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Neural stem cell plasma membrane vesicles as effective nanocarriers for drug delivery into the brain

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Abstract

The blood brain barrier (BBB) constitutes a major obstacle for treatment of neurological disorders as it effectively precludes by a variety of passive and active molecular mechanisms, drug delivery into the brain. The use of nanocarriers, in which appropriate drugs might be efficiently packaged, is therefore currently considered in order to circumvent BBB extrusion mechanisms, and hence to improve drug delivery. In the present work we demonstrate that plasma membrane vesicles (PMVs) derived from C17.2 neural stem cells can be prepared that display the ability to cross the BBB, as shown in an in vitro cell model, i.e., polarized human cerebral microvessel endothelial cells (hCMEC/D3), as well as in vivo, following systemic administration of the PMVs in a BALB/c mouse. Our data reveal that in contrast to the parental C17.2 neural stem cells, which cross the in vitro barrier by transcellular transport, the PMVs enter the endothelial cell layer, which constitutes the actual barrier, primarily by clathrin- and caveolae-mediated endocytosis, when added to the apical medium. Their effective appearance (approx. 25 % of the added dose) in the basolateral medium, in conjunction with control experiments that exclude PMV-induced perturbation of the hCMEC/D3 cell layer integrity, indicate that transcytosis is primarily responsible for translocation. The fact that systemic administration in vivo, which leads to a prominent appearance of PMVs in brain tissue when comparison to other tissues like liver, long and spleen, supports the view that PMVs may act as potent biological nanocarriers for delivery of therapeutics into the brain.
Introduction

Successful therapy of brain-related diseases is severely hampered by the relatively poor delivery of drugs across the blood-brain barrier (BBB), which constitutes a major hurdle for their entry from the blood into brain tissue. This barrier is constituted by a specialized endothelium of brain microvessels that in this manner preserves brain homeostasis. Although small lipophilic molecules may cross the barrier by means of passive diffusion [1,2], other drugs, like cytostatic antitumor compounds are effectively extruded by multidrug resistant proteins that are abundantly present in the plasma membrane of the endothelial cells. Obviously, these limitations have severely frustrated progress in the development of effective treatment of many brain-related disorders [3]. Nevertheless, in recent years, several approaches have been reported, showing promise in overcoming the BBB. One of those relied on the use of a short peptide derived from rabies virus glycoprotein (RVG), which enables the transvascular delivery of small interfering RNA (siRNA) to the brain [4]. Thus after intravenous injection into mice, RVG-9R delivered siRNA to neuronal cells, resulting in specific gene silencing within the brain, although the precise mechanism of transvascular transport was not addressed. Interestingly however, more recently it has been demonstrated that exosomes, i.e., small intracellular vesicles of an endocytic nature that can be subsequently secreted by cells [5], derived from dendritic cells can be loaded with exogenous siRNA by electroporation, and that these particles are indeed targeted to the brain by expression of the neuro-specific RVG peptide [6]. Alternatively, stem cells, engineered to produce therapeutics of choice [7,8], are also emerging as drug delivery vehicles, rationalized by observations that neural stem cells target neurodegeneration and cerebral ischemia upon intracerebral and intracerebroventricular administration [9,10] and brain malignancies [7,11,12]. However, drawbacks were also reported as not all intravenously administered stem cells reach the intended site of delivery in that they may become entrapped and eventually engrafted in lungs, spleen, liver and bone marrow [13-16]. Hence, a different approach is desirable that simultaneously makes use of the intrinsic capacity of neural stem cells to target the brain, but avoids the complications that may arise from their systemic application.

Accumulating evidence suggests that many cell types may shed small vesicles (microvesicles) from their plasma membranes. Once released such microvesicles may interact with a cell they recognize via ligand-receptor interaction, fuse with it, become endocytosed or even transcytosed [17-19]. In this manner microvesicles might transfer cargo, including therapeutics, to the target cells [20]. Likewise, ex vivo manufactured
plasma membrane vesicles might mirror the behavior of these natural vesicular messengers and also transport exogenous cargo to an acceptor tissue. Accordingly, in the present study we have examined the possibility to use artificially prepared plasma membrane vesicles (‘microvesicles’) from neural stem cells as brain targeting device. Both in vitro and in vivo data demonstrate that the vesicles are effectively translocated across the blood brain barrier, and that this system holds great promise as a biological nanocarrier for delivery of therapeutics into the brain.

