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Differential passage of [^{14}C]sucrose and [^{3}H]inulin across rat blood-brain barrier after cerebral ischemia

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Abstract The biophysical nature of blood-brain barrier (BBB) opening after ischemic or hemorrhagic stroke or traumatic brain injury is unresolved. Ultrastructural (electron micrograph) investigations of experimental BBB injury commonly indicate the abnormal presence of vesicles or tubular structures in cerebrovascular endothelial cells, suggesting the likelihood of convective, fluid-phase transport of blood substances into brain. We measured transfer constants (K_is) for the simultaneous passage of two intravenously delivered tracers ([^{14}C]sucrose, mol wt=342; [^{3}H]inulin ≈5,000) across the intact BBB in the rat, and 24 h after global cerebral ischemia (16–20 min duration) or 24, 48 or 72 h after focal ischemia (2 h duration). In both ischemia models, the upward increment in K_i (ΔK_i) for sucrose, indicating the extra injury-related tracer flux into brain, significantly exceeded that for inulin, as might be expected with faster diffusion of the smaller molecule through injury pores or channels. This inequality of ΔK_is did not suggest a major role for convective, fluid-phase transport by endothelial vesicular or tubular structures and a predominance of diffusional transport was indicated.

Keywords Cerebral ischemia · Blood-brain barrier · Cerebrovascular injury · Stroke

Introduction

The special barrier properties of brain microvasculature that control access of circulating molecules to the extracellular space of neurons and glia are usually compromised after ischemia, concussion, and other insults to brain [11]. The common clinical manifestation is vasogenic edema resulting from indiscriminate movement of plasma solutes and water into brain. The biophysical nature of blood-brain barrier (BBB) opening associated with various injuries or disease processes is not clearly understood, but possible mechanisms include widening of the tight-junctional complexes that join cerebrovascular endothelial cells, enhancement of pinocytotic vesicular transport by these cells, and the formation of pore-like lesions in endothelial membranes [16]. Electron micrograph (EM) studies of BBB opening that follows cerebral ischemia in the rat show induction of pinocytotic vesicles and tubular profiles in endothelial cells as evidenced by the disposition of horseradish peroxidase or lanthanum after global or focal ischemia [4, 6, 7], but an exception to this has also been reported, i.e., tight junction opening without vesicular transport after permanent focal ischemia [23]. The EM appearance of fixed tissues cannot be solely definitive of dynamic transport mechanisms however [2, 25], and use of additional methodologies is important. Measurement of the simultaneous BBB passage of low versus high molecular weight tracers provides another way of assessing the dynamics of BBB opening [13, 14, 28], and this was the objective of this study using two rat models for mimicking the effects of ischemic stroke. One procedure produces global cerebral ischemia, as would occur during temporary cardiac arrest and the other mimics the focal effects of thromboembolic blockage of the middle cerebral artery (MCA). The delayed BBB opening and vasogenic edema which develops after such insults to brain play an important role in the evolution of secondary, delayed neurological damage [11] and knowledge of the mechanism of BBB opening is fundamental to the development of therapies for vasogenic edema.

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Materials and methods

Experiments were performed on male Sprague-Dawley rats (340–400 g). Procedures were approved by a local committee for the Canadian Council on Animal Care. Bilateral cerebral ischemia using the two-vessel occlusion model (2VO, modified after [24]) was carried out with mechanical ventilation and physiological

monitoring (tympanic and rectal temperature, blood pH, pCO_2, pO_2 and glucose) and normothermic temperature control of the rat under pentobarbital anesthesia (65 mg/kg i.p.) [17]. Bilateral carotid clamping for 16, 18 or 20 min was combined with blood withdrawal through a tail-artery cannula to maintain arterial pressure at 42–47 mmHg. After blood re-infusion, clamp removal and wound closure rats were maintained normothermic until recovered from anesthetic and then returned to housing. Because of the global nature of the 2VO insult, and potential varying susceptibility of individual animals, several ischemic durations were tested, beginning first with 16-min 2VO. The objective was to achieve substantial, statistically significant BBB opening 24 h later using a minimum number of rats and without causing the moribundity that can result from severe BBB opening and vasogenic edema.

