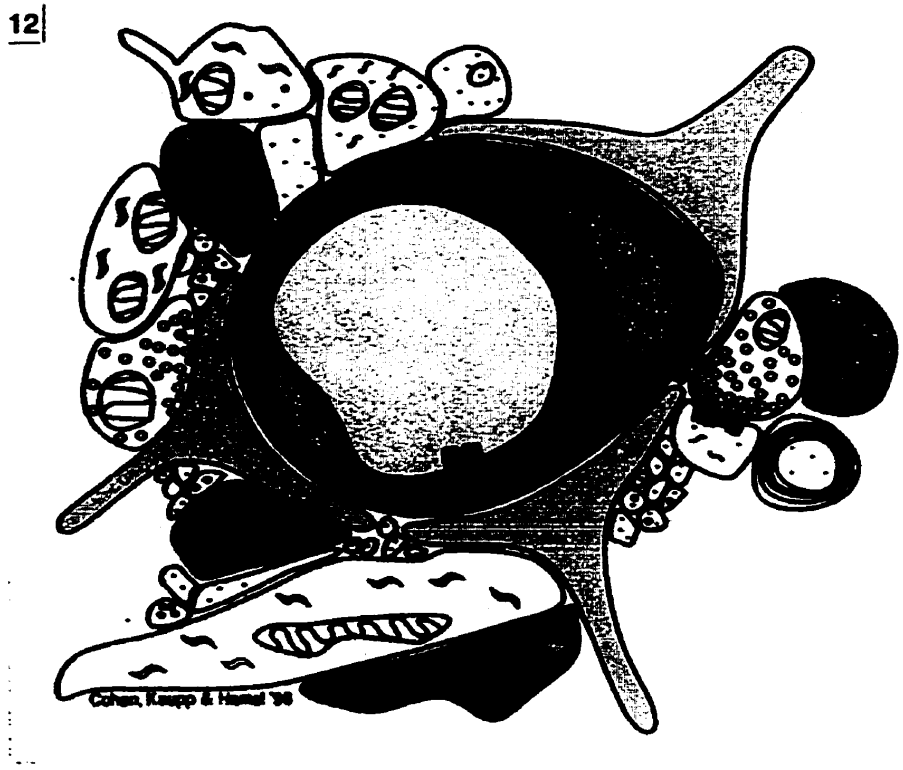

Fig. 12. Diagram illustrating all possible associations between central 5-HT nerve terminals and intraparenchymal microvessels. These vessels are composed of endothelial cells (red) that line the inside of the lumen and smooth muscle cells (brown for arterioles) or pericytes (brown for capillaries) embedded within a basal lamina almost completely surrounded by astrocytic end-feet (yellow). The 5-HT nerve terminals (green) may affect microvascular functions, most notably blood flow regulation, through neurovascular associations that can involve: (i) rarely, direct contact with the endothelium and/or smooth muscle cells; frequently, (ii) interaction with the perivascular astrocytes; and (iii) interaction with a nerve terminals possibly containing other vasoactive substances. The symbols on the astrocytes, endothelial and smooth musscle cells represent 5-HT receptors that have been identified in these different vascular compartements (for details, see Table 6).

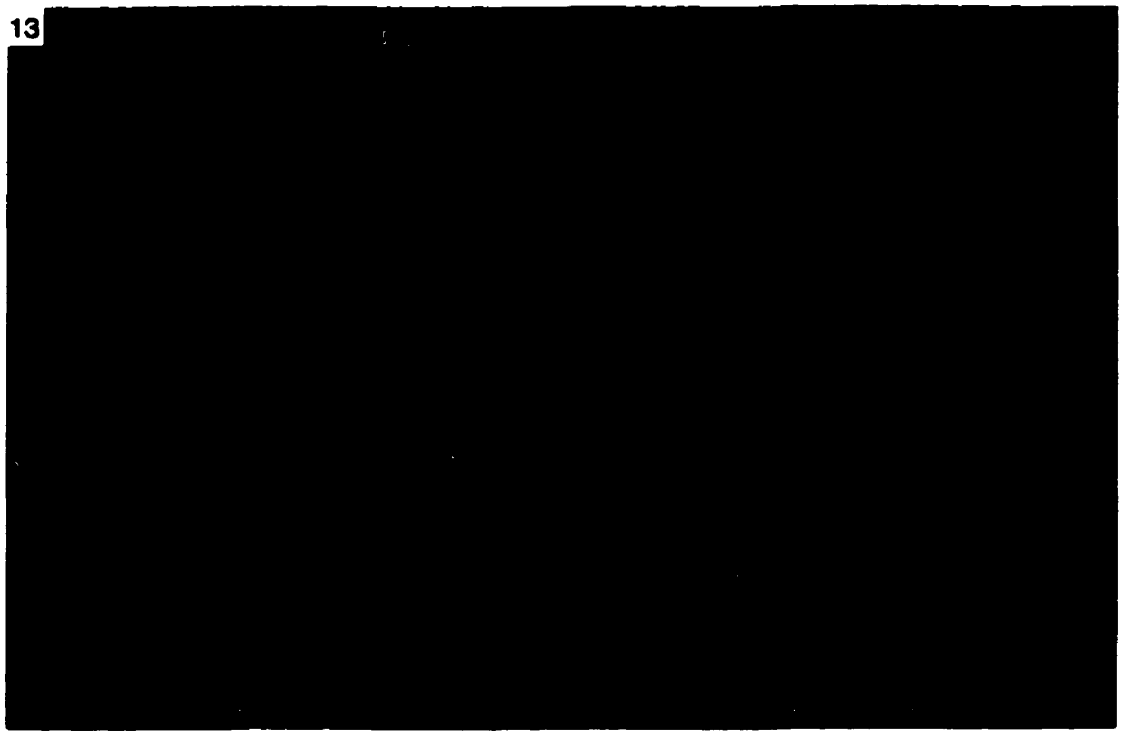

Fig. 13. Coded-color autoradiograms of CBF [(A) and (C)] and CGU [(B) and (D)] in sham [(A) and (B)] and DRN-stimulated anesthetized rats [(C) and (D)]. The color scales for CBF and CGU are represented on the left- and right-hand parts of the figure, respectively. Note the DRN stimulation induced a widespread decrease in CBF with no corresponding metabolic changes, indicating a local uncoupling between the two variables.

Table 7. Effects of Manipulation of the Serotonergic System on the Flow/Metabolism Coupling

Experimental procedure	Treatment	Method	Effects on 5-HT content/release	Effects on CBF/CGU slope (m)	References
			Flow/metabolism coupling		
5-HT lesion	MDA	2-DG/IAP	⇩	⇧	McBean *et al.*, 1990
5-HT_{1A} agonist	8-OH-DPAT	2-DG/IAP	⇩	⇧	McBean *et al.*, 1991
Dorsal Raphe stimulation	Electrical	2-DG/IAP	⇧	⇩	Bonvento *et al.*, 1991b
Dorsal Raphe stimulation	Electrical	2-DG/IAP	⇧	⇩	Cudennec *et al.*, 1993

Symbols: ⇧, increase; ⇩, decrease.
Refer to the list of abbreviations in the text for the different treatments and methods.

crepancies have been explained by the use of anesthetics (Kelly *et al.*, 1995), a delayed impairment in lesioned 5-HT neurons, or compensatory increases in 5-HT levels released from other sources (median raphe) within the brain when the dorsal raphe was acutely lesioned (Underwood *et al.*, 1992). However, when performed in awake animals with proven effective serotonergic lesions, hypercapnia doubled the flow response compared to control condition (Kelly *et al.*, 1995). Therefore, the serotonergic neurons appear to exert a constrictor influence in situations where the cerebrovascular tone is low, as during hypercapnia and in agreement with previous *in vitro* study (Rosenblum and Nelson, 1990). We conclude that the primary cerebrovascular role of the serotonergic system is to modulate or, more generally, to limit the circulatory increases that occur during various situations of activation.

