Acta Physiol Scand 1980, 108: 223–229

Characterization of the in vitro uptake of monoamines into brain microvessels

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HARDEBO, J. E. & OWMAN, CH.: Characterization of the in vitro uptake of monoamines into brain microvessels. Acta Physiol Scand 1980, 108: 223–229. Received 28 May 1979. ISSN 0001-6772. Departments of Histology and Neurology, University of Lund, Sweden.

The ability of brain microvessels to take up and metabolize transmitter monoamines (DA, NA, A and 5-HT) was studied in tissues from several species (rat, rabbit, guinea-pig, cat, dog, baboon, man) in vitro. Following incubation, slices were analyzed by fluorescence histochemistry and isolated microvessels by measurement of radioactivity from tritiated amine. Provided the MAO activity was inhibited a strong accumulation, similar for all animals tested, occurred in the wall (endothelial cells and pericytes) of capillaries and venules—but not in larger vessels—of all species. The degree of amine uptake and accumulation and the conditions under which it was inhibited suggested that it reflected a saturable, energy-dependent process, with characteristics of both the extraneuronal and neuronal type of uptake processes. The mechanism may serve as an inactivation of transmitter monoamines at the blood–brain interphase, thereby assisting in the control of transmitter levels in the cerebral extracellular compartment.

Key words: Blood–brain interphase, brain microvessels, neurotransmitter monoamines, MAO

The existence of a morphologic barrier between the blood and brain parenchyma is well documented (for review, see Rapoport 1976). Another aspect of the barrier function is the presence of an enzymatic blood–brain barrier for monoamine precursors, such as L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan, first shown by Bertler et al. (1966) using fluorometric assay and fluorescence histochemical techniques. Systematically administered L-DOPA and L-5-HTP are taken up into the wall (endothelial cells and pericytes) of cerebral microvessels and is subsequently decarboxylated to the corresponding amine, i.e. dopamine (DA) and 5-hydroxytryptamine (5-HT). Using the same techniques it has been found that only minor amounts of circulating neurotransmitter monoamines, such as DA, noradrenaline (NA), adrenaline (A) and 5-HT accumulate within the endothelial cells and pericytes of cerebral microvessels (Hardebo et al. 1979*a*); the bulk of monoamines is prevented from leaving the brain circulation already at the luminal membrane of the endothelial cells (Bertler et al. 1966, Hardebo et al. 1979*a*). Evidence has been presented for the existence for an enzymatic blood–brain barrier not only for the monoamine precursors but also for the monoamines themselves; they are rapidly broken down by monoamine oxidase (MAO) present in the endothelial cells and pericytes of the brain microvessels (Bertler et al. 1966, Spector, Baird-Lambert & Lai 1977, Hardebo et al. 1977 and 1979*b*) and, in addition, by MAO and catechol-O-methyl transferase (COMT) in the smooth muscle cell layer of pial vessels and brain parenchymal arterioles (Hardebo et al. 1979*b*). On the other hand, little is known about the clearance of neurotransmitter monoamines in direction from the brain parenchyma to the circulation. It can be assumed that the local presence of a sufficient excretory mechanism into the cerebral microcirculation—along with the reuptake into the aminergic nerve terminal and the clearance from the cerebrospinal fluid—will assist in the control of the level of the amine in the brain extracellular compartment. In the present study, a

brain microvascular uptake of various neurotransmitter monoamines is demonstrated and characterized.

MATERIAL AND METHODS

Animals. The study was performed on adult animals of either sex, namely 64 Sprague-Dawley rats, 29 albino rabbits, 7 guinea-pigs, 5 cats, 4 dogs, 4 baboons, and brain tissue from 5 adult female and male patients. The laboratory animals had free access to food and water. These animals were killed under Nembutal anesthesia by perfusion of the vascular system with 0.9% saline to eliminate any error caused by the presence of blood cells. The brains were dissected out and kept in Krebs-Ringer buffer solution on ice (for composition, see below) or, during preparation of microvessel fractions, in cold phosphate buffer. The human tissue (macroscopically intact frontal and temporal cortex) was obtained during neurosurgical lobe resection operations; the tissue was immediately placed in ice-cold buffer solution and transported on ice to the laboratory (within 30 min).