To produce focal ischemia by MCA occlusion (MCAO) the rat was placed under 1.5–2% isoflurane anesthesia, the right temporal skull area exposed without damage to the zygomatic arch, and a 3-mm burr hole was created to reveal the MCA where it traverses the rhinal fissure. A miniature aneurysm clip [3] was applied to the MCA, the wound sutured, and the rat returned to housing. Twenty-four hours later, the rat was briefly anesthetized with isoflurane, and the common carotid arteries were exposed and occluded with vascular clamps to minimize collateral circulation and effect ischemia in the territory of the occluded MCA [27]. Metal wound clips were applied to the neck incision and the rat permitted to recover for 2 h after which, anesthesia was briefly restored, the carotid arteries unclamped, and the skin wound sutured. The rat was returned to its cage and sacrificed 24 h, 48 or 72 h later in a radiotracer experiment, at the termination of which the MCA clip was also retrieved.

To measure BBB permeability rats were anesthetized with sodium pentobarbital, a femoral artery cannula inserted and 300 units sodium heparin solution (0.3 ml) injected directly into the femoral vein followed by a bolus of saline (~1 ml) containing 25 μCi [^{14}C]sucrose (NEC-100X, NEN Dupont) and 65 μCi [^{3}H]inulin (NET 314). A syringe pump connected to the arterial cannula was immediately started (time 0), to withdraw a total 0.5 ml arterial blood sample over the next 30 min. At 0+25 min the right carotid artery was cannulated and at 0+30 min the head vasculature cleared of blood by perfusion of 25 ml saline [18]. Weighed samples of frontoparietal cortex, the entire striatum, both hippocampi (after 2VO) or cerebral hemispheres (MCAO) and 50-μl volumes of plasma were prepared [17] for dual-label liquid scintillation counting. The concentration of each tracer was determined in brain parenchyma (C_{paren}, dpm/g) and plasma (C_{plasma}, dpm/ml). The latter value was corrected for dilution by the syringe/cannula dead-space (~50 μl saline, measured) and multiplied by the circulation time (1,800 s) to provide the time-integrated plasma concentration ($_0I^{1,800}C_{plasma}dt$, dpm.s/ml). Transfer constants (K_is) were calculated from the following relationship [15]: $K_i = C_{paren}/_oI^{1,800}C_{plasma}dt$.

[^{3}H]Inulin used for the above experiments was purified by dialysis using cellulose tubing (3,500 mol wt cutoff) as described previously [17]. Purity of the [^{14}C]sucrose stock was considered acceptable on basis of its focused migration relative to unlabeled sucrose on thin-layer chromatograms and the magnitude of preliminary baseline K_i measurements in rats with intact BBB, which can be unduly elevated by the presence of BBB-permeable radioimpurities [21].

To show histological appearance of cortical damage two rats were subjected to 20-min 2VO and two rats to MCAO. After 24 h, the animals were anesthetized with pentobarbital and subjected to perfusion fixation of brain using saline followed by buffered formalin. Paraffin-embedded coronal brain sections (10 μm) were mounted on slides and stained with hematoxylin and eosin.

Results

Transfer constants measured initially in four non-operated rats (BBB intact) provided baseline data enabling evaluation of the degree of BBB injury measured 24 h after global (2VO) ischemia. As determined by measurements of BBB opening, 2VO ischemias were carried out in the following sequence in ten rats: 16 min 2VO (n=4); 18 min (n=2); 20 min (n=4). Six of these animals (one 16-min, one 18-min, all 20-min) exhibited substantial cortical BBB opening, indicated by sucrose K_is ranging from 10.1 to 25.4 nl.g^{-1}.s^{-1} as compared to four non-operated control rats which had a mean baseline K_i of 2.1±0.25 (± SEM, range 1.9–2.7). The three other 16-min rats showed milder BBB damage (range 5.2–8.6, also see below), while one 18-min animal failed to exhibit cortical BBB opening (K_i=2.3, data not used). The measurements from the six animals with marked cortical BBB injury were combined to compare the simultaneous injury-related flux of inulin vs sucrose in these particular animals (Table 1). Mean regional K_is for sucrose were significantly higher than those for inulin both in four non-ischemic control rats (column 4 vs 1) and in the six post-ischemic rats (5 vs 2), indicating a faster rate of BBB passage for the smaller molecular-weight tracer. With either tracer the BBB leakiness was greater in ischemia-injured cortex compared to striatum or hippocampus as indicated by mean K_i (columns 2 and 5) and by mean K_i increments (ΔK_is, 3 and 6), which signaled the augmented flux of each tracer attributable to the injury per se. In all three regions the upward increments in K_i for sucrose significantly exceeded those for inulin (column 6 vs 3). Analysis of individual experiment ΔK_i ratios for sucrose/inulin ($\Delta K_{i,suc}/\Delta K_{i,inu}$) yielded

