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Histofluorescence Study on Monoamine Entry into the Brain Before and After Opening of the Blood-Brain Barrier by Various Mechanisms

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Summary. The relationship between exogenous, circulating monoamines to the wall of cerebral microvessels, and the entrance of these amines into the cerebral parenchyma was studied by the formaldehyde histofluorescence technique in rats. No monoamine fluorescence could be detected in the wall tissue of the microvessels (pericytes and andothelial cells) unless either MAO or COMT were inhibited; these are integral to the blood-brain barrier mechanisms to monoamines. After transient opening of the morphologic blood-brain barrier by either a hypertonic of hypertensive insult, the amine fluorescence in the walls of the microvessels was intensified compared to that which was noted after monoamine oxidase inhibition by itself. Following opening of the structural blood-brain barrier, the circulating amines also passed through into the neuropil where they were concentrated within neurons, as demonstrated by prior depletion of endogenous monoamine transmitters by reserpine. Thus, both enzymatic and morphologic mechanisms in the blood-brain barrier ar involved in impeding the passage of monoamines into the cerebral parenchyma.

Key words: Blood-brain barrier – Monoamines – Fluorescence histochemistry – Monoamine oxidase – Catechol-O-methyltransferase – Barrier opening

Cerebral microvessels, in contrast to those of the peripheral circulation, are equipped with unique barrier properties that prevent various substances from entering the brain parenchyma. The morphological basis for the blood-brain barrier has been recognized at the ultrastructural level: the cerebral vessels are distinguished by the presence of closed tight junctions between adjacent endothelial cells (Reese and Karnovsky, 1967) along with absence of endothelial fenestrae and a paucity of pincocytotic vesicular transport across the endothelial cells. In this way, the diffusion of substances from the circulation into the brain is severely limited (for review, see Rapoport, 1976).

Another barrier mechanism involves the presence of aromatic amino acid decarboxylase activity in the endothelial cells and pericytes of capillaries and venules (Bertler et al., 1966; Owman and Rosengren, 1967; Hardebo et al., 1976). Monoamine precursors, such as L-dopa and L-5-hydroxytryptophan, are prevented from entering the brain from the circulation by a fascilitated uptake into the endothelial cells and pericytes (Wade and Katzman, 1975; Oldendorf and Szabo, 1976) after which a local decarboxylation to the corresponding monoamine takes place, most probably followed by a breakdown by monoamine oxidase (MAO), also present in these cells (Bertler et al., 1966; MacKenzie et al., 1975).

It is generally considered that there is only a minor, if any, penetration of neurotransmitter monoamines from the circulation into the brain (Axelrod et al., 1959; Weil-Malherbe et al., 1961; Bertler et al., 1966; Oldendorf, 1971). The present fluorescence microscopic study elucidates the ability of such monoamines to get access to the brain parenchyma under conditions when the enzymatic or morphological blood-brain barrier is intact or transiently opened.

Materials and Methods

Animals

The experiments were carried out on 66 adult male Sprague-Dawley rats, weighing 300–350 g and fed by standard pellets and tap water. The animals were initially anesthetized in a jar containing 2–3% halothane. A tracheal cannula was inserted for artificial ventilation with a small animal respirator (Braun); anesthesia was continued with a mixture of 70% N_2O and 30% O_2. The body temperature was kept

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around 37° C as checked by a rectal thermometer. Thin teflon catheters were placed in the femoral artery and vein on one side. The femoral arterial catheter was used for continuous recording of mean arterial blood pressure and anaerobic sampling of blood for measurement of arterial gas tensions (BMS Mk II; Radiometer, Copenhagen). The animals were maintained at normocapnia and normoxia. In those animals in which the blood-brain barrier was opened (see below), the main trunk of the right external carotid artery was cannulated centripetally with the catheter opening placed within 2 mm distal to the carotid bifurcation, and the remaining branches of the ipsilateral external carotid were ligated. Injections through the cannula in the external carotid artery were thus carried into the internal carotid artery directly to the brain (with a minor amount to the territory of the pterygopalatine and occipital arteries).

