BUTYRYLCHOLINESTERASE ACTIVITY, GLUCOSE TRANSPORTER (GLUT-1) AND P-GLYCOPROTEIN IMMUNOREACTIVITY IN ENDOTHELIAL CELLS IN THE AUDITORY BRAINSTEM OF THE YOUNG POSTNATAL RAT

by

Eduardo Krupnik

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ABBREVIATIONS

ACh.....acetylcholinesterase

ANOVA.....analysis of variance

AC.....auditory cortex

AP.....alkaline phosphatase

bFGF.....basic fibroblast growth factor

BBB.....blood-brain barrier

BuChE.....butyrylcholinesterase

CNS.....central nervous system

CC.....cerebral cortex

CSF.....cerebrospinal fluid

CVO.....circumventricular organ

CAM.....chorioallantoic membrane

CP.....choroid plexus

CB.....cytochalasin B

DAB.....diaminobemzidine tetrahydrochloride

ECM.....extracellular matrix

FCS.....fetal calf serum

GFAP.....glial fibrillary acidic protein

GT.....glucose transporter

HRP.....horseradish peroxidase

IC.....inferior colliculus

MG.....medial geniculate nucleus of the thalamus

mab.....monoclonal antibody

NGS.....normal goat serum

NRS.....normal rabbit serum

PAP.....peroxidase-anti-peroxidase

Pgp.....P-glycoprotein

PBS.....phosphate buffered saline

PG.....pineal gland

Pit.....pituitary gland

TBS.....Tris buffered saline

VCN.....ventral cochlear nucleus

ABSTRACT

The presence and localization of three proteins - butyrylcholinesterase, the glucose transporter isoform GLUT-1, and P-glycoprotein- proposed to be blood-brain barrier "markers" , was studied in the vasculature of the brain, as well as in several circumventricular organs and ependymal cells of the rat, during post-natal ages 1, 7, 14, 21, and 28 days, a period during which much of the brain growth and development take place. Our main objectives included: (1) to establish the histochemical and immunohistochemical methods for localizing the three proposed "markers"; (2) to determine whether or not the expression of each marker is exclusive for blood-brain barrier type capillaries; (3) to identify "marker"-containing cell types; (4) to characterize, according to their outer diameter, blood vessels which show immunoreactivity for GLUT-1; and (5) to determine the changes in vascularity as a function of age in the inferior colliculus and ventral cochlear nucleus of the young post-natal rat. Each of the procedures utilized for the visualization of each marker was found to have different fixative requirements. The expression of these three proteins was confirmed in blood-brain barrier type capillaries at all ages examined, a finding demonstrating that immature endothelial cells of the rat brain possess some of the chemical characteristics of mature endothelial cells that form part of the blood-brain barrier. Butyrylcholinesterase activity was also found in meningeal

vessels, nonvascular elements of the pituitary, vessels of the choroid plexus, and in individual neurons within some of the brainstem nuclei. The degree of activity of this enzyme in the meninges, choroid plexus and neurons appeared to be age related. GLUT-1-like immunostaining was found in cells forming blood-tissue barriers and in a number of ependymal cells resembling tanycytes. P-glycoprotein-like immunoreaction was also present in most, if not all, ependymal cells.

Using the results obtained with GLUT-1, we found that the total number of vessel profiles per unit area increased with age in both the ventral cochlear nucleus and the central region of the inferior colliculus of the rat. Even though the increase in vascular density of the ventral cochlear nucleus continued after the onset of hearing (post-natal day 10), the first large increase in vascularization of this nucleus was found between 7 and 14 days of age. Therefore a degree of endothelial cell proliferation may be due to the activation of neurons in the auditory pathway. The ventral cochlear nucleus was found to have a greater vascular density than the inferior colliculus during the first two post-natal weeks of the rat, after which the differences between these two regions were not statistically significant. The number per mm^2 of vessels with an outer diameter less than 8 μm in the inferior colliculus of 21 day-old rats, was found to be similar to that reported in the adult for the central region of this nucleus.

GENERAL INTRODUCTION

A- The Developing Brain

The nervous system develops by a process called neural induction from the ectoderm overlying the mesoderm which later will form the notochord, somites and prechordal plate.

Neural induction starts at the beginning of gastrulation in amphibians and mammals, and is accomplished by the transmission of signals from the inductor, in this case the mesoderm, to the induced tissue, the ectoderm. There is evidence that growth factors are involved in this process. The ectoderm thickens forming the neural plate which follows the notochordal process elongation and it finally extends from the primitive knot to the oropharyngeal membrane. At about the 18^{th} embryonic day in humans, the neural plate starts to fold over in a process called neurulation. Involved in neurulation are localized and oriented cell divisions, cell movements, differential cell adhesion and changes in the shape of the cells. The midline of the neural plate invaginates while the margins elevate forming the neural folds. These folds fuse in the dorsal midline forming the neural tube, from which the future central nervous system develops. The walls of the neural tube give rise to the brain and spinal cord while its lumen develop will into the ventricular system of the brain and the central canal of the spinal cord (Jacobson, 1991).

B- Vascularization of the Rodent's Brain

Developing blood vessels grow from the aortic arch forming a primordial vascular plexus on the basolateral aspect of the avascular neural tube (Strong, 1964). The leptomeningeal (extracerebral) vascular system develops from the vascular plexus (Bär, 1980). The superficial vessels form vascular sprouts which penetrate the external limiting membrane of the early brain vesicles, a process which starts the internal vascularization of the brain (Bär 1983).

1-Internal vascularization of the brain

Starting at embryonic day 12 (E12) in the rat, capillaries grow radially toward the ventricles (Bär, 1983). The capillaries branch by sprouting in the circumventricular zone at about E14. These capillary sprouts consist of solid cords of cells with cytoplasmic pseudopod-like projections at the tips, but lacking a lumen. The capillary lumen is formed by the endothelial cells secre tion of extracellular matrix (ECM) substances. The sprouts later coalesce to form anastomotic capillary loops, which are surrounded by a large extracellular space. The endothelium exhibits fenestrae (Bär, 1972).

In the cerebral cortex, with the increase in volume more vessels penetrate the brain. The new penetrating stem vessels reach only the more recently formed and superficially located layers (Bär, 1983).

Most of the cortical vessels in the rat develop during the

first ten post-natal days, a period in which most of the growth in volume occurs (Caley and Maxwell, 1970).

Between post-natal days eight to 20, there is a proliferation of capillaries that will result in differences in capillary density among the different regions of the brain (Bär, 1978). This proliferation takes place by mitotic divisions of the endothelial cells and by sprouting of preexisting vessels (Bär 1980).

After post-natal day 20 and through adulthood, most of the increase in capillary length is due to the elongation of endothelial cells, a process that decreases the thickness of the capillary wall and therefore its outer diameter.

2- Angiogenic Factors

The formation of new capillaries from preexisting blood vessels (i.e. angiogenesis) involves several steps: the degradation of the capillary basement membrane, the migration and proliferation of the endothelial cells, and the formation of a tube. These processes are regulated by soluble factors which behave as stimulators or inhibitors of angiogenesis. (Klagsbrun and D'Amore, 1991; Folkman et al, 1983).

The basic fibroblast growth factor (bFGF) has been shown to be mitogenic and chemotactic for endothelial cells in vitro and it can perform as an angiogenic factor in vivo (Gospodarowicz et al, 1986). The endothelial cells have been demonstrated to express the bFGF gene and to produce and

release bFGF. Because of this finding, it has been concluded that the formation of new capillaries may be induced by the capillary endothelial cells themselves (Schweigerer et al, 1987). Other endothelial mitogens have been characterized; for example the vascular endothelial growth factor (Conn, et al, 1990) and the platelet-derived endothelial cell growth factor (Ishikawa et al, 1989).

Angiogenesis is also dependent upon interaction with extracytoplasmic matrix (ECM). Laminin and fibronectin, two ECM components found mainly in the vascular basement membrane, are known to promote adhesion and migration of endothelial cells both in vivo and in vitro (Krum et al 1991; Risau and Lemmon, 1988; reviewed in Carey, 1991). These proteins are recognized in the endothelial cells by receptors which belong to the integrin family. Receptors of the integrin family recognize a specific amino acid sequence in the ECM molecule, RGD; endothelial cell attachment to a laminin substrate can be inhibited by a synthetic polypeptide containing this sequence (Tashiro et al, 1991).

C- The Blood-Brain Barrier

The endothelial cells of the brain are different from those in other organs in several aspects (Goldstein, and Betz,

1986). They form a continuous "wall" that helps to control the brain's internal environment by preventing substances in the blood from passing freely into the brain and alter the its milieu. Metabolites necessary for the brain's internal activity are recognized by specific carriers that transport them from the blood to the brain tissue.

1- Historical Survey

At the end of last century, Paul Herlich administrated different dyes intravenously into small animals staining all the organs but the brain tissue. Later Edwin Goldmann by injecting trypan blue into the cerebrospinal fluid stained the brain tissue but not its blood vessels, showing the existence of a blood-brain barrier (BBB) (Goldstein and Betz, 1986). However, it was not until the 1960's that the existence of the BBB was demonstrated.

Like Erlich but this time using horseradish peroxidase (40,000 MW)(HRP), a plant enzyme that can be traced by electron microscopy, Thomas S. Reese and Morris J. Karnovsky (1967) obtained the same results, but this time they were able to see that the peroxidase in capillaries of the heart was able to pass through the endothelial cells, in between endothelial cells or taken up by endothelial cells inside vesicles. In the brain, tight junctions between endothelial cells prevented the passage of peroxidase, with few endothelial vesicles carrying the tracer. Later Reese and

Brightman (1969) repeated Goldmann's experiment by injecting HRP into the brain ventricles. The tracer entered the brain as expected and the tight junctions of the endothelial cells prevented it from leaving the brain. Thus, these two sets of experiments demonstrated that the brain endothelium is the basis of the BBB.

2- The Anatomical Features of the Endothelial Cells of the Brain

There are three possible permeability routes through a blood vessel outside the brain: gaps between endothelial cells, transfer by vesicles, and fenestrae (Stewart and Hayakawa, 1986).

The endothelial cells of the brain are coupled together by tight junctions, or zonulae occludens, from the earliest periods of development (Caley and Maxwell, 1970; Hanna and Nathaniel, 1974; Butt et al, 1990). Sometimes adjacent endothelial cells or the two ends of one endothelial cell surrounding the lumen, simply adjoin. It is also possible to find one endothelial membrane overlapping its neighbour, and as a consequence, a flap formed by one or both cells projects into the lumen (Peters, Palay and Webster, 1991). At the junction, the opposed plasma membranes are separated from each other by a space or junctional cleft of about 10-15 nm. Near the luminal side the adjacent cell membranes appear to fuse, obliterating the intercellular space (Reese and Karnovsky,

1967). The tight junctions between endothelial cells are fundamental to the function of the BBB, and is the major factor for the restrictive permeability in the brain. The electrical resistance provided by these transendothelial junctions is about 1462 Ω cm^2 in the mature capillaries of the rat (Butt, Jones and Abbott, 1990), and is required for the formation of an effective barrier.

Vesicular transfer could be defined as the process by which fluids and solutes are transported from one wall of the cell to the other to empty its contents outside the cell without fusing with any intracellular organelles as the pinocytotic vesicles do (Brightman, 1977). This process has been described in vessels outside the brain (Reese and Karnovsky, 1967) but not to any significant extent in brain capillaries. Furthermore, the few vesicles found to contain peroxidase after intravenous injection of the enzyme, fail to discharge the molecules of peroxidase on the abluminal side of the brain endothelium (Reese and Karnovsky, 1967).

Brain microvessels lack the presence of fenestrae, which are characteristic of vessels in other organs. At the fenestrae, the capillary walls are fused but not perforated, forming a thin membrane through which solutes can permeate (Peters, Palay and Webster, 1991).

Apart from the presence of interendothelial tight junctions, fenestrae and a few transfer vesicles, the majority of the blood vessels in the brain do not differ fundamentally

in morphology from those in other tissues.

During the first ten post-natal days of the rat, the endothelial cells are characterized by a thick wall and a narrow, slit-like lumen into which cytoplasmic pseudopod-like projections can be seen (Hanna and Nathaniel, 1974; Caley and Maxwell, 1970). By the ninth day the blood vessels show a range of different characteristics of maturity and by the 21st post-natal day they cannot be distinguished from those of adult animals (Caley and Maxwell, 1970). The mature endothelial walls are thin except in the region occupied by a flattened and elongated nucleus. Mitochondria are scattered throughout the cytoplasm, whereas free ribosomes are not abundant, but the latter can be found bound to the membrane of the rough endoplasmic reticulum and to the outer membrane of the nuclear envelope (Peters, Palay and Webster, 1991).

The capillaries are surrounded by a rather thick basal lamina separated occasionally from the basal lamina of the brain parenchyma, upon which the end feet of astrocytes repose, by a narrow perivascular space filled with collagen fibers and pericytes. However in most places the two basal laminae fuse obliterating the perivascular space (Peters, Palay and Webster, 1991). The development of the basal lamina is related to the association between astroglia and the capillaries (Caley and Maxwell, 1970). During the first post-natal week of the rat, the vessels become increasingly covered by astrocytic end-feet. By the third week the capillaries are

completely covered by the astrocyte end-feet. This marks the end of the vascular sprouting, and the large extracellular spaces are significantly reduced. The definitive basal lamina of brain capillaries is a result of the fusion of the basal lamina synthesized by the endothelium with the one produced by the perivascular glia (Bär and Wolff, 1972).

3- The Circumventricular Organs

Wislocki and Leduc (1952) identified sites of the brain where the neural tissue became stained after trypan blue was injected into the blood, thus demonstrating that these small sites lack a BBB. These structures, located on the midline of the brain, in the walls of the third and fourth ventricles of vertebrates, are known as the circumventricular organs (CVOs). Included in this group are the pineal gland, pituitary gland, median eminence, subcomissural organ, subfornical organ, area postrema, and organum vasculosum of the lamina terminalis. The capillary endothelial cells in the CVOs differ from those in the rest of the brain. They were found to have more transfer vesicles, fewer mitochondria, fenestrae, wider interendothelial clefts and perivascular space, and less structural support by astrocytes (Coomber and Stewart, 1985). In the pineal gland there is a considerable interspecies variation concerning the characteristics of the capillaries. In the rat the endothelial cells of the capillaries are fenestrated with interendothelial clefts which extend from the

capillary lumen to a wide perivascular space. In the Mongolian gerbil, the capillaries of the gland are all fenestrated and the pineal gland of the Golden hamster presents regional differences with some capillaries being fenestrated and others not (Hewing and Bergmann, 1985). In the posterior lobe of the pituitary (the neural lobe) fenestrations have been described in all the species of vertebrates studied including the human one. In the neural lobe the blood vessels are encircled by a large perivascular space containing collagenous fibers and fibroblasts. This perivascular space is in turn surrounded by two basement membranes: the one that belongs to the endothelial cells on one side, and on the other side, the axon terminals and the pituicytes processes (S.L.Palay, 1955; K.Lederis, 1965). The CVOs are neuroendocrine organs and the increased permeability of their capillaries may enable them to perceive any chemical change in the blood and communicate with other parts of the brain or among themselves.

4- The Blood-Cerebrospinal Fluid Barrier

The choroid plexus is a vascular fold derived partly from the pia mater; it projects into the ventricles of the brain and is covered with epithelial cells responsible for the synthesis of the cerebrospinal fluid (CSF). Blood vessels are present in the connective tissue in the centre of the plexus and in the villi. These blood vessels include small arteries, arterioles, venus sinuses, and capillaries with an unusually large diameter when compared to the capillaries in the rest of

the brain. Like the vessels in the CVOs, the choroidal blood vessels are leaky. The capillaries are fenestrated, have very thin endothelial cells and contain a large number of pinocytotic vesicles. The fenestrae are not punctuated but rather formed by a thin diaphragm, thinner than a single lipid bilayer (Peters, Palay, Webster, 1991). The choroidal epithelial cells are attached to each other by tight junctions similar to the ones found in the BBB endothelium. This epithelium forms what is known as the blood-CSF barrier which restricts the interchange of substances between the blood and the CSF. The CSF covers the outer surface of the brain and hence the necessity for a blood-CSF barrier to maintain controlled the brain's milieu (Cserr, H., 1988).

5- The Molecular Biology of the BBB

The microvessels that form the BBB are also different from those in other organs in the presence of certain enzymes and carriers. The continuous tight junctions formed by the brain capillary endothelial cells, means that, to reach the brain tissue from the blood, a substance must first cross through the endothelial cells. Only plasma membrane soluble substances by virtue of their liposoluble character, would be able to perform this task, even though mechanisms may exist to transport these lipophilic molecules back into the blood stream. The P-glycoprotein found in the brain capillaries and other tissues, is an energy-dependent efflux pump involved in

normal detoxification and transport of lipophilic molecules out of the cells. This glycoprotein has been also found to be overexpressed in tumors and confers resistance to cancer chemotherapy drugs (Cordon-Cardo, 1988)

Biochemical studies of isolated brain capillaries have shown the BBB to be enriched with certain enzymes such as gamma-glutamyl transpeptidase, leucine aminopeptidase, butyrylcholinesterase, glucose-6-phosphatase, GTPase, alkaline phosphatase (AP), adenosine deaminase, and cytochrome c oxidase (Djuricic et al, 1978). For those molecules that are necessary for the brain's internal metabolism, the endothelial cells contain specific carrier systems. On the basis of biochemical and physiological studies, different transport systems have been described in the BBB: the glucose transporter (Crone, 1965) and different aminoacid transporters which includes transporters for neutral, basic, and acidic amino acids (Pardridge, 1977; Banos et al, 1977; Oldendorf and Szabo, 1976). These transporters have been shown to be saturable and stereospecific.

With the aid of polyclonal and specific monoclonal antibodies different brain capillary proteins have been identified as part of the search for proteins relevant to the BBB: the HT7 antigen found only in the chick brain endothelium (Risau et al, 1986b);the neurothelin glycoprotein (Schlosshauer and Herzog, 1990) and the 53- and 45 kilo-Dalton proteins described by Pardridge and his group (1990); however

direct evidence demonstrating the role of these proteins in the maintainance of the BBB is still lacking.

The impermeability to horseradish peroxidase is one way of defining the BBB. It defines the structural or physical anatomy of the BBB. Another way of defining the BBB is given by the different molecules that are enriched in most of the brain vasculature, which could be regarded as "markers" of this type of vessels, and hence the BBB could be defined utilizing histochemical and immunocytochemical techniques by the presence of these "markers".

6- Development of the BBB as defined by impermeability to horseradish peroxidase

One way of studying the development of the BBB is by monitoring the "tightness" of the brain microvessels after intravascular injection of HRP at different developmental stages. With this approach it was shown that "leakiness" of the BBB to HRP is higher in fetal brains than in adult brains (Stewart and Hayakawa, 1986) and that the development is time dependent and species specific. In the chick the BBB is fully impermeable to HRP by E13 (Wakai and Hirokawa, 1978) and in the mouse it is around post-natal day 10 (Stewart and Hayakawa, 1986). The tightening of the brain microvessels has been correlated with an increase in the transendothelial electrical resistance (Butt et al, 1990). Stewart and Hayakawa (1986) have shown that of the 3 possible permeability routes

through the blood vessels: fenestrae, transfer vesicles and junctions; only the latter could be correlated with a decrease in HRP permeability by the brain endothelium of the mouse. These authors demonstrated that the decline in HRP permeability with age,is due to a decrease in the proportion of interendothelial junctional clefts. They claim these clefts are possible paracellular channels that are expanded in developing blood vessels but they gradually close while the occluding junctions increase in extent.

7- Development of the BBB as defined by the presence of specific brain endothelium "markers"

The presence of specific brain endothelium markers appears to correlate with the loss of permeability to HRP of the microvessels; Risau and his group (1986a) have drawn this conclusion after seeing that butyrylcholinesterase, AP, gamma glutamyl transpeptidase, and the transferrin receptor in the mouse are gradually expressed in the brain endothelium in correspondence with the gradual impermeability of this endothelium to HRP. In the chick AP is first detected at E12, a time which also corresponds with the maturation of the BBB, which is between E10-E16 (Stewart and Wiley, 1981b). Also in the chick Risau and his group described by immunocytochemistry the expression of a BBB-chick specific protein as defined by the monoclonal antibody HT7 (Risau et al, 1986b). This protein was first detected at E10; hence it could also be correlated

with the maturation of the BBB as defined by the impermeability to HRP.

From the kind of studies like the ones described above, it was suggested that the maturation of the BBB is an event with a particular spatio- temporal pattern revealed by the sequential expression of BBB related "markers" (Risau et al, 1986a).

8- The Differentiation of Brain Endothelial Cells

One question that has been addressed is how the endothelial cells within the central nervous system develop differently from those in other organs. Is it because they are genetically predetermined to become BBB endothelium, or is it their environment which somehow induces the barrier properties of these cells? To answer this question Stewart and Wiley (1981a) performed a study using quail and chick transplantation chimeras. They transplanted avascular pieces of brain from quail embryos to the coelomic cavity of E3 chick embryos (before the formation of blood vessels has started) to demonstrate whether or not the future vessels to develop in the grafts belonged to the host animal. They also transplanted somites from quail embryos into the brain ventricles of the chick embryos. Analysis of the blood vessels in the grafted brain tissue showed that most of them contained BBB characteristics: expression of butyrylcholinesterase and AP, frequent tight junctions, and a mitochondria density that

resembled the ones found in normal brain capillaries. On the other hand the capillaries in the transplanted somites did not react for butyrylcholinesterase nor for AP; tight junctions were scarce and pinocytotic vesicles were abundant. This experiment demonstrated that the brain environment induced the invading coelomic endothelial cells of the chick to acquire BBB characteristics. In a similar kind of experiment, Risau and his collaborators have found the expression of the HT7 antigen in invading chick endothelial cells from the chorioallantoic membrane (CAM) into transplanted mouse embryonic brain. Mouse brain tissue, which does not express HT7, was able to induce the expression of HT7 in blood vessels which also do not naturally express this antigen (Risau et al, 1986b).

The next question to be answered is which specific cells induce the BBB characteristics in the brain endothelium.

As discussed before, astrocytic end feet completely cover the adult brain endothelium. Phelps (1972) has shown in the rat, that a gliovascular relationship developed completely in the cervical spinal cord before birth and that no further morphological modification of this relationship occurred during post-natal development. In the cerebral cortex of the rat, during the first postnatal week, the proportion of capillaries covered by astroglial cells increases from 66% to 84% and during the second and third postnatal weeks, these cerebral cortex capillaries are completely covered by the

astroglial elements (Bär, 1983). Later it was shown in the developing rat brain, that the activity of glutathione S transferase, an enzyme exclusively localized in astroglial and ependymal cells of the adult rat brain, increases in correspondence with a decrease in HRP permeability by the brain vasculature (Senjo et al 1986). These studies have led others to postulate that astrocytes are involved in inducing BBB formation.

