SUPPLEMENTAL MATERIAL

METHAMPHETAMINE-INDUCED NITRIC OXIDE PROMOTES VESICULAR TRANSPORT IN BLOOD-BRAIN BARRIER ENDOTHELIAL CELLS

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Supplemental Methods

VEC antibodies- The peptide CLYGSDPQEELII, corresponding to the carboxyl-terminus of VEC, was synthesised and, coupled to keyhole limpet hemocyanin, used to immunise rabbits (GenePep SA, St Jean de Védas, France). Anti-VEC antibodies were further purified on immobilized CLYGSDPQEELII peptide, concentrated (to 0.1 to 0.2 mg/ml) and dialysed against PBS. Resulting antibodies were used at 0.2 or 2 μg/ml for immunoblotting and immunocytochemistry, respectively. Anti-VEC antibodies were monospecific for VEC as determined by immunoblotting (Supplemental Figure S3). Additionally, when used for immunocytochemical staining of ECs, anti-VEC revealed junction staining in normal (Figure 2) but not VEC-null cells (data not shown).

MTT reduction assay- Cell viability was analysed by measuring reduction of MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]. Confluent GPNT ECs were treated with increasing concentrations of METH. After treatment, MTT (0.5 mg/ml) was added to each well. After 3 h of incubation (until the formation of crystals), 100 μl of 50% dimethylformamide and 50 mg/ml sodium dodecyl sulfate was added. The cells were kept at 37ºC overnight until all crystals dissolved. Absorbance of each well was measured in a Safire microplate reader (Tecan, Reading, UK) at 570 nm (reference filter at 620 nm).

Supplemental Figure Legends

Supplemental Figure S1. Specificity of VEC antibodies

GPNT BMVECs were lysed and total protein extracts (ca. 50 µg) analysed by immunoblotting with affinity-purified anti-VEC antibodies. Immunodecoration using affinity-purified anti-VEC antibodies revealed a single band with an apparent mass of ca. 130 kDa. Positions of molecular mass markers are indicated on the left.

Supplemental Figure S2. Effect of METH on the organisation of the interendothelial junctions.

(**A-C**) ECs were either left untreated (NT) or treated with METH (1 µM) for 2 or 6 h. Subsequently, cells were fixed and stained for VEC, claudin-5, occludin and ZO-1, and then analysed by confocal microscopy. Shown are representative projections of overlapping 0.35 µm sections spanning the entire cell thickness. METH exposure did not disrupt the continuity of any junction staining. However, additional VEC and occludin staining was also observed at parajunctional areas in METH treated cells (red arrowheads). Enlarged regions of the staining for VEC and occludin are shown in (B). (C) Shows apical and basal sections of BMVEC, treated with METH for 6 h and stained for VEC and claudin-5. White arrowheads depict an uninterrupted interendothelial junction (as determined by the claudin-5 staining), which was also intact as judged by VEC staining. Additional diffuse VEC staining could be found in areas basal to the junction. Scale bars = 20 µm.

Supplemental Figure S3. Determination of optimal HRP loading times.

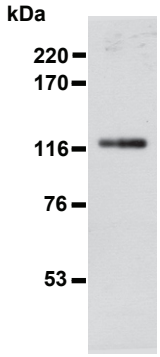
Primary BMVECs were treated with METH (1 µM) for 15 or 30 min as indicated. Subsequently, HRP (1 mg/ml) was added. After the time indicated min cells were extensively washed, cells lysed and intracellular HRP levels determined as described in the methods section. Shown are mean levels of intracellular HRP -/+ SEM (n = 3). From these experiments it was concluded that short (i.e. 5 min) incubation with HRP was just as effective to determine vesicular uptake than any longer times.

Supplemental Figure S4. Effect of METH on cell viability.

GPNT ECs were either left untreated (NT) or treated with the indicated concentrations of METH during 24 h. ECs were then incubated with MTT for 3 h and cellular reduction of MTT determined. The results are expressed as means ± SEM of four replicates from three independent experiments.