Groups of animals received i.p. injections of 20 mg/kg L-noradrenaline (NA; 23 animals), L-adrenaline (A; 10 animals), dopamine (DA; 14 animals) and 5-hydroxytryptamine (5-HT; 13 animals) 4–5 min before killing. In 2 animals, 5-HT (20 mg/kg) was given i.v. 2 min before killing. To avoid an acute rise in the systemic blood pressure – which would have caused a transient opening of the blood-brain barrier (Haas et al., 1975), withdrawal of blood was performed when the blood pressure (MABP) started to increase, about 30 s after the injections, and the MABP was not allowed to exceed 160 mm Hg during the experiment (Johansson and Linder, 1978).

Nineteen of the animals had received nialamide (100 mg/kg i.p.), an inhibitor of both MAO A and B (Neff and Yang, 1974) 1 h before the amine (NA 8 animals, DA 4 animals, A 3 animals and 5-HT 4 animals). Four of the animals receiving NA had been given reserpine (5 mg/kg i.p.) 4 h before nialamide. In another group of 6 animals the catechol-O-methyltransferase (COMT) inhibitor U-0521 (20 mg/kg; Giles and Miller, 1967) was given i.p. 30 min before NA. Twentyone of the animals had received Evans blue i.v. 10–20 min before the amine (1 ml of a 1.5–2.0% solution in Krebs-Ringer buffer); 4 animals received Evans blue alone. At the concentration used, Evans blue binds totally with serum proteins (Freedman and Johnson, 1969) and does, therefore, no penetrate the blood-brain barrier under normal conditions. No marked change in MABP was noticed after administration of nialamide, U-0521 or Evans blue.

Hypertonic Opening of the Blood-Brain Barrier

Just before the administration of the amines, 1 ml of Krebs-Ringer buffer solution (pH 7.4, 37° C) containing 2 M urea was infused (13 animals) during 15 s down the cannulated external carotid artery to reach the brain. Nine of the animals had received Evans blue i.v. 10–20 min beforehand.

Hypertensive Opening of the Blood-Brain Barrier

Just before the administration of the various amines, 1 ml of blood (autotransfusion of blood slowly collected from the femoral artery immediately before) was rapidly injected (9 animals) over 3 s into the cannulated external artery to reach the brain. Five of the animals had been given Evans blue 10–20 min beforehand.

Fluorescence Microscopy

The animals were decapitated (some were perfused with 0.9% saline through the left ventricle of the heart beforehand) and the brain was removed. Selected regions (frontal, parietal, temporal, and occipital cortex with underlying white matter as well as caudate nucleus, thalamus, mesencephalon, and cerebellum) were frozen rapidly in a propane-propylene mixture cooled to the temperature of liquid nitrogen. After subsequent freeze-drying, the specimens were processed for fluorescence microscopy according to the method of Falck and Hillarp, involving exposure of the tissue to gaseous formaldehyde under dry conditions (Björklund et al., 1972). NA, DA, and A display a green fluorescence, the fluorescence of 5-HT is yellow (Björklund et al., 1972), and the Evans blue exhibits a red fluorescence (Steinwall and Klatzo, 1966) under the fluorescence microscopic conditions used.

Drugs

L-Noradrenaline bitartrate monohydrate, L-adrenaline bitartrate, dopamine hydrochloride and 5-hydroxytryptamine creatinine sulphate (all Sigma), nialamide (Niamid, Pfizer), 3′4′-dihydroxy-2-methylpropiophenone (U-0521, Upjohn), reserpine (Serpasil, Ciba), Evans blue (Sigma).