Incubation of tissue slices. Approximately 1 mm thin slices of the parietal cortex (frontal and temporal cortex in man), caudate nucleus, cerebellum, hypothalamus (at the level of the median eminence), spinal cord, and heart from the various animals were cut with a razor blade and transferred to incubation vials containing ice-cold Krebs-Ringer buffer solution. The buffer solution had the following composition (mM): NaCl 118, KCl 4.5, $CaCl_2 \times 2H_2O$ 2.5, $MgSO_4 \times 7H_2O$ 1.0, $NaHCO_3$ 25, KH_2PO_4 1.0, to which was added 1 mg/ml glucose, 0.2 mg/ml ascorbic acid and 0.05 mg/ml EDTA.

The vials were placed in an incubation bath at 37°C for preincubation during 20 min in the presence of the MAO inhibitor, nialamide (10^{-3} M), followed by incubation during 20 min after the addition of various doses (1, 10 or 100 μg/ml) of NA, A, DA, or 5-HT. In addition, slices from the parietal cortex of rat were incubated in the presence of several increasing doses (1, 2, 5, 10, 20, 50 or 100 μg/ml) of NA in an attempt to find an upper limit for the uptake process. The buffer solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2, giving a pH of about 7.4 and a pO_2 around 200 mmHg (as measured on an MRK II blood gas analyzer; Radiometer, Copenhagen). Slices from the parietal cortex (frontal and temporal cortex in man) were also incubated with NA, A, DA or 5-HT (1 or 10 μg/ml) in the absence of nialamide. Control slices were incubated without addition of any drugs.

In order to further characterize the uptake, the incubations of the parietal cortex (frontal or temporal cortex in man) with monoamines were also performed in the presence of either cocaine (10^{-5} or 10^{-3} M), desmethylimipramine (10^{-6} or 10^{-4} M), phenoxybenzamine (10^{-6} or 10^{-5} M), oestradiol-17 β (10^{-6} or 10^{-5} M), theophylline 10^{-5} M), ouabain (10^{-6} or 10^{-5} M), or dinitrophenol (5×10^{-5} or 10^{-3} M). These compounds were added to the bath at the start of the preincubation together with nialamide. Incubation was also performed in a low Na^+/high K^+ milieu (in the buffer solution KCl substituted for all NaCl) and under a combination of anoxic conditions (continuous bubbling with 95% N_2 and 5% CO_2) and deprivation of energy substrate (glucose), and also at 20°C and 0°C.

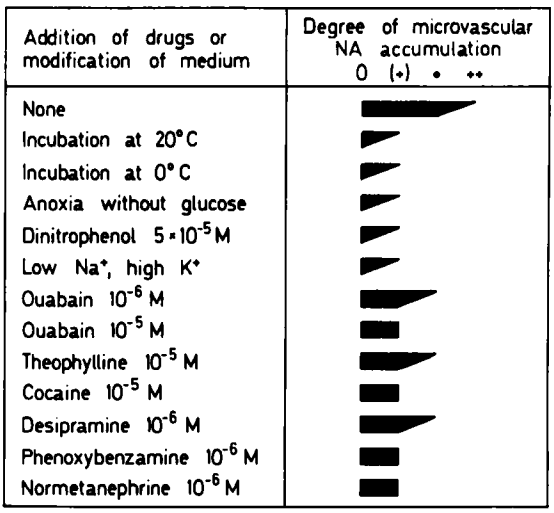

Fig. 1. Accmulation of NA fluorophore in the walls of microvessels in rat cortical slices incubated in the presence of 10 μg/ml noradrenaline following MAO inhibition (10^{-3} M nialamide) and processed according to the formaldehyde histofluorescence method. The degree of microvascular fluorescence intensity above the diffuse, general background fluorescence is expressed arbitrarily, the tapering part of the bars indicating intermediary intensities.