To test the above hypothesis, Janzer and Raff (1987) injected into the anterior eye chamber of adult rats a suspension of glial fibrillary acidic protein (GFAP)-positive cells which were shown to bear type 1 astrocytic characteristics (Eng et al, 1971). The injected cells formed aggregates which were invaded by vessels growing from the iris. These ingrowing vessels were later shown by these authors to be "tight" as intravenously injected Evan's blue was unable to stain the astrocyte aggregates. When meningeal cells of neonatal rats were used instead of astrocytes, their aggregates also became vascularized but by vessels which were found to be permeable to the dye. To further demonstrate that the astrocytes are responsable for inducing the invading capillaries to become "tight", these researchers placed astrocytes onto the CAM of E5 chick embryos. Intravenously injected Evan's blue was unable to penetrate the astrocyte aggregates providing even more evidence that type 1 astrocytes induce the barrier characteristics in the invading non-neural

endothelial cells.

Studies done in vitro have shown that purified bovine endothelial cells developed tight junctions when they were co-cultured with rat astrocytes. But these astrocytes were unable to induce tight junction formation when they were co-cultured with endothelium from pulmonary artery or aorta (Tao-Cheng and Brightman, 1988).

Many questions have arisen about the methodology used to prove the astrocytic induction of the BBB (Holash and Stewart, 1992 personal communications; Barbosa et al, personal communications, 1992). It has been argued that in vivo, astrocyte grafts bear low vascular volume and poor vascular perfusion and the lack of staining by the intravenously injected Evan's blue could be the result of these conditions. The anterior eye chamber contains endogenous GFAP positive cells which could have invaded the implanted grafts. Furthermore, vessels of the iris have been shown to be impermeable to circulating HRP in mice (Smith, R.S., 1971) and to immunostain for the presence of the glucose transporter isoform GLUT-1, another BBB marker, which suggest that these blood vessels do not allow the simple diffusion of glucose thereby exhibiting barrier properties (Harik et al, 1990).

Holash and Stewart (personal comm., 1992), implanted purified rat astrocytes and a mixture of astrocytes and neurons onto the chicken CAM. The invading vessels did not show the morphological features of barrier endothelium and

they did not immunostain for the presence of the BBB markers GLUT-1 or HT7. Thus mature neural cells are not able to induce barrier characteristics, a task that could be accomplished by developing neuroepithelial cells. The question of which cell types are involved in the induction of BBB characteristics, remains open.

9- Three BBB "Markers"

As described above, the cerebral endothelial cell has a series of morphological and biochemical properties that make it different from the non-neural endothelium. Morphologically brain endothelium can be characterized by the presence of complex tight junctions, the lack of fenestrae and a low pinocytotic activity. Biochemically the blood vessels of the brain reveal the enriched presence of certain molecules that can be regarded as markers for this vessel type. The glucose transporter isoform GLUT-1, the P- glycoprotein, and butyrylcholinesterase are three examples of proposed BBB "markers".

Objectives

1- to establish the histochemical and immunohistochemical methods for localizing the three proposed BBB "markers": Butyrylcholinesterase (BuChE), glucose transporter (GLUT-1), and P-glycoprotein (Pgp).

2- to find out whether or not the localization of each possible marker is consistent with it being enriched in BBB type capillaries but not in other type of capillaries.

3- to identify marker-containing cell types in several regions of the rat brain at five different postnatal ages.

4- to establish the usefulness of GLUT-1 as a tool for small vessel quantitation.

5- to determine the changes in vascularity with age in the inferior colliculus and ventral cochlear nucleus of the young post-natal rat, by use of the marker GLUT-1.

A- Rationale for the study of the first four postnatal weeks of the rat:

1- the most rapid vascular growth of the capillary bed in the auditory brain of the rat, occurs during the second and third postnatal weeks (Andrew and Paterson, 1989).

2- occluding junctions increase in extent while junctional clefts decline in mouse during this period of time (Stewart and Hayakawa,1987) therefore differentiation of brain endothelium may be incomplete in neonate rodent's vessels.

B- Rationale for using the auditory brain in this study:

1. - The auditory brain stem of the rat is late to begin its functional development. Rats do not respond to sound until day 9 but by day 12 they respond to loud high pitched sounds.

The functional maturation of the rat auditory system is between day 10 and day 30.

2. The regions chosen for this study include the ventral cochlear nucleus (VCN), the inferior colliculus (IC), the medial geniculate nucleus of the thalamus (MG), and the auditory cortex (AC). In 1920 Craigie described regional variations in capillary density in the brain of the rat. He found the dorsal cochlear nucleus and the superior olive to be among the most vascularized. More recently the IC, MG, and AC were also shown to be among the most richly vascularized regions of the rat brain (Klein et al, 1986; Gross et al, 1986; Borowsky and Collins, 1989).

CHAPTER1: BUTYRYLCHOLINESTERASE

INTRODUCTION

Butyrylcholinesterase (BuChE) was first described by Mendel and Rudney (1943) who purified two esterases, the true cholinesterase or Acetylcholinesterase (AChE) and a pseudocholinesterase, from horse serum and dog pancreas. One of the first extended attempts to compare the two types of cholinesterase in different mammalian tissues was done by Ord and Thompson (1950) who reported the presence of pseudocholinesterase in brain. BuChE, as well as AChE, exists in vertebrates in three different globular forms, and in three different asymmetric polymers in which subunits are attached to a collagen tail (Massoulié and Bon, 1982).

Until Koelle and Friedenwald published their thiocholine histochemical method for localizing cholinesterase activity (1949), there was little information about the exact site of cholinesterase activity. In their method they employed substrates, acetylthiocholine iodide or butyrylthiocholine iodide, which are hydrolyzed at a much more rapid rate by AChE and BuChE respectively, than acetylcholine itself. Several modifications of the original method have been made (Koelle, 1951; Silver, 1974).

Various methods have been worked out to distinguish AChE from BuChE. The selective inhibition of either of them by

specific inhibitors has been one approach. Another method is based on the fact that butyrylthiocholine as a substrate is hydrolysed only by BuChE (Koelle, 1951; Shute and Lewis, 1963).

BuChE activity has been investigated histochemically in a number of tissues and species, including man (Friede, 1967; Kasa et al, 1991). The cell types in which BuChE activity has been localized vary with method and species; these include neural cells in the peripheral (Koelle, 1951) and central nervous system (Friede, 1967) as well as cerebral vascular cells in some species (Kasa et al, 1991; Trancard et al, 1989).

The presence and distribution of BuChE in the rat brain has been studied by a number of authors with both the light and electron microscopes (Flumerfelt et al, 1973; Joó and Csillik, 1966; Koelle, 1954; Trancard et al, 1989). In the rat's nervous system BuChE activity was found in the fibrous astrocytes, smooth muscle of larger blood vessels, the walls of capillaries, and the cytoplasm of certain neurons (Flumerfelt et al, 1973; Joó and Csillik, 1966; Koelle, 1954). No reaction product was found in the fenestrated capillaries of the circumventricular organs in any species except in the area postrema of the rat and guinea pig (Karcsú et al, 1977; Trancard et al, 1989).

In their electron microscopic study of the rat brain, Flumerfelt, Lewis and Gwyn (1973), found the reaction product

for BuChE within the matrix of the basement membrane surrounding the endothelial cells and in the intermembranous space of the endothelial nuclear envelope, and occasionally in the endothelial rough endoplasmic reticulum, but they found no reaction within the endothelial pinocytotic vesicles; the latter observation contradicts the findings of Joó and Csillik (1966). All of these studies were done in adult animals.

As the rat brain microvasculature is rich in BuChE (Djuricic and Mrsulja, 1977), several speculations for a possible role in the function of the blood brain-barrier (BBB) by this enzyme have been given (Gerhart and Drewes, 1987; Hardebo and Owman, 1984; Henderson, 1986; Silver, 1974; Skärby et al, 1979). One group (Hardebo and Owman, 1984) has suggested that endothelial BuChE may act as an enzymatic barrier to cholinergic substances. Another author (Henderson, 1986) suggested that BuChE might be involved in changing the permeability of the capillaries by acting as a 'receptor' molecule to ACh or to the vasoactive intestinal polypeptide which is contained in the cholinergic bipolar neurons of the rat cerebral cortex.

However the absence of BuChE in cat's and gerbil's cerebral microvasculature (Trancard, 1989) and its presence in dog's pericytes but not in endothelial cells (Gerhart and Drewes, 1987), as well as its presence in the area postrema of rat (Karcsú et al, 1977) and guinea pig (Trancard, 1989), are evidence against a functional involvement of this enzyme in

the BBB of most species.

Because most of the descriptive work about BuChE has been done in adult animals, little is known about possible developmental changes in this enzyme in early postnatal brain. The aim of this report is to describe the distribution of BuChE activity during the first four postnatal weeks, in the rat brain microvasculature. The regions chosen for the study include some of the auditory centres: the ventral cochlear nucleus (VCN), the inferior colliculus (IC), the medial geniculate nucleus (MG), and the auditory cortex (AC). Also several regions with fenestrated blood vessels were studied: the pituitary gland (Pit), and the choroid plexus (CP).

Materials and Methods

1- Animals, tissue fixation and preparation

Sprague-Dawley rats of postnatal ages 1, 7, 14, 21, and 28 days (3 per age), were used. Animals of 14, 21, and 28 days of age were anaesthetized by intraperitoneal injection of nembutal (5 mg/gbw); 1 and 7 days old animals were anaesthetized with isoflurane(Forane, Anaquest). Except for those aged one and seven days, the rats were perfused through the ascending aorta with phosphate buffered saline (pH 7.4) to clear the blood, followed by 4% paraformaldehyde in Sorenson's phosphate buffer (pH 7.4) for 10 min. The perfused brains were dissected out and postfixed for 6 hours by immersion in the same fixative. For rats aged 1 and 7 days old, the brains were fixed by immersion only in 4% paraformaldehyde in Sorenson's phosphate buffer (pH 7.4) for 6 hours. After rinsing in a series of phosphate buffers containing 10% and 20% sucrose, the brains were frozen onto chucks in 2-methyl-butane (Mallinckodt) precooled in dry ice. Serial sections 40 μm thick were cut in the coronal plane, mounted on subbed slides and stored at -20 $^{\circ}$C for periods ranging between one day to one month.

2- Histochemistry

For the histochemical reaction, a modification of the Koelle and Friedenwald method (1949) was used. The sections were washed for 15 min. in 0.1M acetate buffer (pH 5.5) at

room temperature, pre-incubated for 1 hour at room temperature in a medium lacking the substrate, and incubated overnight at 4 °C in a solution containing 4.5 mM of Butyrylthiocholine iodide (Sigma, St. Louis, Mo.) as the substrate, with its pH adjusted to 5.5 with the acetate buffer. The incubation time can be reduced to 3 hours if it is carried out at room temperature. The control solution was prepared without the substrate. After the incubation period the slides were rinsed in acetate buffer, and immersed in 4% ammonium sulphide in acetate buffer for 5 min. to convert the copper thiocholine precipitate into a brown deposit of copper sulphide (Koelle and Friedenwald, 1949). The sections were then counterstained in 1% cresyl violet, dehydrated and mounted with Permount. The sections were then studied under the light microscope.

RESULTS

In the auditory brainstem and cerebral cortex, the small-diameter vessels, (capillaries and small venules less than or equal to 12 μm outer diameter), stained heavily for BuChE activity at all the ages, with no apparent difference in the intensity of the stain among the IC (fig. 1), VCN (fig. 2), MG, and AC (fig. 3E) and ages under study. The sections that were incubated with the medium lacking the substrate, did not show any positive staining above the background level of staining.

The larger venules (larger than 12 μm in outer diameter) were also stained positively for BuChE but the stain was less intense when compared to that in the capillaries.

When the stained endothelial cells were viewed at high magnification (100X), the deposits of reaction product were observed in the nuclear envelope and less intensely, in the cytoplasm (Fig. 3A).

In the brainstem nuclei of rats one and 7 days old, a poor vascularization when compared to that in the older animals was observed, and most of the vessels were distributed perpendicular to the brain surface (figs. 1A, B). In the auditory brainstem nuclei of rats at 14 days of age and older the vessels appeared to be more irregularly oriented (figs. 1C, D and 2C, D).

The highly vascular, thick meninges of the 1- and 7-day old

rats showed an intense reaction in all its blood vessels. Relative to this reaction, that in meningeal vessels of the older rats was faint (figs. 3D, E).

The arteries, such as those at the base of the brainstem, were negative in all ages under study.

Blood vessels in the pituitary did not stain but a strong reaction for BuChE was found at the junction of the posterior and intermediate lobes, and in the septae that divide the intermediate lobe (fig. 3F). Activity was also found in a small number of individual pituicytes of the posterior lobe, and in rare individual cells of the anterior lobe.

The choroid plexus showed some staining in the endothelial cells of its blood vessels. This staining appeared to decrease with age, being the most intense at one day of age (fig. 2A) and the least at 28 days old (fig. 3B).

In the brainstem of rats 14 days and older, individual neurons in some nuclei -the superior vestibular nucleus, part of the nucleus of the spinal tract of the trigeminal nerve; the dorsal tegmental nucleus, and the parabrachial nucleus; the nucleus of Edinger-Westphal and the interstitial nucleus of the ventral tegmental decussation- all reacted to the substrate (fig. 3C). In constrat, no neurons were positive in 1 day old rats. At 7 days old only a few cells were BuChE positive in the Edinger-Westphal nucleus.

Fig.1: Distribution of BuChE in the blood vessels of the rat inferior colliculus at 4 different post-natal ages : 1 day (A); 7 days (B); 14 days (C); and 28 days (D). The convex dorsolateral surface of the coronally sectioned inferior colliculus is evident in each photograph. It is possible to notice an increase in vascularization with age and a more radial orientation of the blood vessels in the first two ages. Scale bar = 100 μm.

Fig.2: Distribution of BuChE in the blood vessels of the rat ventral cochlear nucleus at 4 different post-natal ages : 1 day (A); 7 days (B); 14 days (C) and 28 days (D). Most of the VCN in transverse section is shown. The auditory white matter is at right in figures B, C & D. It is possible to notice an increase in vascularization with age. Note in the one day old animal the BuChE reaction product in the meningeal vessels supplying the choroid plexus (A, arrow). Also note BuChE activity associated with vessels in the choroid plexus itself (CP) at one and 14 days. Scale bar = 50 μm.

Fig.3: (A): High magnification showing BuChE reaction product to be located mainly in association with the nuclear envelope of the endothelial cells of capillaries. This capillary is from the IC of a 14-day old rat. Scale bar = 5 μm.

(B): High magnification of two choroidal blood vessels from a 28-day rat showing BuChE activity associated with them.

Scale bar = 5 μm.

(C): Individual neurons in the nucleus of the spinal track of the trigeminal nerve of a 28-day rat stained intensely for the presence of BuChE specially in their ovoid cell bodies. Stained vessels in the trigeminal tract (left) and nucleus are also evident. Scale bar = 50 μm.

(D): Cerebral cortex of a one day old animal showing positive reaction for BuChE in the meningeal and cortical vessels. The hippocampus is at right. Scale bar = 100 μm.

(E): Auditory cortex of a 21-day old animal. Staining for BuChE in the meningeal vessels is faint or absent. Scale bar = 100 μm.

(F): The pituitary gland of a 21-day old animal. Histochemical reaction for BuChE is present in a few pituicytes of the posterior lobe (P), in cells at the junction between the posterior and intermediate lobes (I), in cells of the septae of the intermediate lobe, and in rare individual cells of the anterior lobe (A). Scale bar = 50 μm.

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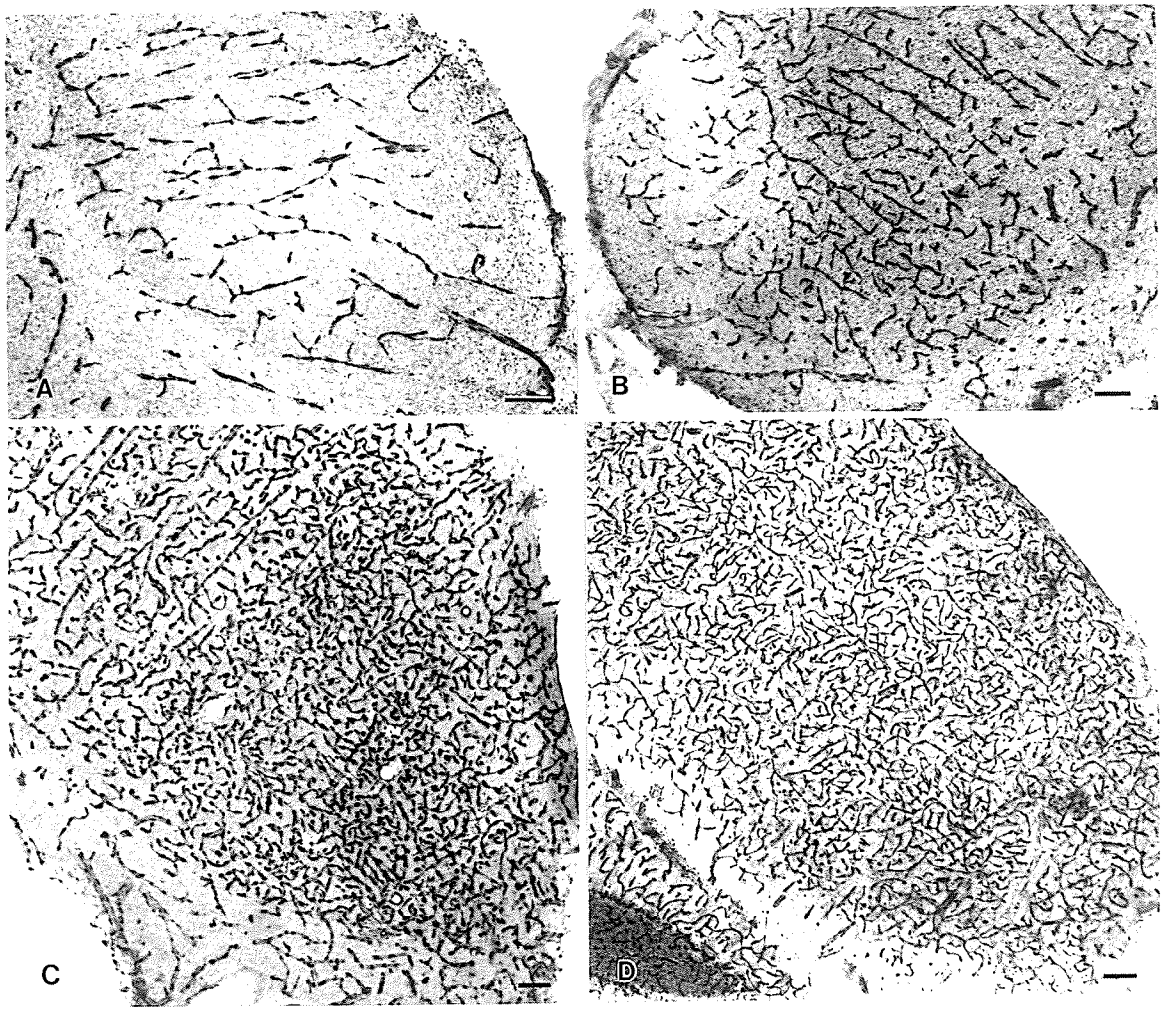