Results

Intact Blood-Brain Barrier

Fluorescence microscopy of brain tissue from control animals receiving no monoamine or Evans blue revealed a greenish background fluorescence of the brain tissue. The wellknown appearance and distribution of the formaldehyde-induced fluorescence in the monoamine neuron systems were recognized. The internal elastic lamina of larger pial arteries had a greenish autofluorescence.

All brain regions taken from animals injected with Evans blue alone showed the same general background fluorescence of the brain tissue. In animals not perfused with saline at killing, the lumen of the vessels contained Evans blue, which could be visualized easily by its bright red fluorescence. No Evans blue was noted in the vascular walls of either perfused or non-perfused animals except for a frequent red fluorescence which was restricted to the intima of the large pial vessels, especially arteries.

In non-perfused animals to which monoamines were administered, but not pretreated with either an MAO or a COMT inhibitor, fluorescence microscopy revealed a fluorescence which was restricted to the lumen of all vessels. Perfusion of the animals with saline did not reveal any wall fluorescence, except for a weak amine fluorescence in the wall of large pial arteries. However, in animals that were pretreated with nialamide or U-0521 (and perfused at killing) a very weak green (yellow for 5-HT) fluorescence was noticed in the endothelial cells and pericytes of microvessel walls (capillaries and venules), but not in the wall of parenchymal arterioles. The neuronal and background fluorescence of the brain parenchyma was unaffected. No refilling of the neuronal stores of monoamines was observed when these had been depleted beforehand by reserpine. However, this does not exclude the possibility that small amounts of amine, left undetected by the histofluorescence method used, have nevertheless

