

Altered Blood-Brain Barrier Permeability and Its Effect on the Distribution of Evans Blue and Sodium Fluorescein in the Rat Brain Applied by Intracarotid Injection

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Summary

The aim was to study the blood-brain permeability according to the distribution in the rat brain of Evans blue (EB) and sodium fluorescein (NaFl) administered by an intracarotid injection. Eighteen animals were divided into six groups according to the state of the blood-brain barrier (BBB) at the moment when the dyes were being applied. In the first two groups, the BBB was intact, in groups 3 and 4 the barrier had been opened osmotically prior to the application of the dyes, and in groups 5 and 6 a cellular edema was induced by hyperhydration before administration of the dyes. The intracellular and extracellular distribution of the dyes was studied by fluorescence microscopy. The histological picture thus represented the morphological correlate of the way BBB permeability had been changed before the application of the dyes.

Key words

Blood-brain barrier • Cellular edema • Intravital dyes • Fluorescence microscopy

Introduction

The blood-brain barrier (BBB) ensures an optimally controlled homeostasis of the brain's internal environment. Its anatomical structure consisting of the gliovascular complex with tight junctions between the endothelial cells enables a very selective transport of substances from the blood to the brain by any of the following routes: 1) paracellular water way through the tight junctions for hydrophilic substances of less than 180 Da molecular weight; 2) transcellular diffusion of lipophilic substances; 3) transcellular route using transport proteins, e.g. glucose, some amino acids; 4) endocytosis mediated by specific receptors, e.g. for

insulin, transferrin; 5) adsorptive endocytosis and transcytosis for cationized plasma proteins, i.e. transport vesicles (Abbot and Romero 1996, Kroll and Neuwelt 1998).

The BBB is studied with a great attention for several reasons, namely to discover more details about its architecture, development, biology and physiology (Pardridge 1999, Rubin and Staddon 1999, Bauer and Bauer 2000, Kniesel and Wolburg 2000, Stewart 2000), and to search for methods of BBB opening safely and reversibly for the application of diagnostic and therapeutic substances, to which an intact BBB is impermeable (Kroll and Neuwelt 1998, Rapoport 2000). Most interest is focused on the BBB under pathological

conditions, particularly on its role in vasogenic brain edema, which is – together with cytotoxic (cellular) swelling – the most frequent cause of life-threatening intracranial hypertension (Betz *et al.* 1989, Klatzo 1994, Marmarou 1994, Kimelberg 1995, Dirnagl *et al.* 1999, Kempinski 2001).

BBB permeability can be tested by different methods. The most widely used one in experimental models is that intravenously applied intravital dyes (tracers, markers) of molecular weight greater than 180 Da preclude passage across an intact barrier. In terms of molecular size, these markers represent a broad spectrum of dyes, in which sodium fluorescein (NaFl, MW 376 Da) is one of the smallest, while Evans blue (EB, MW 961 Da) is one of the largest. In blood circulation, however, the latter becomes strongly, though reversibly, bound to the albumin fraction of proteins to give rise to a high-molecular complex (EBA 68 500 Da) (Broman 1944, Wolman *et al.* 1981, Lafuente *et al.* 1990, Kroll and Neuwelt 1998, Pokorný *et al.* 2002). As Wolman *et al.* (1981) showed, EB would not bind to albumin until after at least five minutes after its application into the blood stream, and the time factor relevance for the development of this bond is likewise stressed by Moos and Mollgard (1993). Hence, in experimental models designed to test BBB permeability, EB is injected i.v. at least 5-10 min before BBB opening, e.g. by an osmotic impact. In such experiments, EB presence in the brain is detected either macroscopically, using an all-brain staining scale, or microscopically where evidence of EB permeation of the BBB is seen in its extravasation or in its presence in the brain capillary system. The presence of a sodium fluorescein extravasate in the brain tissue is consistent with low-molecular NaFl passage across the barrier (Lafuente *et al.* 1990, Kroll and Neuwelt 1998). According to Greenwood *et al.* (1988), if EB is applied by the arterial route (EB application into the ascending aorta 2 min after opening the BBB with mannitol) it will pass through the barrier as a “pure” dye (MW = 961 Da) without bonding to albumin. Invasion of the barrier was rated microscopically as EB extravasation into the brain tissue.