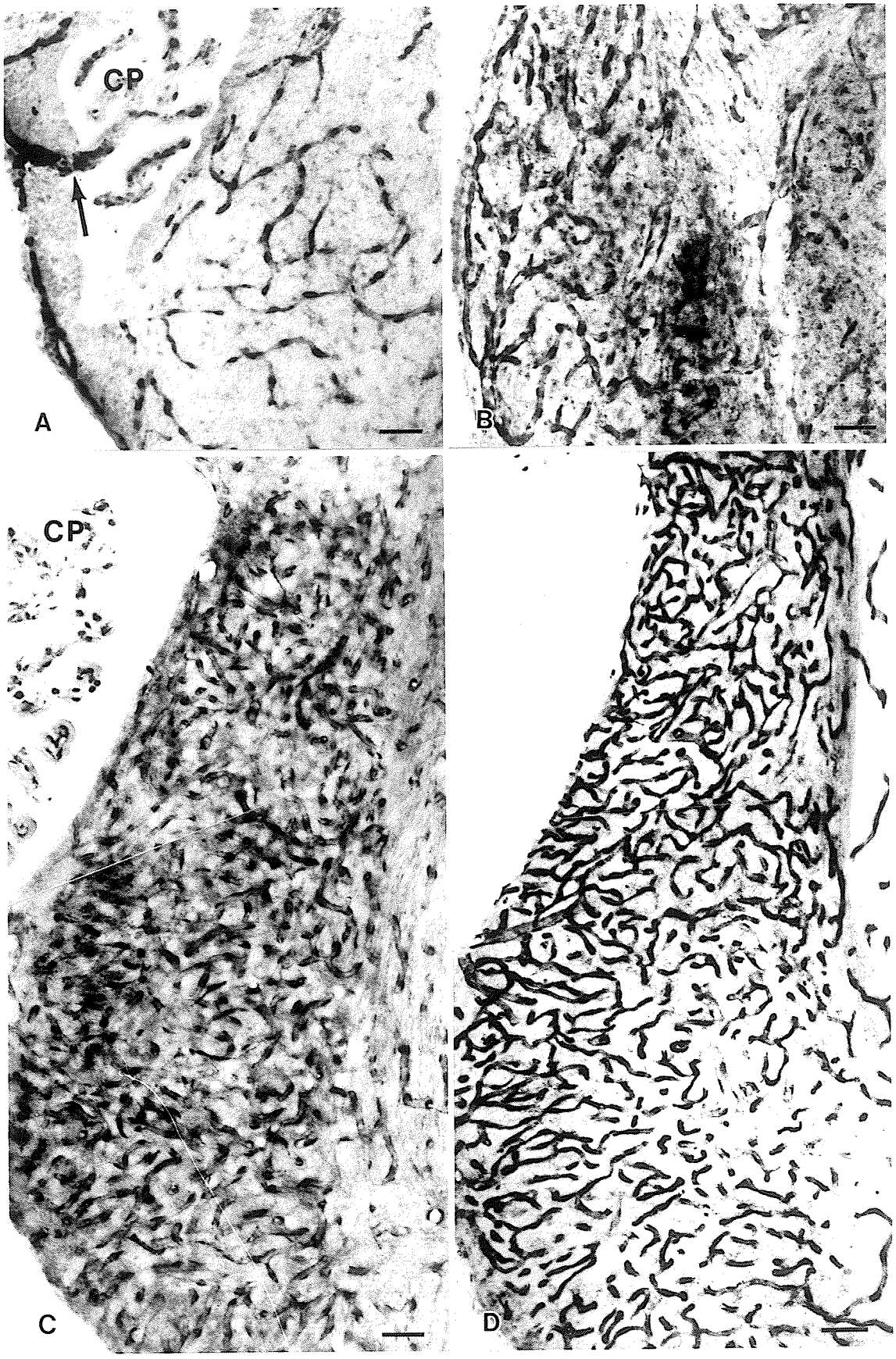
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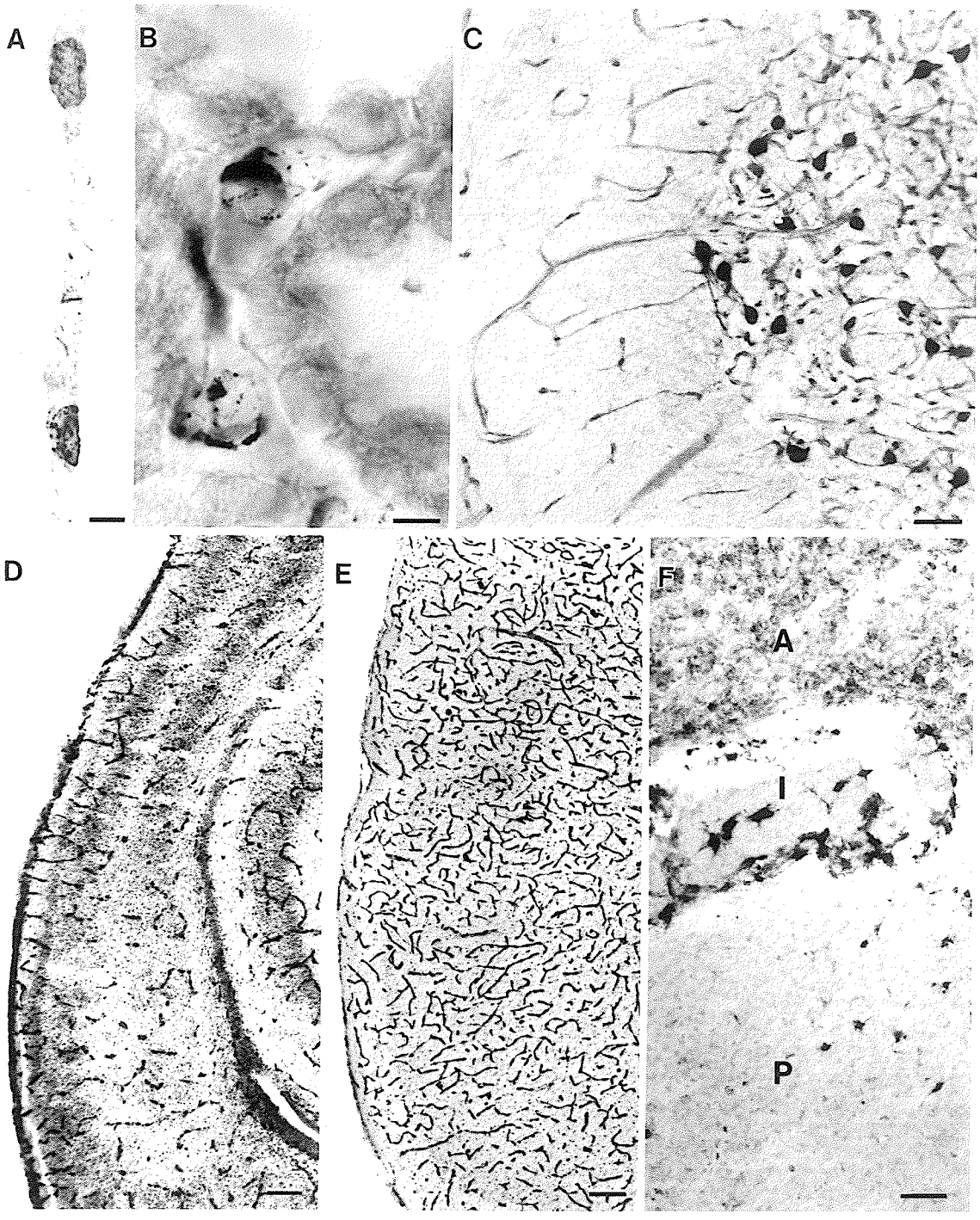


DISCUSSION

A- Histochemical Method

The histochemical method used here selectively demonstrates BuChE activity. This is based on the fact that Butyrylcholine is hydrolysed by BuChE at a much higher rate than acetylcholine [Agustinsson 1949 cited in Ref. 13]. In rats butyrylcholine iodide does not appear to be hydrolysed by AChE even after a long incubation time, as the addition of AChE inhibitor to butyrylthiocholine substrate does not affect the staining when compared to that of the butyrylthiocholine by itself (Shute and Lewis, 1963).

B- Localization of BuChE in the developing rat brain microvasculature

In this study we have found the presence of BuChE activity in brain microvessels of rats aged one to 28 days, with no apparent difference in the intensity of the stain among the different regions and ages under study.

In a previous study (Joó et al, 1967), no BuChE activity was found in brain capillaries of rat embryos; the first appearance of BuChE activity was detected in 3 day-old rats. However the work of Risau and collaborators (1986a) with developing mice demonstrated BuChE activity in stem vessels within the brain as early as embryonic day 17.

Like Flumerfelt and associates (1973) working with adult

rats, we have found that in the older rats (14, 21, and 28 days old), the staining of the larger blood vessels that penetrate the brain from the pial surface is less intense than the reaction found in the capillaries. But in the younger animals, 1 and 7 days old, we have found that these penetrating meningeal vessels are strongly stained. The presence of BuChE in early forming capillaries as well as in the radially penetrating stem vessels from the leptomeningeal plexus, as suggested here, was also indicated by the work of Risau and his group (1986a), who detected BuChE activity in the perineural plexus of the mouse at all ages of development.

At high magnification, the subcellular localization of BuChE seems to be mainly at the nuclear envelope, a finding which corresponds with the ultrastructural localization done by Flumerfelt and coworkers (1973). The latter group has described reaction product to be present also in the matrix of the basement membrane.

Our findings in the three lobes of the pituitary agree with those of Whitaker and La Bella,(Whitaker and La Bella, 1972; Whitaker et al, 1974) who, in light and electron microscope studies, have described the presence of BuChE activity in the same pituitary structures as we have. These authors have also found reaction product to be present weakly in some endothelial cells of the three lobes of the pituitary.

Individual neurons in several nuclei were found to be positive for BuChE activity. Friede (1967) in his comparative

study of the histochemical distribution of BuChE in four species (man, monkey, cat, and rat) found neuronal nuclei with high BuChE activity but with a considerable species variation in regional and cellular distribution. Friede suggests that because of the enzyme variation among the different species, BuChE cannot be involved in basic metabolic or functional processes common to all neurons.

In our study we found that the presence of BuChE in the neurons was age-related, as we were not able to find BuChE positive neurons in the 1 day old animals. Although the onset of differentiation of these neurons has likely begun before birth, our findings suggests that the presence of this enzyme in specific neurons does not occur until the second postnatal week, probably after their migration within the brainstem.

An unexpected finding of this study was the presence of enzymatic activity in the choroid plexus, apparently in endothelial cells of fenestrated capillaries. Other authors (Flumerfelt et al, 1973; Joó and Csillik, 1966; Trancard et al, 1989) reported no positive reaction in this area. Trancard and his group (1989) have shown that in the guinea-pig area postrema the fenestrated microvessels are stained for BuChE, but did not find any staining in the choroid plexus of the rat. An electron microscopic study would be necessary to find the exact site of the reaction of BuChE in the choroid plexus.

C- Significance of BuChE

The physiological function of BuChE is largely unknown.

Because in the rat there was thought to be a very close relation between the presence of this enzyme and the BBB, a functional role in the maintenance of the barrier was suggested by several authors (Gerhart and Drewes, 1987; Hardebo and Owman, 1984; Joó and Csillik, 1966; Silver, 1974; Skärby et al, 1979). But the immense variation in different species and among different regions in the same species, not only in the distribution of the enzyme but also in its presence or absence, makes it difficult to propose a general role for BuChE in the maintenance of the BBB. For instance it was not possible to demonstrate BuChE in the adult cat's cerebral microvessels; in adult dogs the enzyme is restricted only to vessels of the hippocampus and the cerebellar cortex (Trancard et al, 1989). BuChE activity was revealed in the area postrema of the guinea-pig(Trancard et al, 1989 and the rat (Karscú et al, 1977), and this region is considered to be "outside" the BBB. Because of our results in the choroid plexus, where we found BuChE activity in the blood vessels, we cannot support the hypothesis of a role for BuChE only in the maintenance of the BBB.

BuChE activity has been measured in the cerebrospinal fluid of patients, both those with no neurological disorders and others with such diseases as Alzheimer's, where elevated levels of this activity relative to AChE activity were found,

mainly in early-onset Alzheimer's disease (Arendt et al, 1984). Disturbances in cholinesterase metabolism in human brain may be reflected by changes in relative levels of BuChE activity in the CSF. Our finding of BuChE activity in the rat choroid plexus may be relevant to this clinical observation. If this localization is true in man, then choroidal as well as neuronal or vascular sources of BuChE may contribute to the relatively high levels of activity of this enzyme in the CSF of early-onset Alzheimer's patients.

CHAPTER 2: THE GLUCOSE TRANSPORTER GLUT-1

INTRODUCTION

Glucose is one of the main sources of metabolic energy. It also serves as the key precursor for many other carbohydrate compounds in several biochemical pathways.

The molecules of glucose reach the blood stream from the liver which stores them as glycogen or which can synthesize glucose by hepatic glycogenesis and gluconeogenesis. The diet is another way these molecules can reach the blood. The raise in blood glucose levels is detected by the pancreatic ß-cells which release insulin to stimulate the muscle and adipose cells to absorb glucose and to induce the storage of glucose in the liver. As the level of blood glucose drops, the pancreatic cells release glucagon which stimulates glucose release from the liver.

A- How glucose enters the cell

In order to enter the cell from the blood, the glucose molecules must pass the plasma membrane, but the high polarity of these molecules makes the plasma membrane highly impermeable for them to pass by simple diffusion. In the CNS in order to get to the brain tissue, any material must first cross the plasma membrane of the endothelial cells because of the interendothelial tight-junctions which form the physical structure of the blood-brain barrier (Reese and Karnovsky,

1967; Bradbury, 1984).

C. Crone (1965a) established a hierarchy of a series of non-electrolytes based on their ability to cross the brain capillaries. He found that only 3-5% of fructose and glycerol leave the blood and enter the brain, while glucose has a percentage of permeability of 10% even though its chemical structure is very similar to those of fructose and glycerol. Later (1965b) he showed that the passage of glucose into the brain depends on its blood concentration, and demonstrated the existence of a saturable mechanism of glucose transport.

All of these findings made Crone conclude that a transporter must exist to facilitate the transfer of glucose across the endothelial cell membrane and the cell membranes of all the other cells. This transport is today known as the glucose transporter (GT).

B- Molecular structure of the GT

In 1977, M. Kasahara, and P.C. Hinkle isolated the first GT from human erythrocytes (cited in Lienhard et al, 1992). Later, monoclonal and polyclonal antibodies raised against the human-erythrocyte GT, recognized by western blotting a glycosylated protein of 55,000 Daltons that becomes a 46,000 Daltons one after the oligosaccharide is removed.

The amino acid sequence and the first model of the glucose transporter were defined by M. Mueckler, H.F. Lodish and co-workers (1985) from a human hepatoma cell line (HepG2). They

accomplished it by first screening a HepG2 complementary DNA expression library with rabbit antiserum to the human erythrocyte glucose transporter. Once the cDNA was isolated and its nucleic acid sequence determined, the amino acid sequence of a 492-residue protein was deduced by applying the genetic code. From the analysis of the sequence, they predicted the existence of 12 transmembrane segments linked by 13 hydrophillic segments with both carboxyl and amino ends present in the cytoplasm and the presence of one N-linked oligosaccharide situated in the Asn^{45} residue located in the first exoplasmic segment (see figure of model in appendix 2-1 from Andersson and Lundahl, 1988).

Lienhard, Slot, James and Mueckler (1992) have speculated a structural model for the glucose transporter based on the folding arrangement of the protein and the analysis of the amino acid sequence of the transmembrane segments. Spectroscopic studies show that each transmembrane segment is helical and in 5 of them -3,5,7,8 and 11- the hydrophobic amino acids are on one side of the helix and the hydrophillic ones on the other. These authors suggest that these 5 segments form a pore with the hydrophobic amino acids facing away from their common axis and the hydrophillic amino acids would form the inner layer of the pore giving the right environment for the molecule of glucose.

C- A family of glucose transporters

Because each tissue has its own needs for glucose, it is fair to assume that they might have a different glucose transporter. By using the DNA sequence of the human/erythrocyte glucose transporter as a probe, cDNA clones encoding different transporters were isolated. Based on their order of isolation, the transporters were named Glut-1, Glut-2, Glut-3, Glut-4, Glut-5. These transporters form a family of structurally related proteins.

All of these integral membrane proteins transport glucose down its concentration gradient and therefore are also called the facilitative glucose transporter family.

Because the facilitative human/erythrocyte GT was the first one to be characterized, it was designated as GLUT-1. GLUT-2 or liver type was found in liver, ß-cell, kidney and small intestine; GLUT-3 was found mainly in the neurons but also in the human placenta and kidney; GLUT-4 is the muscle/fat type and its major sites of expression are skeletal muscle, brown and white fat, and heart, (this is the only insulin-responsive type of the facilitative glucose transporter family); GLUT-5 is the small intestine type and is located in the upper jejunum (Bell and associates, 1990).

D- The biochemistry of the facilitative Glucose transporter

The biochemical properties of the erythrocyte GT were determined in experiments in which the carrier was

incorporated into liposomes (Thorens et al, 1990). With this procedure it was found that GLUT-1 and GLUT-3 have similar affinity for glucose and under physiological conditions the glucose uptake by these proteins is near maximal allowing the tissues where they are present, a continuous flux of glucose. The uptake of glucose by GLUT-2 found in hepatocytes and pancreatic ß-cells, increases with increasing blood glucose levels so that the ß-cells would be able to respond to any change in blood glucose concentration.

Insulin causes a 20- to 40- fold increase in glucose uptake by the muscle and fat cells which express the GLUT-4 isoform. It seems like the stimulus of insulin would involve the recruitment of transporters from transporter-containing vesicles within the cell to the cell membrane. This elevation in the number of carriers would account for the raise in uptake instead of a change in the carrier's affinity for glucose (see Kasanicki and Pilch, 1990 for review).

The transport can be inhibited by phloretin, $HgCl_2$, and the fungal metabolite cytochalasin B (CB) (see Wheeler and Hinkle for review, 1985). Binding of CB can be inhibited by glucose competition (Sogin and Hinkle, 1980). Crystallographic studies have shown that CB is structurally similar to the molecule of glucose and thus it would bind the glucose site of the transporter (Kaplan, Pring and Passow, 1983). Radioactive CB can be used to label the GT by using ultraviolet to cause a covalent bond between the GT's inhibitor and its binding

site. The reversible and irreversible binding properties of CB provide a way to design quantitative studies for GT expression. It is now accepted that the transport of D-glucose is saturable, stereospecific, sodium and energy independent, and not influenced by insulin except in adipocytes and cardiac and skeletal muscles which contain the insulin-respondant GLUT-4 isoform.

E- The erythrocyte/brain GLUT-1

The mammalian GLUT-1's share a remarkable sequence homology among species. Birnbaum and his co-workers (1986) characterized the cDNA of the rat brain GLUT-1 which predicts that it is a protein containing 492 amino-acid residues and a protein that has >97% sequence identity with the human erythrocyte glucose carrier. GLUT-1 is also known as the erythrocyte/brain GT.

There was even found sequence homology between mammalian and bacterial GT's (Maiden et al, 1987).

1- Tissue expression of GLUT-1

The tissue localization of GLUT-1 has been studied with a number of methods. The table below shows the major sites of expression of the carrier by 2 different methods: northern blot and immunocytochemistry.

Tissue	Northern Blot[1]	Immunocytochemistry[2]
brain	+	+
testis	+	+
heart	+	-
kidney	+	+
fat	+	-
liver	-	-
skeletal muscle	-	-

[1]: From Charron et al, 1989.

[2]: Harik et al, 1990; Farrell et al, 1992; Gerhart et al, 1989.

2- Expression of GLUT-1 gene in the brain

GLUT-1 protein has been found to be highly enriched in the brain microvessels (Flier, Mueckler, McCall and Lodish, 1987). Immunohistochemical and [^{3}H]CB-binding methods were used to show a high density of GT protein in the human brain microvessels with minimal staining of the neuropil (Kalaria et al, 1988). By Western blot analysis, Pardridge and his group (1990) have demonstrated that GLUT-1 accounts for almost 100% of the GT binding sites in isolated bovine brain microvessels. Using riboprobes against the bovine brain capillary GT cDNA this group has confirmed the selective expression of the GLUT-1 gene in the endothelial cells of the brain which form the physical structure of the BBB with minimal expression in the neurons and glia. S. Harik and his associates have confirmed immunohistochemically these findings in rats where they have

shown the presence of the GLUT-1 protein in all the vessels of the brain and they found no specific immunostaining in the neuropil (1990). Based on these findings it would appear that GLUT-1 is both synthetized and utilized within the endothelial cells of the brain microvessels which form part of the BBB.

GLUT-1 has also been found by immunocytochemistry in the choroid plexus epithelium, which also forms tight junctions (Kalaria et al, 1988), and in the tanycytes in the floor of the third ventricle (Harik, et al 1990). The staining of the choroid plexus epithelium has been shown to be confined to the basolateral membranes of this epithelium (Farrell, Yang and Pardridge, 1992).

3- Ultrastructural localization in the endothelial cell of the brain microvasculature

Based on their quantitative electron microscopic immunogold studies, Farrell and Pardridge (1991) have demonstrated that the GLUT-1 protein is expressed asymetrically within the plasma membrane with expression being about 4-fold higher in the ablumenal membrane than in the lumenal membrane and about 40% of the total immunogold reactive GLUT-1 protein was located in the cytoplasm.

4- GLUT-1 during development

GLUT-1 mRNA is expressed in mouse embryos from the preimplantation stages (Hogan et al, 1991). In rats at E12 only the neuroepithelium is immunoreactive to the anti-GLUT-1 antibodies. With the development of tightness as defined by intravascularly applied horseradish peroxidase in the intracerebral blood vessels, there is a decrease in the expression of GLUT-1 in the neuroepithelium and GLUT-1 immunoreactivity is located only in the vasculature(Dermietzel et al, 1992). This gradual decrease in GLUT-1 immunoreactivity in the neuroepithelium is also true at the mRNA level. The GLUT-1 mRNA from the neuroepithelium is finally retained in the ependymal cells lining the ventricles.

5- The Na^+-dependent glucose transporter

The facilitative glucose transporter family differs from another class of glucose carriers that pulls the molecule of glucose against its concentration gradient by coupling its transport with the transport of a sodium ion along its own gradient providing the driving energy for the uphill uptake of glucose. Na^+ also induces a conformational change in the carrier increasing its affinity for the molecule of glucose (Peerce and Wright, 1984). Thus this glucose carrier is called the Na^+-glucose co-transporter and is found in the epithelial cells of the small intestine and proximal tubule of kidney. The DNA sequence of the co-transporter revealed no homology

between the Na^+-glucose co-transporter and either of the facilitative glucose transporter families, suggesting no evolutionary relationship (Hediger et al, 1987).

Materials and Methods

1- Animals, tissue fixation and preparation: Sprague-Dawley male rats at post-natal days 1, 7, 14, 21, and 28 were used for immunolocalization of GLUT-1. Except for the 1 day-old ones, the animals were anesthetized with nembutal and perfused through the heart with cold PBS buffer (pH 7.4) for 1 minute and then perfusion-fixed with Bouin's solution for 10 minutes. After perfusion the brains were removed and post-fixed in the same fixative for 1 hour. One day-old rats were anesthetized with isoflurane and immersion-fixed in Bouin's solution for 24 hours to ensure good penetration of the fixative. The brains were stored for cryoprotection in 10% sucrose in Sorenson's buffer for 1 hour and in 20% sucrose in the same phosphate buffer overnight. Also brains fixed in 4% paraformaldehyde, pH 7.4, and unfixed brains were used for this study. The fresh brains, after being dissected from the skull, were immediately immersed in 2-methyl butane precooled to -50 °C in dry ice. The frozen sections (10 μm) from the unfixed brains, were fixed in cold 100% acetone. For fixed brains, both slide-mounted (10 μm) and free-floating (40 μm) sections were made. A few free floating sections were embedded in Epon after the immunocytochemical staining was performed, in order to localize the staining in semi-thin sections (1 μm). Tissue from one animal was embedded in paraffin and 4 μm sections were cut. The tissues collected include the ventral cochlear

nucleus (VCN), inferior colliculus (IC), medial geniculate of the thalamus (MG), auditory cortex (AC), pineal gland (PG), choroid plexus (CP), and the pituitary gland (Pit).

2- Antibodies: The primary antibody used is the monoclonal B315:32 directed against the human erythrocyte glucose transporter and which recognizes the carboxy end of the carrier. This monoclonal antibody, contained in the hybridoma supernatant provided by L. Andersson, has been well characterized (Andersson and Lundahl, 1988). A 1:50 dilution of the supernatant containing the monoclonal antibody (in 0.1M TRIS pH 7.6) for the frozen sectioned tissues, and 1:2 for the tissues fixed in Bouin's solution and embedded in paraffin, were used. In control experiments to show the specificity of immunostaining, the primary antibody was 1) omitted or 2) replaced by 5% fetal calf serum (FCS) in 0.1M TRIS or 3) adsorbed with the synthetic polypeptide P2 (Andersson and Lundahl, 1988), which is the C terminus of the erythrocyte GT, before immunoassay.

3- Immunohistochemical staining: The Avidin-biotin-peroxidase method was performed (Hsu et al, 1981) using the ABC (Elite) kit (Vector Laboratories, California). A detailed description of this method can be found in appendix 2-2.

4- Classification of vessel-profile types: Vessel profiles in the central nucleus of the IC and in the VCN were classified according to their outer diameter size into 4 groups: 1)outer diameter less than 8 μm, which includes capillaries; 2) outer

diameter equal to 8 μm, 3) outer diameter between 8 and 12 μm, which includes the penetrating branches from the pial circulation, small venules, and small arterioles, and 4) outer diameter greater than 12 μm, a group that includes venules, arterioles, veins, and arteries.

5- Density of vessel profiles: The total number of vessel profiles per mm^2 was calculated from counts of vessel profiles in a sampled area of a section through the central nucleus of the IC, or through the VCN. The area used was measured from photographs by use of a Zeiss Interactive Digital Analysis System (Zidas, Carl Zeiss, Inc.).