Material and Methods

Cell lines
Human cerebral microvessel endothelial cells (hCMEC/D3) [21] were grown in flasks, coated with 100 μg/mL rat tail collagen type-I (BD Biosciences, Franklin Lakes, NJ), in endothelial basal medium-2 (EBM-2; Lonza Group, Basel, Switzerland) supplemented with 2.5 % fetal bovine serum, 100 μg/mL penicillin/streptomycin and an EGM-2-MV bullet kit (Lonza) that contained vascular endothelial growth factor, R3-insulin-like growth factor-1, human epidermal growth factor, human fibroblast growth factor-basic, hydrocortisone. For experiments the cells were differentiated in EBM-2 basal medium supplemented with 1 μmol/L dexamethasone (Sigma Aldrich, St Louis, MO Aldrich) and 1 ng/mL bFGF (Invitrogen, Carlsbad, CA), as described in Georgieva et al [22]. Cells were maintained at 37 °C under an atmosphere of 5% CO₂. Unless indicated otherwise, the experiments were performed in EBM-2 medium without additives.

C17.2 neural stem cells (generously provided by Dr. Evan Y. Snyder, Stem cell and Regeneration Program, Burnham Institute for Medical Research, CA) were grown in high glucose Dulbecco's modified Eagle's medium, DMEM, (Invitrogen, Paisley, UK) supplemented with 10 % fetal calf serum (BODINCO B.V. The Netherlands), 5 % horse serum (Invitrogen, Paisley, UK), 100 mg/L ampicillin (Invitrogen, Paisley, UK), and 100 mg/L streptomycin (Invitrogen, Paisley, UK) at 37°C under a 5 % CO₂ atmosphere, and passaged twice a week (1:10 dilution).

Crude Membrane Preparation
Crude plasma membranes of C17.2 NSCs were prepared as follows. Cells were grown to confluency in T162 flasks and washed once with 20 ml of Hank's buffered salt solution (HBSS, Invitrogen, Paisley, UK), pH 7.4. The cells were detached by incubation at 37 °C for 10 min with 20 ml 5mM EDTA. Cells were collected by centrifugation at 9.000 g for 10 min. The cell pellet was diluted at least 40 times with
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Lysis buffer [5 mM sodium phosphate buffer, pH 7.4 and 1 mM EDTA with protease inhibitors PMSF (0.1 mM), Leupeptin (0.5 μg/ml) and Aprotinine (1 μg/ml), Sigma], and lysed by stirring for 90 min on ice. The lysate was centrifuged for 45 min at 100,000 g, 4°C. The resulting pellet was resuspended in 30 ml ice-cold resuspension buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM EDTA with protease inhibitors) and homogenized in a 30 ml Dounce B potter (50 strokes with tight pastille) with 3 μl Benzonase (Merck Chemicals, Darmstadt, Germany) to fragment DNA. Portions of 7 ml of the homogenate were overlaid on 4 ml 38% w/w sucrose solution with protease inhibitors and centrifuged at 280,000 g for 2 hours at 4°C. The interface was collected, and washed by centrifugation for 45 min at 100,000 g at 4°C in ice-cold resuspension buffer. The pellet was finally resuspended in approximately 2 ml of resuspension buffer. The protein content of the plasma membrane preparations was determined by the bicinchoninic acid assay (Sigma Aldrich, St Louis, MO), using bovine serum albumin as a standard. The resuspended membranes were stored in aliquots at -80°C.

**Vesicles extrusion and fluorescent labeling**

C17.2 NSC plasma membranes, prepared as described in the previous paragraph, were resuspended in isotonic buffer (50 mM Hepes pH 7.4 with 100 mM NaCl and 1 mM EDTA), and vesicles were prepared by passing the membranes through a Liposofast™ vesicle extruder (200 nm filter, Avestin, Ottawa, Canada). The vesicles were spun down at 70,000 g for 20 min at 4°C. The protein concentration was determined by the bicinchoninic acid assay (Sigma Aldrich, St Louis, MO), and the vesicles were suspended in isotonic buffer at a protein concentration of approximately 2 mg/ml.