Table 1 Comparison of regional transfer constant (K_i) for simultaneous passage of inulin and sucrose across the blood-brain barrier 24 h after cerebral ischemia in the rat (2VO model) and in non-operated controls. Values are mean ± SEM for n=4 control rats and n=6 rats subjected to 16–20 min of cerebral ischemia 24 h earlier. ΔK_is were obtained by subtracting the mean control values from K_is obtained in individual post-ischemic rats (*2VO* two-vessel occlusion model)

	Inulin K_i (nl.g^{-1}.s^{-1})			Sucrose K_i (nl.g^{-1}.s^{-1})		
	(1) Control	(2) Ischemia	(3) ΔK_i	(4) Control	(5) Ischemia	(6) ΔK_i
Cortex	0.84±0.05	5.09±0.71[a]	4.25±0.71[a]	2.11±0.25	15.43±2.38[a]	13.33±2.38[ab]
Striatum	0.74±0.08	3.09±0.28	2.34±0.28	1.62±0.21	8.33±0.91	6.71±0.91[b]
Hippocampus	1.03±0.36	2.42±0.37	1.39±0.37	1.71±0.38[c]	7.19±0.96	5.47±0.96[b]

Within columns: [a]P<0.05 comparing cortex to striatum or hippocampus (ANOVA plus Tukey-Kramer test). Within region (*row*): [b]P<0.005 comparing ΔK_is in column 6 vs 3 (Student's pair *t*-test). Within region (*row*): P<0.05 (unmarked) for all comparisons of K_is in columns 2 vs 1, 5 vs 4, 5 vs 2, 4 vs 1 ([c]exception P=0.15) based on Student's *t*-test for paired or unpaired observations

Table 2 Transfer constants (K_i) for simultaneous blood-brain barrier passage of sucrose and inulin measured in cerebral hemispheres of rats at 24 h (n=5), 48 h (n=3) or 72 h (n=3) after 2 h of focal ischemia in right MCA territory and comparing lesion vs contralateral, intact side. Values are mean ± SEM (*MCA* middle cerebral artery)

	Inulin K_i ($nl.g^{-1}.s^{-1}$)			Sucrose K_i ($nl.g^{-1}.s^{-1}$)		
	(1) Intact	(2) Lesion	(3) ΔK_i	(4) Intact	(5) Lesion	(6) ΔK_i
24 h	0.67±0.05	2.47±0.27	1.80±0.26	1.93±0.14	6.20±0.91	4.27±0.93
48 h	0.65±0.05	4.40±0.34[ab]	3.76±0.30[ab]	1.98±0.06	12.48±1.11[a]	10.50±1.06 [a]
72 h	0.65±0.03	3.09±0.26	2.44±0.24	1.96±0.07	8.74±1.11	6.78±1.07

Within rows: $P<0.05$ (unmarked) for all comparisons of ΔK_is in column 6 vs 3 (Student's t-test for paired data). Within column, between rows: [a]$P<0.01$ compared to 24 h, [b]$P<0.05$ compared to 72 h (ANOVA plus Tukey Kramer test)

Table 3 Transfer constants (K_i) for simultaneous blood-brain barrier passage of sucrose and inulin measured in frontoparietal cortex (48 or 72 h after focal ischemia in right MCA territory and comparing lesion vs contralateral, intact side). Values are mean ± SEM from n=3 rats per time point. Data are from same experiments as in Table 2 but based on radiotracer counts in frontoparietal cortex only (mean sample weight 172±4 mg)

	Inulin K_i ($nl.g^{-1}.s^{-1}$)			Sucrose K_i ($nl.g^{-1}.s^{-1}$)		
	(1) Intact	(2) Lesion	(3) ΔK_i	(4) Intact	(5) Lesion	(6) ΔK_i
48 h	0.53±0.05	8.52±0.56	7.99±0.55	1.95±0.13	26.37±2.25	24.42±2.19
72 h	0.63±0.12	6.73±1.14	6.10±1.22	2.04±0.15	20.88±4.37	18.84±4.45[*]

Within row and unmarked, $P<0.05$ for all comparisons of sucrose vs inulin (columns 4 vs 1. 5 vs 2, 6 vs 3) except [*]$P=0.06$, Student's t-test for paired data

mean values (± SEM) of 3.1±0.2 (cortex), 2.9±0.3 (striatum) and 4.6±0.6 (hippocampus). These values were greater than 1.0 ($P<0.005$, Student's 1-sample t-test).