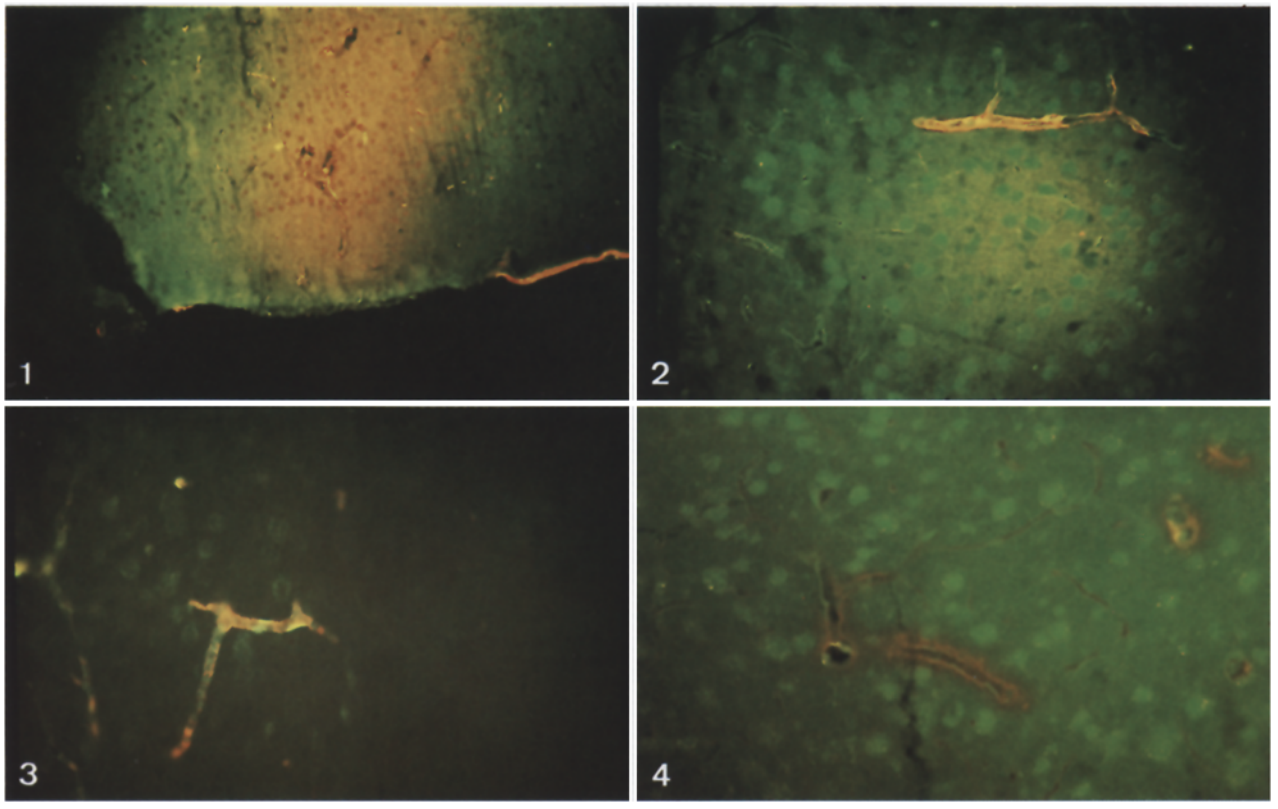

Fig. 1. Fluorescence photomicrograph of a section from the right hemisphere of a brain removed 10 min after the injection of a bolus of 2 M urea into the right internal carotid artery. The area of diffuse reddish parenchymal fluorescence represents extravasation of the Evans blue-albumin complex from the vessel seen in the center. Many of the neurons within and adjacent to the stained region are loaded with Evans blue. × 65

Fig. 2. In areas reached by the concentrated urea bolus, but showing little or no macroscopic or microscopic extravasation, a reddish fluorescence was sometimes seen in the walls of small vessels which indicates damage to the walls. In addition, a weak penetration into the parenchyma is seen. × 175

Fig. 3. Rat pretreated with urea and nialamide and injected with 20 mg/kg NA, 4–5 min before killing. In addition to the reddish fluorescence of Evans blue, a distinct green NA fluorescence is visible in the microvascular endothelial cells and pericytes. Neurons storing endogenous catecholamine in their cytoplasm are well visible. × 175

Fig. 4. Nialamide- and reserpine-pretreated rat, in which the blood-brain barrier was opened by a hypertensive insult, showing the entrance of exogenous circulating NA 120 mg/kg i.p., 4–5 min before killing, into the microvessel walls, and into the brain parenchyma (note the diffuse greenish background fluorescence) and neurons which have become loaded with NA fluorescence (including the nuclear region). In addition, reddish fluorescence of Evans blue in vessel walls and in the surrounding parenchyma. × 175

leaked into the brain after inhibition of MAO or COMT. In animals that received both the monoamine and Evans blue no extravasation of Evans blue was noticed either macro- or microscopically; this would indicate that the blood-brain barrier mechanisms to protein were unaffected by the drug administration.

Opened Blood-Brain Barrier

In rats to whom Evans blue was administered after the intracarotid injection of 2 M urea, a patchy blue staining was seen macroscopically; these patches were somewhat more prominent in superficial than in deep structures of the affected hemisphere, whereas the contralateral hemisphere and the brain stem (including cerebellum) remained largely unstained. In a few animals localized, discrete segments of the pial arterial walls were seen to blue. Likewise, after a rapid intracarotid infusion of blood, a punctuate blue staining was seen macroscopically, more commonly in the superficial than in the deep structures of the affected hemisphere. As in the experiments with urea, segments of the pial arterial walls were stained blue. The signs of extravasated Evans blue correspond to the regions reached by the initial high concentration of urea, or by the bolus of blood, i.e., the territory of the anterior and middle cerebral artery (the proximal part of the anterior cerebral artery is unpaired, which means that the urea and the bolus of blood reaches the supply of this artery in both hemispheres). In a few animals treated with the

rapid blood infusion, extravasation of Evans blue was also seen in the cerebellar hemisphere, indicating a transient redistribution in flow due to the high-pressure infusion.