For the purpose of fluorescence analysis and visualization, the PMVs were labeled with either one of two fluorophores. N-Rhodaminephosphatidylethanolamine (N-Rh-PE) (Avanti Polar Lipids, Inc., Alabaster, AL), which was employed for histological examination, was introduced just prior to vesicle extrusion. In short, 0.2 nmol of N-Rh-PE in 1 μl of 100% ethanol was pipetted under vortexing into 100μl membrane suspension containing ~ 500 μg protein after which the fluorescently-labeled membrane fraction was extruded as described above. Alternatively, extruded vesicles were spun down at 70,000 g for 20 min at 4°C and resuspended in 100μL 0.25 mg/ml fluorescein isothiocyanate (FITC, Sigma Aldrich, St Louis, MO) solubilized in 0.3 M carbonate / bicarbonate buffer pH 8.6, which was followed by a 1 hour incubation at room temperature in the dark. The volume of the vesicle suspension was adjusted to 3
ml by isotonic buffer, and excess FITC was then removed by chromatographic separation over a desalting column (Econo-Pack 10DG; Bio-Rad, Hercules, CA). The vesicle fraction was collected by centrifugation at 70,000 g for 20 min at 4°C.

**Measurement of vesicle size and zeta potential**

The size, polydispersity index and zeta-potential of the C17.2 NSC derived plasma membrane vesicles were measured, using a Malvern zetasizer Nano-Z (Malvern Instruments, Worcestershire, UK). The measurements were performed in PBS (Invitrogen, Paisley, UK).

**Interaction of C17.2 NSC derived PMVs with hCMEC/D3 cells**

hCMEC/D3 cells were seeded at a concentration of $2 \times 10^5$ cells/cm$^2$ on glass coverslips, precoated with 100 μg/ml collagen type-I, and grown to confluency as described previously [22]. On the day of the experiment the cells were washed and incubated in EBM-2 at 37°C for 30 min. When indicated, inhibitors (purchased from Sigma Aldrich, St Louis, MO) of the various endocytotic pathways were included in the medium at this stage. Applied effective concentrations, as verified in our previous studies [22-24], were as follows: as inhibitor for clathrin-mediated endocytosis: chlorpromazine (5 μg/ml); as inhibitor for caveolae-mediated internalization: filipin III (1 μg/ml; Sigma Aldrich, St Louis, MO Aldrich); as inhibitor of macropinocytosis: dimethylamiloride (40 μM); as an inhibitor of dynamin dynamics (involved in both clathrin- and caveolin-mediated endocytosis): dynasore 80 μM. The cells, irrespective of the presence of inhibitors, were incubated with 10 μg/ml of FITC labeled PMVs during 2 hours. At the end of the incubation the coverslip-attached cells were washed two times with HBSS, fixed with 4% formaldehyde, washed again and mounted on glass slides with Faramount aqueous mounting medium (Dako, Glostrup, Denmark). The experiments were done three times in duplicate. Images of at least five random fields of view were acquired, using a Leica TCS SP-2 confocal microscope. The number of PMVs per field of view was quantified using Particles Tracking plug-in for ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij).

**Transcytosis assay**

To determine the transcytotic transport efficiency of the C17.2 NSC PMVs, hCMEC/D3 cells were seeded at a density of $2 \times 10^5$ cells/cm$^2$ on 3-μm filter inserts in Transwell chamber plates (Corning, Corning, NY), coated with collagen type-I. Media were changed two times a week and the transepithelial electrical resistance value was
measured using a Millicell-ERS (Millipore, Billerica, MA). The experiments were performed when hCMEC/D3 monolayers reached a transepithelial electrical resistance value of 50 Ω/cm² i.e., after 14–15 days in culture.

For the purpose of monitoring their transcytosis, C17.2 NSCs were labeled with 5 μM CellTracker Green CMFDA (Molecular Probes, Inc., Eugene, OR) for 30 min according to the manufacturer protocol for adherent cells. FITC labeled PMVs were applied to the apical compartment at a concentration of 10 μg protein per ml. After an incubation of 18 hours, the media from apical and basolateral compartment were collected, while the cell-supporting filters were cut out and soaked in 1ml of distilled water. The fluorescence in the various samples thus obtained was quantified by measurement in a Perkin Elmer LS5 luminescence spectrometer, using FL WinLab 4.0 software in the time drive mode over a time period of 20 s with 0.1 s data collection interval at the following settings: excitation wavelength 480 nm, excitation slit 2.5 nm, emission wavelength 520 nm, emission slit 2.5 nm The results are presented as % of total fluorescence per fraction, based upon data collected from three independent experiments, carried out in duplicate.

In order to verify maintenance of the integrity of the D3 monolayer during PMVs treatment, TRITC-labeled 65-85 kDa dextran (Sigma, St louis, MO) was used. [25]. To this end, 500 μl TRITC dextran (3 mg/ml) was applied to the apical chamber of the D3 monolayer, either in the absence or presence of the PMVs. After 18 hours of incubation, samples were taken and processed in an identical manner as described for determining the transcytotic capacity of the PMVs. The fluorescence in each fraction was measured at the following settings: excitation wavelength 560 nm, excitation slit 2.5 nm, emission wavelength 580 nm and emission slit 2.5 nm.