Supplemental Figure S5. Plasmalemmal caveolar structures in primary BMVECs.

Primary rat BMVECs were fixed and processed for transmission EM. Shown is a representative image (and a magnified section in b), which demonstrated the presence of caveolar structures (outlined in red) on the apical plasma membrane. Scale bars = 200 nm.



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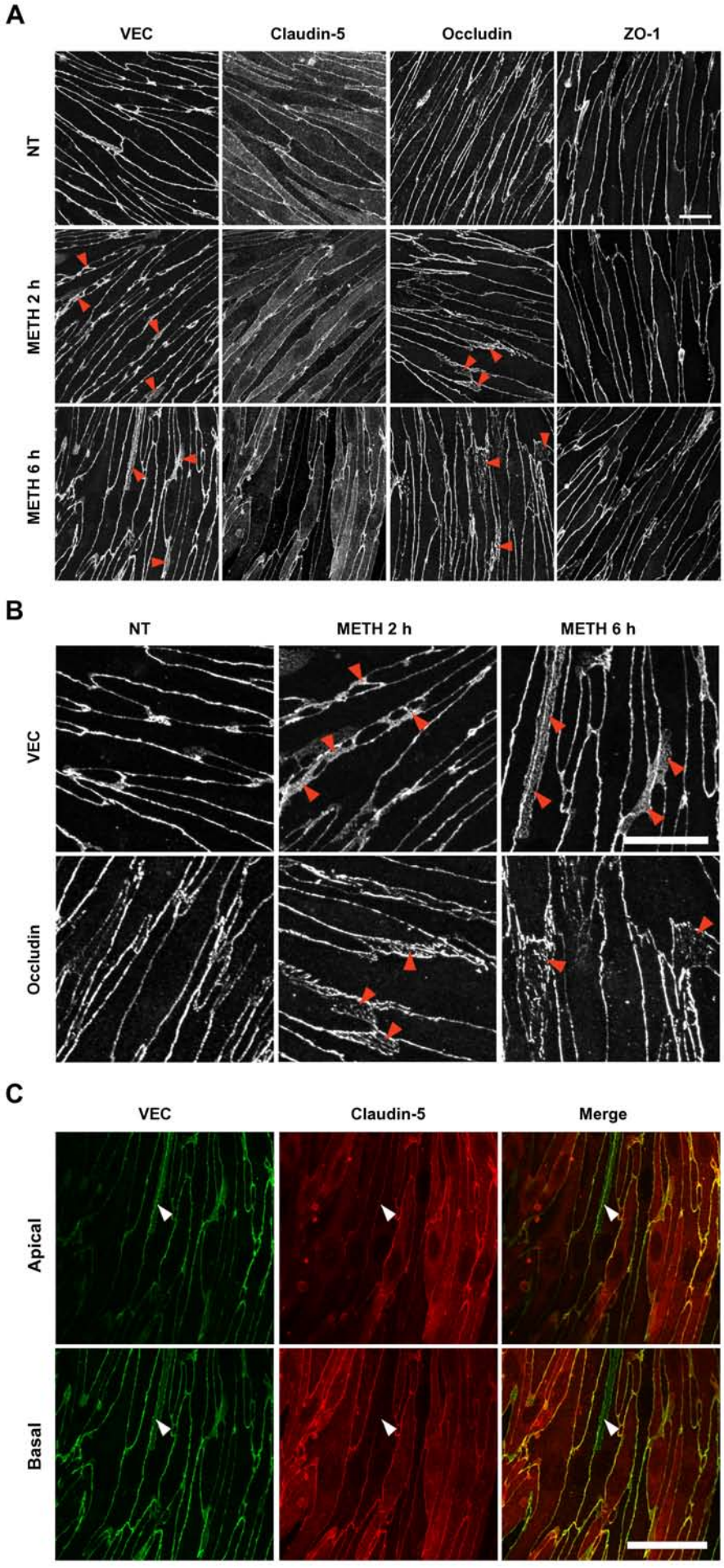
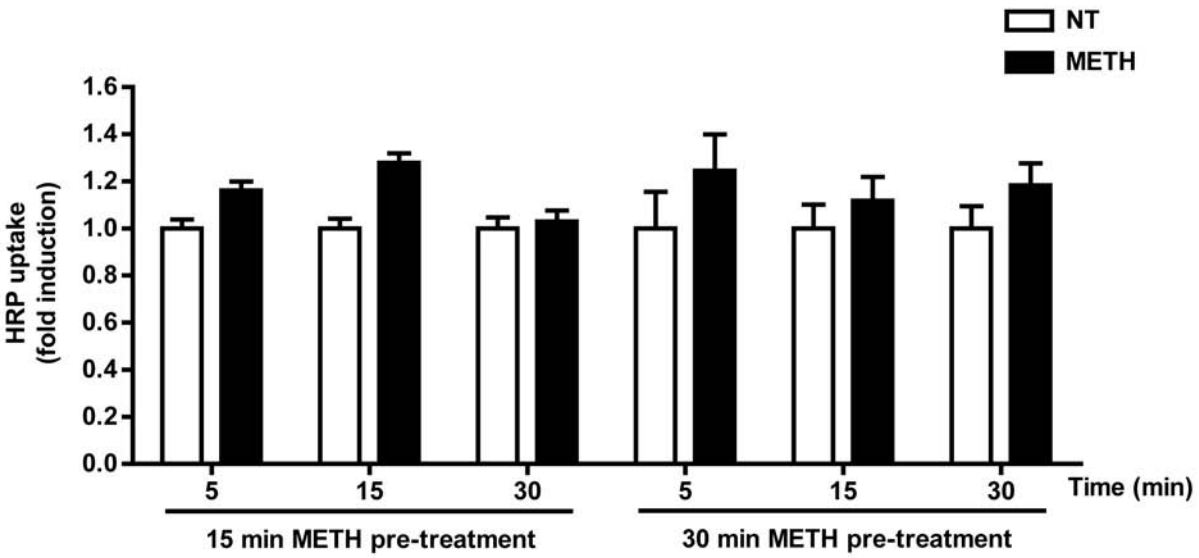
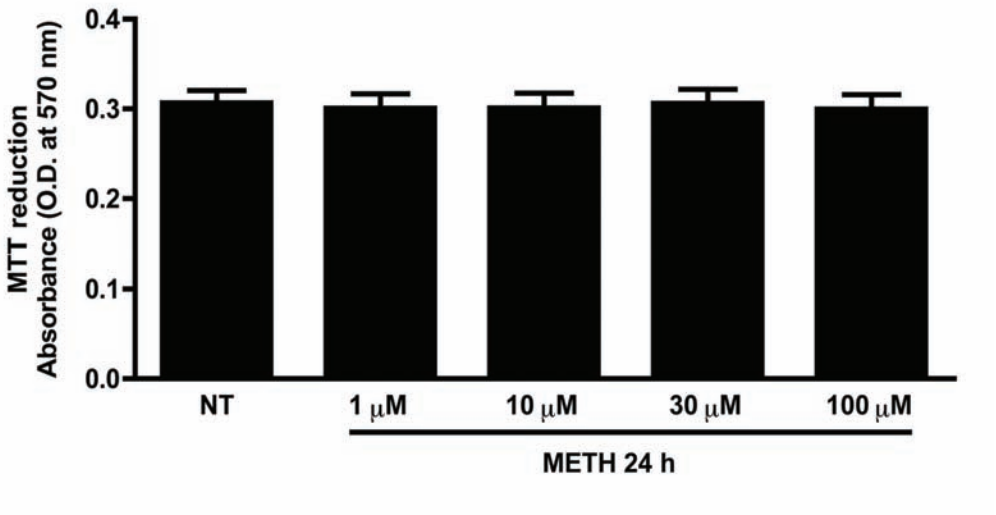