In the fluorescence microscope a red fluorescence of patchy distribution was seen around arterioles, capillaries and venules in all parts of the parenchyma in those regions which showed a macroscopically visible staining (Fig. 1). The green formaldehyde-induced neuronal fluorescence in these regions of extravasation was not as well visible as in the remainder of the parenchyma; the neurons were even seen to be loaded with red-fluorescent material (Fig. 1; Flodmark et al., 1969; Blomstrand et al., 1975). Occasionally, a red fluorescence was also seen in the wall of parenchymal vessels, particularly in capillaries and in the wall of pial arteries and arterioles, not only in areas showing extravasation (Fig. 2).

Fluorescence microscopy of brain tissue from animals receiving monoamines after pretreatment with nialamide and given urea or a rapid infusion of blood showed a weak to moderate fluorescence (weaker for 5-HT than catecholamines) in the walls of microvessels and pial veins, and occasionally in pial and parenchymal arterioles (Fig. 3). Only when the neuronal stores of monoamines had been depleted by reserpine was it possible to show any local spread of the monoamine into the brain parenchyma, reflected by a refilling of the neurons. A further indication of an impaired blood-brain barrier function was evidenced by extravasation of the additionally given Evans blue (Fig. 4).

Discussion

It is generally considered that there is only a minor, if any, penetrability of monoamines across the blood-brain barrier (for review, see Edvinsson and MacKenzie, 1976). It can be assumed that a sufficient barrier to transmitters, such as NA and 5-HT, at the blood-brain interface is necessary for an adequate function of neurotransmission in the brain. Only in the newborn animal, in which the barrier is not yet fully developed, has a substantial uptake of circulating neurotransmitter monoamines been demonstrated (Loizou, 1970); the exogenous amines accumulated not only in the brain neurons but also in endothelial cells and pericytes of cerebral microvessels. In the adult animal, fluorescence microscopy after systemic administration of DA has indicated that the passage of amines is greatly impeded already at the luminal surface of the brain vessel (Bertler et al., 1966). After systemic injection of NA, an accumulation in the adventitia of pial arteries has been found by autoradiography (Samorajski and Marks, 1962). Evidence has been presented for a minor passage into the brain parenchyma of circulating 5-HT (Bulat and Supek, 1968; Welch et al., 1972), NA and A (Weil-Malherbe et al., 1961; Zakusov et al., 1972). In line with this, a small percentage (3–5%) of trace amounts of exogenous NA, A, DA, and 5-HT is extracted from the brain circulation during a 5–15 s circulation time (Oldendorf, 1971; Hardebo and Nilsson, 1979a). The method used to determine the "brain uptake index" for these amines does not reveal whether the amine accumulates only in the vascular wall or, in addition, leaks further into the parenchyma. Since the neurotransmitter monoamines are fairly small molecules (MW 153–183), it is probable that their poor lipid solubility and high polarity, rather than their molecular size, is the main limiting factor for their poor penetrability across the barrier. The low penetrability across the intact barrier of circulating amines, such as NA, DA, and 5-HT, may be one of the reasons for the weak effect on total cerebral blood flow seen after intravascular administration (for review, see Edvinsson and MacKenzie, 1976).

That monoamines in fact may accumulate in the cerebrovascular walls, although to a small extent, after systemic administration to animals with an intact barrier, is supported by the present findings. Only after pretreatment with an MAO (nialamide) or COMT inhibitor (U-0521) was any detectable accumulation found in the microvessel wall of animals with an intact morphological barrier. Further, the accumulation in animals with an impaired barrier function was greater in those given nialamide than in animals not subject to this treatment. One explanation for these findings is that inhibition of the peripheral break-down of the monoamine will result in higher concentrations available in the cerebral circulation. However, taken together with findings by others (MacKenzie et al., 1975; Spector et al., 1977; Hardebo et al., 1979c), an additional explanation is the presence of MAO acting as an enzymatic barrier mechanism to monoamines in the cells of the cerebral microvessel wall.