**Immunocytochemistry**

Cover slips with cells were washed two times with HBSS, fixed with 4% formaldehyde, and subsequently permeabilized with 0.2% Triton X-100 at room temperature for 5 minutes. Nonspecific sites were blocked by 60 min incubation at room temperature with 10% FCS. Thereafter, the cells were stained with mouse anti-human PECAM-1 antibody, clone EN4 (Sanbio, Am Uden, The Netherlands) at a dilution 1:50 in 1% FCS for 90 min at room temperature. The cells were washed three times with PBS and incubated with goat anti-mouse AlexaFluor 633 (Molecular probes, Eugene, OR) at a dilution of 1:1000 for another 30 min at 37°C. The cells were washed three times and mounted onto microscopic slides with Faramount aqueous mounting medium (Dako,
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Glostrup, Denmark). Images were acquired by confocal microscopy [Leica TCS SP-2 (Accusto-optical beam splitter)].

**Transmission electron microscopy**

hCMEC/D3 cells were grown in 12-wells plates, precoated with collagen type-I. Either 10 μg/mL of PMVs or 1x10^5 C17.2 NSCs were added to the confluent monolayers and incubated for 18 hours. Subsequently, cells were washed with HBSS and fixed in 2% paraformaldehyde plus 0.2% glutaraldehyde, buffered with 100 mmol/L sodium cacodylate for 2 hours on ice. Postfixation with 2% osmium tetroxide was carried out for 30 minutes at 4 °C. Gradual dehydration was performed with increasing ethanol concentrations from 30 to 100%. The samples were embedded in EPON, and ultrathin sections were prepared and contrasted with uranyl acetate and lead citrate, according to standard procedures.

Samples were examined in a Philips CM 100 electron microscope, operated at 80 kV.

**In vivo fate of plasma membrane vesicles**

To demonstrate that PMVs can cross the blood brain barrier in vivo, they were injected i.v. in male BALB/c mice obtained from Harlan (Horst, The Netherland). The experiment was approved by the Animal Ethics Committee of the University of Groningen, The Netherlands and performed by licensed investigators in accordance with the Law on Animal Experiments of The Netherlands.

The mice were sacrificed 24h later. Blood was collected for blood smears. Spleen, liver, lung and brain were isolated, and snap frozen. 8μm sections were prepared with a LEICA cryostat. To visualize cell nuclei, sections were stained with DAPI.

**Results**

**Translocation of neural stem cells across polarized hCMEC/D3 cells occurs via the paracellular route**

In vivo, the infiltration of immune cells into the brain via transport across the BBB has been well documented and both transcellular migration across the endothelium and paracellular diapedesis have been identified as underlying migration mechanisms [26]. Presumably, neural stem cells, that show an intrinsic tropism for brain malignancies [27], might similarly adhere to and interact with microvascular endothelium. Obviously, the effectiveness of such an interaction, likely driven by a mechanism involving cell surface localized adhesion molecules, would be a prerequisite for
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potential successful transcellular transport of stem cell-derived plasma membrane vesicles. Accordingly, we first investigated the interaction of C17.2 NSCs, using an in vitro BBB model constituted by polarized monolayers of human brain endothelial hCMEC/D3 cells, to verify whether such interactions may occur as well in vitro. To this end the stem cells were fluorescently labeled with CMFDA as described, and were subsequently added to apical side of the hCMEC/D3 cells, cultured in the transwell system. Microscopic analysis of the cells was carried out after an overnight incubation. In order to visualize cell-cell contacts in the hCMEC/D3 monolayer, the filter-grown cells were fixed at the end of the incubation, and immunostained for PECAM-1, a specific cell surface localized cellular adhesion molecule present on endothelial cells [28]. As revealed by fluorescence microscopy, when visualized from the apical surface, the neural stem cells were found engrafted in the hCMEC/D3 monolayer (Fig. 1a), while visualization of the monolayers at the basolateral surface demonstrated the occasional protrusion of the green-labeled stem cells and the presence of whole cell bodies (Fig 1b).