4.2. Other Functions

4.2.1. *Blood–Brain Barrier Permeability*

Besides their postulated role in relaying vasomotor information, astrocytes could possibly serve as intermediates in transmitting other neurally mediated microvascular functions via their processes that surround brain microvessels (see Section 3.3). In fact, the tripartite unit consisting of serotonergic neurons–astrocytes–intraparenchymal vessels is most likely to participate in the formation, maintenance, functioning and integrity of the BBB (for review, see Cancilla *et al.*, 1993). Such an interaction between astrocytes and endothelial cells is well documented and 5-HT could potentially exert some influence at this level. For example, astrocytes induce the specific endothelial enzyme, δ-glutamyltranspeptidase, and increase glucose uptake by the endothelium (Cancilla *et al.*, 1993). On the other hand, serotonin can induce transport across cerebral endothelial cells (Westergaard, 1975; see Sharma *et al.*, 1991 and references therein). The mechanism of action for such 5-HT-induced changes in permeability is unknown but it may rely on the presence of specific 5-HT receptors, as evidenced by a change in BBB permeability following administration of 5-HT_2 receptor antagonists (Sharma *et al.*, 1990). The recent demonstration of several types of 5-HT receptors in human endothelial cells (see Section 3.4.2), namely $5\text{-HT}_{1D\alpha}$, 5-HT_{2B} and 5-HT_7, adds further evidence that 5-HT may be involved in brain capillary functions. In addition, as recently reported in the skin of the mouse, 5-HT may promote increased permeability through the interaction of NO (Fujii *et al.*, 1994).

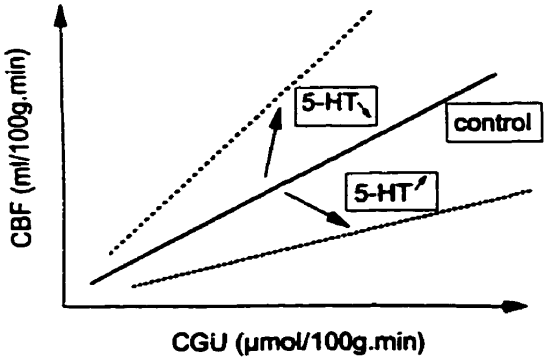

Fig. 14. Schematic representation of the correlation between CBF and CGU in control condition (control) and following experimentally induced increase (↑) or decrease (↓) in 5-HT neurotransmission. Note that an increased 5-HT release is accompanied by a decrease of the CBF/CGU slope, whereas the opposite is observed when the 5-HT transmission is diminished. These changes favor a vasoconstrictor role for endogenously released 5-HT.

4.2.2. *Glycogenolysis*

Along with an interaction between 5-HT and astrocytes in the regulation of the BBB permeability are several lines of evidence that link 5-HT to specific metabolic functions taking place within perivascular astrocytes. In mouse cerebral cortical slices, serotonin promotes glycogenolysis (Quach *et al.*, 1982), a metabolic pathway exclusive to brain astrocytes which are the only cells within the CNS that store and contain measurable amounts of glycogen (for review, see Magistretti *et al.*, 1993). Originally thought to be mediated by 5-HT_{2C} receptors (Zhang *et al.*, 1993), glycogenolysis recently has been attributed to 5-HT_{2A} receptors (Poblete and Azmitia, 1995) present on astrocytes. Interestingly, as described in Section 3.4.2, molecular biology data have shown expression of mRNA for the 5-HT_{2A} receptor in human microvascular fractions to be exclusive to the astrocytes. It appears that enhancing glycogenolysis in the astrocytes would provide energy either for the astrocyte itself or for the activated neurons via the release of lactate and its uptake into the neuronal compartments (Magistretti *et al.*, 1993). Challenging 5-HT neurotransmission thus could have direct repercussions on the availability of energy stores for maintaining adequate neuronal functions.

Table 8. Effects of Raphe or Serotonergic Lesion on Cerebrovascular Hypercapnic Response

Experimental paradigm	Treatment	Method	Effects	References
		Hypercapnic challenge		
5-HT lesion	5,7-DHT	Xe	–	Dahlgren *et al.*, 1981
5-HT lesion	5,7-DHT (cortex)	H_2	⇑	Itakura *et al.*, 1985
Raphe lesion	5,7-DHT (raphe)	LDF	–	Underwood *et al.*, 1992
5-HT lesion	MDA	IAP	⇑	Kelly *et al.*, 1995

Symbols: –, no change; ⇑, increase.
Refer to the list of abbreviations in the text for the different treatments and methods.

Alternatively, astrocytes could intervene in the process of inactivating endogenously released 5-HT by taking up the indoleamine from the extracellular space (Anderson *et al.*, 1992) and metabolizing it to 5-HIAA before it can be removed from the CNS into the blood (Kimelberg *et al.*, 1993). Finally, activation of astrocytic 5-HT receptors can modify K^+ channel activity via an initial increase in intracellular calcium (for review, see Kimelberg, 1995), a mechanism of possible importance in blood flow regulation (see Section 3.3; Kuschinsky, 1996).

4.2.3. *Vascular and Neuronal Growth*

Serotonin also has been considered as a possible growth factor in various cell types. The 5-HT is able to stimulate mitogenesis in aortic vascular smooth muscle cells (Nemecek *et al.*, 1986) and lung fibroblasts (Seuwen *et al.*, 1988). This results in an increase in [^{3}H]-thymidine incorporation, as also observed in smooth muscle from the basilar artery (Kent *et al.*, 1992). Stimulation in DNA synthesis is thought to be mediated through 5-HT_{1B} receptors in rodent fibroblasts (Seuwen *et al.*, 1988) while in blood vessels, other than in man and guinea pig (Cohen *et al.*, 1995b; Ullmer *et al.*, 1995), 5-HT_{2A} receptors may possibly be involved (Kent *et al.*, 1991). In addition, the indoleamine, through activation of 5-HT_{1A} receptor, can release from astrocytes the neuronal growth factor, S-100β (Whitaker-Azmitia *et al.*, 1993), possibly involved in the migration and maturation of growing serotonergic processes and in synaptic turnover in adult brain. Thus, 5-HT neurons could monitor their own development by stimulating the release of beneficial trophic factors and appear able to promote vascular growth as well.

The privileged anatomical and functional relationships between the different compartments of the microvascular tripartite unit, namely 5-HT neurons–astrocytes–intraparenchymal blood vessels, could insure a multitude of vascular related physiological functions within the CNS.

5. PATHOPHYSIOLOGICAL IMPLICATIONS

5.1. Subarachnoid Hemorrhage and Cerebral Vasospasm

Subarachnoid hemorrhage (SAH) occurs when blood leaks between the layers of the pia-arachnoid membrane. Arteries and veins passing through this space are both possible sources of bleeding, but the vast majority of SAHs arise from ruptured aneurysms or arterio-venous malformations. Cerebral vasospasm is one of the most important causes of death and disability in patients surviving the first critical days of SAH. This condition is known to lead to progressive ischemia and neurological deficits. The precise mechanisms involved in cerebral vasospasm after SAH remains obscure, but several vasoactive substances, most likely vasoconstrictive agents, may be implicated in the pathogenesis of this condition. A number of these substances have been detected in the cerebrospinal fluid (CSF) in patients following intracranial aneurysm rupture and include oxyhemoglobin, thrombin, prostaglandins, endothelin, and 5-HT (Chehrazi *et al.*, 1989; Seifert *et al.*, 1995).