6- Statistical analysis: For the measurements of vascular density, and of percentages of different vessel profile types, the one-way analysis of variance (ANOVA) was done in order to compare age-related differences, using the means for three rats (N = 3) (SigmaStat, Jandel Scientific, S. Rafael, CA). For comparisons of vascular density between the different brain regions of the same rats within one age group, the paired t-test (N = 3) was done (NCSS, Dr. J. Hintze,Kaysville, Utah). A value of less than 0.05 probability was used to reject the null hypothesis.

Whenever multiple comparisons were necessary to isolate which ages differ from the others, the Student-Newman-Keuls method was used, and a value less than 0.05 probability was required for significance.

For the statistical analysis, the vessel-profile type

groups 2) and 3) were combined into a single one.

Results

The immunoreactivity of the GLUT-1 was found to be damaged by paraformaldehyde, and immunoreaction on fresh frozen tissue gave poor results as heavy background overshadowed the staining. The use of Bouin's solution improved significantly the immunostaining by lowering the background and increasing the intensity of the stain; therefore the results to be described in this section are those ones obtained with tissue fixed in Bouin's solution.

1- GLUT-1 in the auditory brainstem

In the auditory brainstem and cerebral cortex, GLUT-1-like immunoreactivity was observed in the endothelial cells of most, if not all, capillaries, and small venules (less than or equal to 12 μm in outer diameter) at all the postnatal ages examined (figs. 4 and 5).

Many but not all large venules (more than 12 μm in outer diameter) as well as a few arterioles of small calibre, showed GLUT-1 immunoreactive endothelia, but their staining was not as intense as the adjoining capillaries.

Arteries were negative in all ages under study.

At high magnification (100 X) the immunostaining of the endothelial cells appeared to be located in the plasma membrane and less intensely in the cytoplasm (fig. 6a).

In the experiments where the primary antibody had been

omitted, or replaced by 5% FCS, or adsorbed with the synthetic polypeptide P2, the sections did not show any specific immunostaining in small vessels. The "background" pale amber staining in the neuropile of the 21-day and 28-day old rats was shown to be nonspecific, as were deposits of stain at the edges of some sections (figs. 4f and 5f).

2- GLUT-1 in other brain regions

The microvessels of the posterior lobe of the pituitary and pineal gland did not show GLUT-1 immunostaining (fig. 6b). Blood vessels in the choroid plexus were not immunostained but in the choroidal epithelium, GLUT-1-like immunoreaction was observed (figs. 6 c-d).

Certain ependymal cells, lining the fourth ventricle, the lateral foramen of the fourth ventricle, the cerebral aqueduct and the third ventricle, showed GLUT-1 immunoreactivity. The GLUT-1 immunoreaction in the ependymal cells of the cerebral aqueduct, and of the fourth ventricle and its lateral foramen, seems to be located in the apical and basolateral membranes, and a more faint immunoreaction can be noticed in the cytoplasm. Some of these cells revealed a more intense immunostaining than others but there were also some that were not immunostained. The scattered positive cells were seen in both 1 day old and 28 day old rats (figs. 6e and 7).

Most if not all of the tanycytes lining the floor of the third ventricle showed a strong GLUT-1-like immunostain in

their lateral membranes and basal processes (fig. 6f).

3- Quantitative studies

Since most if not all the capillaries can be detected by immunoreactivity to GLUT-1, this "marker" was used for quantitative analysis of the vascular profiles of the IC and VCN.

a- Percentage of total vessel profiles for each group based on outer diameter

At every age, in both the VCN and the IC, the small capillaries with an outer diameter less than 8 μm formed the largest proportion of the total vessel profiles (Tables 1 a,b and 2 a,b).

In the VCN the percentage of these small-vessel profiles increased with age ($P < 0.018$). When a multiple comparison procedure was used to isolate which age(s) differ from the others, a difference was found between rats of 7 and 21 days, and 7 and 28 days ($P < 0.05$) but no significant difference was found in other pairwise comparisons. The percentage of medium calibre vessels, outer diameter equal to 8 or less or equal to 12 μm, decreased with age ($P < 0.007$) especially between 7 and 14 days. The largest-diameter group formed a very low proportion (about 2%) of the total profiles at every age, and so age-comparisons were not done.

In the IC the percentage of small-calibre vessel profiles

did not show a statistically significant difference among the different ages under study ($P > 0.05$) nor did the percentage of medium-calibre vessels. However the pattern of change resembled that observed for the VCN.

b- Vascular growth

The number of total vessel profiles per mm^2 increased with age in both the IC and VCN (ANOVA, $P < 0.001$) (Tables 3 a,b and 4 a,b). The first statistically significant difference (in pairwise comparisons) was found between rats of 7 days and 14 days of age ($P < 0.05$) in both regions. The one day old value did not differ from the 7-day value, but differed significantly from that of 14-day and older rats. In the IC, the vascularity of the 14 days rats, differed significantly from the 21 days rats while in the VCN these two groups did not differ with each other. When the IC and VCN were compared for vascular density at different ages, more vessel profiles per mm^2 were found in the VCN than in the IC in rats of aged one ($P < 0.0012$) and 7 days ($P < 0.0213$). For rats of 14 days or older, no difference in vascular density was observed ($P > 0.05$) between the two regions (Table 5). By 21 days, the numerical density of total vessels profiles had reached 481 per mm^2 in the VCN and 530 mm^2 in the IC, while the numerical density of vessel profiles with an outer diameter under 8 μm had reached by this age 463 per mm^2 in the VCN and 456 per mm^2 in the IC. The latter are average values calculated separately

by taking the number of vessel profiles with an outer diameter under 8 μm from each one of the 21 day-old rats (tables 1b and 2b) and divide them by the real areas calculated in tables 3b and 4b.

Fig. 4: Immunohistochemical staining with the monoclonal anti-GLUT-1 antibody of the rat inferior colliculus at 4 different post-natal ages: 1 day (**A**); 7 days (**B-C**); 14 days (**D**); and 28 days (**E**). The convex dorsolateral surface of the coronally sectioned inferior colliculus is evident in each photograph. Note an increase in vascularization with age, and a more radial orientation of the blood vessels in the first two ages than in the older ones. (**C**) 40 μm thick section of a 7 day-old rat in which the radially penetrating vessels from the leptomeningeal plexus are more evident than in the 10 μm thick ones (**A,B,D,E**). (**F**) Omission control of **E** showing no evident immunostaining. Scale bars = 200 μm

Fig. 5: Immunohistochemically-stained frozen sections (10 μm thick) of the rat ventral cochlear nucleus at 5 different post-natal ages: 1 day (**A**); 7 days (**B**); 14 days (**C**); 21 days (**D**); and 28 days (**E**). There is a notable increase in vascularization with age. (**F**) No specific staining is seen in this control experiment of **D** in which the primary antibody was adsorbed with the GLUT-1 C-terminal peptide P2. Scale bars = 100 μm

Fig. 6: (**A**): High magnification view of a frozen section illustrating the localization of GLUT-1 immunoreactivity in the capillary of a 1 day-old rat inferior colliculus. Note that the reaction seems to be restricted to the capillary endothelium with no apparent staining of the neuropil. Scale bar = 20 μm

(B): GLUT-1 immunostaining of a 28 day-old rat pineal (P) and the adjacent cerebral cortex (CC). Immunostaining is evident in capillaries of the cerebral cortex but not in the pineal gland. Scale bar = 20 μm

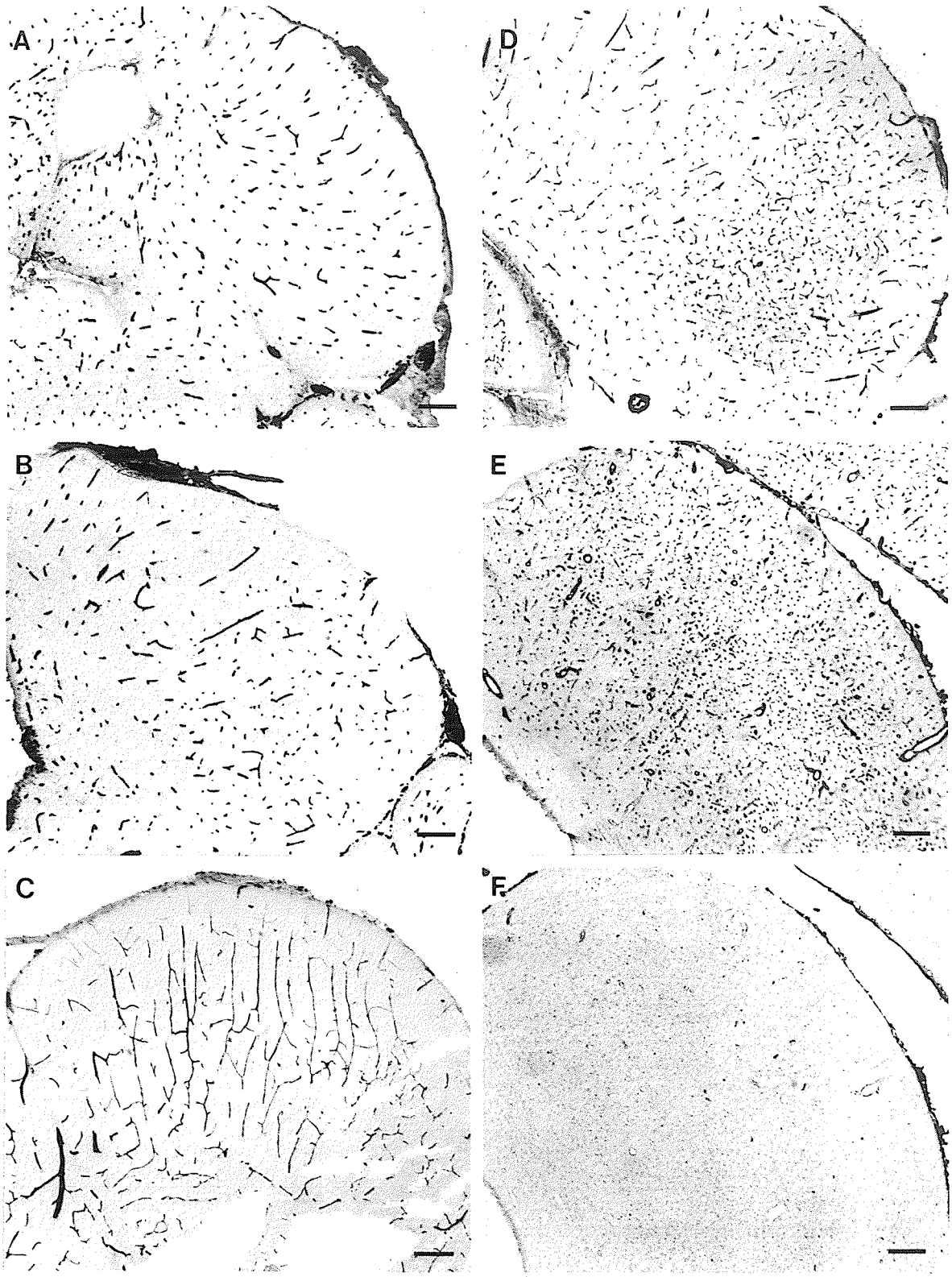
(C): High magnification view of a frozen section of the choroid plexus of a 28 day-old rat immunostained for the presence of GLUT-1. GLUT-1-like immunoreaction is found in the choroidal epithelium but not in the choroidal microvascular endothelium. **(D)** Adjacent section of choroid plexus stained in the same way as for **C** but in which anti GLUT-1 was replaced with fetal calf serum; this section shows no immunoreactive sites. iv, fourth ventricle. Scale bars **C,D** = 5 μm

(E): Immunostaining of GLUT-1 in ependymal cells lining the cerebral aqueduct (ca) of a 7 day-old rat. Notice that while some cells strongly stained, others did not show any immunoreactive sites. Scale bar = 20 μm.

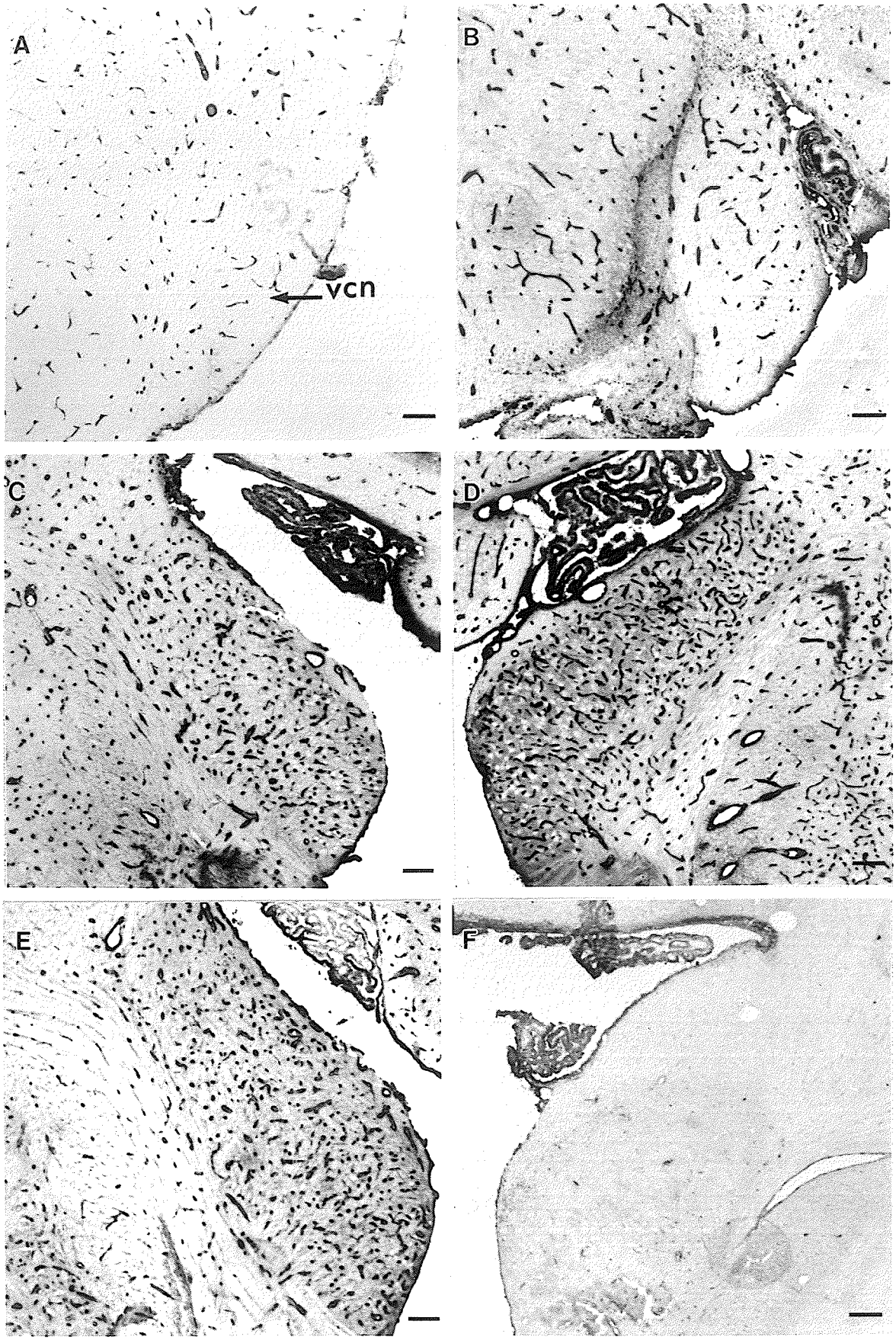
(F): Immunostaining of GLUT-1 in tanycytes of a 21 day-old rat at the level of the infundibular recess. Note that most, if not all, of the tanycytes stained in their membranes and basal processes. Capillaries also appear stained. iii, third ventricle. Scale bar = 20 μm.

Colour microphotograph: Immunostaining with antibodies to GLUT-1 in ependymal cells lining the fourth ventricle of a 1 day-old rat. Notice that only a few cells strongly stained (empty arrow). The endothelial cells of a microvessel are also immunostained (full arrow). Scale bar = 6.5 μm.

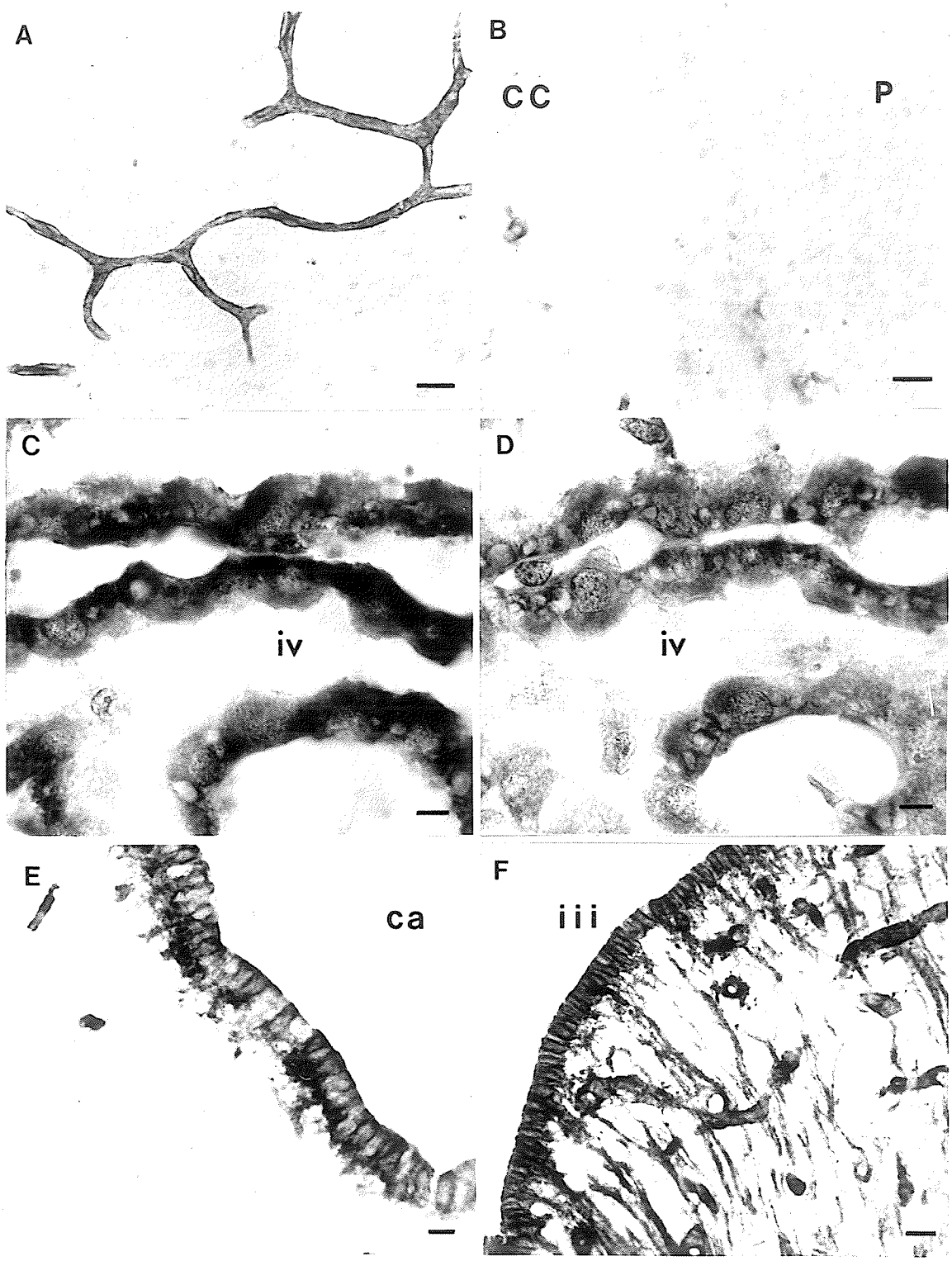
F. 4



F.5



F. 6



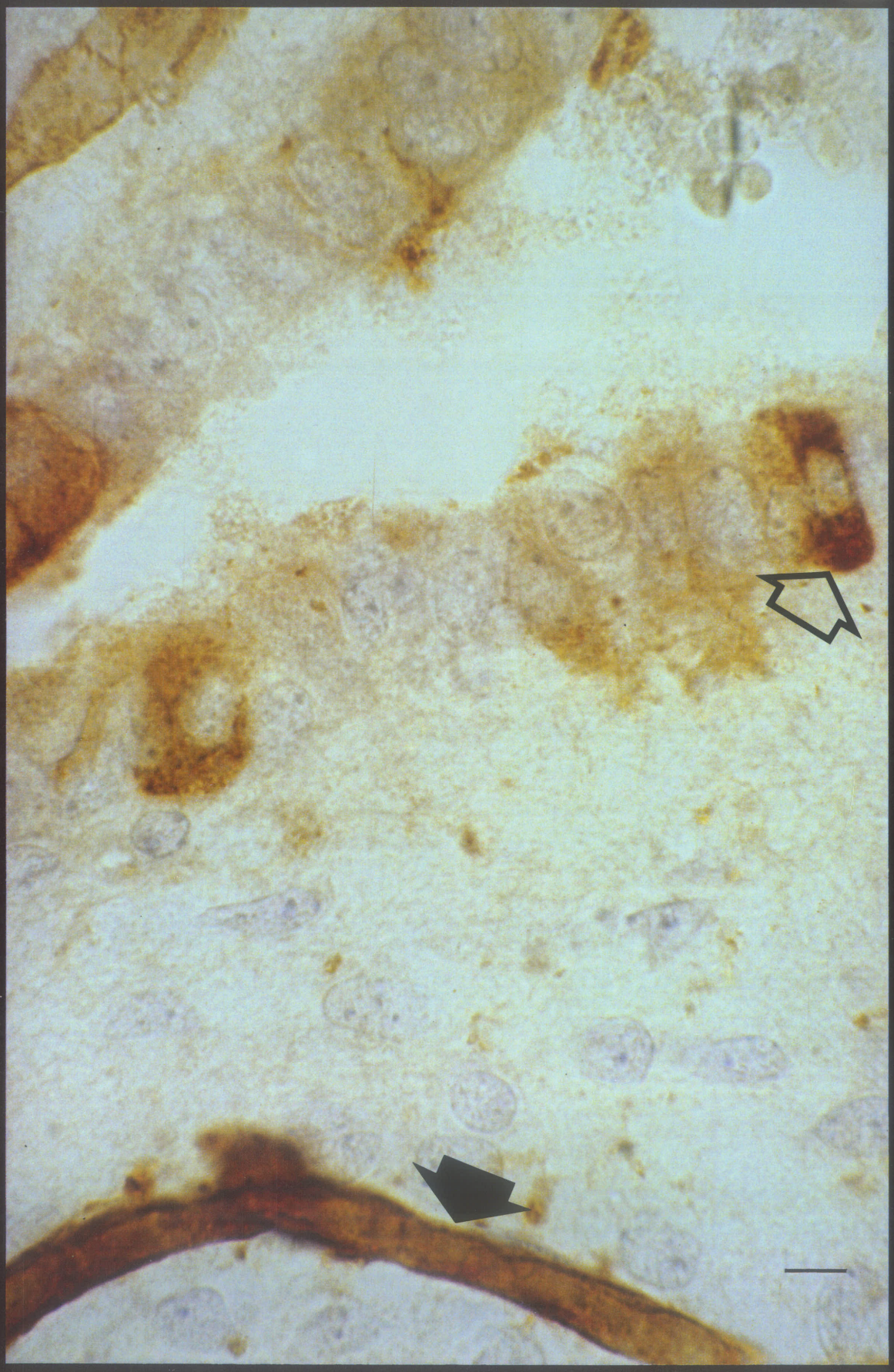


TABLE 1a. DISTRIBUTION OF OUTER DIAMETERS OF IMMUNOSTAINED VESSELS IN VENTRAL COCHLEAR NUCLEUS

AGE (days)		1				7			14		
RAT #		9162	9163	9166	9217	9164	9165	9239	9167	9208	9250
X < 8μm											
	#	28	31	29	38	46	60	40	170	135	289
	%	70.0	70.5	65.9	73.1	65.7	65.9	46.5	95.5	71.0	67.4
mean ± std. dev.		69.9 ± 3.0				59.4 ± 11.1			78.0 ± 15.3		
X = 8μm											
	#	5	6	8	6	12	11	5	2	17	47
	%	12.5	13.6	18.2	11.5	17.1	12.1	5.8	1.1	8.9	11.0
mean ± std.dev.		14.0 ± 3.0				11.7 ± 5.7			7.0 ± 5.2		
8μm < X ≤ 12μm											
	#	6	7	6	8	12	18	37	6	36	76
	%	15.0	15.9	13.6	15.4	17.1	19.8	43.0	3.4	19.0	17.7
mean ± std. dev.		15.0 ± 1.0				26.6 ± 14.2			13.4 ± 8.7		
X > 12μm											
	#	1	0	1	0	0	2	4	0	2	17
	%	2.5	0.0	2.3	0.0	0.0	2.2	4.7	0.0	1.1	4.0
mean		1.2				2.3			1.7		
TOTAL #		40	44	44	52	70	91	86	178	190	429