Fluorescence microscopy. Following the incubation, the various tissue pieces were frozen to the temperature of liquid nitrogen and further processed for fluorescence monoamine histochemistry according to the standard Falck-Hillarp method (Björklund, Falck & Owman 1972). The paraformaldehyde used in the histochemical procedure had previously been equilibrated with air of 70% humidity. The formaldehyde-induced, histochemically visible fluorophores of NA, A and DA are indistinguishable in the sense that they have the same spectral characteristics and exhibit a green light under the optical conditions used, whereas 5-HT exhibits a yellow, more rapidly fading light (Björklund et al. 1972). Fluorescence microscopy was carried out on 6 μm thick sections using a Zeiss fluorescence microscope equipped with an Osram HBO 200 mercury lamp, and Schott BG 12 and OG 4 primary and secondary filters, respectively. The relative fluorescence intensity in the microvessel walls was evaluated according to an arbitrary scale (see Fig. 1) using coded preparations. The different preparations to be compared after incubations at the various conditions (see below) were always freeze-dried and further processed simultaneously.

Isolation of cerebral microvessels. Whole brains from rats and rabbits were used. After removal they were kept in an ice-cold 67 mM phosphate buffer (pH 7.4) throughout the procedure (see Hardebo et al. 1979*b*). The meninges, including the pial membrane and its vessels,

were carefully torn off and the choroid plexuses were removed. White matter, including the whole brain stem, was dissected away. Tissue obtained from 4–6 rats or 2–3 rabbits was chopped with a razor blade, and then gently further disrupted by some 10 strokes up and down through three different 10–20 ml plastic syringes equipped with a nylon net (pore size 1 000 μm, 500 μm and 280 μm, respectively) glued to their open cut end. The material was subsequently homogenized by hand with a loosely fitting Teflon pestle in a smooth glass tube (0.1 mm clearance). The homogenate was washed through one nylon sieve with 225 μm pore size. The material remaining on the sieve, after extensive washing, was re-homogenized and re-sieved 3–4 times through the same, carefully washed sieve. The tissue passing these sieves was collected and sieved through another sieve with 75 μm pore size. The material remaining on this sieve after washing was re-homogenized and re-sieved in the same, carefully washed sieve. The tissue fraction remaining on the last sieve, after extensive washing, consists of capillaries, venules and a few larger vessels, whereas most of the larger vessels and vessels branching off into clusters of small vessels, as well as a few small clusters of neurons and glia, remain on the 225 μm sieve (Hardebo et al. 1979*b*). Neurons, glia, small microvessel segments, and subcellular fragments pass the 75 μm sieve. The capillary fraction is pure with regard to vessels and is only contaminated with a few glial endfeet, stuck onto the vessel wall (Hardebo et al. 1977). The yield of microvessels is less than 1/1000 of the original wet weight of the grey matter. Brendel, Meezan & Carlson (1974) have shown that a mixed fraction of intracerebral vessels, prepared under similar conditions, is metabolically active.

Incubation of isolated microvessels. The microvessel fractions were transferred to incubation vials containing ice-cold Krebs-Ringer buffer solution (composition as above). The vials were placed in an incubation bath and allowed to equilibrate for 20 min at 37°C before incubation, which was started by adding ^{3}H-NA to the solution. The tissue preparations were incubated for 15 min at a temperature of 37°C. The buffer solution was continuously aerated with 95% O_2 and 5% CO_2. The incubation was terminated by transferring the tissue into ice-cold buffer solution. The microvessel fraction was collected by centrifugation at 4°C at 110 g and washed twice in isotope-free cold buffer for each 10 min.