Since the initial demonstration of 5-HT as a potent vasoconstrictor of pial vessels, several studies have proposed that the amine is a causative factor of SAH. No study has demonstrated unequivocally that 5-HT is directly involved in the etiology of SAH and the accompanying vasospasm, but there is circumstantial evidence to support such a role. In the ependyma of the cerebral ventricles, immunoreactivity of 5-HT fibers is markedly reduced after SAH (Hara and Kobayashi, 1993), suggesting that the depletion of the indoleamine is somehow implicated in the generation of cerebral vasospasm as a result of SAH. The concentrations of 5-HT found in the CSF of SAH patients are very high and able to induce potent contractions of isolated basilar arteries, a response which can be blocked by methysergide (Allen *et al.*, 1974). Similarly, peri-aneurysmal CSF from SAH patients induces constriction of isolated intracranial arteries, an effect which is blocked by ketanserin (Tagari *et al.*, 1983). After experimental SAH, the uptake of 5-HT into perivascular fibers is altered and results in a major increase in serotonin content in major cerebral vessels, suggesting that the deleterious vasomotor effects of SAH and its consequences could be attributed to 5-HT itself (Jackowski *et al.*, 1989b). However, it is possible also that high concentrations of 5-HT potentiate the release of other potent vasoconstrictive substances such as noradrenaline, neuropeptide Y and endothelin, which are commonly detected in cerebral vasospasm. The new development of selective and potent compounds that would induce 5-HT-related vasodilatation, such as 5-HT_{2B} receptor agonists and perhaps 5-$HT_{1D\beta}$ receptor antagonists (see Section 3.4), may help in establishing the exact role of 5-HT in the progression of SAH into cerebral vasospasm.

5.2. Cerebral Ischemia

The precise role of 5-HT in the etiology of cerebral ischemia is not fully understood, but several lines of evidence have been advanced that link the indoleamine to this dysfunction. Depletion of 5-HT from cerebral tissues results in a decreased incidence of ischemia, suggesting that a lower availability of 5-HT for release onto collateral vessels could protect from the cascade of events leading to infarction (Welch *et al.*, 1977). Numerous reports have confirmed the protective effects of serotonergic related compounds in the pathophysiology of ischemic brain damage (Bielenberg and Burkhardt, 1990; Nakata *et al.*, 1992; Prehn *et al.*, 1993). These compounds share their ability to reduce brain serotonergic neurotransmission and include 5-HT_{1A} receptor agonists (8-OH-DPAT and ipsapirone), 5-HT_2 receptor antagonists [ketanserin, ritanserin, and (S)-emopramil] and 5-HT reuptake inhibitors (citalopram and clomipramine).

In many animal models, elevated extracellular levels of 5-HT and 5-HIAA are detected in regions that are vulnerable to transient global ischemia (Globus *et al.*, 1992), a finding consistent with an earlier study that showed increased 5-HT concentrations in the CSF of stroke patients (Meyer *et al.*, 1973). It has been suggested that such an increase in extracellular 5-HT levels causes the detrimental effects observed in the neuronal parenchyma. The neuronal damage induced by ischemia is thought to result from an imbalance between excitatory and inhibitory mechanisms and serotonin reportedly potentiates neuronal excitation induced by amino acids (Globus *et al.*, 1991). It is thus conceivable that the accumulated serotonin creates an overexcitation leading to the damage. The demonstration that 5-HT_{1A} receptor agonists (known to reduce 5-HT release by inhibiting cell firing) and 5-HT_2 receptor antagonists markedly reduce neuronal damage (Zivin and Venditto, 1984; Prehn *et al.*, 1993) offers compelling evidence that 5-HT is involved in the progression of the ischemic insult.

Another property of 5-HT that could contribute to the pathology of cerebral ischemia is its ability to induce potent vasoconstrictions in cerebral arteries, pial vessels and, possibly, cortical microarterioles. Following an ischemic insult, there is an increase in vascular leakage through the endothelial cells, which would allow binding of serotonin to smooth muscle receptors, thus decreasing blood flow in the affected region and aggravating the neuronal damage. The observation that 5-HT_2 receptor antagonists such as ketanserin and ritanserin, which increase CBF in cortical areas remote from thrombotic infarction (Dietrich *et al.*, 1989), can reduce ischemic damage in the rat is consistent with 5-HT_2 receptors mediating vasoconstriction in this species. Interestingly, in the rat, ketanserin prevented the CBF decreases elicited by raphe nucleus stimulation (Cao *et al.*, 1992). In view of the species-related differences in 5-HT receptors mediating cerebral vasoconstriction, a similar scenario in man would be likely to be mediated by the 5-$HT_{1D\beta}$ receptor subtype (see Section 3.4).

5.3. Migraine

The initial work by Graham and Wolff (1938) suggested that the pain associated with migraine headache results from dilatation of cranial blood vessels. However, recent work has suggested that vessel dilatation may not be the exclusive source of pain and it has been proposed that a neurogenic inflammatory response in dural and meningeal vessels contribute significantly, if not exclusively, to headache pain (Moskowitz, 1992). This neurogenic inflammation transmitted through the trigeminovascular fibers is characterized by plasma protein extravasation and mast cell degranulation, and would result from the release of the vasoactive neuropeptides calcitonin gene related peptide (CGRP) and substance P onto the vessel wall (Moskowitz, 1993).

Serotonin has been implicated in the pathogenesis of migraine since Sicuteri and his colleagues (Sicuteri, 1959; Sicuteri *et al.*, 1961) reported increased levels of 5-HIAA, the major metabolite of 5-HT, in urine of migraine patients and showed that administration of the 5-HT receptor antagonist, methysergide, prevented migraine headaches. Almost 40 years later, and despite the overwhelming evidence implicating 5-HT in the etiology of migraine, its precise role still remains elusive. It is undeniable, however, that the pathogenesis of this condition is caused by a perturbed 5-HT neurotransmission and that stabilization of this system alleviates the manifestation and/or the symptoms of migraine attacks (Raskin, 1993).

In migraine prophylaxis, several of the most effective drugs appear to relieve migraine through their ability to suppress serotonergic activity in the dorsal raphe neurons, while others will target specific 5-HT receptors. For years, some 5-HT_2 antagonists were proven effective in preventing migraine headache while others such as ketanserin and mianserin were not (Mylecharane, 1991). Only recently has some indication emerged regarding their possible mechanism of action. Based on the cloning of the 5-HT_{2B} receptor (Schmuck *et al.*, 1994; Kursar *et al.*, 1994), the known ability of the 5-$HT_{2B/2C}$ receptor agonist, *m*-chlorophenylpiperazine (*m*-CPP), to trigger migraine headache in susceptible patients (Brewerton *et al.*, 1988) and the efficacy of only certain 5-HT_2 receptor antagonists in migraine prophylaxis, Fozard and Kalkman (1994) have recently proposed the involvement of 5-HT_{2B} receptors in the triggering events that lead to a migraine attack. They suggest that activation of endothelial 5-HT_{2B} receptors in cranial blood vessels would induce the synthesis and release of NO, which would then diffuse to the adjacent smooth muscle to induce a potent vasodilatation. Concurrently, NO would stimulate the perivascular trigeminovascular sensory afferents which transmit pain. To support the above hypothesis, mRNA transcripts for the 5-HT_{2B} receptor recently have been detected in human brain endothelial cells (Table 6; Cohen and Hamel, 1996). However, their expression was not restricted to the endothelial cells and other cellular contributors may be involved in the vasomotor and inflammatory responses.