Although three of the foregoing 16-min 2VO rats exhibited a lesser degree of BBB opening (not included in Table 1), they too showed discrimination in the BBB leakiness to sucrose versus inulin. The upward injury-induced increments in K_i ($nl.g^{-1}.s^{-1}$) above Table 1 mean control values ± SEM were as follows for sucrose versus inulin, respectively (ΔK_is): cortex 4.44±1.02 vs 0.96±0.41 (P= 0.03); striatum–5.31±0.82 vs 1.22±0.05 (P=0.04); hippocampus–3.49±1.49 vs 0.65±0.43 (P=0.12, Student's paired t-test).

At 24, 48 or 72 h after focal ischemia, the lesioned hemispheres showed a significant elevation in uptake of both sucrose and inulin and elevated K_is compared to those for the contralateral hemispheres taken as control measurements (Table 2 columns 2 vs 1, 5 vs 4). The upward increment in K_i (mean K_i difference between lesion vs control hemisphere) was significantly greater for sucrose than inulin at all three time points (column 6 vs 3) with the largest increments for either tracer being at 48 h. The mean ratios $\Delta K_{i,suc}/\Delta K_{i,inu}$ calculated from individual experiments were 2.3±0.2 at 24 h, 2.8±0.1 (48 h) and 2.7±0.2 (72 h) and were significantly elevated above 1.0 ($P<0.05$).

In the foregoing experiments at 48 or 72 h post-MCAO K_i values were also calculated for slabs of frontoparietal cortex which had been dissected and counted separately from the remaining ipsilateral cerebral tissue. The ΔK_i values for frontoparietal cortex (Table 3, columns 3 and 6) were about double the magnitude of values calculated for the entire ipsilateral cerebrum (3 and 6 in Table 2). The data in Table 3 also indicate the differential passage of the two tracers with the injury related flux of sucrose significantly exceeding that for inulin. The mean ratios $\Delta K_{i,suc}/\Delta K_{i,inu}$ for frontoparietal cortex, calculated from individual experiments were 3.1±0.1 (48 h) and 3.0±0.1 (72 h) and different from 1.0 ($P<0.005$). Counts of the remaining cerebral tissue obtained in the 48 and 72 h experiments indicated statistically significant BBB injury, although to a lesser degree than in the frontoparietal region, and also differential permeation of sucrose versus inulin (data not shown).

Hematoxylin and eosin-stained brain sections obtained 24 h after MCAO showed an extensive zone of injury extending throughout the right frontoparietal cortex with widespread neuronal pyknosis (Fig. 1A) as compared to contralateral cortex, which had been subjected to carotid but not MCAO (Fig. 1B). 2VO rats also showed extensive neuronal pyknosis along with prominent tissue vacuolation affecting cortex (Fig. 1C), hippocampus and striatum (not shown).

Discussion

In a previous study [19] we reported that 10 min of 2VO ischemia produced progressive opening of the BBB in striatum and hippocampus, but not cortex, characterized by a severalfold increase in sucrose K_i by 6 h post-2VO and then largely recovering by 24 h. With prolonged ischemia (20 or 25 min) by contrast, striatum and hippocampus remain BBB damaged at 24 h, but the cortical BBB markedly deteriorates between the 6 and 24 h time points, showing much greater elevations in K_is at 24 h than other