The purpose of our study was to obtain a histological picture of the propagation of sodium fluorescein (MW 376 Da) and pure Evans blue (MW 961 Da) in some of the areas of the rat brain after injection into the carotid system with altered BBB permeability. Changes of permeability were induced in a number of ways (see below).

Methods

Eighteen adult Wistar rats weighing 400-450 g were divided into six groups of three animals each according to the technique of dye application and change in BBB permeability. Group 1: the dyes were injected into the right common carotid artery (CCA) with the BBB intact. Group 2: the dyes were injected into the right internal carotid artery (ICA) with the BBB intact. Group 3: the dyes were injected into the right CCA two minutes after the application of mannitol. Group 4: the dyes were injected into the right ICA two minutes after the application of mannitol. Group 5: the dyes were injected into the right ICA of hydrated rats (see below). Group 6: the dyes were injected into the right ICA two minutes after mannitol application to hydrated rats. The rats were treated in compliance with valid guidelines for work with laboratory animals – EU Guidelines 86/609/EEC. For general anesthesia, each rat received 4 mg/100 g of thiopental applied intraperitoneally. Using the conventional technique, the right-sided CCA, ICA and arteria carotis externa (ECA) were exposed from a linear incision between the sternal manubrium and mandible. A polyethylene catheter fixed by ligation was introduced into the bifurcation through a small arteriotomy of the CCA. The ICA application groups (groups 2, 4, 5, 6) had, in addition, the ECA ligated close behind the bifurcation. Prior to the application of the dyes, rats of groups 5 and 6 were hyperhydrated with distilled water given intraperitoneally in quantities equal to 15 % of their body weight. The total amount was divided into three doses given at intervals of 8 h by intraperitoneal injections during 24 h before BBB permeability testing (Olson *et al.* 1994). Rats of groups 1, 2 and 5 had only the dyes – 2 % EB at a dose of 2 ml/kg, and 10 % NaFl, 0.6 ml/kg – applied from one syringe at a rate of 0.45 ml/min (Saris *et al.* 1988). Rats of groups 3, 4 and 6 had 20 % mannitol (200 g ad 1000 ml aqua pro inj., 1098 mosmol/l) applied through the same catheter two minutes before dyes application at a dose of 5 ml/kg and a rate of 0.12 ml/s (Rapoport 2000). After the application, the catheter was removed and the CCA ligated below and above the arteriotomy. The operation wound was closed in a single layer, and the spontaneously ventilating rat was placed in a heated box (37 °C). Within 20-30 min after the end of intracarotid injection, the brain was fixed by transcardial perfusion with a 4 % paraformaldehyde solution in pH 7.4 phosphate buffer for a period of 15 min, and then fixed in the same solution for another 24 h. Each brain was then sliced on a vibratome into