TABLE 1b. DISTRIBUTION OF OUTER DIAMETERS OF IMMUNOSTAINED VESSELS IN VENTRAL COCHLEAR NUCLEUS

AGE (days)		21			28		
RAT #		9210	9211	9243	9231	9253	9254
X < 8μm							
	#	187	87	371	183	248	322
	%	87.4	87.0	80.8	91.5	81.8	81.9
mean ± std. dev.		85.1 ± 3.7			85.1 ± 5.6		
X = 8μm							
	#	7	4	27	4	15	17
	%	3.3	4.0	5.9	2.0	4.9	4.3
mean ± std. dev.		4.4 ± 1.3			3.7 ± 1.5		
8μm < X ≤ 12μm							
	#	13	8	46	12	35	45
	%	6.1	8.0	11.3	6.0	11.5	11.5
mean ± std. dev.		8.5 ± 2.6			9.7 ± 3.2		
X >12μm							
	#	7	1	9	1	5	9
	%	3.3	1.0	2.0	0.5	1.7	2.3
mean		2.2			1.5		
TOTAL #		214	100	459	200	303	393

TABLE 2a. DISTRIBUTION OF OUTER DIAMETERS OF IMMUNOSTAINED VESSELS IN INFERIOR COLLICULUS

AGE (days)		1			7			14		
RAT #		9162	9166	9217	9164	9165	9239	9167	9208	9250
X < 8μm										
	#	107	43	33	93	154	81	196	150	101
	%	66.5	71.7	62.3	70.4	85.6	56.6	94.7	77.3	67.3
mean ± std. dev.		66.8 ± 4.7			70.9 ± 14.5			79.8 ± 13.9		
X = 8μm										
	#	23	7	8	21	11	15	5	15	7
	%	14.3	11.7	15.1	15.9	6.1	10.5	2.4	7.3	4.7
mean ± std. dev.		13.7 ± 1.8			10.8 ± 4.9			4.8 ± 2.4		
8μm < X ≤ 12μm										
	#	29	9	12	18	14	45	6	28	35
	%	18.0	15.0	24.5	13.6	7.8	31.5	2.9	14.4	23.3
mean ± std. dev.		19.2 ± 4.9			17.6 ± 12.4			13.5 ± 10.2		
X > 12μm										
	#	2	1	0	0	1	2	0	1	7
	%	1.2	1.7	0.0	0.0	0.6	1.4	0.0	0.5	4.7
mean		1.0			0.7			1.7		
TOTAL #		161	60	53	132	180	143	533	194	150

TABLE 2b. DISTRIBUTION OF OUTER DIAMETERS OF IMMUNOSTAINED VESSELS IN INFERIOR COLLICULUS

AGE (days)		21			28		
RAT #		9210	9211	9243	9231	9253	9254
X < 8μm							
	#	215	201	267	337	291	298
	%	86.0	90.1	80.4	93.6	80.8	82.8
mean ± std. dev.		85.5 ± 4.9			85.7 ± 6.9		
X = 8μm							
	#	12	7	23	3	16	17
	%	4.8	3.1	6.9	0.8	4.4	4.7
mean ± std. dev.		4.9 ± 1.9			3.3 ± 2.2		
8μm < X ≤ 12μm							
	#	19	15	37	17	46	35
	%	7.6	6.7	11.1	4.7	12.8	9.7
mean ± std. dev.		8.5 ± 2.3			9.1 ± 4.1		
X > 12μm							
	#	4	0	5	3	7	10
	%	1.6	0.0	1.5	0.8	1.9	2.8
mean		1.0			1.0		
TOTAL #		250	223	332	360	360	360

TABLE 3a. CHANGE WITH AGE IN NUMERICAL DENSITY OF ALL GLUT-1-POSITIVE VESSEL PROFILES IN VENTRAL COCHLEAR NUCLEUS

AGE (days)	1 day			7 days			14 days		
RAT #	9162	9166	9217	9164	9165	9239	9167	9208	9250
picture area (mm^2)	598.6	4209	916.7	8006	12650	1540	1282	1222	10472
Magnification	54 X	135 X	54 X	135 X	135 X	54 X	54 X	54 X	135 X
real area (mm^2)	0.20	0.23	0.31	0.44	0.69	0.53	0.44	0.42	0.57
vessel profiles (#)	40	44	52	70	91	86	178	190	287
vessel profiles / unit area	200.0	191.3	167.7	159.1	131.9	162.3	404.5	452.4	503.5
average vessel profiles / unit area	186.3 ± 16.7			151.1 ± 16.7			453.5 ± 49.5		

TABLE 3b. CHANGE WITH AGE IN NUMERICAL DENSITY OF ALL GLUT-1-POSITIVE VESSEL PROFILES IN VENTRAL COCHLEAR NUCLEUS

AGE (days)	21			28		
RAT #	9210	9211	9243	9231	9253	9254
picture area (mm^2)	1380	460.9	2434	1090	1443	2222
magnification	54 X	54 X	54 X	54 X	54 X	54 X
real area (mm^2)	0.47	0.16	0.83	0.37	0.49	0.76
vessel profiles (#)	214	100	459	200	303	393
vessel profiles / unit area	455.3	434.8	553.0	540.5	594.1	517.1
average vessel profiles / unit area	481.0 ± 63.2			550.6 ± 39.5		

TABLE 4a. CHANGE WITH AGE IN NUMERICAL DENSITY OF ALL GLUT-1-POSITIVE VESSEL PROFILES IN INFERIOR COLLICULUS

AGE (days)	1			7			14		
RAT #	9162	9166	9217	9164	9165	9239	9250	9167	9208
picture area (mm^2)	5098	2504	2745	3697	4918	3466	2291	17030	1823
Magnification	54 X	54 X	54 X	54 X	54 X	54 X	54 X	135 X	54 X
real area (mm^2)	1.75	0.86	0.94	1.27	1.69	1.19	0.79	0.93	0.62
vessel Profiles (#)	161	61	53	132	167	143	150	207	194
vessel profiles/ unit area	92.0	70.9	56.4	103.9	98.8	120.2	189.9	222.6	312.9
average vessel profiles/ unit area	73.1 ± 17.9			107.6 ± 11.2			241.8 ± 63.7		

TABLE 4b. CHANGE WITH AGE IN NUMERICAL DENSITY OF ALL GLUT-1-POSITIVE VESSEL PROFILES IN INFERIOR COLLICULUS

AGE (days)	21			28		
RAT #	9210	9211	9243	9231	9253	9254
picture area (mm^2)	1406	6704	2072	12460	2196	1643
magnification	54 X	135 X	54 X	135 X	54 X	54 X
real area (mm^2)	0.48	0.37	0.71	0.68	0.75	0.56
vessel profiles (#)	250	223	332	360	360	360
vessel profiles/ unit area	520.0	602.7	467.6	529.4	480.0	642.86
average vessel profiles/ unit area	530.1 ± 68.1			550.7 ± 83.5		

TABLE 5. COMPARISON IN VASCULAR DENSITY BETWEEN THE VCN AND THE IC

age (days)	average total vessel profiles / unit area		P level
	VCN	IC	
1	186.3 +/- 16.7	73.1 +/- 17.9	0.0011
7	151.1 +/- 16.7	107.6 +/- 11.2	0.0212
14	453.5 +/- 49.5	241.8 +/- 63.7	0.0821
21	481.0 +/- 63.2	530.1 +/- 68.1	0.5734
28	550.6 +/- 39.5	550.7 +/- 83.5	0.9983

Discussion

The brain microvessels of the rat have been shown to contain GLUT-1-like immunoreactivity in all the ages under study. The specificity of the monoclonal antibody B315:32 for the C-terminal region of the human red cell glucose transporter (GLUT-1) was demonstrated by the absence of staining following adsorption controls with the P2 peptide. This antibody did not appear to cross-react with GLUT-3, which was proposed to be the major neuronal glucose transporter in rodents (Maher et al, 1991 and 1992). Such a cross-reaction is unlikely since the C-terminal sequences of both isoforms are quite different and GLUT-3 does not contain any other obvious sequence similarity with the C-terminus of GLUT-1 (Bell et al, 1990).

A- Vessel classification and changes with age in the vascularization of the VCN and IC

The monoclonal antibody against the human erythrocyte GLUT-1, has been proven to be a good marker of rat brain small blood vessels as most, if not all, capillaries and small venules (less than or equal to 12 μm in outer diameter) have been found to show GLUT-1 immunoreactive sites. Hence this antibody could be used as a tool for quantitative studies in brain vascularization.

Bär (1980) classified capillaries in the CNS as having an

internal diameter equal or less than 7.5 μm in cross section. In our study the majority of the vessel profiles appeared to fall into this category. Our statistical analysis revealed that the percentage of small vessel profiles increased with age in the VCN while the percentage of the medium calibre vessel profiles decreased with age in this nucleus. The newly-forming vessels belong to the category with outer diameters equal or less than 8 μm, and their relative number increases rapidly during the first post-natal month. Bär (1980) observed for rat cerebral cortex that the range of internal diameters of microvessels in neonatal rats was approximately 3 to 12 μm whereas the range for adult rats was more narrow (peaks beteewn 4.5 and 6 μm). Also the vessel walls in the younger animals are thicker than those in the older ages. Most of the increase in capillary length takes place after post-natal day 20 in cerebral cortex, as a result of endothelial cell elongation (Bär, 1980), a process that brings down the thickness of the capillary wall and therefore its outer diameter. Even though we did not find these statistically significant differences in the IC, the pattern of change was very similar to the one observed in the VCN.

We have also noticed a change with age in the vascular density of the VCN and IC. Bär (1978) indicated that between post-natal days 8-20 there is a proliferation of capillaries by the processes of endothelial mitosis, elongation and by sprouting of preexisting vessels, that will determine the

differences in vascularity among the different regions of the brain. Gross and colleagues (1987) found that in adult rats the average number of capillary profiles in the IC is 433 per mm^2. In our study we found the average number of vessel profiles with an outer diameter under 8 μm to be around 456 in 21 day-old rats and hence the adult levels of vascularity in the rat had been reached in the IC by post-natal day 21.

When the vascularization of the VCN was compared to that of the IC, the former region showed a greater vascular density during the first post-natal week but thereafter there were no differences between both regions (table 5). As it was pointed out before, hearing did not appear to trigger vascular growth in the IC (Andrew and Paterson, 1989) as the increase in the number of vessels per unit area began before the onset of hearing which is at about 10 days of age. From the present results it would appear that this timing may also be true in the VCN. However the first significant difference in vascularization was found between 7-14 days of age and most of the vascular growth occurred during the second and third post-natal weeks and therefore some of the endothelial cell proliferation could be related to the activation of neurons in the auditory pathway.

B- GLUT-1 immunoresponse in other brain regions

Microvessels with fenestrae and gaps between cells, like the ones in the pineal and pituitary glands and choroid

plexus, did not contain immunoreactive sites detectable by the anti-GLUT-1 antibody. These have not been unexpected results; in non-barrier vessels polar molecules like glucose are able to diffuse passively across the wall; in barrier vessels such an important metabolite would need a specific carrier to cross the capillary wall. This was first predicted by Crone (1965) and confirmed by others (Harik et al, 1990; Pardridge et al, 1990).

While the choroidal vessels did not stain for GLUT-1, the choroid epithelium did stain in its basolateral membranes. This epithelium forms tight junctions providing a barrier separating the cerebrospinal fluid (CSF) from the blood (Brightman, 1977). It would appear that GLUT-1 is the isoform characteristic of barrier tissues as its presence has been described in renal tubules (Thorens et al, 1990), epidermis of the skin, and perineurium of peripheral nerves (Harik et al, 1990). The HT7 antigen, another BBB marker, has been also described to be present in the choroid plexus epithelium as well as in the renal tubules (Risau et al, 1986).

C- GLUT-1 in tanycytes

The presence of GLUT-1 has also been described in such structures lacking tight junctions as the capillaries of the testis (Harik et al, 1990) and in ependymal cells in the floor of the third ventricle (Harik et al, 1990; Farrell et al, 1992). In agreement with the latter finding, we have also

described the presence of GLUT-1 in certain ependymal cells not only in the floor of the third ventricle but also in the cerebral aqueduct, fourth ventricle and in the lateral foramen of the fourth ventricle.

The specialized ependymal cells in the ventral portion of the third ventricle, the tanycytes, feature long, radially oriented basal processes (for reviews see Bruni et al, 1985; Peters, Palay and Webster, 1991) that extend into cell groups of the hypothalamus and to the surfaces of blood vessels of the hypophysial-portal vasculature (Bleier, 1971). These processes are separated from the endothelial cells by two basal laminae and the perivascular space in between them. In considering the functions, tanycytes were described to have a supporting role in the brain, to be involved in the secretion of neurohormones and to provide an anatomical link for bidirectional transport of hormones between the CSF and the hypophysial-portal vasculature that may provide adenohypophysial regulation (Bleier, 1971; Bruni et al, 1985; Ugramov 1990; Peters et al 1991).

Tanycytes have also been encountered in the walls of the cerebral aqueduct, the fourth ventricle and the spinal canal (Burnett and Felten, 1981; Felten, Cummings and Burnett, 1981; Rafols, 1986 cited in Peters, Palay and Webster, 1991). This may explain our findings of certain ependymal cells to be GLUT-1 immunoreactive, in the cited regions.

The immunoresponse of the specialized ependymal cells to

GLUT-1 antibody shows that some kind of specific transport ought to exist in these cells, and as GLUT-1 was proposed to be the glucose transporter isoform of barrier tissues (Harik et al, 1990), tanycytes could be involved in the CSF-brain barrier.

CHAPTER 3: THE P-GLYCOPROTEIN

INTRODUCTION

A major problem facing chemotherapeutic treatments of human cancers is the cross-resistance of human tumors to multiple chemotherapeutic agents. While some cancers are curable by chemotherapy, others are not, and sometimes a patient who initially responds well to the chemotherapeutic treatment, has a relapse (Ling, 1989). The selection of tissue cultured cells for resistance to a hydrophobic natural drug such as colchicine, actinomycin D, or Adriamycin, results in a pleiotropic cross-resistance to these agents as well as to a variety of other drugs to which the cells were never exposed (Gottesman and Pastan, 1988). This discovery has led to speculations that similar multidrug-resistance tumor cells arise in vivo and selectively grow leading to a non-responsive type of cancer to any combination of chemotherapeutic agents (Ling, 1989).

More than a decade ago, Juliano and Ling (1976) isolated Chinese hamster ovary cells in vitro selected for resistance to colchicine. These cells also displayed pleiotropic cross-resistance to a range of other hydrophobic drugs. One feature of these cells is that they accumulated less drug than the drug-sensitive parental cell line. Surface labelling studies revealed the presence of a 170,000 daltons glycoprotein,

designated P-glycoprotein (Pgp), in the drug-resistance cells and a strong correlation was observed between the amount of Pgp and the degree of drug resistance. Pgp was proposed to be an energy-dependent drug efflux system which exports outside the cells hydrophobic drugs that have previously entered the cell by simple diffusion.

A- Identification of the Pgp gene

Multidrug-resistant Chinese hamster cells were shown to have amplified a common region of DNA which was found to contain a transcript unit that encodes a 4.5 Kb mRNA and the expression of this mRNA was correlated with multidrug-resistance in hamster cells (Gros, et al, 1986). The transcriptionally active DNA sequence was designated mdr. Later it was observed that human KB (HeLa) carcinoma cells, selected for resistance to either colchicine, vinblastine, or Adriamycin, contained two different DNA sequences similar to the hamster mdr, which were designated mdr1 and mdr2 (Roninson et al, 1986). The human cells that expressed mdr1 gene were also shown to express Pgp, and cDNA probes encoding Pgp cross-hybridized with mdr1 cDNAs (Ueda et al 1986). Transcripts of mdr2 were found in human liver but its function is yet not known (Gottesman and Pastan 1988). The conclusion was that Pgp is the product of the mdr1 gene in human cells. Both mdr1 and mdr2 were assigned to chromosome 7q (Fojo et al, 1986). The Pgp gene family consists of three genes in hamsters and mice

but only two in primates (Ng et al, 1989).

Gros and others (1986) have demonstrated that when isolated mdr cDNA extracted from mouse drug-sensitive cells is transfected to other drug-sensitive cells, the latter become multidrug-resistant. Because the cDNA was isolated from drug-sensitive cells, overexpression of the mdr genes was associated with the multidrug-resistant phenotype.

B- Molecular biology of Pgp

As noted above, the human mdr1 gene is the one responsible for encoding Pgp. The cDNA sequence of this gene was obtained from a multidrug-resistant KB cell line (Chen et al 1986). The analysis of the protein encoded by the mdr1 gene, revealed a 1280-amino acid polypeptide that consists of two homologous parts which share 43% identity. The analysis also predicted 12 transmembrane domains. Two intracytoplasmic segments, one in each half of the molecule, were found to be highly homologous. These segments were described as potential ATP-binding sites (Chen et al, 1986). The deduced sequence of the mouse Pgp was found to be 80 % identical to that of the human and the greatest homology was in the putative ATP-binding sites and in other cytoplasmic regions perhaps involved in drug binding (Gottesman and Pastan, 1988). Glycosylation sites were located in the first extracytoplasmic loop of the polypeptide (Chen et al, 1986).

C- Homology to bacterial transport proteins

Extensive homology between Pgp and several bacterial transport proteins have been detected, like hisP (Salmonella) and HlyB (E. coli) (Gerlach et al, 1986; Chen et al, 1986). An allpairwise alignment of Pgp, hisP, and HlyB proteins, indicated that the highest level of homology was observed in the putative ATP-binding sites. The bacterial proteins are membrane-associated components of high-affinity, substrate-binding dependent, active transport systems, suggesting that Pgp may also couple ATP hydrolysis to transport drugs outside the cell in a similar way to the bacterial transport systems.

D- Functional model of Pgp

The predicted transmembrane domains, the presence of ATP-binding sites, and the homology with the bacterial active-transport system, are consistent with the proposed function of Pgp in the plasma membrane as an efflux pump for the export of molecules from the cell, and through a channel formed by the transmembrane segments, by coupling energy obtained from the hydrolysis of ATP (Gerlach et al, 1986; Chen et al, 1986). The orientation of Pgp in the plasma membrane, with the C-terminus in the cytoplasmic side of the cell, was based on evidence that monoclonal antibody C219 is directed against a conserved cytoplasmic domain in the C-terminal region of Pgp (Kartner et al, 1985) and on the assumption that the ATP binding sites are also cytoplasmic.

E- Expression of Pgp in normal tissues and in tumors

Using the human mdr1 cDNA as a probe, the levels of mdr1 mRNA in normal tissues, were detected to be high in the kidney and the adrenal gland; intermediate in the liver, lung, lower jejunum, colon and rectum; and low in the brain, prostate and skin (Fojo et al, 1987). With the same procedure, more than 400 human cancers were tested for the presence of mdr1 mRNA and four groups of cancers were identified: a) non-treated cancers that usually express high levels of mdr1 mRNA including colon carcinoma, renal cell carcinoma and hepatoma; b) non-treated cancers that occasionally express high levels of mdr1 mRNA like acute lymphocytic leukemia, non-Hodgkin's lymphoma and neuroblastoma; c) non-treated cancers with low levels of the mdr1 gene transcript product - breast cancer, bladder cancer and non-small cell lung cancer-; and d) cancers with high levels of mdr1 mRNA expression after chemotherapeutic treatments and included in this group are non-Hodgkin's lymphoma, neuroblastoma and breast cancer (Goldstein et al, 1989). The conclusion drawn from the last study, was that Pgp expression can be detected in a variety of different cancers and that the measurement of mdr1 mRNA can be used as a tool in designing or altering chemotherapeutic protocols.

With the production of specific monoclonal antibodies, it was possible to study the expression of the mdr1 gene product in normal tissues and in a number of cancers. The table below

presents normal tissues in which Pgp was detected by immunohistochemistry.

Tissue and cell type	**Species**	**Ref.**
Skin		
sweat glands	human	*
papillary dermis endothelial cells	human	*,**
acrosyringeum	human	*
brain		
endothelial cells	human, rat, hamster	*,**,æ, ⌂ ¥
Reproductive system		
testes endothelial cells	human, rat	**, ⌂
	hamster	¥
seminal vesicles	human, rat	⌂
	hamster	⌟
uterus	human, hamster	*,¥
Breast		
mammary glands	human	*
Lung		
bronchial cells	human	*
Digestive system		
small intestine (lining epithelium)	human, hamster	*,¥
large intestine (lining epithelium)	human, rat	*,⌂
	hamster	¥,⌟
Liver		
bile canaliculi	human, rat	*,⌂
hepatocytes	human, hamster	*,¥,⌟
Pancreas		
acinar cells	human, rat	*,⌂
ductal cells	human	*
Kidney		
proximal tubular epithelium	human, rat	*,æ,⌂
Adrenal gland		
cortical cells	human, hamster	*,æ,⌂, ¥,⌟
Skeletal muscle	human, rat	*,⌂
	hamster	¥,⌟
Cardiac muscle	human, rat	*,⌂
	hamster	¥
Placenta		
trophoblast	human	*,æ

¥ Bradley et al, 1990
* Cordon-Cardo et al, 1990
** Cordon-Cardo et al, 1989
⌟ Georges et al, 1990
æ Sugawara et al, 1990

⌂ Thiebaut et al, 1989

F- Functional role of Pgp in normal tissues

The physiological role of Pgp in normal tissues, can be seen as a protective system against a wide range of exogenous and endogenous toxic substances, and it may function in a similar way as in multidrug-resistant cells.