**Figure 1.** Translocation of neural stem cells across BBB endothelial cells in vitro occurs by paracellular transport. (a) CMFDA-labeled C17.2 neural stem cells (green) intercalate in and squeeze through a monolayer of hCMEC/D3 cells, grown on transwell filters. The cell boundaries of hCMEC/D3 cells were immunolabeled for the cell surface marker PECAM (red); apical view — top of the filter. (b) Projections of the neural stem cells are apparent at the basolateral surface, while some cells have completely transversed the monolayer and filter support; basal view — bottom of the filter. (c) Focal points of adhesion between an endothelial cell and neural stem cell (white arrows), scale bar 2 µm. (d) Tight junctional connections between hCMEM/D3 cells remain intact at places where the monolayer is not invaded by C17.2 NSCs. (e) Consecutively acquired images, combined in a panorama view, show a neural stem cell, filling the paracellular space between two endothelial cells.

To further clarify the route of migration of the C17.2 NSCs across an in vitro BBB model, the process was also examined by transmission electron microscopy. For that
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purpose the stem cells were allowed to interact with hCMEC/D3 cells plated on 12-wells plate. As shown in Fig. 1c, hCMEC/D3 cells are characterized by the presence of a relatively large nucleus (N). Invasive cells, as indicated by their cellular boundaries, are readily distinguished as C17.2 NSCs, and it is apparent (Fig 1c and 1e) that the C17.2 neural stem cells use the paracellular route to migrate through hCMEC/D3 monolayers. It should be noted that at places where the monolayer is not invaded by C17.2 NSCs (Fig. 1d) tight junctional connections between hCMEM/D3 cells remain intact (Fig 1d). Together, these data indicate that neural stem cells display a capacity to migrate across an in vitro BBB model. Next, we therefore set out to prepare NSC-derived plasma membrane vesicles to verify whether such particles would display similar properties and hence may display potential of acting as a biological nanocarrier.

**Preparation and characterization of NSC-derived plasma membrane vesicles**

Crude plasma membranes were obtained from lysed cells by pelleting and subsequent fractionation on a sucrose gradient, as described in Material and Methods. Plasma membrane vesicles were obtained by resuspending the membrane fraction in isotonic buffer and subsequent passage of the suspension through a LiposofastTM vesicle extruder, using a 200 nm filter. As detailed in the Methods and Materials section, to allow visualization for microscopic analysis and detection by fluorometry, the obtained vesicle fraction was labeled with either fluorescein isothiocyanate (FITC) or the fluorescent lipid marker N-Rh-PE, the latter being incorporated into the membrane preparation prior to its extrusion. Typically, application of this procedure resulted in the assembly of vesicles with a diameter varying between 150-200 nm, displaying a slightly negative charge, as determined by light scattering and surface potential measurements, respectively, summarized in Table 1. Consistently, a similar range in diameters could be visualized upon examination of the NSC-derived vesicles by electron microscopy (Fig. 2). It should be noted that the presence of clusters is an artifact, resulting from vesicular staining (and subsequent clustering) with uranyl acetate. We subsequently employed these preparations to study their potential of transcellular transport capacity across the in vitro BBB model.
NSC-derived plasma membrane vesicles are internalized by endothelial hCMEC/D3 cells

To investigate the interaction of NSC plasma membrane vesicles with hCMEC/D3 cells, FITC labeled vesicles (FITC-PMV) were incubated with the endothelial cells, grown on coverslips or 12-wells plate as described in Materials and Methods. To appreciate individual cells in the confluent monolayer of the brain endothelium, cellular boundaries were labeled for PECAM-1. After an incubation for 18 h, FITC-PMV appear intracellularly, as visualized by fluorescence (Fig 3a) and electron microscopy (Figs 3b and c), showing unique intracellular vesicular structures with a typical double membrane structure. Over this incubation period the PMVs localized in the cytosol, and no association with lysosomal compartments could be detected. Most interestingly however, occasionally the FITC-PMV colocalized with PECAM-1 (Fig. 3a, white arrow). Since PMVs accessed the BBB endothelium from the apical site whereas PECAM-1 is sorted basolaterally, i.e., opposite of the apical surface, these data may support the notion that apically added PMVs engage in transcytosis, i.e.
transcellular transport from the apical to the basolateral surface. To obtain further supportive evidence for these observations we next investigated in further detail the pathways of entry of the PMVs.