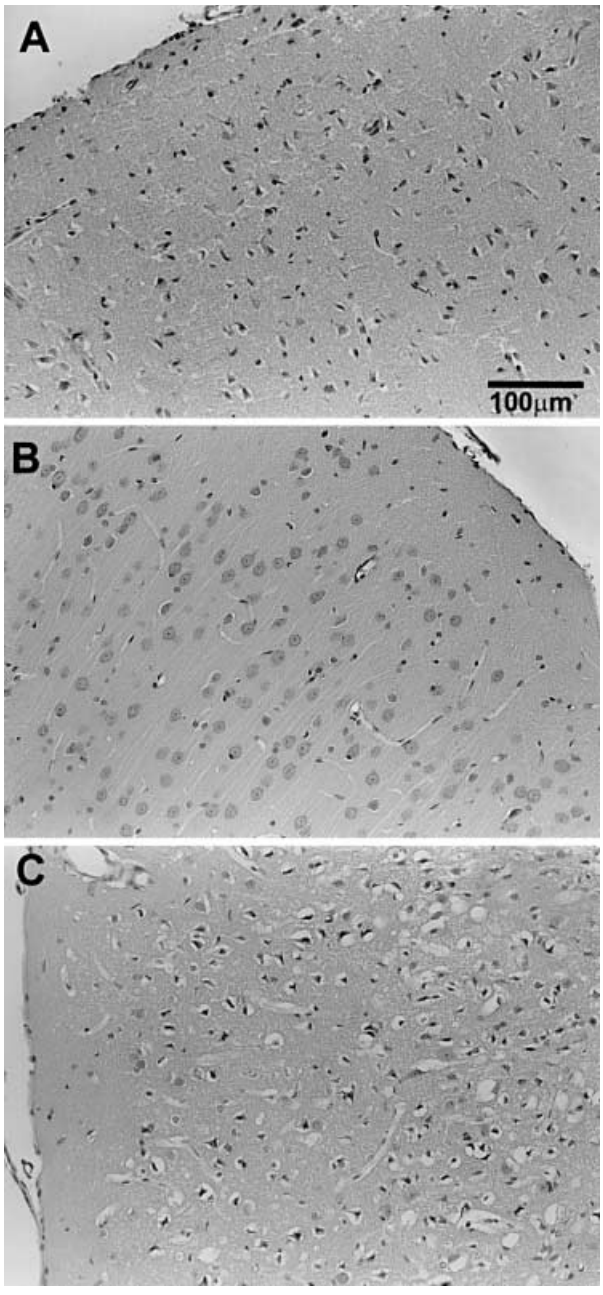

Fig. 1 Hematoxylin and eosin-stained sections of parietal cortex 24 h after focal or global ischemia. **A** After focal ischemia (MCAO) neurons throughout the focal infarct zone appeared shrunken, eosinophilic, and exhibited small, darkly stained, pyknotic nuclei. **B** By comparison, the majority of neurons in the contralateral non-infarcted cortex (same coronal section) exhibited large round nuclei of normal staining and appearance. **C** (global, 20 min 2VO) shows extensive neuronal injury as in **A** and many pale areas of vacuolation (*MCAO* middle cerebral artery occlusion, *2VO* two-vessel occlusion model). *Bar* **A**–**C** 100 μm

tissues [19, 20]. The 2VO durations tested in the current experiments were meant to evoke the aforementioned 'overnight' cortical BBB deterioration. Six of the ten 2VO rats clearly demonstrated such injury, as indicated by the magnitudes of increased K_i in cortex relative to striatum and hippocampus, and enabled comparison of simultaneous inulin and sucrose flux related to this BBB opening.

The two-step procedure for focal ischemia [27] is meant to dissociate potential complications associated with the surgery for MCA exposure (prolonged anesthesia, brain exposure and cooling) from the ischemic period. The premise that the clip alone does not cause an infarction, and that temporary (2 h) removal of collateral circulation by carotid occlusion infarcts the MCA territory by 24 h has been confirmed separately in this laboratory by presence and absence, respectively, of red formazan staining after 2,3,5-triphenyltetrazolium chloride incubation of fresh brain slices (unpublished observations). We have previously profiled the time course of BBB opening to radiosucrose after 2 h MCAO [9] using the same radiotracer methodology as present, and as with prolonged 2VO [19, 20], observed that a marked intensification of cortical BBB injury develops by 24 h and worsens by 48 h. Magnitudes of sucrose K_i increments measured in cerebral hemispheres of the MCAO rats (Table 2) are not as great as regional cortical values reported previously [9]. However, the significance of this relates at least in part to sampling because measurements of the whole hemisphere, while offering consistent sample geometry, would have encompassed considerable amounts of tissue that was either not injured or injured to a lesser degree than the MCA territory. The supplementary K_i calculations in 48- and 72-h rats (Table 3) based on separate counts of the frontoparietal MCA supply territory yielded higher K_i increments as expected and still confirmed the differential passage of the two radiotracers.