In order to further characterize the uptake, microvessel fractions were also incubated in the presence of ouabain (10^{-5} M), under combined anoxia and absence of glucose, and also at 0°C.

Measurement of radioactivity. After incubation, the microvessel samples were weighed and transferred to liquid scintillation vials. They were solubilized in 0.5 ml Soluene (Packard), and liquid scintillation counting was performed in 10 ml Instagel (Packard), as were appropriate samples (25 μl) of the incubation medium. Quench corrections were obtained according to conventional principles.

Drugs. Dopamine hydrochloride (Sigma); L-noradrenaline bitartrate monohydrate (Sigma), L-adrenaline bitartrate (Sigma), 5-hydroxytryptamine creatinine hydrochloride (Sigma), cocaine hydrochloride (ACO), ouabain (Sigma), 2,4-dinitrophenol (Chroma), theophylline (Sigma), desmethyl imipramine (Desipramine, Geigy), phenoxybenzamine hydrochloride (Dibenzyline; Smith, Kline and French), nialamide (Pfizer) and L-norepinephrine-7-^{3}H (New England Nuclear; 9.1 Ci/mmol).

Statistics. Mean values were compared according to the Student's *t*-test for unpaired data.

RESULTS

Incubation of tissue slices. Fluorescence microscopy of the various brain regions from control slices incubated in buffer solution alone showed a dense network of delicate catecholamine-containing nerve terminals emitting a green fluorescence; in the cerebellum only a small number of isolated green-fluorescent axons were present. The parenchymal blood vessels were equipped with varying amounts of sympathetic nerves. In the heart tissue adrenergic nerves were seen in the myocard and in association with the blood vessels. All tissues showed a slight diffuse, non-specific greenish background fluorescence. The vessel wall proper was in all regions essentially non-fluorescent, except for the intense autofluorescence of the internal elastic membrane of larger pial arteries.

After incubation with the various monoamines an uptake (as reflected in an accumulation of fluorophore well above the diffuse background fluorescence) was seen in the brain microvessel walls (endothelial cells and pericytes of capillaries, and small veins) in all species studied. The intensity of the general background fluorescence was also increased. No clearcut accumulation above the diffuse background fluorescence had occurred in the walls of the brain parenchymal arterioles or heart vessels. Possibly, a slight uptake and accumulation was seen in the wall of pial arteries. Inhibition of MAO by nialamide clearly enhanced the accumulation of the monoamines in the microvessel wall, as evidenced by a stronger fluorescence intensity in relation to the background fluorescence. At an identical concentration in the incubation bath DA, NA and A all induced a similarly strong fluorescence in the microvessel wall, whereas the fluorescence induced by 5-HT was weaker. This discrepancy in fluorescence intensity is at least partly explainable by the varying fluorescence yield in the standard formaldehyde reaction (Björklund et al. 1972). An upper limit of fluorescence intensity in brain microvessel walls could be disclosed when the tissue