Consistent with its proposed function, Pgp expression was found in the luminal lining of the large and small intestines and on the biliary ducts (see table).

An interesting observation was to see the presence of the mdr1 gene product in such glandular cells as the adrenal cortical cells, and in intestinal lining epithelial cells, epithelial cells of the proximal tubules of the kidney, hepatocytes, all of which are thought to secrete hormones and metabolites. In the adrenal gland, Pgp was proposed to be involved in steroid transport (Thiebaut from Bradley, 1987), but Bradley and co-authors (1990) noticed in hamster a sex-dependent immunoexpression of Pgp in the adrenal gland cortical cells; the strong expression of Pgp was only seen in the adrenal cortex of male hamsters. This finding argued against a general function of Pgp in steroid transport. These researchers suggested instead that Pgp may act as a more specific steroid transporter or it may be involved in an unknown sex-dependent transport function of the adrenal cortex.

In cardiac and skeletal muscle Pgp may function in their

membranes as a transporter of metabolites (Bradley et al, 1990).

The most relevant observation to this thesis was the presence of Pgp in the capillaries at blood-tissue barriers, like the central nervous system, the testes and the papillary dermis, and it has been suggested that in barrier capillaries Pgp may play a role as a general detoxification system by preventing the entrance of harmful hydrophobic molecules, such as certain xenobiotics, from the blood into the tissues(Cordon-Cardo et al 1989).

In the human brain, Pgp-like-immunoresponse was found on the endothelial cells of capillaries but not on the endothelium of arterioles,larger blood vessels, and choroid plexus (Cordon-Cardo et al, 1989). In the hamster, Pgp immunostaining was observed not only in the capillaries but also in small arterioles and venules but it was not detected in large arterioles and venules (Bradley et al, 1990).

All of these studies were done on adult specimens. In this work we will report a detailed description of the sites of Pgp immunoreactivity in the brain of the young postnatal rat.

Materials and methods

1- Animals, tissue fixation and preparation: Three Sprague-Dawley rats for each of five postnatal ages- 1, 7, 14, 21, and 28 days- were used. The animals were anaesthetized with isoflurane (Forane, Anaquest) and the brains removed and immediately frozen onto chucks in 2-methyl-butane (Mallinckodt) precooled in dry ice. Frozen sections (10 μm) were cut in the coronal plane, mounted on subbed slides, fixed in 100% cold acetone for 10 minutes and air-dried. Alternatively, brain sections from animals that were perfused through the ascending aorta with 4% paraformaldehyde, were also cut. The tissues collected included the VCN, IC, MG of the thalamus, AC, choroid plexus. Sections of the liver and P-glycoCHECK control slides (Centocor, Malvern, PA) were used as tissue controls to confirm that the reagents worked. The P-glycoCHECK control slide contains two acetone fixed cell preparations from human leukemia cell lines, one is a positive staining control (drug- resistant) and the other is a negative staining control (drug-sensitive).

2- Immunohistochemical staining: Alternatively, the Peroxidase anti-peroxidase (PAP) (Sternberger, 1979) and the Avidin-biotin-peroxidase (Hsu, 1981) using the ABC (Elite) kit (Vector Laboratories, CA), methods were performed. A detailed description of the PAP method can be found in appendix 3-2.

3- Antibodies: The primary antibody used was the mouse monoclonal antibody (mab) C219 (Centocor, Malvern, PA)

directed against a conserved intracytoplasmic domain in the C-terminal region of Pgp. This mab, provided as an ascites (100 μg/ml specific antibody), was used for immunohistochemistry at final concentrations of 10 μg/ml or 20 μg/ml in 0.1 M PBS (pH 7.4). As a positive control, purified mouse monoclonal antibody against glial fibrillary acidic protein (GFAP), obtained as an ascites from Amersham (U.K.), was used. As negative controls, the primary antibody was omitted, or replaced by irrelevant ascites fluid obtained from a mouse, or replaced by purified mouse IgG (Zymed) used at the same working dilution as the mab C219. The secondary and tertiary antibodies used for the PAP method were (i) goat anti-mouse IgG and (ii) mouse peroxidase anti-peroxidase (Jackson Immunoresearch Laboratories, PA). For the Avidin-biotin-peroxidase method the secondary antibody used was the biotinylated rabbit anti-mouse IgG (Zymed, CA) followed by the avidin-biotin-peroxidase complex (Vector Laboratories, CA).

Results

We utilized the mouse monoclonal antibody C219 to immunolocalize Pgp expression in brain sections from the young postnatal rat. Pgp immunoreactivity was positive in fresh frozen tissues but it was lost in paraformaldehyde fixed tissues; therefore the results to be described in the sections below, correspond to the ones obtained with fresh frozen tissues post-fixed in cold acetone. No apparent difference in the intensity of the stain was observed between the two different antibody concentrations used.

1- Immunohistochemical method: variable results according to commercial lot of C219 monoclonal antibody

The immunohistochemical methods that we used to study Pgp expression included the PAP and the Avidin-biotin-peroxidase. Both methods have given satisfactory results -low nonspecific staining and good specific signal when compared to control experiments- and nonsatisfactory results -heavy background, poor vessel staining, and nonspecific neuronal staining. One interpretation for these results, is that fresh frozen tissues tend to be more "sticky" than the fixed tissues and hence there is an increase of the nonspecific staining. But our results obtained using sections treated in parallel with the monoclonal antibody to GFAP, were in most of the cases clean in background and with a strong signal in the positively

immunostained cells. This result suggested that the primary antibody rather than the subsequent steps may have been the source of nonspecific stain. Many of our most reliable results were obtained from one lot of mab C219 (obtained as a gift from Dr. McKenna). With the other three lots of mab C219, we obtained heavy nonspecific staining, and poor or no staining on the endothelial cells. Improvement in the staining was accomplished when Triton 100 X was included in the blocking step. The epitope for mab C219 is located on the cytoplasmic side of the plasma membrane, and so it is necessary to permeabilize the cells to make the internal epitope accessible to the antibody. However, the efforts to do so by delipidization with alcohols did not improve Pgp detection.

2- Pgp in the auditory brainstem and cerebral cortex

In the auditory brainstem and cerebral cortex of the rat, Pgp-like immunoreactivity was observed on the endothelial cells of most, if not all, capillaries, and also in some arterioles and venules of up to 15 μm of outer diameter, at all postnatal ages under study. No specific immunoreaction was detected in arteries and veins, nor in neurons. Glia cells were found not to contain antigenic sites detectable by the mab C219 (figs. 7 and 8).

At high magnification (100 X) the immunostaining of the endothelial cells appeared to be located on the plasma membrane (fig 8a).

In the experiments where the primary antibody was omitted, or substituted by irrelevant ascites fluid or purified mouse IgG, the sections did not show any specific immunostaining. Serial sections to the ones stained for Pgp expression, were stained in parallel with the mouse mab anti-GFAP, used as a positive control for the reagents as well as to evaluate the specificity of the mouse mab C219. In the brainstem of the rat, GFAP-positive cells were found at all ages under study, forming the glia limitans under the pia mater, and in the neuropil surrounding the blood vessels. Endothelial cells, neurons and ependyma cells, were found to be GFAP-negative (figs. 1d, 2b, and 2e). Positive staining with mab C219 was obtained in the positive drug-resistant tumor cells on the control slides while no staining of the negative drug-sensitive cells was observed. In the liver, the bile ducts were found to be Pgp immunoreactive (fig. 2c). These controls helped confirm the validity of immunostaing with the mab C219.

3- Pgp in other brain regions

Blood vessels of the choroid plexus and pineal gland did not show any specific Pgp-immunostaining.

The ependymal cells lining the third ventricle, cerebral aqueduct, the fourth ventricle and its lateral foramen, showed a strong Pgp immunoreaction, which was located in the apical membrane of these cells (figs. 7c and 8d). This immunoreaction on the ependymal cells, seems to be age-dependent as ependymal

cells in 1 day-old animals did not show Pgp-like immunoreactivity.

Fig. 7: Immunoperoxidase staining of Pgp in early post-natal rat brain. (**A**) Inferior colliculus of a one day-old rat. (**B**) Inferior colliculus of a 21 day-old rat. (**C**) Ventral cochlear nucleus of a 28 day-old rat. The brain capillary endothelium is immunoreactive for Pgp at all the ages under study. The arrow in **C** shows immunostaining in ependymal cells of the lateral foramen of the fourth ventricle. (**D**) Immunostaining of GFAP in the ventral cochlear nucleus of a 28 day-old rat. Scale bars = 100 μm.

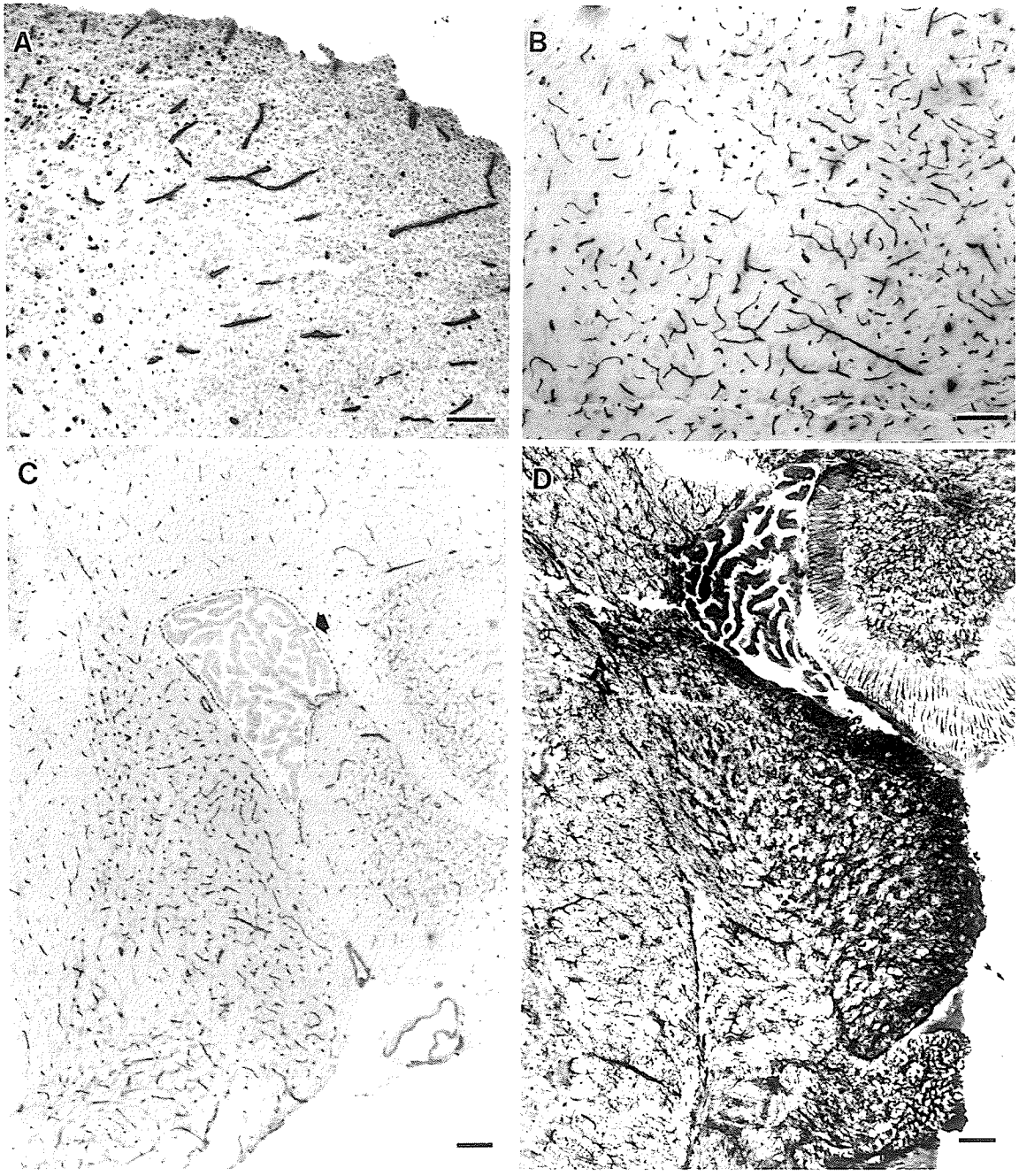
Fig. 8: (**A**) High magnification view of a frozen section of a one day-old inferior colliculus in which capillary profiles are immunostained for Pgp. Scale bar = 20 μm.

(**B**) High magnification view of GFAP-positive astrocytes surrounding a brain capillary in the pons of a 14 day-old rat. Scale bar = 20 μm.

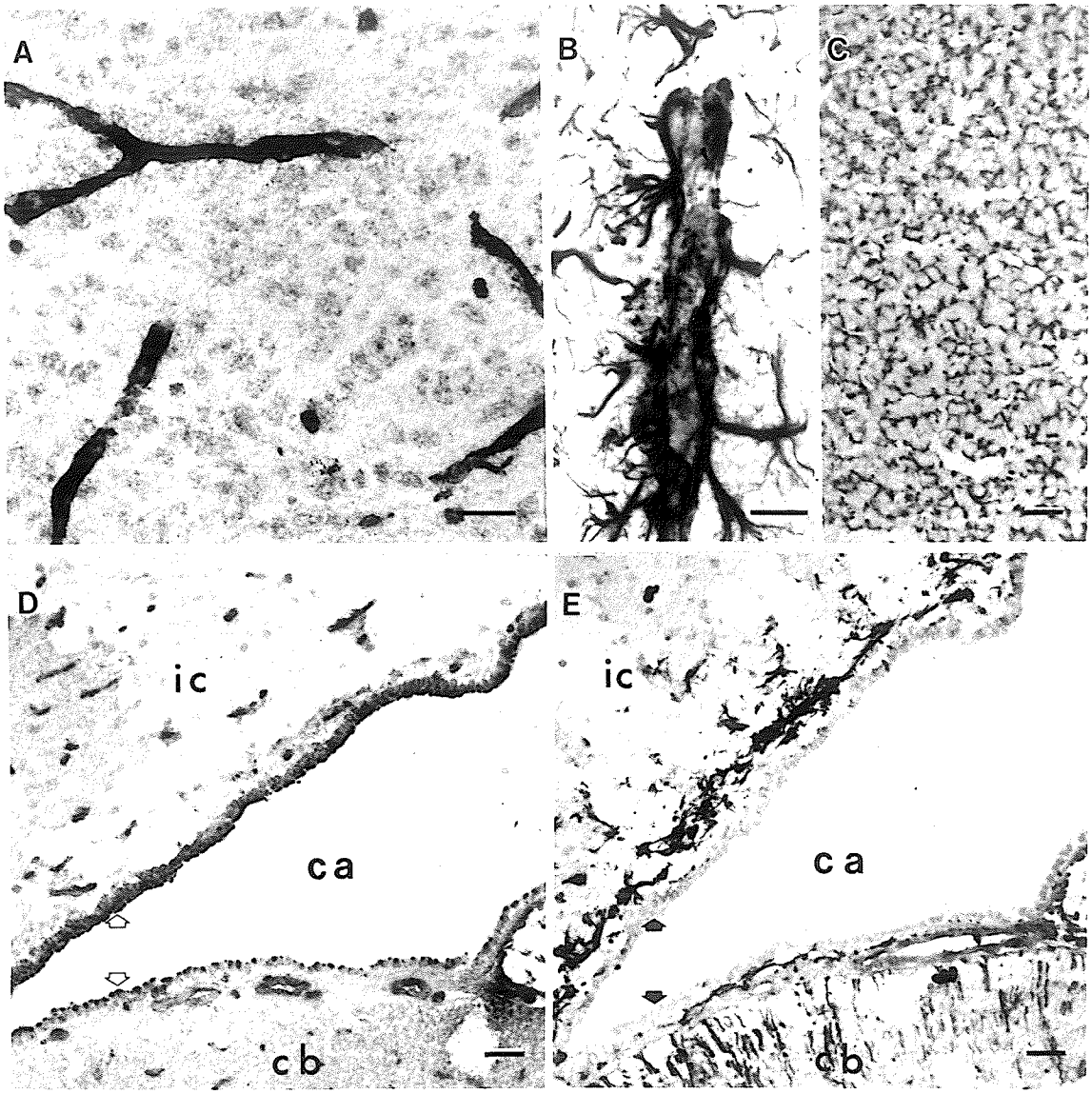
(**C**) Pgp-like immunostaining of a 28 days rat liver showing immunoreactive sites in the bile ducts. Scale bar = 100 μm.

(**D**) Ependymal cells lining the cerebral aqueduct of a 21 day-old rat showing immunoreactivity for Pgp at their apical end (empty arrows). (**E**) Serial section stained for GFAP. Staining is found in cells under the ependymal epithelium but not in the ependymal layer itself (full arrows). ca, cerebral aqueduct; cb, cerebellum; ic, inferior colliculus. Scale bars **D,E** = 100 μm.

F. 7



F. 8



Discussion

A- Immunohistochemical method

Weinstein and co-workers (1990) observed that Pgp immunoexpression in normal colon and ureter, as detected with the mouse mabs C219 and JSB-1 directed against different internal cytoplasmic epitopes, correlated to tissues obtained from blood group A individuals. Later another study demonstrated that some of the lots of mabs C219 and JSB-1 contained antibodies against group A determinants, which are carbohydrate structures (Finstad et al, 1991). Gooi and Feiji (1982 cited in Finstad et al 1991) showed that mouse sera and ascites fluid, contain naturally occurring anti-carbohydrate antibodies and it is quite possible that these antibodies were co-purified with the commercial Pgp mabs supplied in ascites fluid. As it was pointed out by Finstad and associates (1991) endothelial cells and most epithelial cells are known to carry the A,B, and/or O (H) blood groups antigens and this may explain the apparent staining of neurons obtained with three of our four working lots of mabs. The vascular staining was consistently present with all four lots. The suggestion was to run complementary methods such as in situ hybridization using an mdr1 cDNA probe to confirm the results obtained for Pgp expression using mabs.

The Pgp antigen appears to be susceptible to paraformaldehyde fixation, alcohols, and enzymatic digestion

to unmask fixed antigens (Cordon-Cardo, 1990). Therefore when using the mab C219 for the immunodetection of Pgp, it is recommended to use fresh frozen tissues.

B- Immunolocalization of Pgp in the developing rat brain

In this study we have found the presence of Pgp-like-immunoreaction in the brain microvessels of rats aged one to 28 days.

Previous work has not studied the expression of Pgp during the first post-natal month of the rat.

Like Cordo-Cardo and associates using human tissues (1989), we have found expression of Pgp on endothelial cells of capillaries at the blood-brain barrier sites, but unlike them and in agreement with work done in hamsters (Bradley et al, 1990), we have also found mab C219 immunoresponse in small arterioles and venules, no larger than 15 μm of outer diameter. As pointed by Bradley, Georges and Ling (1990), this finding is surprising as arterioles and venules are not expected to be involved in the blood-brain barrier.

Another unexpected finding of the present work, was the presence of Pgp-like-immunoreaction in ependymal cells of the third ventricle, cerebral aqueduct, and the fourth ventricle and its lateral foramen. It looked like most if not all the ependymal cells immunoreacted in their apical surfaces. Ependymal cells proper, to differentiate from the specialized tanycytes, are not known for being a barrier tissue as they

are not joined together by tight junctions but they were shown to have in their cilia, endogenous brain lectin which may allow the cilia to trap elements within the CSF that should not normally be there (Peraud et al, 1988, cited in Peters, Palay and Webster, 1991). Pgp may serve in the ependymal cells to transport back into the CSF noxious substances that have previously diffused into these cells.

General Discussion

A- Histochemical and immunohistochemical methods

Each of the procedures utilized for the visualization of the three markers under study, was found to have individual requirements in order for the method to work well.

BuChE was found to be susceptible to the time of incubation and to the pH of the incubation medium. Like Whitaker and La Bella (1972) we have found that an increase in the incubation time, gave an excessive accumulation of reaction product in sites like the intermediate lobe of the pituitary, vessels of the choroid plexus, and neurons, which made difficult the visualization of the results. Similar results were obtained when the pH was increased. Therefore, for tissues with high BuChE activity, like the brain, a pH of 5.5 and an incubation time of 18 hrs. at 4 ^{0}C, are recommended.

Because the antigens GLUT-1 and Pgp are located in the cytoplasmic side of the plasma membrane (Gerlach et al, 1986; Andersson and Lundahl, 1988), it was not surprising to find an improvement in the immunohistochemical staining with the addition of a detergent like Triton 100 X in the blocking step. This improvement became more evident in the older ages (14, 21, and 28), when more nerve fibres are covered by myelin. The myelin fibres were found to increase the level of "noise", or nonspecific staining, and to mask the reaction in the blood vessels that run across the white matter.

Antigenic sites detectable by mab C219 were found to be damaged by paraformaldehyde fixation, ethanol, and methanol. Immunolocalization of GLUT-1 was also found susceptible to damage with paraformaldehyde fixation alone, and like mab C219, localization of GLUT-1 was consistent using fresh-frozen, acetone fixed tissues. Considerable improvement in GLUT-1 immunostaining was achieved with the introduction of the Bouin's solution as the fixative: the ratio of the "signal" of the reaction to "noise" was higher than the one obtained with fresh-frozen tissues and higher than the one obtained with mab C219. BuChE histochemistry had also given a high "signal"/"noise" ratio, but because it was performed on 40 μm thick sections and the reaction product mainly localized in the nuclei of the endothelial cells, BuChE is not a good marker for the identification of blood vessel profiles. Based on these observations, GLUT-1 is the most suitable marker for the identification of blood vessel profiles in the rat brain; hence it was the marker of choice for our quantitative studies.