K_i values obtained using this radiotracer methodology approximate the product of cerebrovascular permeability, P, and exchange area, A, [15]. The advantage as here of measuring K_is for sucrose and inulin simultaneously in the same animals is that variations in A affect both tracers equally, and data such as that in Tables 1, 2, and 3 should reflect on their relative permeabilities. If the BBB damage after ischemia involved pores or channels broad enough to allow unrestricted diffusion of the tracers, the ratio of ΔK_is for sucrose versus inulin would approximate the ratio of their free diffusion coefficients, i.e., $D_{f,suc}/D_{f,inu}$ = 2.9 in H_2O at 38°C [1]. The two- to fivefold ratio we observed in ΔK_is for sucrose/inulin after ischemia allows the deduction that the most of the extra tracer movement across the BBB, resulting from its injury, involved a diffusional process, as opposed to bulk fluid-phase transport by pinocytosis for which the ΔK_i ratio would approximate unity. Theory indicates [5] that pores of a size sufficient to allow only partially impeded or relatively free diffusion of inulin would be comparatively large, e.g., radius 10–100 times that of the inulin molecule, 1.31 nm [1]. Such pores might also allow appreciable convective solute transport by water, tending to reduce the ΔK_i ratio (sucrose: inulin) towards unity. In previous studies we have measured a significant increase in tissue water content associated with elevated sucrose K_is after MCAO and prolonged 2VO [9, 20]. Our impression is that dissected brain

samples did appear edematous after 2VO or MCAO in the present study, and involvement of a convective component of tracer flux (in vesicles or channels) cannot be ruled out in our present K_i measurements. However, the magnitudes and ratios of ΔK_is for sucrose versus inulin indicate a predominant involvement of tracer diffusion through pores or channels, quite possibly of dimensions offering some steric hindrance to the tracer molecules. Steric hindrance, having a greater impact on the larger molecule, inulin, would account for a ΔK_i ratio (sucrose/inulin) greater than the ratio of their free diffusion coefficients ($D_{f,suc}/D_{f,inu}$).

We have earlier demonstrated [17] a pore-like nature of the regionally selective BBB injury that develops in hippocampus and striatum 6 h after 10-min 2VO and then largely recovers by 24 h [19]. It is noteworthy that the 24-h opening reported here in the cortex after prolonged 2VO (16–20 min), is also pore-like given the differences in regionality and timing [19], and possibly in the neurochemical disturbances involved. We did not examine later times after prolonged 2VO because of the global nature of the vasogenic edema, which we know intensifies between 24 and 48 h to cause seizures (unpublished observations). That the opening after focal ischemia was also pore-like might be expected, seeing that global ischemia gave this result. However, in another study [22] we have also found that a sustained period (>1 week) of BBB leakiness following mechanical trauma to the rat striatum also exhibits differential opening to sucrose and inulin, with differences in ΔK_is qualitatively similar to those we report here for 2VO and MCAO ischemia.

We cannot ascribe a major role for pinocytosis and bulk vesicular transport in these openings in context of the concept that this mode of BBB injury will provide equal transport to molecules as different-sized as sucrose and inulin [13, 14, 16, 28]. EM studies suggest the presence of enhanced vesicular transport after brain trauma [12, 26], hemorrhage [10] and both focal and global ischemia [4, 6, 7]; however, tubular structures within endothelial cells were also reported [4, 6, 7, 12]. A predominance of diffusional over convective transport in such structures may possibly account for the differential, injury-related passage of radiolabeled sucrose versus inulin seen in the present experiments.

The measurements in the present study took place at post-ischemic times 24–72 h, when inflammatory events are thought to contribute to parenchymal damage, e.g., eicosanoid production, edema, neutrophil infiltration, have been shown to reach peak levels 24–72 h after MCAO in rat brain [8]. Here we show (Fig. 1), confirming previously unpublished histology, that neuronal destruction is well developed by 24 h in both ischemia models. The radiotracer data presented here indicate that pore-like BBB opening and diffusive blood-to-brain transport are a predominant feature of the accompanying cerebrovascular pathology in these ischemic injury models.

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