Figure 3. Intracellular localization of PMVs in polarized hCMEC/D3 cells. PMVs were added from the apical site to hCMEC/D3 cells, grown to confluency on coverslips or 12-wells plates. After 18 hrs of incubation at 37°C, the cells were fixed and processed for fluorescence or electron microscopy, as described in Materials and Methods). (a) PECAM-1 (pseudocolored in blue) marks the cell boundaries of single cells. FITC-PMVs (pseudocolored in green) are detected as round shaped objects within the endothelial cells. Occasionally, PMVs colocalize with PECAM-1 (white arrow). Scale bar, 20 µm. (b) Intracellular localization of PMVs (arrows), showing a typical appearance of double membrane vesicular structures in the cytoplasm of the cells. Scale bar, 500 nm. (c) Magnification from (b).
The internalization of PMVs is energy dependent and occurs via different endocytic pathways

While paracellular transport appears to be the primary mechanism by which NSCs were translocated across the polarized hCMEC/D3 cells, as described above (see Fig. 1), significant paracellular transfer of the membrane vesicles was not observed. Accordingly, we anticipated, supported by the observations reported in Fig. 3, that endocytotic internalization mechanisms mediate the potential translocation in case of the PMVs. To investigate the involvement of these pathways, a variety of inhibitors were employed, previously verified to effectively inhibit the entry of distinct ligands along either of these pathways. Since all these pathways are energy-dependent, the cells were depleted of ATP by exposing the hCMEC/D3 cells to 10 mM sodium azide (NaN₃) and 10 mM 2-deoxyglucose for 30 min in a calcium- and magnesium-containing PBS medium [29]. The uptake of FITC-PMVs was subsequently determined after incubation for 2 h, in the presence of inhibitors, i.e., conditions which did not significantly affect cell viability. As shown in Fig. 4, energy depletion inhibited PMV uptake by approximately 80%, indicating that their internalization is an active, energy-dependent process, consistent with endocytosis as the mechanism of entry and hence, its potential involvement in transcellular transport of the PMVs.

Figure 4. Effect of metabolic inhibitors on the uptake of PMVs by hCMEC/D3 cells. hCMEC/D3 cells, grown on coverslips, were preincubated with azide/deoxyglucose (NaN₃/2DG) to deplete the cells of energy, dynasore (dyn), a dynamin inhibitor, dimethylamiloride (DMA), an inhibitor of macropinocytosis, nystatin (nys) and filipin (FIII) – inhibitors of caveolae-mediated endocytosis, and chlorpromazine (CPM), an inhibitor of clathrin-mediated endocytosis for 30 min at 37 °C. Subsequently, 10 mg/ml of plasma membrane vesicles were included in the incubation medium, while the inhibitors were present during the entire incubation period. After 120 min, the cells were washed and fixed, after which the number of vesicles per field of view was quantified. The uptake obtained in the absence of inhibitors was set at 100% (control).
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To distinguish between the different endocytotic pathways, the effect of the various inhibitors was investigated at identical conditions at which the energy depletion experiment were carried out. As demonstrated in Fig. 4, dynasore, an inhibitor that interferes with dynamin GTPase activity and affects both clathrin- and caveolin-mediated endocytosis, accounts for approximately 50% inhibition of the internalization of the labeled PMVs (Fig. 4). Neither dimethylamiloride (Fig. 4) nor wortmannin (data not shown) affected PMVs internalization, indicating that macropinocytosis as mechanism of internalization can be excluded. By contrast, chlorpromazine, an inhibitor of clathrin-mediated endocytosis and, nystatin and filipin III, inhibitors of caveolae-mediated endocytosis, blocked PMV internalization by approximately 45, 45 and 35%, respectively. Taken together these data indicate that multiple endocytic pathways, including clathrin- and caveolae-mediated endocytosis are involved in the internalization of PMVs, derived from NSC by hCMEC/D3 cells. Next, we examined the extent to which this mode of internalization subsequently contributed to transcellular transport, i.e., the processing of PMVs across the in vitro BBB cell model from the apical medium/cell surface to the basolateral surface and ensuing secretion into the basolateral medium.