In the acute phase of migraine, i.e. when the

headache has developed, drugs such as dihydroergotamine and sumatriptan have been shown to be highly effective in aborting migraine headache. Whether their anti-migraine activity is due to their ability to induce cerebral vasoconstriction (Humphrey *et al.*, 1988) and/or to inhibit trigeminovascular inflammation (Moskowitz, 1992) is not known (see Hamel, 1996, for review). Recent pharmacological and molecular biological studies suggest that these effects are mediated by a 5-HT_{1D} receptor (for review, see Connor and Beattie, 1996). More specifically, the vasoconstriction would be mediated by the 5-$HT_{1D\beta}$ subtype (Hamel *et al.*, 1993; Bouchelet *et al.*, 1996; Ullmer *et al.*, 1995), while the participation of 5-$HT_{1D\alpha}$ and/or 5-$HT_{1D\beta}$ receptor subtypes in the trigeminovascular response has not been unequivocally identified (Rebeck *et al.*, 1994; Bouchelet *et al.*, 1996; Hamel, 1996; Connor and Beattie, 1996). The development of new drugs that would be selective to the 5-$HT_{1D\alpha}$ subtype would be greatly needed to unmask which, if any, of the vascular or neuronal mechanism alleviates migraine. The presence of sumatriptan-sensitive 5-HT_{1D} receptors in the human brain microcirculation, however, deserved further investigation (Table 6). Similarly, the possibility that 5-HT receptors other than the 5-HT_{1D} may be involved in the efficacy of sumatriptan to relieve migraine has not been excluded (Yu *et al.*, 1995; Bouchelet *et al.*, 1996; Hamel, 1996).

6. CONCLUSION AND PERSPECTIVE

In the present review, we have discussed the functional and anatomical evidence that support a role for brainstem raphe nuclei 5-HT neurons in the regulation of the cerebral microcirculation. We presented arguments in favor of an *uncoupling* between brain metabolic activity and perfusion, the vascular changes being consecutive to a direct neurogenic control of the microvascular bed by intracerebrally released 5-HT. The underlying morphological neurovascular associations were identified and shown to vary in frequency and/or tightness depending on the brain region and whether or not their local perfusion is influenced by brain intrinsic 5-HT pathways. Detailed ultrastructural analysis of the neurovascular interactions re-emphasized the strategic position of the perivascular astrocytes in the interactions between brain neurons and microvessels (Andriezen, 1893). Using quantitative cerebral maps of metabolic and circulatory activities, together with morphological and molecular correlates, we refined and extended this original concept to suggest that the functional tripartite unit *neuron–astrocyte–microvessel* may indeed represent the elementary unit whereby the local regulation of cerebral blood flow is exerted. This implies that neuronal release of 5-HT can affect the local circulation directly, but most frequently through interactions with the perivascular astrocytes or other vasoactive perivascular neuronal systems. We have shown that the vascular and astroglial compartments of the tripartite functional unit harbor selective and specific populations of 5-HT receptors able to translate the neuronal signal into a precise vascular response related either to vasomotricity, permeability and possibly cellular growth.

Interestingly, the functional microvascular unit as defined here for the serotonergic system, also applies to other brain intrinsic neurotransmitters known to exert a direct control on the local microvascular bed, such as the well-defined basalocortical cholinergic neurons (Sato and Sato, 1992; Chédotal *et al.*, 1994; Vaucher and Hamel, 1995). Thus, it is tempting to suggest that these neuronal–glial–vascular interactions constitute a general basis for the neurogenic control of the cerebral microcirculation. A better understanding of the mechanisms involved in the transmission of the capillary and microarteriole signals to local resistance vessels is still needed in order to explain the considerable changes observed in local brain perfusion following manipulation of brain intrinsic neurons.

We conclude that any significant change in the status of brain 5-HT neurons is likely to have vascular repercussions in specific brain regions. These neurovascular interactions may form the basis of normal blood flow regulation and, when dysfunctional, may lead to inadequate blood supply to the brain.

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In Vivo-Synthesized Radioactively Labelled α-Methyl Serotonin as a Selective Tracer for Visualization of Brain Serotonin Neurons

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ABSTRACT To investigate the use of α-[^{3}H]methyl tryptophan (α-[^{3}H]MTrp) as a tracer for the in vivo study of brain serotonergic neurons, we examined whether α-[^{3}H]MTrp and its metabolite α-[^{3}H]methyl serotonin (α-[^{3}H]M5-HT) selectively label serotonergic neurons and whether once accumulated in these neurons, the radioactive metabolite behaves like endogenous serotonin. Rats received a systemic injection of 1–5 mCi of α-[^{3}H]MTrp and 24 h later their brains were immediately removed or fixed by perfusion before removal. Tissue sections in which serotonergic neurons had been immunostained for 5-HT or its synthesizing enzyme, tryptophan hydroxylase, were processed for radioautography at the light and electron microscopic level. In another group of rats, the release of radioactivity from different brain areas was studied both under basal and depolarizing conditions. In the dorsal raphe nucleus, the light microscopic examination revealed almost complete colocalization between serotonergic neurons and those that accumulated radioactivity, with a heterogeneity in the content of α-[^{3}H]M5-HT among the various cells. At the ultrastructural level, immunoidentified serotonergic perikarya and dendritic processes in the dorsal raphe nucleus, as well as nerve terminals in the cerebral cortex were also found to contain α-[^{3}H]M5-HT. Under basal conditions, radioactivity was released from the brainstem raphe region and from projection areas such as the striatum and hippocampus. The basal output of α-[^{3}H]M5-HT increased approximately twofold after a depolarizing 50 mM KCl solution was added to the perfusion fluid. These findings suggest that newly synthesized α-[^{3}H]M5-HT can be released both at somatodendritic and terminal sites. In sum, the present results demonstrate the selectivity of α-[^{3}H]MTrp as a tracer for serotonergic cells, and further suggest that α-[^{3}H]MTrp radiolabelling provides for a direct assessment of the in vivo dynamics of brain serotonergic neurons at the cellular level.

INTRODUCTION

Different approaches have been used to investigate the anatomical distribution of 5-hydroxytryptamine (5-HT, serotonin)-containing neurons in various species including man. These include histofluorescence (Anden et al., 1966; Dahlström and Fuxe, 1964; Léger et al., 1979), radioautographic detection of uptaken radiolabelled 5-HT (Beaudet and Descarries, 1979; Chan-Palay, 1982; Parent et al., 1981), 5-HT immunocytochemistry (Steinbusch, 1981; Takeuchi et al., 1982) and immunocytochemical localization of the 5-HT synthesizing enzyme, tryptophan hydroxylase (TPH; Weissmann et al., 1987). None of these techniques, however, provides information on the in vivo dynamics of 5-HT metabolism within serotonergic cells. Recently, we described in the rat (Diksic, 1991; Nagahiro et al., 1990a) a radioautographic method suitable for measuring the rate of serotonin synthesis in vivo. It provides information on TPH activity in contrast to other approaches allowing biochemical and/or anatomical analyses of TPH content and turnover rate (Poncet et al., 1993; Richard et al., 1990; Weissmann et al., 1990; see also Boadle-Biber, 1993). This new method uses a radioactively labelled tryptophan analog, α-methyl-L-tryptophan (α-MTrp), which once injected systemically, is readily taken up into the brain where it is converted

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into labelled α-methyl serotonin (α-M5-HT). Preliminary data suggest that this conversion occurs primarily at the level of 5-HT nerve cell bodies and is followed by anterograde transport of α-M5-HT to terminal areas as observed for endogenous 5-HT (Diksic et al., 1990; Nagahiro et al., 1990b). If this proves to be the case, α-MTrp would qualify as a direct and easy tool for obtaining information on the intrinsic activity of brain 5-HT neurons (Tsuiki et al., 1994). Furthermore, the chemical nature of α-MTrp, as well as its biological and radiochemical properties, would make it a tracer of choice for mapping serotonergic pathways in vivo within the human brain by positron emission tomography (Diksic et al., 1991; Diksic, 1992).