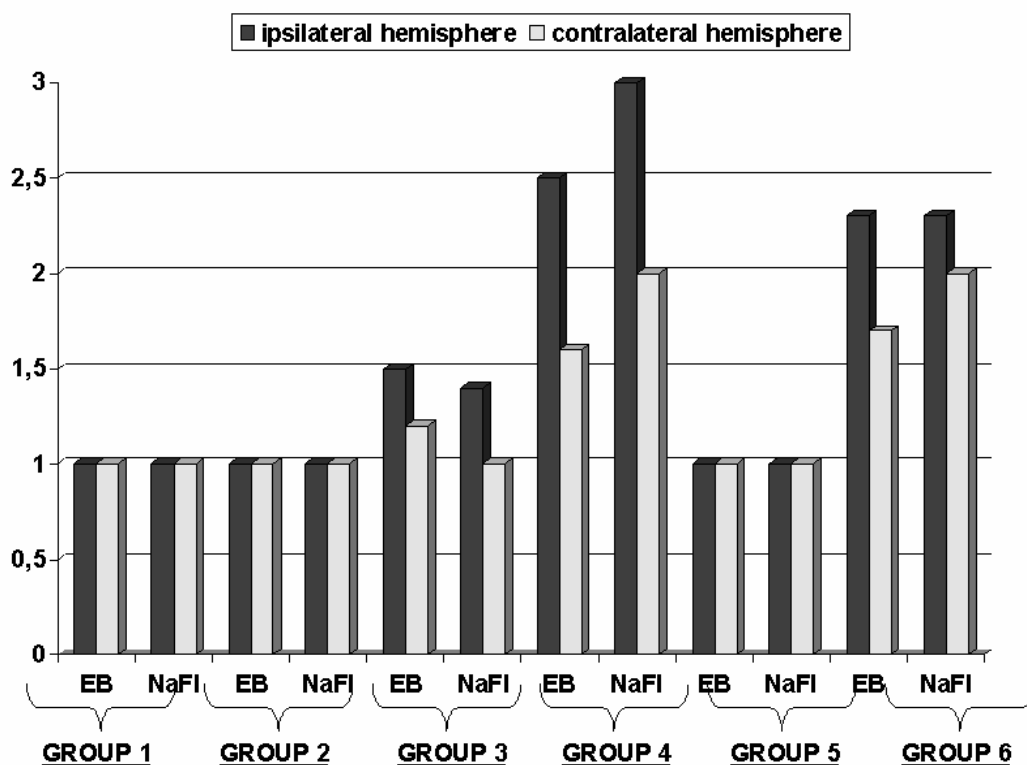
coronary sections 30 µm thick and then, without further staining, placed on slides for microscopic examination. The sections were studied under a fluorescence microscope for staining intensity, intracellular and extracellular distribution of the dyes in the cortex, in the CA1, CA3 areas and in the hippocampal gyrus dentatus (GD) – both in the ipsilateral (right) and in the contralateral (left) hemispheres. For rating of staining intensity, we used a three-degree scale of 1 to 3 (1 = faint, 2 = medium, 3 = intense). The ratio of intracellular/extracellular distribution of the two dyes in each estimated area was expressed in per cent (e.g. 62.5 %:37.5 % representing the intracellular/extracellular

distribution in the given area). The results represent the average of all the data obtained in three rats of each particular group. As plasma natremia reflects the degree of hyperhydration, hyponatremia confirms osmotic cellular edema. The respective blood samples were obtained from a catheter in the CCA before any of the applications.

Results

Results of overall staining and intracellular/extracellular ratio of dyes obtained in each group are summarized in Figures 1, 2A, 2B and 2C.

Fig.1: Overall intensity of staining



EB - Evans Blue, NaFl - sodium fluorescein

Rats of groups 1 and 2 had the dyes applied by intracarotid injections with the BBB intact. In spite of that, there was some weak staining of the target tissue, when the dye was injected into the ICA (Fig. 2A).