B- Localization of each "marker" in BBB type capillaries

BBB type capillaries have been shown to express BuChE activity, and to be GLUT-1 and C219 immunoreactive at all the post-natal ages examined in the brain of rat. The expression of these proteins in early post-natal ages in the microvessels of the rat, before the BBB, as defined by vascular

impermeability to horse radish peroxidase (Reese and Karnovsky, 1967), is fully developed, shows that the young endothelial cells of the rat brain already have some of the chemical characteristics of the mature endothelial cells that form part of the BBB. The brain environment seems to induce the differentiation of endothelial cells. Stewart and Wiley (1981a) have demonstrated that endothelial cells that have invaded transplanted brain tissue, acquired brain-endothelium characteristics. The expression of BuChE, GLUT-1, and Pgp in the brain endothelial cells, might be induced by the surrounding brain tissue during BBB development, as early as E13 in the rat (Dermietzel, 1992). There is evidence that the developing neuroepithelial cells induce BBB development (Holash and Stewart, 1992, personal communications).

BuChE was the only "marker" to be found in non-BBB type microvessels, as its activity was localized in the blood vessels of the choroid plexus. BuChE end product was also demonstrated in blood vessels of the area postrema of the rat (Karcú et al, 1977) and the guinea pig (Trancard et al, 1989) so that a parallelism between BBB function and BuChE activity can not be concluded.

C- Localization of BuChE, GLUT-1, and Pgp expression in other brain cell types

Not only endothelial cells were stained for the presence of these "markers", but also other brain cell types did.

Pgp was mainly found in brain capillaries but immunoreactivity was also noticed in most, if not all, ependymal cells of rats aged 14, 21 and 28 days, and a few in 7 day-old rats, although the immunostaining at this age seems to be weaker than in the older ages. Thus Pgp expression is not exclusive for barrier tissues. Horseradish peroxidase injected into the brain ventricles, fails to cross the ependymal tanycytes overlying the median eminence and the area postrema but this marker permeates between ependymal cells elsewhere in the brain ventricles (Reese and Brightman, 1968). The reason for this difference in horseradish peroxidase permeability is that simple ependymal cells are connected by gap-junctions (Brightman and Reese, 1969) while tight junctions as well as gap-junctions connect tanycytes to each other (Reese and Brightman, 1968). Therefore in brain regions in which the capillaries do not have BBB characteristics, like the choroid plexus, median eminence and area postrema, it is the overlying epithelium which has barrier properties to maintain separation of the CSF from blood (Brightman and Reese, 1969). The presence of Pgp in ependymal cells, would give to this epithelium a "barrier" characteristic that may serve for the protection of the underlying neural tissue from the exposure to noxious substances that may reach the CSF.

Apart from being found in brain and choroid plexus microvessels, BuChE end-product was also detected in some neuronal nuclei of the brain stem, in cells in the

intermediate and neural lobes of the pituitary, and in meningeal blood vessels. BuChE activity in the choroid plexus and meningeal blood vessels as well as in the neurons, seems to be age related. In the first two regions the younger the rat the stronger the BuChE activity found, while in the neurons, BuChE activity was first seen at post-natal day 7.

Table 6 shows that of the three proteins under study, GLUT-1 was the only one to be detected exclusively in barrier tissues of the brain: BBB type capillaries, choroid plexus epithelium, ependymal tanycytes located in the ventral portion of the third ventricle. GLUT-1 immunoreaction was also described in the epithelium of the ascending loop of Henle, and the epidermis of the skin, both of which possess occluding junctions,and in the testis capillaries proposed to have some barrier properties also (Harik et al, 1990).

TABLE 6. EXPRESSION OF BuChE, GLUT-1, AND Pgp IN DIFFERENT CELL TYPES OF THE YOUNG RAT BRAIN

age (days) ▸	1			7			14 - 21 - 28		
Cell types ▾	BuChE	GLUT-1	Pgp	BuChE	GLUT-1	Pgp	BuChE	GLUT-1	Pgp
endothelial (BBB-type)	+	+	+	+	+	+	+	+	+
endothelial (non-BBB type)	+	-	-	+	-	-	+	-	-
ependymal	-	+	-	-	+	±	-	+	+
neurons	-	-	-	+	-	-	+	-	-
glial	-	-	-	-	-	-	-	-	-
ChPl epithelial	-	+	-	-	+	-	-	+	-

+ strong staining, ± weak staining, - undetectable staining.

Conclusions

1- The presence and localization of three proteins - butyrylcholinesterase, the glucose transporter isoform GLUT-1, and the P glycoprotein- proposed to be BBB "markers" was studied in the brain of the rat during post-natal ages 1, 7, 14, 21, and 28 days, a period during which much of the brain growth and development take place.

2- In the brain of the rat,

- The expression of these "markers" was confirmed in BBB-type capillaries at all ages examined;
- BuChE activity was not found to be present exclusively in BBB capillaries, as blood vessels in the choroid plexus have also expressed BuChE activity. Also individual neurons in the vestibular nucleus, the nucleus of the spinal tract of the trigeminal nerve, the dorsal tegmental nucleus, the parabrachial nucleus, the Edinger-Westphal nucleus and the interstitial nucleus of the ventral tegmental decussation,have been demonstrated to have BuChE activity;
- GLUT-1-like immunostaining was found exclusively in blood-tissue barriers;
- Pgp-like-immunoreaction was present only in BBB type capillaries, although a few small arterioles and venules (no more than 15 μm of outer diameter) have also immunoreacted, and in ependymal cells lining the third ventricle, cerebral aqueduct, and the fourth ventricle and

its lateral foramen.

3- Quantitative studies obtained with GLUT-1 have shown that:

. most of the immunostained blood vessels of the rat brainstem are capillaries (outer diameter less than 8 μm),

. The number of vessel profiles with an outer diameter less than 8 μm in the inferior colliculus of 21 day-old rats, was similiar to that reported for the central region of this nucleus in the adult. It was concluded that adult levels in vascularity have been achieved in the rat inferior colliculus by post-natal day 21,

. there was an increase with age in the total number of blood vessel profiles per unit area in both the ventral cochlear nucleus and the central region of the inferior colliculus,

. the vascular density of the VCN is greater than the vascular density of the IC only during the first two post-natal weeks of the rat (differences statistically significant). By the beginning of the third post-natal week of age, the differences in vascular density between these two regions were not significantly different.

Appendix 1-1: Solutions for BuChE Histochemistry

1) 0.1 M Acetate buffer

Stock solution A:

Sodium Acetate	1.36 gm
Distilled water	100.0 ml

Stock solution B:

Glacial Acetic Acid	0.58 ml
Distilled water	100.0 ml

Mix 86 ml of solution A and 14 ml of solution B

Adjust to Ph 5.5.

2) Substrate solution

Butyrylthiocholine iodide (Sigma)	43 mgm
distilled water	1.8 ml
0.1 M Cupric Sulphate (MW 249.686)	0.6 ml

Centrifuge for 20 min. Save supernatant. Store at 4 °C.

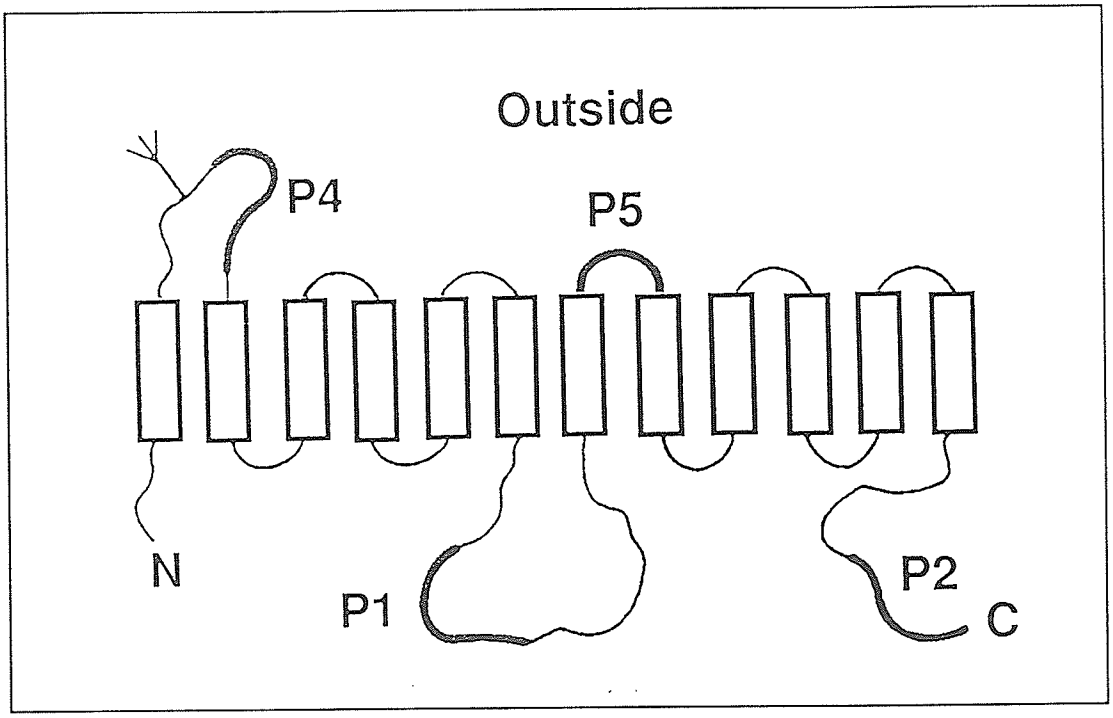
3) Incubation Medium

0.1 M acetate buffer	10.0 ml
substrate solution	1.6 ml
3.75% Glycine	0.4 ml
0.1 M cupric sulphate	0.4 ml
distilled water	7.6 ml

4) Developing Solution

Ammonium sulphide	1.0 ml
0.1 M acetate buffer	25.0 ml

Appendix 2-1: Structural model of the glucose transporter isoform GLUT-1



Model for orientation of the erythrocyte/brain type glucose transporter GLUT-1 in the cell membrane. The rectangles represent the twelve putative transmembrane domains. The location of binding epitopes for monoclonal antibodies against GLUT-1, are shown as segments P1, P2, P_4, and P_5. The monoclonal antibody utilized in this study, B315:32, is directed towards the C terminus of the glucose transporter (P2).

Appendix 2-2: The Avidin-biotin-peroxidase immunostaining method for GLUT-1

1. If the tissue sections are stored in the freezer, bring them to room temperature.
2. Wash the slides in cold 0.1M TRIS (pH 7.6), 3 changes of 10 minutes each, in order to wash the fixative away.
3. Remove endogenous peroxidase reactivity that can be found in the tissues by storing the slides in 0.3% hydrogen peroxidase in TRIS for 25 min. at room temperature.
4. Wash in cold 0.1 M TRIS (pH 7.6) 2 times, 2 minutes.
5. Block the possible nonspecific protein interactions between the tissue and the link antibody by incubating the slides for 1 hour at room temperature in 5% of the normal serum from the animal used to produce the link antibody. The dilution is made in TRIS containing 0.1% Triton X100 (Rohn and Haas).
6. Dilute the primary antibody 1:50 for frozen sections, or 1:2 for the paraffin sections, in 0.1M TRIS (pH 7.6). Prepare 5% of fetal calf serum in TRIS and dilute it 1:50 in the same buffer. For the absorption control, place 50 μg of P2 peptide in a tube containing 0.5 ml of the primary antibody dilution. Shake for 2 hours at room temperature and centrifuge in cold for 30 min. Store the supernatant in the fridge until use.
7. Drain the blocking solution from the slides and immediately place the primary antibody and control solutions onto the slides. Store them in a humid chamber at 4 degrees C for 18

hours.

8. Rinse thoroughly with cold TRIS, 3 times at 10 minutes each. Keep controls and experimental slides separate during rinses.

9. Incubate the slides with biotinylated rabbit anti-mouse IgG (Zymed, California), diluted 1:100 in TRIS, for 1 hour at room temperature in a humid chamber.

10. Wash in cold TRIS 3 times, 10 min. each. Controls and experimental slides can be washed together again at this point. 11. While washing, prepare the ABC reagent by adding 2 drops of the Avidin reagent to 5 ml of TRIS, then add 2 drops of the biotin reagent to the same mixing bottle, shake and allow to stand for 30 min. before use.

12. Incubate the sections with the ABC reagent for 1 hour at room temperature.

13. Wash the slides 3 times, 10 min. each in cold TRIS.

14. During the washing dissolve diaminobenzidine tetrahydrochloride (DAB) powder in 0.1M TRIS to make a solution that is 0.025% DAB, in a brown bottle, and stir. The solution should be a pale straw colour without precipitate.

15. Just before the slides are to put into the DAB solution, add to it the correct volume of 3% hydrogen peroxide to make 0.01% hydrogen peroxide, and stir.

16. Add the DAB solution to the slides. Stop the reaction after 3 minutes by transfering the slides into a jar containing 0.1M TRIS.

17. Rinse the slides in water for 5 minutes.

18. Counterstain lightly with 0.1% cresyl violet.

19. Dehydrate in a series of increasing concentrations of ethanol and xylene. Mount with Pro-Tex (Lerner).

Appendix 3-1: The Peroxidase-anti-peroxidase immunostaining method for Pgp and GFAP

1. If the tissue sections are stored in the freezer, bring them to room temperature.
2. Wash the slides in cold 0.1M PBS (pH 7.4), 3 changes of 10 minutes each, in order to wash the fixative away.
3. Remove endogenous peroxidase reactivity that can be found in the tissues by storing the slides in 0.3% hydrogen peroxidase in PBS for 15 min. at room temperature.
4. Wash in cold 0.1 M PBS (pH 7.4) 2 times, 2 minutes.
5. Block the possible nonspecific protein interactions between the tissue and the link antibody by incubating the slides for 1 hour at room temperature in 5% of the normal serum from the animal used to produce the link antibody, in this case it is normal goat serum (NGS). The dilution is made in PBS containing 0.1% Triton X100 (Rohn and Haas).
6. Dilute the primary antibody, C219, to a final concentration of 10 μg/ml or 20 μg/ml in antibody diluent 1% NGS in 0.1 M PBS). The mouse monoclonal anti-GFAP antibody was used at a final concetration of 1/100 in the same diluent solution. Prepare 10 μg/ml or 20 μg/ml of normal mouse ascites fluid or purified mouse IgG in antibody diluent.
7. Drain the blocking solution from the slides and immediately place the primary antibody and control solutions onto the slides. Store them in a humid chamber at 4 degrees C for 18

hours.

8. Rinse thoroughly with cold PBS, 3 times at 10 minutes each. Keep controls and experimental slides separate during rinses.

9. Incubate the slides with the "link" antibody for the PAP technique, goat anti-mouse IgG (Jackson laboratories, CA) diluted 1:40 in PBS, for 1 hour at room temperature in a humid chamber.

10. Wash in cold PBS 3 times, 10 min. each. Controls and experimental slides can be washed together again at this point.

11. Incubate the slides with mouse peroxidase anti-peroxidase complex (Jackson laboratories, CA), 1:100 in antibody diluent (1% NGS in PBS) for one hour at room temperature in a humid chamber.

12. Wash the slides 2 times, 10 min. each in cold TBS (pH 7.4) and once more in cold 0.1 M TRIS for another 10 minutes.

13. During the washing dissolve diaminobenzidine tetrahydrochloride (DAB) powder in 0.1M TRIS to make a solution that is 0.025% DAB, in a brown bottle, and stir. The solution should be a pale straw colour without precipitate.

14. Just before the slides are to put into the DAB solution, add to it the correct volume of 3% hydrogen peroxide to make 0.01% hydrogen peroxide, and stir.

15. Add the DAB solution to the slides. Stop the reaction after 3 minutes by transfering the slides into a jar

containing 0.1M TRIS.

16. Rinse the slides in water for 5 minutes.

17. Counterstain lightly with 0.1% cresyl violet.

18. Dehydrate in a series of increasing concentrations of ethanol and xylene. Mount with Pro-Tex (Lerner).

Appendix 4: Working immunohistochemical solutions, dilutions and buffers

1000 μl = 1 ml

5% Normal Rabbit Serum (NRS) 0.1% Triton

Add 50 μl of NRS plus 10 μl of 10% Triton in 1 ml of buffer

Dilutions of the different monoclonal antibodies

GLUT-1:	1:50	10 μl serum	+ 490 μl 0.1 M Tris (pH 7.6)
C219:	10 μg/ml	50 μl ascites*	+ 450 μl 0.1 M PBS (pH 7.4)
	20 μg/ml	100 μl ascites	+ 400 μl 0.1 M PBS (pH 7.4)
GFAP:	1:100	5 μl ascites**	+ 495 μl 0.1 M PBS (pH 7.4)

* stock concentration is 100 μg ab/ml; ** stock concentration not provided by commercial source

0.025% DAB solution plus 0.01% H_2O_2

Dissolve 25 mgm of DAB powder in 100 ml of 0.1 M Tris. Just before the slides are to be incubated, add 330 μl of H_2O_2 from a fresh 3% stock.

0.1 M Tris (pH 7.6)

1. Prepare 0.5 M Tris stock solution:
   - 60.57 gms Tris in 500 mls. of double distilled water (ddw)
   - add HCl to bring pH to 7.6
   - dilute to one litre with ddw

2. Take 200 mls of the 0.5 M Tris and add 600 mls of ddw. Adjust pH to 7.6 with HCl and dilute to one litre with ddw to make 0.1 M Tris.

Tris Buffered Saline, TBS (pH 7.4)

100 ml	0.5 M Tris buffer
900 ml	0.9% NaCl (9 gms. NaCl in 1 litre of ddw)

0.1 M Phosphate Buffered Saline, PBS

Dissolve in 800 ml of ddw, 8.77 gms. of NaCl, 1.24 gms. of $Na_2 HPO_4$, and 0.18 gms. of $Na\ H_2PO_4 .H_2O$. Adjust pH to 7.4 with HCl and bring up to one litre with ddw.

REFERENCES

Andersson, L. and Lundahl, P., C-Terminal-Specific Monoclonal Antibodies against the Human Red Cell Glucose Transporter, J. Biol. Chem., 263 (1988) 11414-11420.

Andrew, D.L.E. and Paterson, J.A., Postnatal Development of Vascularity in the Inferior Colliculus of the Young Rat, Am. J. Anat., 186 (1989) 389-396.

Atack, J.R., Perry, E.K., Bonham, J.R. and Perry, R.H., Molecular Forms of Acetylcholinesterase and Butyrylcholinesterase in Human Plasma and Cerebrospinal Fluid, J. Neurochem., 48 (1987) 1845-1850.

Baños, G., Daniel, P.M. and Pratt, O.E., Saturation of a Shared Mechanism Which Transports L-Arginine and L-Lysine Into the Brain of the Living Rat, J. Physiol. 236 (1974) 29-41.

Bär, Th. and Wolff, J.R., The Formation of Capillary Basement Membranes During Internal Vascularization of the Rat's Cerebral Cortex, Z. Zellforsch. 133 (1972) 231-248.

Bär, Th., Morphometric Evaluation of Capillaries in Different

Laminae of Rat Cerebral Cortex by Automatic Image Analysis: Changes During Development and Aging. In Pathology of Cerebrospinal Microcirculation, J. Cervos-Navarro, E. Betz, G. Ebhardt, R. Ferszt and R. Wüllenweber, eds., Raven Press, New York, Adv. Neurol., 20 (1978) 1-9.

Bär, Th., The Vascular System of the Cerebral Cortex, Adv. Anat. Embryol. Cell Biol., 59 (1980) 1-59.

Bär, Th., Patterns of Vascularization in the Developing Cerebral Cortex. In Develpment of the Vascular System, J. Nugent and M.O. O'Connor, eds. Pitman Books, London, (1983) pp. 20-36.

Bell, G.I., Kayano, T., Buse, J.B., Burant, C.F., Takeda, J., Lin, D., Fukumoto, H. and Seino, S., Molecular Biology of Mammalian Glucose Transporters, Diabetes Care, 13 (1990) 198-208.

Birnbaum, M.J., Haspel, H.C. and Rosen, O.M., Cloning and Characterization of a cDNA Encoding the Rat Brain Glucose-Transporter Protein, Proc. Natl. Acad. Sci. USA, 83 (1986) 5784-5788.

Borowsky, I.W. and Collins, R.C., Metabolic Anatomy of Brain: A Comparison of Regional Capillary Density, Glucose

Metabolism, and Enzyme Activities, J. Comp. Neurol., 288 (1989) 401-413.

Bradbury, W.B., The Structure and Function of the Blood-Brain Barrier, Fed. Proc., 43 (1984) 186-190.

Bradley, G., Georges, E. and Ling, V., Sex-Dependent and Independent Expression of the P-Glycoprotein Isoforms in Chinese Hamster, J. Cell. Physio., 145 (1990) 398-408.

Brightman, M.W. and Reese, T.S., Junctions Between Intimately Apposed Cell Membranes in the Vertebrate Brain, J. Cell Biol., 40 (1969) 648-677.

Brightman, M.W., Morphology of the Blood-Brain Barrier, Exp. Eye Res. (suppl.), 25 (1977) 1-25.

Bruni, J.E., Del Bigio, M.R. and Clattenburg, R.E., Ependyma: Normal and Pathological. A Review of the Literature, Brain Res. Reviews, 9 (1985) 1-19.

Butt, A.M., Jones, H.C. and Abbott, N.J., Electrical Resistance Across the Blood-Brain Barrier in Anaesthetized Rats: A Developmental Study, J. Physio. 429 (1990) 47-62.

Caley, D.W. and Maxwell D.S., Development of the Blood Vessels

and Extracellular Spaces During Postnatal Maturation of Rat Cerebral Cortex, J. Comp. Neurol., 138 (1970) 31-48.

Carey, D.J., Control of Growth and Differentiation of Vascular Cells by Extracellular Matrix Proteins, Annu. Rev. Physiol., 53 (1991) 161-177.

Charron M.J., Brosius, F.C. III, Alper, S.L. and Lodish, H.F., A Novel Glucose Transport Protein Expressed Predominately in Insuline-Responsive Tissues, Proc. Natl. Acad. Sci. USA, 86 (1989) 2535-2539.

Chen, Ch., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M., Roninson, I.B., Internal Duplication and Homology with Bacterial Proteins in the mdr-1 (P-Glycoprotein) Gene from Multi-Drug Resistant Human Cells, Cell, 47 (1986) 381-389.

Conn, G., Bayne, M.L., Soderman, D.D., Kwok. P.W., Sullivan, K.A., Palisi, T.M., Hope, D.A. and Thomas, K.A., Amino Acid and cDNA Sequences of a Vascular Endothelial Cell Mitogen that is Homologous to Platelet-Derived Growth Factor, Proc. Natl. Acad. Sci. USA, 87 (1990) 2628-2632.

Coomber, B.L. and Stewart, P.A., Morphometric Analysis of CNS

Microvascular Endothelium, Microvascular Research, 30 (1985) 99-115.

Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R. and Bertino, J.R., Multi-Drug Resistant Gene (P-Glycoprotein) Is Expressed By Endothelial Cells at Blood-Brain Barrier Sites, Proc. Natl. Acad. Sci. USA, 86 (1989) 695-698.