The applicability of radiolabelled α-MTrp hinges on the demonstration that the tracer and its metabolite, α-[^{3}H]M5-HT, are selectively taken up and retained within brain serotonergic neurons. To this end, we have combined high resolution radioautographic detection of α-[^{3}H]M5-HT to the immunocytochemical visualization of serotonergic neurons at the light and electron microscopic level. In order to functionally assess the use of this tracer, we have also examined the in vitro-evoked release of radioactivity from different brain areas from rats injected with α-[^{3}H]MTrp. These experiments show colocalization of labelled α-M5-HT with endogenous 5-HT and its synthesizing enzyme, TPH, at the cellular level, as well as the ability of freshly synthesized radiolabelled α-M5-HT to be released from brain tissue upon stimulation. Furthermore, the results indicate that serotonergic neurons within the raphe nucleus exhibit differential rate of 5-HT (or α-[^{3}H]M5-HT) synthesis.

MATERIALS AND METHODS

Injection of α-[^{3}H]MTrp

Information relative to the preparation of the animals prior to α-[^{3}H]MTrp injection has been described at length elsewhere (Diksic et al., 1990; Nagahiro et al., 1990a). In short, adult Wistar rats (~200 g) were fasted overnight and the following day, the femoral vein and artery were catheterized under 1.5% halothane anesthesia. Arterial pH, $PaCO_2$, PaO_2, blood pressure, and hematocrit were measured prior to tracer injection. Rectal temperature was maintained at about 37°C. Approximately 2 h after they had recovered from the anesthesia, the rats were injected with 1 ml saline containing α-[^{3}H]MTrp ([^{3}H] label was in the CH_2 group next to the indol; S.A. was about 10 Ci/mmol; prepared by Amersham Co., Arlington Heights, IL) using a metering pump for delivery over 5 min at a constant rate. Following the injection, the rats were again lightly anesthetized with 1.5% halothane, the physiological parameters recorded before removal of the catheters and then returned to their cages.

Simultaneous detection of α-[^{3}H]M5-HT and endogenous 5-HT or TPH

Two different antisera were used to immunocytochemically visualize serotonergic nerve cell bodies, dendrites and/or terminals. The first was a polyclonal antibody, raised in a rabbit against a serotonin-glutaraldehyde-protein conjugate (Immunotech, Marseille, France) and was used only for light microscopic studies. The second antibody, also polyclonal, was raised in the sheep against isolated rat brain TPH (Institut Jacques Boy S.A., Reims, France) and was used both at the light and electron microscopic levels.

Tissue preparation

For immunocytochemical detection of endogenous 5-HT, three rats were injected with 1–2 mCi of α-[^{3}H]MTrp (tracer amount). Twenty-four hours later, they were anesthetized with sodium pentobarbital (65 mg/kg body weight, i.p.) and injected with pargyline (75 mg/ml, 0.1 ml/100 g body weight, i.p.) one hour prior to perfusion through the ascending aorta with 700 ml of 3.5% glutaraldehyde in 0.05 M Sorensen buffer. The brains were removed and postfixed (2 h) in the same solution, and coronal sections (30 μm) were made on an Oxford vibratome at the level of the DRN (dorsal raphe; interaural 1.00–1.70 mm; Paxinos and Watson, 1986). For immunocytochemistry of TPH, 24 hours after being injected with 1.5 mCi (light microscopy, LM) or 5 mCi (electron microscopy, EM) of radiolabelled tracer, seven Wistar rats were deeply anesthetized as above and perfused with 100 ml of cold 0.1 M sodium phosphate buffer (NaPB, pH 7.4) containing 20 mM $MgCl_2$, followed by 750 ml of 4% paraformaldehyde (PF) in NaPB with 15% picric acid in the case of LM studies. For EM, the perfusion consisted of a 500 ml mixture of 4% PF and 0.025% glutaraldehyde followed by 750 ml of 4% PF alone. All brains were removed and immersion-fixed (2 h) in the 4% PF solution. For light microscopy, brains were cryoprotected (30% sucrose), frozen and sliced (40 μm) at the level of the cerebral cortex and DRN on a freezing sliding microtome. Following the postfixation period, brains used for electron microscopy were immediately cut (60 μm) on a vibratome at the level of the DRN and the frontoparietal regions of the cerebral cortex.

Immunocytochemistry of 5-HT and TPH

5-HT. Tissue sections were washed in Tris saline (0.1 M, pH 7.6) containing 0.1 M lysine, then incubated in 3.3% normal goat serum (NGS) and rinsed in 0.1 M Tris saline before overnight incubation with the 5-HT antibody (diluted 1/500 in 1% NGS) at 4°C. The following day, sections were rinsed in 1% NGS and subsequently incubated in a goat anti-rabbit IgG (45 min). After a rinse with 1% NGS, the tissue was treated with rabbit peroxidase anti-peroxidase (PAP, 45 min), rinsed

with 0.1 M Tris saline, and the peroxidase activity revealed (~6 min) with a 0.1 M Tris-HCl (pH 7.6) solution of 3,3-(±)-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) containing 0.01% H_2O_2. The sections were mounted on gelatinized slides and dehydrated.

TPH. For both light and electron microscopy, free-floating sections were collected in 0.1 M phosphate-buffered saline (PBS, pH 7.4), and rinsed in PBS supplemented with 0.2% gelatin (porcine skin, Sigma) and 0.001% sodium azide. They were then incubated overnight with the TPH antiserum (1/1,000 in the PBS/gelatin/sodium azide) at room temperature under mild agitation. For light microscopy, all solutions also contained 0.1% Triton X-100. The next day, the sections were thoroughly rinsed in PBS/gelatin and then in PBS alone before incubation (1 h) with a biotinylated rabbit anti-sheep immunoglobulin (1/100, Vector Laboratories, Burlingame, CA). After being washed, they were incubated (1.5 h) with the avidin-biotin peroxidase complex (ABC, 1/100, Vectastain Kit, Vector Laboratories), washed extensively, and the ABC complex revealed (~6 min) with the DAB/H_2O_2 solution prepared as described above. The sections were briefly rinsed (0.1 M Tris-HCl) and those processed for light microscopy were mounted on gelatin-coated slides, dehydrated and air-dried overnight.

Tissue sections used for ultrastructural analysis were postfixed (1.5 h) with 2% osmium tetroxide and then extensively rinsed in 0.1 M NaPB. They were dehydrated in alcohols and acetone before being flat-embedded between two plastic coverslips in pure Araldite 502 resin for 48 h at 60°C. Small blocks comprising the DRN and upper layers of the frontoparietal subdivision of the cerebral cortex were cut and reembedded in Araldite-filled BEEM capsules (again 48 h at 60°C). Ultrathin sections (90–120 nm, straw color) were made on a Reichert-Jung ultramicrotome, deposited on slides coated with a celloidin film (2% parloidin strips in amyl acetate), stained with 10% uranyl acetate and Reynold's lead citrate, and then lightly vaporized with carbon.

Radioautography of α-[^{3}H]M5-HT

Light microscopy. Following dehydration, immunocytochemically stained sections were dipped in either NTB-2 or K-5 nuclear emulsion (diluted 1:1) and stored in light-proof boxes for 3–6 weeks. The radioautographs dipped in NTB-2 were developed in Dektol (17°C for 1.5 min) and fixed in Ektaflo (4°C for 10 min) while D-19 (16°C for 4 min) and sodium thiosulfate (4°C for 10 min) were used for the K-5 emulsion. All slides were coverslipped, and the silver grains and immunocytochemical reactions observed under a Leitz Aristoplan light microscope and the appropriate regions eventually photographed.