Transcytosis of PMVs across hCMEC/D3 cells

Thus far, we showed that PMVs, derived from neural stem cells, are efficiently internalized by BBB endothelium. To investigate their transcytotic potential we therefore added the FITC-labeled vesicles (10 μg/ml) to the apical chamber of the transwell system in which the hCMEC/D3 cells were cultured. After an incubation of 18 h, fluorescence was determined by fluorometry in both the apical and basolateral medium and that associated with the cells, present on the filter. The data thus obtained were expressed as % fluorescence present in each fraction, relative to total fluorescence. As quantified in Fig. 5a, approximately 25% of the labeled NSC-derived plasma membrane vesicles had reached the basolateral medium, presumably via transcytosis, after 18 hrs. Moreover, the fluorescence intensity that was measured in the basal compartment, originates from intact FITC-PMVs, as evidenced by examination of the basolateral medium by fluorescence microscopy. As shown in Fig 5b the fluorescence was found to be associated with particles of a size that was reminiscent to those of the extruded vesicles.
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Importantly, to exclude that the presence and processing of the PMVs may have affected the integrity of the endothelial monolayer, and which might have resulted in leakage of PMVs across the monolayer, we verified in the presence of non-labeled PMVs the transport of high molecular weight dextran, as a marker for paracellular leakage. As shown in Fig. 5c, while the fraction of paracellular leakage of dextran was less than 10 %, the presence of the PMV did not significantly modify the integrity of the hCMEC/D3 monolayer as leakage of the dextran marker was indistinguishable from the control.

Together these data indicate that PMVs, derived from neural stem cells can be effectively translocated across a monolayer of hCMEC/D3 cells, serving as a model for the BBB in vitro. To examine whether PMVs might also engage in transvascular transport across the BBB in vivo, this possibility was examined next.

**Intravenously administrated PMVs reach the brain parenchyma in vivo**

To determine whether PMVs could cross the BBB in vivo and accordingly reach brain tissue, N-Rh-PE-labeled vesicles were prepared, which were systemically administered by injection into the tail vein of BALB/c mice. The fate of the labeled PMVs was
subsequently established qualitatively by analyzing the presence of fluorescence in distinct fractions and/or organs. As shown in Fig. 6a, twenty four hours after injection, a substantial fraction of the PMVs were still localizing in the circulation as evidenced by the presence of N-Rh-PE fluorescence in a blood sample. In addition, sections from lung, spleen and liver, also revealed the presence of PMVs in these organs (Fig. 6d, e and f). Most importantly, a major fraction of the injected PMVs was detectable in multiple brain coronal sections, demonstrating a considerable accumulation in brain parenchyma (Fig. 6b and c).

Figure 6. Histological analysis of the in vivo body distribution of N-Rhodamine PE-labeled PMVs in BALB/c mouse. Labeled PMVs were systemically administered into a BALB/c mouse and after 24 h a blood sample was taken and the animal sacrificed. Blood and the indicated organs were analyzed by fluorescence microscopy for the presence of labeled PMVs. (a) PMVs (red) still circulated in the blood plasma, 24 hrs after systemic administration. PMVs were detected in lung (d), spleen (e) and liver (f). However, note the prominent accumulation of PMVs in brain parenchyma (b and c).
Discussion

Neural stem cells, when injected intravenously, have been shown to be able of crossing the blood brain barrier and migrate into brain lesions. The aim of the present study was to exploit this capacity by investigating whether relatively small plasma membrane vesicles could be prepared from NSCs that could potentially be used in this manner as nanocarriers for brain delivery of therapeutic agents. Here we have demonstrated that such membrane vesicles can be generated from C17.2 NSC, and that these vesicles can indeed cross the blood brain barrier as revealed both in an in vitro human vascular endothelial cell model for the BBB, as well as in vivo. Our in vitro data show that the mechanism of transcellular transport relies on an energy-dependent mechanism involving internalization by endocytosis at the apical cell surface and subsequent secretion at the basolateral surface. Although the mechanism of transcellular transport into the brain of BALB/c mice, following systemic administration, remains to be determined, the data highlight the potential of applying PMVs as genuine biological nanocarriers.

In recent years a great number of different nanocarriers have been developed and applied for delivery of genes and therapeutics. Many of these systems can be categorized as a viral- or non-viral based delivery systems, each of them having advantages and disadvantages, such as a higher or lower delivery efficiency, or immunological and toxicological hazards. However, overall, by far the majority of these systems appeared highly inefficient in crossing the blood brain barrier, implying that for successful delivery of therapeutics into the brain more appropriate systems are still very much desired. One of the approaches to overcome the blood brain barrier, the major hurdle in reaching brain tissue, relies on the use of neural stem cells, carrying a gene of interest. Thus neural stem cells have been engineered to produce glial cell line-derived neurotrophic factor [31]. The delivery of this protein was found to occur in therapeutic quantities, preventing the degeneration of dopaminergic neurons in a mouse model of Parkinson’s disease. Alternatively, cells engineered to continuously express ciliary neurotrophic factor were encapsulated in alginate polymers. When such polymeric systems were implanted in the ventricle these microcapsules were able to halt cognitive deterioration in a mouse model of Alzheimer’s disease [32]. However, given their large diameter (>15µm), the i.v. application of whole cells might be less desirable and indeed, after systemic application, a significant fraction of the NSCs becomes entrapped in lung capillaries, due to a first pass effect [33,34], consistent with our observations when using of C17.2 NSCs [35]. Nevertheless, those cells that reach and interact with vascular endothelial cells are capable of crossing the BBB. Our
transwell experiments showed that C17.2 NSCs integrate into the hCMEC/D3 monolayer and examination of the interaction on the level of fluorescence and electron microscopy revealed that C17.2 NSCs invade the monolayer at the level of endothelial cell-cell contacts leading to their paracellular migration. These observations are in line with previous studies. For example, it has been shown that also mesenchymal stem cells can pass endothelial monolayers via the paracellular route both in coculture and in isolated heart perfusion model systems [36,37], and in this manner invade surrounding tissue.