MAO and COMT activity has been demonstrated in brain vessels (Spector et al., 1977; Hardebo et al., 1979c). The fractions of isolated small brain vessels mainly consists of capillaries and venules, but are inevitably contamined with parenchymal arteries, arterioles and veins. The enzyme (MAO) activity is not significantly influenced by elimination of the perivascular sympathetic innervation. Thus, both enzymes have primarily an extraneuronal localization, and may be present in the endothelial cells and/or smooth muscle cells. The endothelial cells in cerebral arteries, arterioles and veins, in contrast to those of capillaries and venules, may lack MAO and COMT activity, since not

even inhibition of these enzymes will reveal any accumulation of the amines in the former cells in vitro (Hardebo et al., 1979b) or in vivo, as shown in the present study. However, in analogy with the situation in cerebral capillaries and venules, a minor amount of monoamines may well pass the luminal endothelial membrane of the arteries and arterioles – and, in the absence of an enzymatic breakdown mechanism within this cell, also the abluminal membrane of the endothelial cell – thereby reaching the smooth muscle cell layer. The monoamines will now be efficiently taken up and metabolized by MAO and COMT in the vascular smooth muscle cells (for review, see Spector et al., 1972), in the perivascular sympathetic nerves, and probably also in perivascular mast cells (Edvinsson et al., 1977). This uptake of amine may well be left undetected by the fluorescence method used because it does not accumulate in an intracellular store (smooth muscle cells) or it mixes with the endogenous amine (nerve terminals and mast cells). Hence, the perivascular smooth muscle cell layer may act as a blood-brain barrier mechanism for monoamines.

It has been shown in vitro (incubation of brain slices) that neurotransmitter monoamines accumulate in the endothelial cells and pericytes of brain microvessels due to a saturable, energy-dependent uptake mechanism (Hamberger, 1967; Hardebo et al., 1979b). The finding that a more intense monoamine fluorescence develops in these cells in vitro and at situations with a opened, defect or circumvented morphological blood-brain barrier (see also Bertler et al., 1966; Fuxe and Ungerstedt, 1968; Flodmark et al., 1969; Loizou, 1970), compared to the intensity after systemic administration with an intact barrier, indicates that the amine is taken up primarily at the abluminal side of the cells. The efficient accumulation at this site – apart from the subsequent intracellular inactivation (i.e., deamination) – may be looked upon as yet another mechanism limiting the entrance of circulating monoamines into the brain parenchyma and, in addition, as an inactivation mechanism for excess of neurotransmitter monoamines present in the brain extracellular compartment. Endothelial cells of vessels in regions outside the central nervous system are, however, with few exceptions not capable of accumulating these amines (Bertler et al., 1966; Owman and Rosengren, 1967). It should be noted that high local concentrations of neurotransmitter monoamines may actually per se cause an impairment of the morphological barrier function (Westergaard, 1975).

Inhibition of MAO in the cerebral vessels should result in an enhanced access of monoamines to the brain parenchyma. A metabolic effect within the brain of baboons can in fact be obtained by circulating noradrenaline after pretreatment with the MAO A inhibitor, clorgyline (MacKenzie et al., 1975). Similarly, following pretreatment with the MAO B inhibitor, deprenyl – for which phenylethylamine is a substrate – a significant reduction in cerebral blood flow and metabolism is obtained in the baboon after administration of a dose phenylethylamine that is without effect in normal animals (McCulloch and Harper, 1978). In vitro studies have indicated that human brain MAO is primarily type B, and that dopamine in human brain is a type B substrate (Youdim, 1977; Glover et al., 1977). Deprenyl has recently been added to the combined L-dopa and decarboxylase inhibitor treatment in Parkinson's disease (Birkmayer et al., 1977). The therapeutic improvement resulting from this treatment is believed to be caused by an enhanced amount of DA available at DA receptor sites, through inhibition of brain MAO. If the MAO present in human brain microvessels also is of the B type, an additional explanation would be an inhibition of the enzymatic barrier mechanism to DA.

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