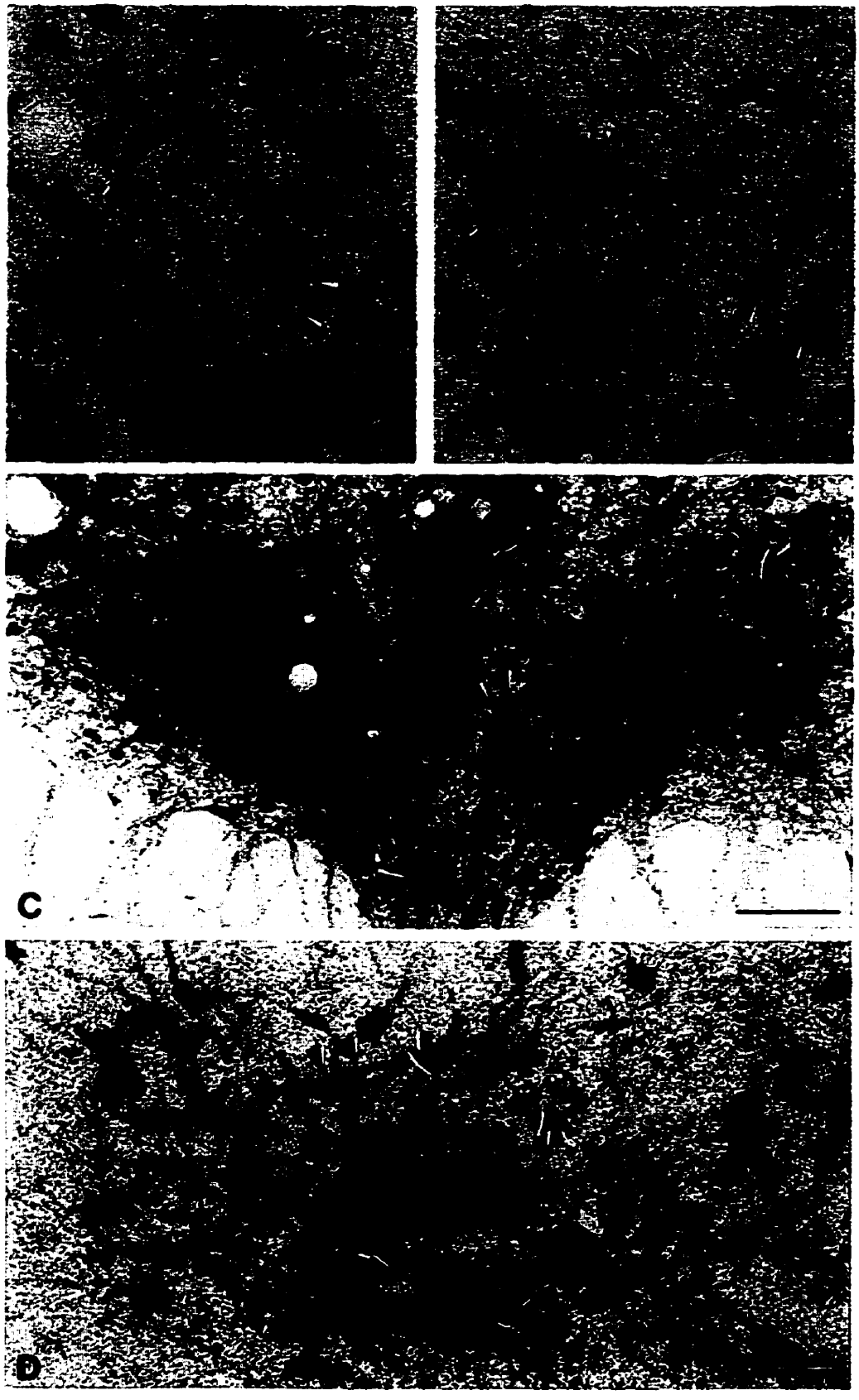
Electron microscopy. The α-[^{3}H]M5-HT-labelled and TPH-immunostained ultrathin sections were coated by dipping in Ilford emulsion L-4 (diluted 1:4 with water). Following a six-month exposure period, they were developed in D-19 (1:5 dilution) and fixed in sodium thiosulfate. The celloidin film was peeled from the slide, 200 mesh copper grids placed over the ultrathin sections and the celloidin membrane thinned with amyl acetate. The grids were examined for silver grains (corresponding to α-[^{3}H]M5-HT) and TPH immunocytochemistry under a JEOL CX 100II electron microscope at a working magnification of 8,000–14,000.

Release experiments

Approximately 24 h after tracer injection (1 mCi), brains from three different rats were removed, the striatum, hippocampus and brainstem raphe area dissected out and put into an oxygenated (5% CO_2 in 95% O_2) Krebs solution (in mM: NaCl 118; KCl 4.7; NaH_2PO_4 1.0, $CaCl_2$ 1.3; $MgCl_2$ 1.2; $NaHCO_3$ 25 and glucose 11.1; pH 7.4) supplemented with 0.2 mM ascorbic acid and 10^{-6} M fluoxetine. The tissues were minced with scissors and the Krebs solution replaced every 5 min for a 20 min period during which the tissues were brought to 37°C. Following a 15 sec centrifugation, 1 ml of freshly oxygenated Krebs solution was added and the experiment started. The Krebs solution was replaced every 2 min as above and pooled for radioactivity determination at intervals of 6 min. Depolarization was induced twice by a 2 min exposure to a 50 mM K^+ Krebs solution. At the end of the experiments, the tissue from each chamber was recovered, solubilized with 0.5 ml of 0.5N NaOH and proteins determined according to Lowry et al. (1951). Results were expressed as the amount of radioactivity released/mg protein during every 6 min interval.

RESULTS

Tissue sections from the DRN radioautographed for α-[^{3}H]M5-HT and immunostained for endogenous 5-HT showed a very good correlation between the cellular localization of the two markers, as observed at the light microscopic level (Fig. 1A,B). Similarly, the cellular distribution of α-[^{3}H]M5-HT matched that of immunoreactive TPH within neurons of the DRN (Fig. 1C,D). Silver grains (corresponding to α-[^{3}H]M5-HT) were almost exclusively concentrated over the somata and large dendritic profiles of neurons immunostained for either 5-HT or TPH. Radiolabelling densities, however, varied considerably between the different 5-HT- or TPH-immunostained cells, some cells being heavily labelled (Fig. 1, arrows) and others showing only moderate to low densities of silver grains (Fig. 1, arrowheads). Only few 5-HT- or TPH-immunoreactive cells were devoid of α-[^{3}H]M5-HT radiolabelling. Similarly, only rare radioautographically labelled neuronal perikarya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were devoid of either 5-HT or TPH immunoreactivity (Fig. 1B–D; curved arrows). These immunonegative cells showed no particular morphological or distributional features. They were scattered within the ventral aspect and the lateral wing of the DRN and were intermingled with cells immunoreactive for 5-HT or TPH (Fig. 1). In cerebral cortex, α-[³H]M5-HT radiolabelling was detected at the light microscopic level in the form of individual silver grains scattered over the entire cortical surface. These silver grains overlaid numerous TPH-immunostained varicose fibers distributed across all six cortical layers (data not shown).

At the electron microscopic level, double labelled sections from the DRN indicated that α-[³H]M5-HT radiolabelling was confined to TPH-immunopositive perikarya and dendrites. (Fig. 2A,B). On no occasion did we see at the ultrastructural level α-[³H]M5-HT-labelled somata or dendritic processes that were TPH-immunonegative. In cerebral cortex, silver grains that were seemingly distributed at random in light microscopy material were found to clearly overlay TPH-immunopositive nerve terminals, these being mostly nonsynaptic and filled with small, round and clear synaptic vesicles as illustrated here for molecular layer I of the frontoparietal cortex (Fig. 2C). However, not all TPH-immunopositive varicosities were radiolabelled for α-[³H]M5-HT.