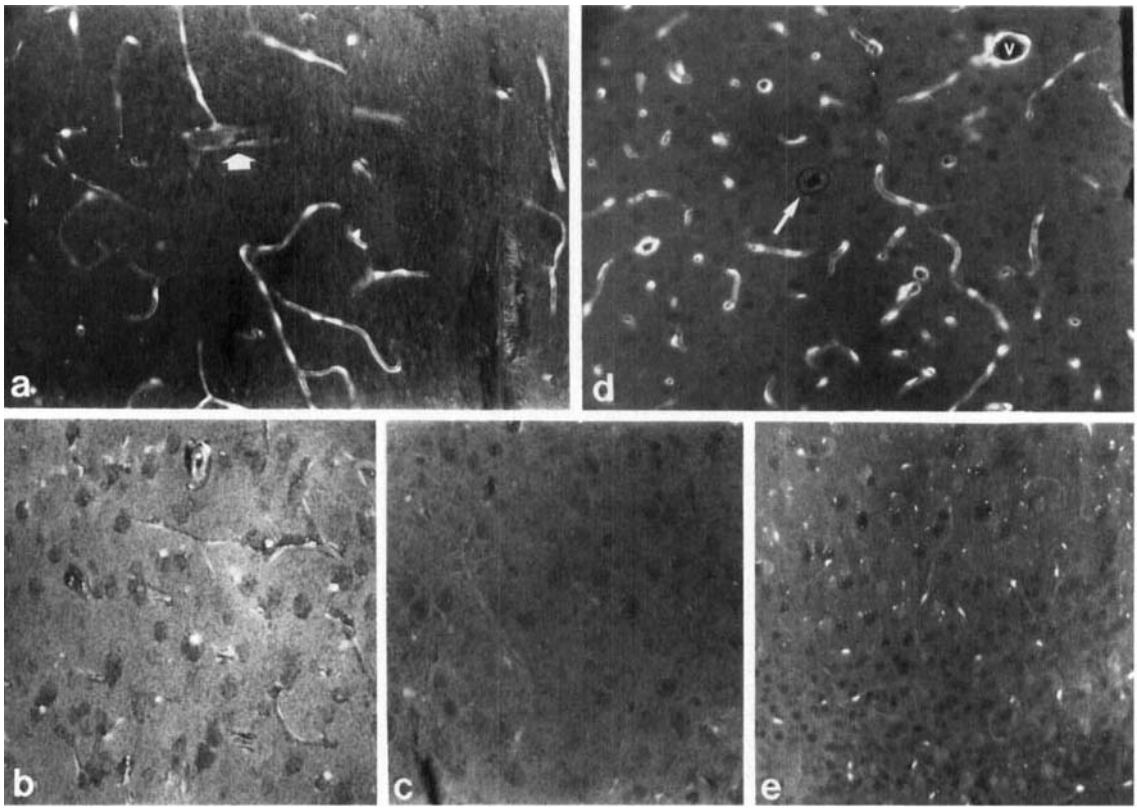

Fig. 2. Fluorescence photomicrographs of brain tissue slices following incubation in the presence of catecholamine after previous inhibition of MAO (10^{-3} M in the medium). (*a*)–(*c*). Human temporal cortex. ×90. (*a*) Intense fluorescence in microvessel walls, but no accumulation in arterioles (arrow), after incubation with 10 μg/ml noradrenaline. (*b*) In the presence of ouabain 10^{-5} M the degree of noradrenaline accumulation is markedly reduced. (*c*) During hypothermia (0°C) only very little noradrenaline accumulates in the microvessel walls. (*d*)–(*e*) Rat parietal cortex. (*d*) Intense fluorescence in the walls of capillaries and venules (*v*), but not in arterioles (arrow), after incubation with 10 μg/ml dopamine. ×110. (*e*) Desipramine (10^{-6} M) reduces the microvascular accumulation so that only the more voluminous nuclear region is clearly visible. ×75.

slices, pretreated with nialamide, were incubated in the presence of increasing concentrations of NA: a maximum fluorescence was seen at the 20 μg/ml concentration. It has been shown in model experiments that there is a linear relationship between fluorescence intensity and the catecholamine concentration. Due to quenching of the fluorescence at high catecholamine concentrations, a further increase in the concentration above a certain level gives an unchanged or even slightly diminished intensity (Ritzén 1966, Jonsson 1971). However, our finding most probably reflects an upper limit for accumulation in the microvessel wall rather than being the result of quenching, which occurs only at very high local concentrations.

The inhibitory influence on the uptake of NA by various drugs or by modification of the medium are listed in Fig. 1 and illustrated in Fig. 2. The pattern of inhibition was the same for the other monoamines studied except for 5-HT, which uptake was resistant to desipramine treatment.