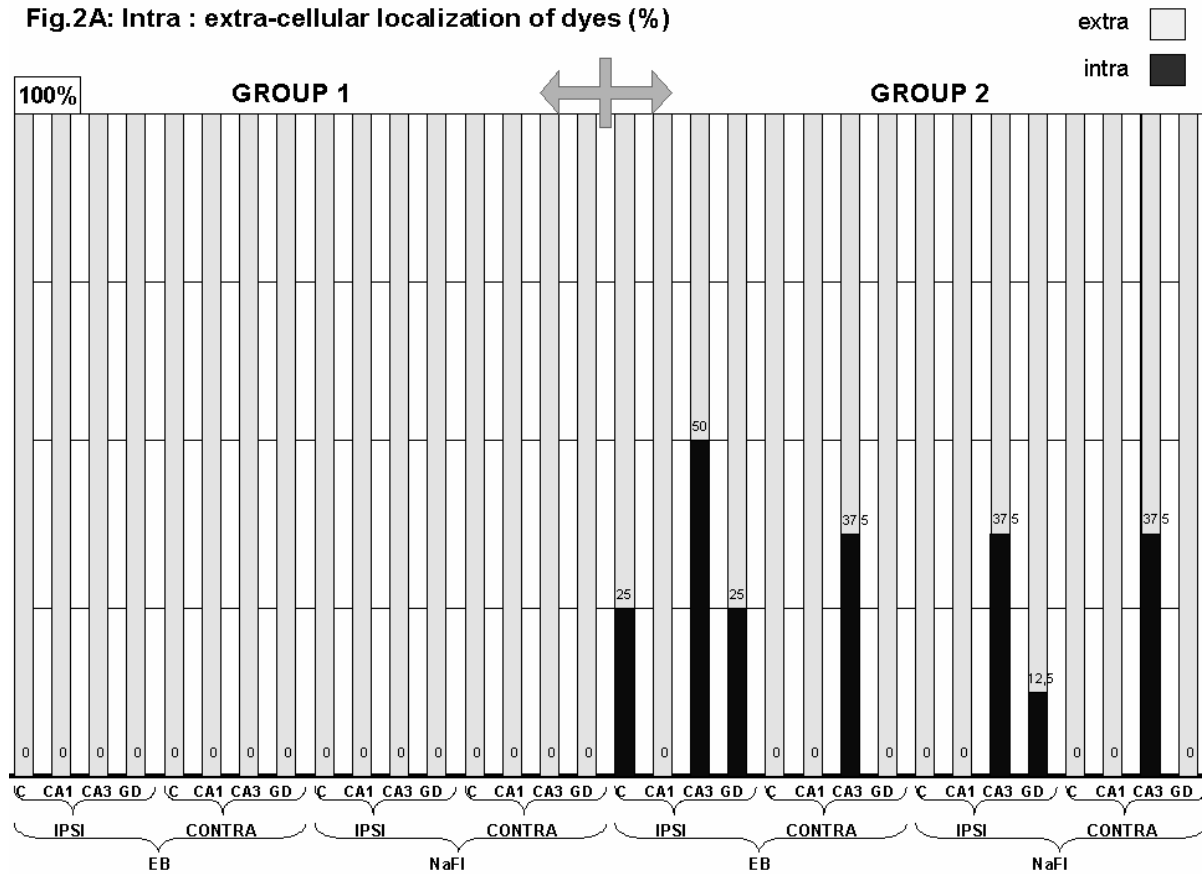
Animals of groups 3 and 4 had the dyes applied after the BBB had been opened with mannitol. An

experiment with osmotic opening of the BBB was first accomplished by Rapoport (1970). It was long after the blood-brain barrier had been opened with an intra-arterially applied hypertonic solution as an undesirable side effect of a contrast medium in cerebral angiography (Broman and Olsson 1949). BBB opening in response to

a single intracarotid administration of a hyperosmolar solution results in endothelial cell shrinkage thus considerably expanding increasing tight junction spacing. This mechanism of BBB opening is the outcome of cerebrovascular dilatation, dehydration of endothelial cells, and contraction of their cytoskeleton solely under the effect of the osmotic impact without participation by other, energy-dependent mechanisms of BBB permeability enhancement (Greenwood *et al.* 1988, Rapoport 2000). The experimental opening of the BBB by means of an osmotic impact is performed by selective injection of mannitol into the internal carotid (ICA). Mannitol injected into the common carotid (CCA) results in only a small or no opening of the BBB (Kroll and Neuwelt 1998, Rapoport 2000) because in this case most of the solution escapes into the ECA system, thus losing

the concentration and osmolality required for opening the barrier. This experience is confirmed by our results (Fig. 2B) showing an intensive intracellular distribution of the dyes after their injection into the ICA.