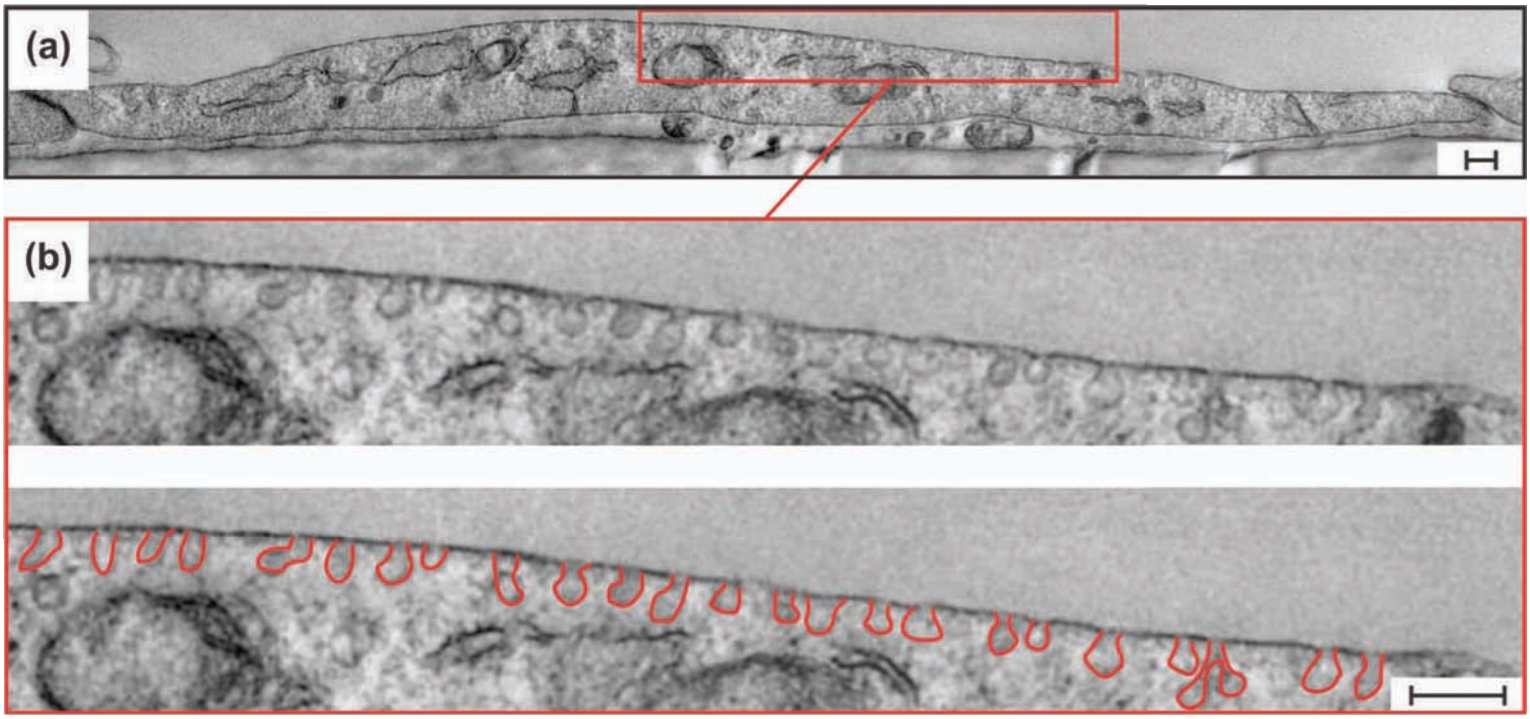
Martins et al., Suppl. Figure S2



Martins et al., Suppl. Figure S3



Martins et al., Suppl. Figure S4



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