Cordon-Cardo, C., O'Brien, J.P., Boccia, J., Casals, D., Bertino,J.R. and Melamed, M.R., Expression of the Multidrug Resistance Gene Product (P-Glycoprotein) in Human Normal and Tumor Tissues, J. Histochem. Cytochem., 38 (1990) 1277-1287.

Craigie, E.H., On the Relative Vascularity of Various Parts of the Central Nervous System of the Albino Rat, J. Comp. Neurol., 31 (1920) 429-464.

Crone, C., The Permeability of Brain Capillaries to Non-Electrolytes, Acta Physiol. Scand., 64 (1965) 407-417.

Crone, C., Facilitative Transfer of Glucose from Blood into the Brain Tissue, J. Physiol., 181 (1965) 103-113.

Cserr, H.F., The Blood CSF-Barrier. In Biology of the Blood-Brain Barrier, Grass Instrument Co. 1988.

Dermietzel, R. and Krause, D., Molecular Anatomy of the Blood-Brain Barrier as Defined by Immunocytochemistry, Intl. Review of Cytology, 127 (1991) 57-109.

Dermietzel, R., Krause, D., Kremer, M., Wang, C. and Stevenson, B., Pattern of Glucose Transporter (Glut 1) Expression in Embryonic Brains is Related to Maturation of Blood-Brain Barrier Tightness, Dev. Dyn.,193 (1992) 152-156.

Djuricic, B.M. and Mrsulja, B.B., Enzymatic Actuvity of the Brain: Microvessels vs. Total Forebrain Homogenate, Brain Research, 138 (1977) 561-564.

Djuricic, B.M., Rogac, Lj., Spatz, M., Rakic, Lj.M. and Mrsulja, B.B., Brain Microvessels. I. Enzymic Activities. In Adv. in Neurol., J. Nervos-Navarro, E. Betz, G. Ebhardt, R. Ferszt, R. WÜllenweber, eds., 20 (1978) 197-205.

Eng, L.F., Vanderhaeghen, J.J., Bignami, A. and Gerstl, B., An Acidic Protein Isolated From Fibrous Astrocytes, Brain Res., 28 (1971) 351-354.

Farrell, C.L. and Pardridge, W.M., Blood-Brain Barrier Glucose Transporter is Asymmetrically distributed on brain capillary Endothelial Lumenal and Ablumenal Membranes: An Electron Microscopic Immunogold Study,Proc. Natl. Acad. Sci. U.S.A.,

88 (1991) 5779-5783.

Farrell, C.L., Yang, J. and Pardridge, W.M., GLUT-1 Glucose Transporter is Present Within Apical and Basolateral Membranes of Brain Epithelial Interfaces and in Microvascular Endothelia With and Without Tight Junctions, J. Histochem. Cytochem., 40 (1992) 193-199.

Finstad, C.L., Yin, B.W.T., Gordon, C.M., Federici, M.G., Welt, S. and Lloyd, K.O., Some Monoclonal Antibody Reagents (C-219 and JSB-1) to P-Glycoprotein Contain Antibodies to Blood Group A Carbohydrate Determinants: A Problem of Quality Control for Immunohistochemical Analysis, J. Histochem. Cytochem., 39 (1991) 1603-1610.

Flier, J.S., Mueckler, M., McCall, A.L. and Lodish, H.F., Distribution of Glucose Transporter Messenger RNA Transcripts in Tissues of Rat and Man, J. Clin. Invest., 79 (1987) 657-661.

Flumerfelt, B.A., Lewis, P.R. and Gwyn, D.G., Cholinesterase Activity in the Rat Brain. A light and Electron Microscopic Study, Histochem. J., 5 (1973) 67-77.

Fojo, A., Lebo, R., Shimizu, M., Chin, J.E., Roninson, I.B., Merlino, G.T., Gottesman, M.M. and Pastan, I., Localization of

Multidrug Resistance-associated DNA sequences to Human Chromosome 7, Somatic Cell Mol. Genet., 4 (1986) 415-420.

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. and Pastan, I., Expression of a Multidrug- Resistance Gene in Human Tumors and Tissues, Proc. Natl. Acad. Sci USA, 84 (1987) 265-269.

Folkman, J., Langer, L., Linhardt, R.J., Haudenschild, C. and Taylor, S., Angiogenesis Inhibition and Tumor Regression Caused by Heparin or a Heparin Fragment in the Presence of Cortisone, Science, 221 (1983) 719-725.

Friede, R.L., A Comparative Histochemical Mapping of the Distribution of Butyrylcholinesterase in the Brains of Four Species of Mammals Including Man, Acta Anat. 66 (1967) 161-177.

Georges, E, Bradley, G., Gariepy, J. and Ling, V., Detection of P-Glycoprotein Isoforms by Gene-Specific Monoclonal Antibodies, Proc. Natl. Acad. Sci. USA, 87 (1990) 152-156.

Gerhart, D.Z. and Drewes, L.R., Butyrylcholinesterase in Pericytes Associated With Canine Brain Capillaries, Cell Tissue Res, 247 (1987) 533-536.

Gerhart, D.Z., LeVasseur, R.J., Broderius, M.A. and Drewes, L.R., Glucose Transporter Localization Using Light and Electron Immunocytochemistry, J. Neuroscience Res., 22 (1989) 464-472.

Gerlach, J.H., Endicott, J.A., Juranka, P.F., Henderson, G., Sarangi, F., Deuchars, K.L. and Ling, V., Homology Between P-Glycoprotein and a Bacterial Haemolysin Transport Protein Suggest a Model for Multidrug Resistance, Nature 324 (1986) 485-489.

Goldstein, G.W. and Betz, A.L., The Blood-Brain Barrier, Sci. Am. 255 (1986) 74-83.

Goldstein, L.J., Galski, H., Fojo, A., Willingham, M., Lai, S.L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G.M., Lieber, M., Cossman, J., Gottesman, M.M. and Pastan, I., Expression of a Multidrug Resistance Gene in Human Cancers, J. Natl. Cancer Inst., 81 (1989) 116-124.

Gospodarowicz, D., Neufeld, G. and Schweigerer, L., Molecular and Biological Characterization of Fibroblast Growth Factor, an Angiogenic Factor which also Contrlos the Proliferation and Differentiation of Mesoderm and Ectoderm Derived Cells, Cell. Diff. 19 (1986) 1-17.

Gottesman, M.M. and Pastan, I., The Multidrug Transporter, a

Double-Edged Sword, J. Biol. Chem., 263 (1988) 12163-12166.

Gros, P., Croop, J.M., Roninson, I.B., Varshavsky, A. and Housman, D.E., Isolation and Characterization od DNA Sequences Amplified in Multidrug-Resistant Hamster Cells, Proc. Natl. Acad. Sci. USA, 83 (1986) 337-341.

Gros, P., Neriah, Y.B., Croop, J.M. and Housman, D.E., Isolation and Expression of a Complementary DNA that Confers Multidrug Resistance, Nature, 323 (1986) 728-731.

Gross, P.M., Sposito, N.M., Pettersen S.E., Panton, D.G. and Fenstermacher, J.D., Topography of Capillary Density, Glucose Metabolism, and Microvascular Function Within the Rat Inferior Colliculus, 7 (1987) 154-160.

Hannah, R.S. and Nathaniel, E.J.H., The Postnatal Development of Blood Vessels in the Substantia Gelatinosa of Rat Cervical Cord: An Ultrastructural Study, Anat. Rec., 178 (1974) 691-710.

Hardebo, J.E. and Owman, C., Perivascular Cholinesterase and Morphological Blood-Brain Barrier Function, Acta Neurol. Scand., 70 (1984) 438-442.

Harik, S.I., Kalaria, R.N., Andersson, L., Lundahl, P. and

Perry, G., Immunocytochemical Localization of the Erythroid Glucose Transporter: Abundance in Tissues With Barrier Functions, J. Neurosci. 10 (1990) 3862-3872.

Harik, S.I., Kalaria, R.N., Whitney, P.M., Andersson, L., Lundahl, P., Ledbetter, S.R. and Perry, G., Glucose Transporters are Abundant in Cells With "Occluding" Junctions at the Blood-Eye Barriers, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4261-4264.

Harik, S.I. and Roessmann, U., The Erythrocyte-Type Glucose Transporter in Blood Vessels of Primary and Metastatic Brain Tumors, Ann. Neurol., 29 (1991) 487-491.

Hediger,M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M., Expression Cloning and cDNA Sequencing of the Na^+/Glucose Cotransporter, Nature, 330 (1987) 379-381.

Henderson, Z., Is there a Link Between Choline Acetyltransferase-Containing Neurons and Butyrilcholinesterase in Rat Cerebral cortex? Trends in Neurosciences, 9 (1986) 20.

Hewing, M. and Brightmann, M., Differential Permeability of Pineal Capillaries to Lanthanum Ion in the Rat (Rattus norvegicus, Gerbil (Meriones unguiculatus) and Golden Hamster

(Mesocricetus auratus), Cell Tissue Res., 241 (1985) 149-154).

Hogan, A., Heyner, S., Charron, M.J., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Thorens, B. and Schultz, G.A., Glucose Transporter Gene Expression in Early Mouse Embryos, Development, 113 (1991) 363-372.

Hsu, S.M., Raine, L. and Fanger, H., A Comparative Study of the Peroxidase-Antiperoxidase Method and an Avidin-Biotin Complex Method for Studying Polypeptide Hormones With Radioimmunoassay Antibodies, Am. J. Clin. Pathol., 75 (1981) 734-738.

Ishikawa, F., Miyazono, K., Hellman, U., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C.H., cDNA Cloning and Expression of a Novel Angiogenic Factor - Platelet-Derived Endothelial Cell Growth Factor, Nature, 338 (1989) 557-562.

Jacobson, M., Developmental neurobiology, Plenum Press, N. Y., 1991.

Janzer, R.C. and Raff, M.C., Astrocytes Induce Blood-Brain Barrier Properties in Endothelial Cells, Nature, 325 (1987) 253-257.

Joo, F. and Csillik, B., Topographic Correlation Between the Hemato-Encephalic Barrier and the Cholinesterase Activity of Brain Capillaries, Experimental Brain Research, 1 (1966) 147-151.

Juliano, R.L. and Ling, V., A Surface Glycoprotein Modulating Drug Permeability in Chinese Hamster Ovary Cell Mutants, Biochim. Biophys. Acta, 455 (1976) 152-162.

Kalaria, R.N., Gravina, S.A., Schmidley, J.W., Perry, G. and Harik, S.I., The Glucose Transporter of the Human Brain and Blood-Brain Barrier, Ann. Neurol., 24 (1988) 757-764.

Karcsú, S., Jancsó, G. and Tóth, L., Butyrylcholinesterase Activity in Fenestrated Capillaries of the Rat Area Postrema, Brain Res., 120 (1977) 146-150.

Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V., Detection of P-Glycoprotein in Multidrug-Resistnat Cell Lines by Monoclonal Antibodies, Nature, 316 (1985) 820-823.

Kasa, P., Pakaski, M., Joo, F. and Lajtha, A., Endothelial Cells from Human Fetal Brain Microvessels May Be Cholinoceptive, but Do Not Synthesize Acetylcholine, J. Neurochem., 56 (1991) 2143-2146.

Kasanicki, M.A. and Pilch, P.E., Regulation of Glucose-Transporter Function, Diabetes Care, 13 (1990) 219-227.

Klagsbrun, M. and D'Amore, P.A., Regulators of Angiogenesis, Annu. Rev. Physiol., 53 (1991) 217-239.

Klein, B., Kuschinsky, W., Schröck, H. and Vetterlein, F., Interdependency of Local Capillary Density, Blood Flow, and Metabolism in Rat Brains, Am. J. Physiol., 251 (1986) H1333-H1340.

Koelle, G.B., The Elimination of Enzymatic Diffusion Artifacts in the Histochemical Localization of Cholinesterases and a Survey of their Cellular Distributions, j. Pharmacol. and Exp. Therap., 103 (1951) 153-171.

Koelle, G.B. and Friedenwald, J.S., A Histochemical Method for Localizing Cholinesterase Activity, Proc. Soc. Exper. Biol. & Med., 70 (1949) 617-622.

Koelle, J.B., The Histochemical Localization of Cholinesterases in the Central Nervous System of the Rat, J. comp. Neurol., 100 (1954) 211-235.

Krum, J.M., More, N.S. and Rosenstein, J.M., Brain Angiogenesis in Vascular Basement Membrane Glycoprotein

Immunoreactivity, Exp. Neurol. 111 (1991) 152-165.

Lederis, K., An Electron Microscopical Study of the Human Neurohypophysis, Z. Zellforsch., 65 (1965) 847-868.

Lienhard, G.E., Slot, J.W., James, D.E., and Mueckler, M.M., How Cells Absorb Glucose, Sci. Am., 266 (1992) 86-91.

Ling, V., Does P-Glycoprotein Predict Response to Chemotherapy?, J. Natl. Cancer Inst. 81 (1989) 84.

Maiden, M.C.J., Davis, E.O., Baldwin, S.A., Moore, D.C.M. and Henderson, P.J.F., Mammalian and Bacterial Sugar Transport Proteins are Homologous, Nature, 325 (1987) 641-643.

Massoulié, J. and Bon, S., The Molecular Forms of Cholinesterase and Acetylcholinesterase in Vertebrates, Annu. Rev. Neurosci., 5 (1982) 57-106.

Mendel, B. and Rudney, H., Studies on Cholinesterase: I. Cholinesterase and Pseudo-cholinesterase, Biochem. J., 37 (1943) 59-63.

Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard G.E. and Lodish, H.F., Sequence Structure of a Human Glucose Transporter,

Science, 229 (1985) 941-945.

Ng W.F., Sarangi, F., Zastawny R.L., Veinot-Drebot, L. and Ling, V., Identification of Members of the P-Glycoprotein Multigene Family, Mol. Cell. Biol., 9 (1989) 1224-1232.

Oldendorf, W. and Szabo, J., Amino Acid Assignment to One of Three Blood-Brain Barrier Amino Acid Carriers, Am. J. Physiol., 230 (1976) 94-98.

Ord, M.G. and Thompson, R.H.S., The Distribution of Cholinesterase Types in Mammalian Tissues, Biochem. J., 46 (1950) 346-352.

Palay, S.L., An Electron Microscope Study of the Neurohypophysis in Normal, Hydrated, and Dehydrated Rats, The Anatomical Record, 121 (1955) 138.

Pardridge, W.M., Kinetics of Competitive Inhibition of Neutral Amino Acid Transport Across the Blood-Brain Barrier, J. Neurochem., 28 (1977) 103-108.

Pardridge, W.M., Yang, J., Buciak, J.L. and Boado, R.J., Differential Expression of 53- and 45-kDa Brain Capillary-Specific Proteins by Brain Capillary Endothelium and Choroid Plexus in Vivo and by Brain Capillary Endothelium in Tissue

Culture, Mol. Cell. Neuro., 1 (1990) 20-28.

Pardridge, W.M., Boado, R.J. and Farrell, C.R., Brain-type Glucose Transporter (GLUT-1) is Selectively Localized to the Blood-Brain Barrier. Studies With Quantitative Western Blotting and In Situ Hybridization, J. Biol. Chem., 265 (1990) 18035-18040.

Peerce, B.E. and Wright, E.M., Conformational Changes in the Intestinal Brush Border Sodium-Glucose Co-Transporter Labelled With Fluorescein Isothiocyanate, Proc. Natl. Acad. Sci. USA, 81 (1984) 2223-2226.

Peters, A., Palay, S.L. and Webster H.deF., The Fine Structure of the Nervous System: Neurons and their Supporting Cells, Oxford University Press, New York, 1991.

Phelps, C.H., The Development of Glio-Vascular Relationships in the Rat Spinal Cord, Z. Zellforsch., 128 (1972) 555-563.

Reese , T.S. and Karnovsky, M.J., Fine Structural Localization of a Blood-Brain Barrier to Exogenous Peroxidase, J. Cell Biol., 34 (1967) 207-217.

Reese, T.S. and Brightman, M.W., Similaruty in Structure and Permeability to Peroxidase of Epithelia Overlying Fenestrated

Cerebral Capillaries, Anat. Rec., 160 (1968) 414. (Abstr.)

Risau, W., Hallmann, R. and Albrecht, U., Differentiation-dependent Expression of Proteins in Brain Endothelium During Development of the Blood-Brain Barrier, Dev. Biol., 117 (1986a) 537-545.

Risau, W., Hallmann, R., Albrecht, U. and Henke-Fahle, S., Brain Induces the Expression of an Early Cell Surface Marker for Blood-Brain Barrier Specific Endothelium, EMBO, 5 (1986b) 3179-3183.

Risau, W. and Lemmon, V., Changes in the Vascular Extracellular Matrix During Embyonic Vasculogenesis and Angiogenesis, Dev. Biol. 125 (1988) 441-450.

Risau, W., Induction of Blood-Brain Barrier Endothelial Cell Differentiation, Ann. N. Y. Acad. Sci., 633 (1991) 405-419.

Roninson, I.B., Chin, J.E., Choi, K., Gros, P., Housman, D.E., Fojo, A., Shen, D., Gottesman, M.M. and Pastan, I., Isolation of Human mdr DNA Sequences Amplified in Multidrug-Resistant KB Carcinoma Cells, Proc. Natl. Acad. Sci. USA, 83 (1986) 4538-4542.

Rubin, L.L., The Blood-Brain Barrier in and out of Cell

Culture, Current Opinion in Neurobiology, 1 (1991) 360-363.

Schlosshauer, B. and Herzog, K.H., Neurothelin: An Inducible Cell Surface Glycoprotein of Blood-Brain Barrier-Specific Endothelial Cells and Distinct Neurons, J. Cell Biol., 110 (1990) 1261-1274.

Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C. and Gospodarowicz, D., Capillary Endothelial Cells Express Basic Fibroblast Growth Factor, a Mitogen that Promotes their Own Growth, Nature, 325 (1987) 257-259).

Senjo, M., Ishibashi, T., Terashima, T. and Inoue, Y., Correlation Between Astrogliogenesis and Blood-Brain Barrier Formation: Immunohistochemical Demonstration by Using Astroglia Specific Enzyme Glutathione, Neurosci. Letts. 66 (1986) 39-42.

Shute, C.C.D. and Lewis, P.R., Cholinesterase-Containing Systems of the Brain of the Rat, Nature, 199 (1963) 1160-1164.

Silver, A., The Biology of Cholinesterases, North-Holland Publ. Co., Amsterdam, 1974,

Sivitz, W., DeSautel, S., Walker, P.S. and Pessin, J.E., Regulation of the Glucose Transporter in Developing Rat

Brain, Endocrinology, 124 (1989) 1875-1880.

Skärby, T., Larsson, B. and Edvinsson, L., Effects of Carbacholine and Physostigmine on the Cerebrovascular Bed. Acta Neurol. Scand. 60 [Suppl] (1979) 148-149.

Smith, R.S., Ultrastructural Studies of the Blood-Aqueous Barrier. I: Transport of an Electron-Dense Tracer in the Iris and Ciliary Body of the Mouse, Am. J. Ophtalmol. 71 (1971) 1066-1077).

Sogin, D.C. and Hinkle, P.C., Binding of Cytochalasin B to Human Erythrocyte Glucose Transporter, Biochemistry, 19 (1980) 5417-5420.

Sternberger, L.A., Immunocytochemistry, Wiley Medical, N. Y., Chichester, Brisbane, Toronto, 1979.

Strong, L.H., The Early Embryonic Pattern of Internal Vascularization of the Mammalian Cerebral Cortex, J. Comp. Neur., 123 (1964) 121-138.

Sugawara, I., Hamada, H., Tsuruo, T. and Mori, S., Specialized Localization of P-Glycoprotein Recognized by MRK 16 Monoclonal Antibody in Endothelial Cells of the Brain and the Spinal Cord, Jpn. J. Cancer Res., 81 (1990) 727-730.

Tao-Cheng, J.H. and Brightman, M.W., Development of Membrane Interactions Between Brain Endothelial Cells and Astrocytes In Vitro, Intl. J. Devl. Neuroscience, 6 (1988) 25-37.

Tashiro, K., Sephel, G.C., Greatorex, D., Sasaki, M., Shirashi, N., Martin, G.R., Kleinman, H.K. and Yamada, Y.,The RGD Containing Site of the Mouse Laminin A Chain is Active for Cell Attachment, Spreading, Migration and Neurite Outgrowth, J. Cell. Physiol., 146 (1991) 451-459.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I. and Willingham, M.C., Immunohistochemical Localization in Normal Tissues of Different Epitopes in the Multidrug Transport Protein P170: Evidence for Localization in Brain Capillaries and Crossreactivity of One Antibody with a Muscle Protein, J. Histochem. Cytochem., 37 (1989) 159-164.

Thorens, B., Charron, M.J. and Lodish, H.F., Molecular Physiology of Glucose Transporters, Diabetes Care, 13 (1990) 209-18.

Trancard, J., Delamanche, I.S., Ruiz, G. and Bouchaud, C., Interspecific Variations of Cerebral Endothelial Cholinesterases in Rodents and Carnivores, Brain Res, 476 (1989) 213-219.

Ueda, K., Cornwell, M.M., Gottesman, M.M., Pastan, I., Roninson, I.B., Ling,V. and Riordan, J.R., The mdr1 Gene for Multidrug- Resistance, Codes for P-Glycoprotein, Biochem. Biophys. Res. Commun., 141 (1986) 956-962.

Ugrumov, M.V., Development of the Median Eminence During Ontogenesis (Morpho-Functional Aspects), Progress Brain Res. 91 (1992) 349-356.

Wakai,S. and Hirokawa, N., Development of the Blood-Brain Barrier to Horseradish Peroxidase in the Chick Embryo, Cell Tissue Res., 195 (1978) 195-203.

Weinstein, R.S., Kuszac, J.R., Jakate, S.M., Lebovitz, M.D., Kluskens, M.F. and Coon, J.S., ABO Blood Type Predicts the Cytolocalization of Anti-P-Glycoprotein Monoclonal Antibody Reactivity in Human Colon and Ureter, Hum. Pathol., 21 (1990) 949-958.

Wheeler, T.J. and Hinkle, P.C., The Glucose Transporter of Mammalian Cells, Annu. Rev. Physiol., 47 (1985) 503-517.

Whitaker, S. and La Bella, F.S., Electron Microscopic Histochemistry of Cholinesterases in the Posterior, Intermediate, and Anterior Lobes of the Rat Pituitary, Z. Zellforsch., 130 (1972) 152-170.

Whitaker, S., La Bella, F.S. and Prasher, M., Cholinesterase in the Posterior Pituitary, Cell Tiss. Res., 149 (1974) 577-586.

Wislocki, G.B. and Leduc, E.H., Vital Staining of the Hematoencephalic Barrier by Silver Nitrate and Trypan Blue, and Cytological Comparisons of the Neurohypophisis, Pineal Body, Area Postrema, Intercolumnar Tubercle, and Supraoptic Crest, J. Comp. Neurol., 96 (1952) 371-413.