The three brain regions obtained fresh from rats injected with α-[³H]MTrp exhibited a spontaneous efflux of tritium which was relatively stable after the 20 min wash period (time 0 on graph). When exposed to a depolarizing 50 mM K^+ solution, the evoked release of tritium corresponded to at least twice that of the basal release in striatum and brainstem, but was less (about 60% increase) in the hippocampus (Fig. 3). Following depolarization, the release returned to basal levels within approximately 12 min under normal Krebs solution. At that time, the tissues were still responsive to stimulation with 50 mM K^+ (Fig. 3), although this second evoked release of radioactivity was much weaker.

DISCUSSION

The present study shows that intravenous injection of tracer amount of α-[³H]MTrp in the rat results in a selective accumulation of radioactivity in serotonergic cell bodies and dendrites in the DRN as well as in their terminal fields, exemplified here in the cerebral cortex. Furthermore, our biochemical data show that newly synthesized α-[³H]M5-HT (Diksic et al., 1990; Nagahiro et al., 1990a) is releasable upon depolarization from perikarya and dendrites in the DRN, as well as from axon terminals in target areas such as the hippocampus and striatum. Taken together, these data indicate that radiolabelled α-MTrp is a selective and specific anatomical and functional marker of brain serotonergic neurons.

Colocalization of α-[³H]M5-HT with TPH or endogenous 5-HT

The basis of our experimental approach rests with the fact that radiolabelled α-MTrp is a selective substrate for the serotonin-synthesizing enzyme, TPH, which is converted in vivo into labelled α-M5-HT (Diksic et al., 1990; Madras et al., 1965; Roberge et al., 1972). Indeed, our previous high performance liquid chromatography studies have shown that labelled α-M5-HT is detected in the brain as soon as 2.5 hours after intravenous injection of tracer amount of α-MTrp (Diksic et al., 1990). Earlier studies further indicated that α-MTrp is readily converted in vivo into α-M-5-HT and that the latter remains in the brain for extended periods of time since it is not a substrate for monoamine oxidase (Missala and Sourkes, 1988). Since TPH is restricted to serotonergic neurons (Kuhar et al., 1972; Weissmann et al., 1987), α-MTrp would allow identification and visualization of neurons involved in the synthesis of α-M5-HT and hence of 5-HT during the course of the experiment.

In keeping with this predictive model, both light and electron microscopic studies showed a very good correspondence between the distribution of the radioactivity and that of endogenous 5-HT or TPH in perikarya and dendrites of the DRN, as well as in axon terminals in 5-HT target zones such as the cerebral cortex where their ultrastructural characteristics corresponded closely to those previously reported for serotonergic varicosities (Cohen et al., 1995; Séguéla et al., 1989). We found that only a few TPH (or 5-HT)-immunonegative cells had accumulated radioactivity, or conversely, TPH (or 5-HT)-immunopositive cells were devoid of radioactive material. A possible interpretation for the former is that the cellular concentration of TPH (or 5-HT) was beyond the sensitivity of immunocytochemical detection, even though the enzyme was in a high enough active form to permit conversion of α-[³H]MTrp into α-[³H]M5-HT. Alternatively, these might be cells that are not serotonergic, but actively take up 5-HT (or α-[³H]M5-HT) in order to regulate 5-HT levels within this region (Adell et al., 1993; Bel and Artigas, 1992; Celada and Artigas, 1992). It is also possible that these radioactive TPH (or 5-HT)-immunonegative cells correspond to neurons that possess somatodendritic 5-HT receptors that internalize together with α-[³H]M5-HT.

Fig. 1. Photomicrographs of nerve cell bodies in the dorsal raphe nucleus double-stained for α-[³H]M5-HT (in the form of black silver grains) and endogenous 5-HT (A,B) or TPH (C,D). Twenty-four hours after α-[³H]MTrp injection, brain sections were obtained and processed for radioautography and immunocytochemistry as described in Materials and Methods. Radioactivity almost exclusively concentrated over immunoidentified serotonergic neurons. Note that some 5-HT- (A,B) or TPH-(C,D) immunopositive cells contain no or few silver grains (arrowheads) while others were very densely labelled (arrows). Rare α-[³H]M5-HT-radiolabelled perikarya (curved arrows) were devoid of 5-HT (A,B) or TPH (C,D) immunostaining. Bars = 50 μm.

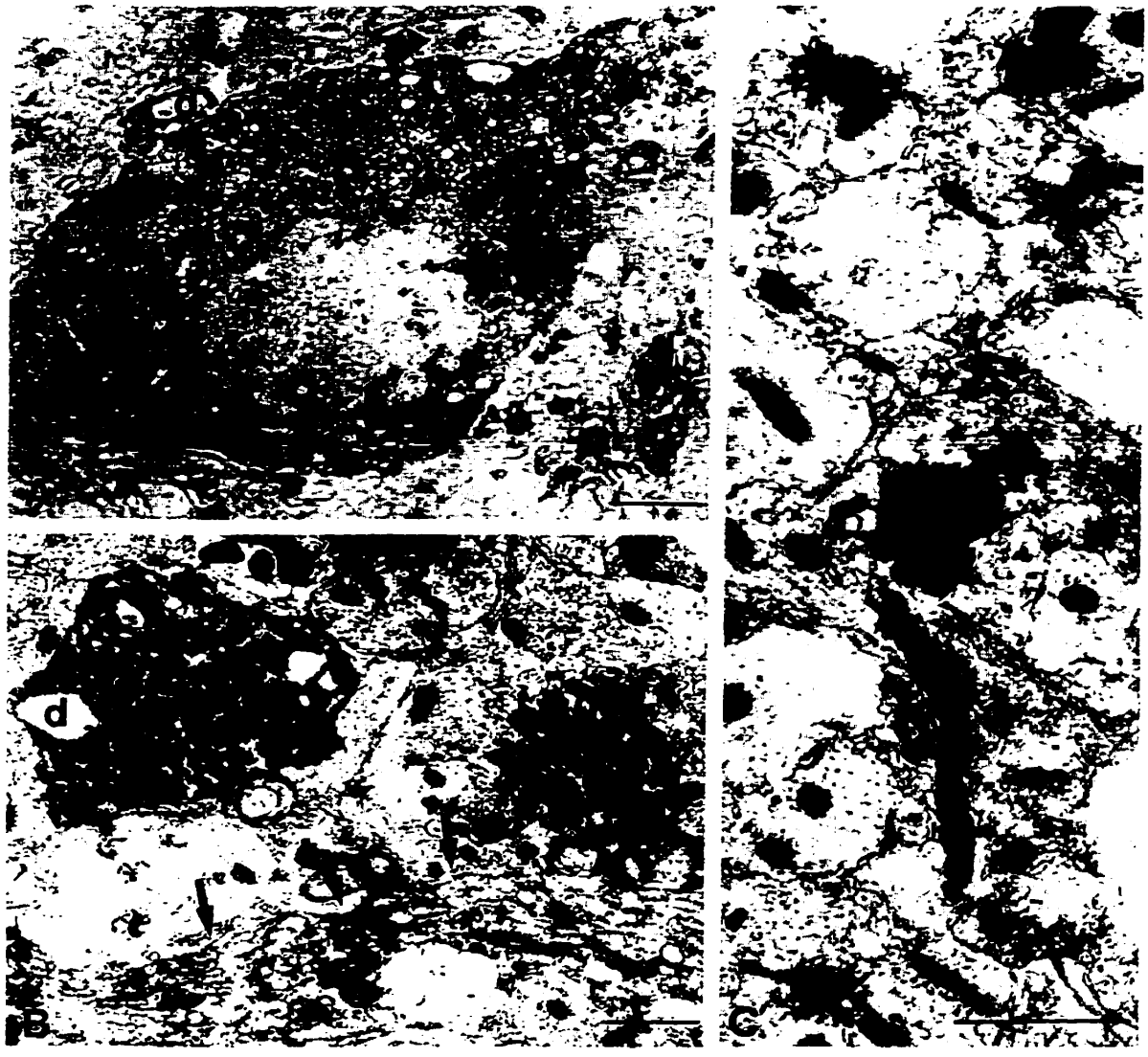

Fig. 2. Electron photomicrographs illustrating the correspondence between TPH-immunoreactive neuronal elements and those containing α-[^{3}H]M5-HT. In the dorsal raphe nucleus, accumulation of α-[^{3}H]M5-HT was confined to TPH-immunopositive cell soma (A), longitudinal axons (small arrows in B) and multiple dendritic (d) branches (A,B). In the frontoparietal cortex, one TPH-immunoreactive nerve terminal containing small spherical vesicles is also radiolabelled (C). Bars = 3 μm (A) and 1 μm (B,C).