In groups 5 and 6, we simulated a model situation of a pathological condition – cellular osmotic edema. This is due, among other factors, to intoxication with water where hyponatremia features as the pathophysiological mechanism of its development. The animals of these groups had been hyperhydrated with distilled water applied in the course of the 24-h period preceding the application of the dyes, and the blood samples did, indeed, show plasma hyponatremia. Plasma natremia in rats of groups 5 and 6 was, on average, 20 mmol/l lower than that in rats of remaining groups.



C - cortex, CA1 - area of hippocampus, CA3 - area of hippocampus, GD - gyrus dentatus, IPSI- ipsilateral hemisphere, CONTRA - contralateral hemisphere, EB - Evans Blue, NaFl - sodium fluorescein, extra - extracellular localization, intra - intracellular localization

Fig.2B: Intra : extra-cellular localization of dyes (%)

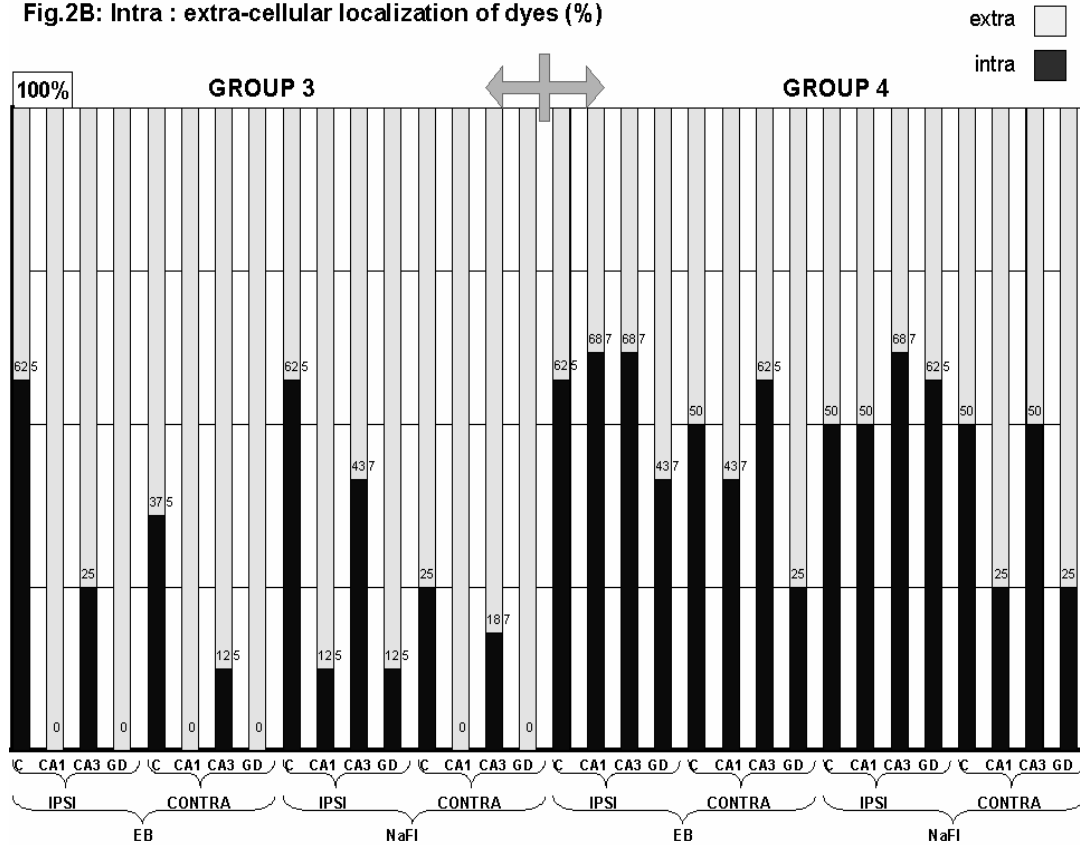
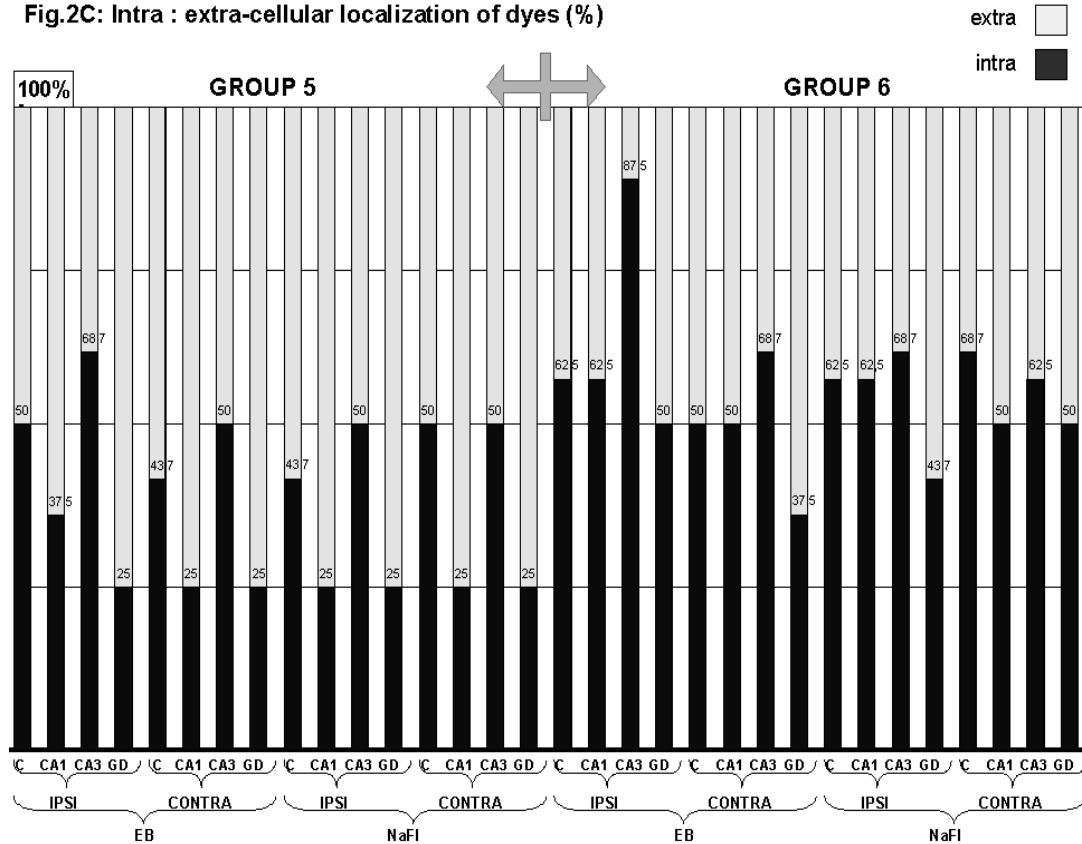