Incubation of microvessels. An uptake and accumulation of radioactivity was obtained in the microvessel fraction after incubation in the presence of tritiated NA, as evidenced by a considerably higher radioactivity than in the incubation medium (Fig. 3). The uptake was significantly reduced by blockade of Na^+/K^+-dependent ATPase activity with ouabain, by incubation under anoxia and absence of glucose, or at 0°C (Fig. 3).

the cells of the microvessel wall is clearly distinguishable (Flodmark et al. 1969, Hardebo et al. 1979*a*). Under these conditions the monoamines may enter the cytoplasm of the endothelial cells by pinocytosis (Hansson, Johansson & Blomstrand 1975), or it may pass between (or through) the endothelial cells to reach the abluminal side of the endothelial membrane and from here enter the endothelial cell as well as the pericyte. It can be assumed that a sufficient inactivating mechanism for extraneuronal monoamines in the brain parenchyma is of fundamental value for maintaining an adequate brain function; a clearance process via the brain microvessel walls may work as a local complement to the neuronal re-uptake mechanism and enzymatic breakdown. It is therefore not unexpected that the active uptake of monoamines into the microvessel walls is almost exclusively working across the abluminal membrane of the endothelial cell, in direction from the brain into the cytoplasm of this cell (and of the pericyte). This would explain why monoamines, following intraparenchymal (Bertler et al. 1966) or intraventricular infusion (Fuxe & Ungerstedt 1968, and own unpublished observations), accumulate in the microvessel walls in a narrow zone around the stich channel and periventricularly, respectively.

Pretreatment with the MAO inhibitor, nialamide, enhanced the in vitro accumulation of monoamine into the endothelial cells and pericytes of the microvessel wall. This finding offers further support for the presence of MAO in these cells (Bertler et al. 1966, Spector et al. 1977, Hardebo et al. 1977 and 1979*b*).

The study has shown the presence of a saturable, energy-dependent transport of monoamines into brain microvessel walls. The process shares characteristics with both neuronal and extraneuronal monoamine uptake mechanisms and resembles the microvascular transport of NA and 5-HT in the lung. Once entering the microvessel wall, the monoamines are effectively metabolized by the locally present MAO. Although monoamines penetrate the luminal membrane of the cerebral microvessel only to a minor extent, MAO will provide an inactivation of those who do enter. However, the major inactivation mechanism at the level of the microvessel is accomplished by the transport and metabolism of amines in the direction from the brain parenchyma. Both aspects of this inactivation at the blood-brain interphase may assist in securing an adequately low level of neurotransmitter in the extracellular compartments. However, in certain pathological conditions—marked impairment of the morphologic barrier capacity and brain ischemia-anoxia—this inactivation mechanism may be inefficient. The transient increase in neurotransmitter monoamine levels that occurs in the brain extracellular fluid compartment initially during ischemia-anoxia (Meyer et al. 1973, 1974, see also Wurtman & Zervas 1974)—that may be detrimental to the brain by increasing the metabolism in the ischemic-anoxic brain area—may in part be due to an impaired uptake of the transmitter bulk into the microvascular wall locally.

This study was supported by the Swedish Medical Research Council (Grant No. 04X-732), and the Swedish Medical Doctors Association.

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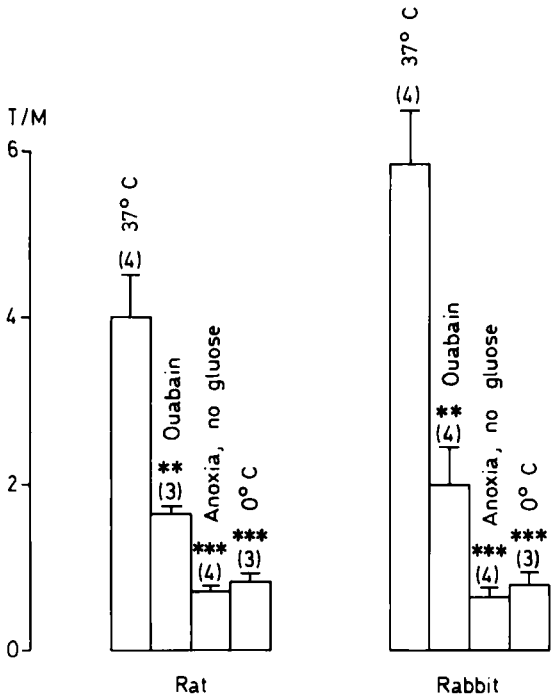