Indeed, it is well known that α-[^{3}H]M5-HT can interact with various populations of 5-HT receptors both in vivo (Missala and Sourkes, 1988; Montine and Sourkes, 1989) and in vitro (Ismaiel et al., 1990).

The apparent lack of α-[^{3}H]M5-HT uptake in a small fraction of neurons that were immunopositive for either 5-HT or TPH, on the other hand, is probably a reflection of the intrinsic activity of the cells at the time of experiment. It has been shown that the firing of raphe neurons will determine the extent to which Trp is taken up by serotonergic cells (Denizeau and Sourkes, 1977) and actively transformed into 5-HT (Boadle-Biber et al., 1986). Our observation of serotonergic cells not containing radioactivity would therefore indicate that these cells were not involved in any firing or synaptic activity at the time of experimentation and thus did not need to actively take up α-[^{3}H]MTrp for 5-HT synthesis. Indeed, it has been suggested that while most serotonergic cells are engaged in 5-HT synthesis, the rate at which they proceed in this activity varies depending whether they fire tonically (Vandermaelen, 1985) or are engaged in synaptic activity (Boadle-Biber, 1993). Similarly, the variations in the intensity of α-M5-HT radiolabelling between individual 5-HT- or TPH-immunoreactive cells (as reflected by the density of silver grains over individual cells) likely reflect different rates of 5-HT synthesis between the various cells of the DRN. This interpretation is congruent with the results

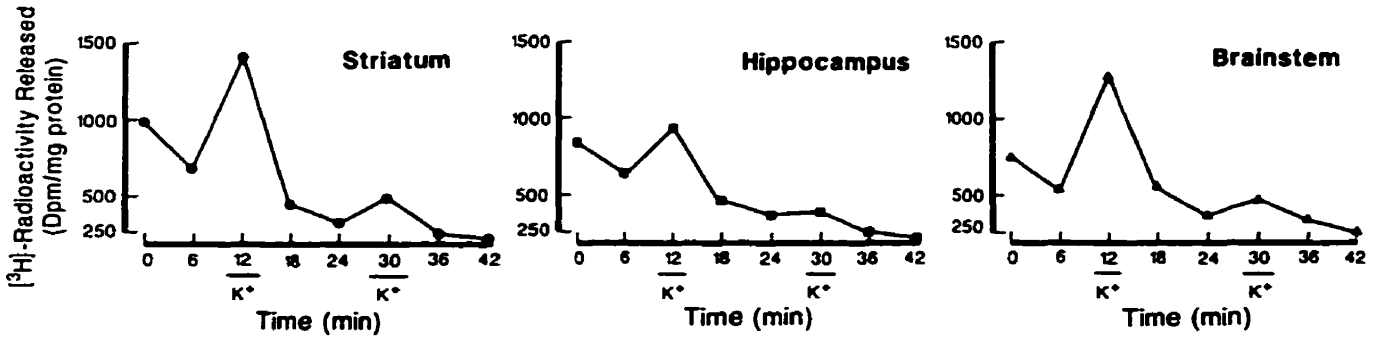

Fig. 3. Release of α-[³H]M5-HT from striatum, hippocampus, and brainstem area obtained from the rats injected with α-[³H]MTrp. An evoked release of radioactivity corresponding to about twice that under basal conditions, was obtained when first exposing the tissues to a depolarizing solution of K^+ (50 mM^+). The small horizontal line marked K^+ indicates the two stimulation periods (2 min each) for which slices were in the presence of 50 mM K^+, at 12 and 30 min, respectively. Repeated release of α-M5-HT was possible, but the second exposure resulted in a minor increase in radioactivity content.

of previous studies showing that cellular TPH activity is tightly regulated by various factors but primarily by neuronal activity (for a recent review, see Boadle-Biber, 1993). That the variation in α-[³H]M5-HT labelling was independent from the TPH or 5-HT content, as qualitatively evaluated by the density of the immunostaining, further suggests that the densities of α-[³H]M5-HT radiolabelling is a better index of TPH activity than of TPH contents.

Release of α-[³H]M5-HT

We found that significant amounts of radioactivity could be released from the striatum, the brainstem and less so from the hippocampus of rats injected with α-[³H]MTrp in response to depolarization with K^+. The striatum and hippocampus are regions which, like the cerebral cortex, receive a direct innervation from serotonergic raphe neurons (Steinbusch, 1981) and which have been shown to release 5-HT through a calcium-dependent mechanism (Adell et al., 1993; Kalen et al., 1988). The fact that α-[³H]M5-HT may be released from these terminal areas suggests that it is incorporated in the releasable pool of 5-HT (Montine and Sourkes, 1989; Regunathan and Sourkes, 1990) which has been shown previously to correspond to the newly synthesized fraction (Héry et al., 1983). Indeed, the evoked release and the synthesis of 5-HT are reportedly coupled by neuronal firing (Boadle-Biber, 1993). The slightly lower release of radioactivity in the hippocampus most likely reflects the lower density of, and/or a smaller releasable pool within serotonergic neuronal elements in this brain area. In any event, the present demonstration that α-[³H]M5-HT located in terminal areas can be released following neuronal depolarization further suggests that α-[³H]M5-HT synthesized in vivo behaves much like endogenous 5-HT. In support of this is the observation that α-M5-HT has physiological properties (e.g., in vivo synthesis, transport along serotonergic pathways by axonal transport, release from synaptosomes, uptake system in synaptosomes) similar to those of endogenous 5-HT (Montine and Sourkes, 1989; Nagahiro et al., 1990a; Regunathan and Sourkes, 1990; Tsuiki et al., 1994). Altogether, these characteristics would agree with the suggestion that α-M5-HT could act as a substitute neurotransmitter within the brain (Missala and Sourkes, 1988; Sourkes and Diksic, 1993). In the present study, significant amounts of radioactivity were also released from the brainstem raphe nuclei area, which is known to contain only a few axonal varicosities (Chan-Palay, 1982; Descarries et al., 1982). Although we cannot exclude the presence of serotonergic nerve terminals in neighboring brainstem structures, this finding concurs with the increasing evidence for somatodendritic release of 5-HT in the rat raphe nuclei (Adell et al., 1993; Héry et al., 1986).

In conclusion, we have provided anatomical and biochemical evidence that indicate that labelled α-MTrp is a useful tracer for in vivo investigation of brain serotonergic pathways. The data demonstrate that newly synthesized radiolabelled α-M5-HT is colocalized with endogenous 5-HT and its synthesizing enzyme, TPH, in serotonergic cells and nerve terminals from which it can be released upon depolarization. From the results, it can be concluded that this tracer provides information not only on the localization of brain serotoninergic neurons but it also appears as a reliable index of the metabolic activity of these neurons at the cellular level. We conclude that α-MTrp should therefore be applicable to the in vivo investigation of the activity of human brain serotonin systems by positron emission tomography (Diksic et al., 1991; Diksic, 1992).

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