To avoid the pulmonary first-pass effect, and to take advantage of the natural propensity of NSCs to target the brain, we set out to prepare plasma membrane derived vesicles from C17.2 cells. Based upon the procedure as described we obtained membrane vesicles with a diameter of approximately 200 nm, which is very reminiscent of diameters of chemically produced and commonly applied nanocarriers. Evidently, the PMVs thus prepared were highly efficiently translocated across hCMEC/D3 monolayers, up to approximately 25 % of the added dose. Although systemic administration in vivo revealed that a fraction of the PMVs did localize in lung tissue, a qualitative analysis of samples taken from distinct organs revealed that a substantial fraction had reached brain parenchyma, implying that efficient translocation across the BBB in vivo similarly occurred. In terms of the mechanism of endothelial translocation, migration of neural progenitors, similarly to leukocytes, is a multistep process that involves clustering of surface adhesion molecules (CD44, VCAM-1, ICAM-1). This clustering remodulates the cortical actin cytoskeleton via members of the ezrin-radixin-moesin family [38,39], and causes the formation of endothelial docking structures, required for diapedesis of the neural progenitors [40]. Being devoid of the cytoskeleton, it is unlikely that PMVs activate the entire machinery, necessary for cell extravasation. However, it is possible, that both C17.2 NSCs and the PMVs derived thereof initially engage with the same cell surface receptors, which leads to internalization of the vesicles, whereas paracellular processing seems to be the predominant transcellular pathway of the C17.2 NSCs. As revealed by the effect of several pharmacological inhibitors, the primary pathways of PMVs entry appear to be the clathrin- and caveolae-dependent pathways. The latter pathway in particular may then contribute to transcellular transport of the vesicles as caveolea-mediated endocytosis in BBB endothelial cells precludes lysosomal delivery and rather, may eventually lead to transcytosis of the cargo [41]. Importantly, transcytosis of PMVs across the BBB model is an essentially non-toxic process as at these conditions paracellular permeability, as verified by potential leakage of 70 kDa dextran, was virtually negligible.
At present it is unclear whether the PMVs express ligands that are specifically recognized by vascular endothelial cells, thereby providing specific targeting towards of the PMVs to the BBB. Clearly, such a targeting mechanism would be beneficial in terms of the potential efficiency of delivery. In fact brain targeting of drug vehicles applied thus far frequently suffer from this shortcoming, thus showing an inefficient crossing the BBB and hence a poor accumulation in the brain. Although several endothelial targeting molecules (transferrin, RI7217, COG133, angiopep-2, and CRM197) [8,42-45] have been proposed, only RI7217 showed to be effective in brain accumulation of liposomes after intravenous administration [46]. Furthermore, although OX23, an anti–transferrin receptor antibody which has also been used for brain targeting of liposomes, displays a high ligand/receptor binding affinity at the level of brain endothelium, the latter appears to interfere with efficient transcytosis of the vehicle [47]. Here we demonstrated that PMVs accumulate in brain parenchyma, after intravenous injection in mice. Notably, close examination of the data indicates that the PMVs occupy perinuclear regions in brain cells, suggesting their active endocytosis following endothelial transcytosis in recipient cells localized within the brain, presumably neurons. These observations thus highlight the proof of principle of employing PMVs, derived from NSC, as potential vehicles for brain delivery. From a fundamental point of view it will be of interest to further characterize the detailed molecular mechanisms underlying overall brain delivery, including efforts aimed at specific targeting of these vehicles. In addition, accomplishing the actual delivery of drugs entrapped within the vehicles will be another challenge, and merits further work.

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