Fig. 3. Tissue/medium ratio (T/M; mg tissue per μl medium) following incubation of isolated cerebral microvessels in the presence of ^{3}H-NA under control conditions (37°C), and in the presence of ouabain (10^{-5} M), under combined anoxia and deprivation of glucose, or at low temperature (0°C). Values are means + S.E.; number of experiments within parenthesis. Control vs. experimental according to Student's *t*-test: ** $0.001<p<0.01$; *** $p<0.001$.

DISCUSSION

The present study provides evidence for the existence in various species including man of a saturable, energy-dependent uptake mechanism for neurotransmitter monoamines in the endothelial cells and pericytes of brain microvessels, in contrast to the endothelium of brain parenchymal arterioles. For comparison, in e.g. the erythrocyte these amines enter by facilitated diffusion (Blakeley & Nicol 1978). The present study does not show whether the uptake occurs across the luminal or the abluminal side of the microvessel wall or both. An uptake of monoamines into brain microvessel walls of the rat has been reported by estimation of formaldehyde-induced fluorescence following incubation of brain DA, NA and their alpha-methylated analogues (Hamberger 1967). In the latter study it was found that the uptake was prevented by desmethylimipramine, *d*-amphetamine and cocaine to a similar extent as the uptake into NA-storing nerve terminals of reserpine-pretreated animals.

The efficiency of the ^{3}H-NA uptake, as reflected by the ratio of radioactivity between the microvessel fraction and the medium, was of the same order as that reported for the uptake into sympathetic nerves under similar conditions (Edvinsson & Owman 1977, Alm et al. 1979), but considerably higher than the extraneuronal uptake in smooth musculature (Alm et al. 1979). The energy-dependency of the microvessel uptake was confirmed by the inhibition accomplished by hypothermia, combined anoxia and glucose deprivation, or uncoupling of the oxidative phosphorylation with dinitrophenol. The results from the incubations with low sodium and high potassium, as well as in the presence of ouabain, suggest that the uptake is linked with a Na^+/K^+-dependent ATPase. The uptake was inhibited both with compounds that are known to affect the neuronal (cocaine, desipramine, phenoxybenzamine) and extraneuronal (normetanephrine, oestradiol, phenoxybenzamine) transport of monoamines. This shows that, although the accumulation in brain microvessels is extraneuronal, it has several features in common with the axonal uptake process, however without the subsequent cytoplasmic retention of the amine by a reserpine-sensitive storage mechanism (Bertler et al. 1966). Thus, the uptake and accumulation of amines in the cerebral microvessels in many respects resembles the uptake shown to occur in the wall of pulmonary microvessels (for review, see Gillis 1976), though a difference in the degree of uptake between the various amines could not be established.

The extraction of trace-amounts of neurotransmitter monoamines from the brain circulation during a single capillary passage is only about 3–5% (Oldendorf 1971, Hardebo et al. 1977, Hardebo & Nilsson 1979). Only at high circulating concentrations, and after inhibition of the MAO activity, is it possible by fluorescence microscopy to visualize a weak accumulation of monoamines in the brain microvessel wall (Hardebo et al. 1979*b*). The poor penetrability across the luminal endothelial membrane is due to the unity of the structural blood–brain barrier (Reese & Karnovsky 1967), which to a large extent impedes the passage of these water-soluble and polar substances. On the other hand, when the blood–brain barrier is opened experimentally, an uptake and accumulation of monoamines in

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