Fig.2C: Intra : extra-cellular localization of dyes (%)



C - cortex, CA1 - area of hippocampus, CA3 - area of hippocampus, GD - gyrus dentatus, IPSI- ipsilateral hemisphere, CONTRA - contralateral hemisphere, EB - Evans Blue, NaFl - sodium fluorescein, extra - extracellular localization, intra - intracellular localization

Discussion

In rats of groups 1 and 2, the entry of the dye into the brain can be attributed to a number of factors which together influence the intact barrier on its luminal side. These concern a rapid increase in intravascular and, consequently, hydrostatic pressure (Barzó *et al.* 1997, Betz *et al.* 1989, Marshall *et al.* 1969), sudden hypervolemia in a limited area (Rapoport 2000), presence of a hypertonic solution on the luminal side of the BBB (Rapoport *et al.* 1972), and lastly, the direct effect of the staining agent. The last resembles the side effect of an angiographic contrast medium (Broman and Olsson 1949). A considerable amount of the dyes escaping into the ECA system during injection into the CCA can explain their intracerebral presence when they are injected into the ICA.

In rats of group 3 and 4, the presence of the dye in cells shows a loss of cell membrane integrity resulting from changes in the composition of the extracellular compartment in response to the opening of the barrier. A prominent role is played by loss of the physiological function membrane Na,K-ATPase (Avert *et al.* 1984, Barzó *et al.* 1997, Kempinski 2001). In response to the osmotic impact, the BBB opens gradually according to the blood circulation rather than instantaneously in all regions of the brain. This accounts for the differences found in ipsilateral hemisphere in contrast to the contralateral hemisphere (Saris *et al.* 1988, Betz *et al.* 1989, Kroll and Neuwelt 1998, Rapoport 2000). The difference in the intracellular/extracellular ratio of the dye distribution in the studied areas is due to the greater vulnerability of some cells, the membranes of which react with greater sensitivity to changes in the extracellular compartment composition in the wake of BBB opening. It applies mainly to cells in the areas of CA 3 and hilus (GD) of the hippocampus (Dhillon *et al.* 1999) where a more intensive intracellular distribution was also noticed in our study.

The current classification of brain edema is a follow-up of the original division into vasogenic (BBB opened) and cytotoxic swellings (BBB intact) (Klatzo 1967), although there are some terminological changes. Instead of the originally defined entity of cytotoxic edema (a model of TET intoxication), the term cellular edema is now used since the toxic noxae leading to the development of purely cellular edema with the BBB

intact may comprise, e.g. ischemia, trauma or hyponatremia. Moreover, none of these pathological conditions is associated with only one type of edema. On the contrary, these can change during the time, so that the initially pure vasogenic edema gradually results in the development of cellular edema just as much as cellular edema can induce BBB opening and enable the development of vasogenic edema (Betz *et al.* 1989, Klatzo 1994, Marmarou 1994, Kimelberg 1995, Barzó *et al.* 1997, Kempinski 2001). This experience is also documented by our results in group 5 (dyes injected into the ICA of hydrated rats) as distinct from group 2 (dyes applied into the ICA with the BBB intact) (Fig. 2A, C). Cellular edema induced in our study by hyperhydration and hyponatremia brings about an osmotic imbalance on the cell membrane followed by intracellular permeation of sodium and simultaneous accumulation of water. The intracellular flow can convey some of the water-soluble dye, which makes these cells visible in light microscope. This primary impact will set off a cascade of processes leading to a subsequent increase in BBB permeability. The cascade includes: a loss of calcium and potassium homeostasis (Siesjo 1993, Kimelberg 1995, Barzó *et al.* 1997), release of excitotoxic amino acids (Bullock *et al.* 1994, Kimelberg 1995, Barzó *et al.* 1997), release of free oxygen radicals (Kontos 1989, Kimelberg 1995, Barzó *et al.* 1997), and induction of intracerebral tissue acidosis (Siesjo *et al.* 1993, Kimelberg 1995, Barzó *et al.* 1997).

The different histological picture of EB and NaFl propagation after the same mode of application in group 2 unlike group 5 is, in our view, evidence of increased BBB permeability as a consequence of induced cellular edema. Group 6 in our study represents a combination of BBB opening from the luminal side (mannitol) and, at the same time, from the abluminal side (cellular edema). For obvious reasons, the largest proportion of intracellular distribution of the two dyes was found just in this particular group (Fig. 2C).

In conclusion, the purpose of the present study was to draw attention to some of the options opened up by *in vivo* experiments for the study of BBB permeability changes. We regard the method employed as a useful approach mainly because the histological picture of EB and NaFl distribution under the fluorescence microscope is the only constant under scrutiny. This picture represents a morphological correlate of BBB permeability changes under preset conditions, including the fact that at

the end of the application all the rats had their CCA occluded with a ligature. As the rat has a completely developed Willis circuit, the occlusion of one carotid in experimental ischaemia models results in no histological alterations (Seta *et al.* 1992). We have chosen the

intracarotid mode of intravital dye application to simulate application of other, e.g. neuroprotective substances.

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Reprint requests

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