

Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma

Marcel Grapp (1), Arne Wrede (2), Michaela Schweizer (3), Sabine Hüwel (4), Hans-Joachim Galla (4), Nicolas Snaidero (5), Mikael Simons (5,6), Johanna Bückers (7), Philip S. Low (8), Henning Urlaub (9,10), Jutta Gärtner (1), Robert Steinfeld (1)

(1) Department of Pediatrics and Pediatric Neurology, University Medical Center Göttingen, 37075 Göttingen, Germany.

(2) Prion and Dementia Research Unit, Institute of Neuropathology, University Medical Center Göttingen, Göttingen, Germany.

(3) Centre for Molecular Neurobiology, University of Hamburg, 20251 Hamburg, Germany

(4) Institute of Biochemistry, University of Münster, 48149 Münster, Germany.

(5) Max-Planck-Institute of Experimental Medicine, 37075 Göttingen, Germany.

(6) Department of Neurology, University Medical Center Göttingen, 37075 Göttingen, Germany.

(7) Department of Nanobiophotonics, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany.

(8) Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, USA.

(9) The Bioanalytical Mass Spectrometry Group, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany.

(10) Department of Clinical Chemistry, University Medical Center Göttingen, 37075 Göttingen, Germany

Correspondence to: Robert Steinfeld, Department of Pediatrics and Pediatric Neurology, University Medical Center Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany. E-mail: rsteinfeld@med.uni-goettingen.de

ABSTRACT

Loss of folate receptor alpha (FR α) function is associated with cerebral folate transport deficiency and childhood-onset neurodegeneration. To clarify the mechanism of cerebral folate transport at the blood-CSF-barrier, we investigate the transport of 5-methyltetrahydrofolate in polarized cells. Here we identify FR α -positive intraluminal vesicles within multivesicular bodies and demonstrate the directional co-transport of human FR α and labelled folate from the basolateral to the apical membrane in rat choroid plexus cells. Both the apical medium of FR α -transfected rat choroid plexus cells and human CSF contain FR α -positive exosomes. Loss of FR α -expressing CSF exosomes correlates with severely reduced 5-methyltetrahydrofolate concentration corroborating the importance of the FR α -mediated folate transport in the CSF. Intraventricular injections of FR α -positive and -negative exosomes into mouse brains demonstrate FR α -dependent delivery of exosomes into the brain parenchyma. Our results unravel a new pathway of FR α -dependent exosome mediated folate delivery into the brain parenchyma and opens new avenues for cerebral drug targeting.

Folates are a group of essential micronutritional compounds that are found in bacteria and plants. Folic acid is a chemically synthesized, oxidized derivative that is converted to 5-methyltetrahydrofolate (5MTHF) by several enzymatic reactions. 5MTHF is the major biologically active form that functions as a cofactor in many methylation reactions. In particular, the conversion of homocysteine to methionine depends on 5MTHF. This reaction is required for the generation of S-adenosylmethionine (SAM) the central methyl-donor in most tissues and subcellular compartments. 5MTHF is distributed by the blood stream to all major organs where it can transverse cell membranes by at least three major transport systems¹. The reduced folate carrier (RFC, SLC19A1) is an organic anion antiporter that exchanges 5MTHF with other inorganic or organic anions². The influx Km of the RFC for 5MTHF and other reduced folates is 2-7 μM . The uptake of folates into enterocytes in the small intestine is mediated by the proton-coupled folate transporter (PCFT, SLC46A1). This transporter has a pH optimum of 5.5 and functions as proton cotransporter with a Km of about 1 μM ³. Two glycosylphosphatidylinositol (GPI)-anchored receptors, folate receptor alpha (FR α) and beta (FR β), mediate endocytosis of folates after binding them with high affinity (Kb 1-10 nM) at neutral pH^{4, 5}. The function of the secreted folate receptor gamma (FR γ) and the very low affinity folate receptor delta (FR δ) are currently not understood. In addition, several organic anion transporters (OAT) and ABC exporters (MRP1-5 and ABCG2) are capable of transporting folates with low affinity^{6, 7}.

We have recently described cerebral folate transport deficiency, a childhood neurodegenerative disease that is caused by mutations in the *FOLR1* gene leading to a functional loss of FR α ^{8, 9}. Several other reports have confirmed the characteristics of this genetic disorder^{10, 11}. Since the functional loss of FR α is associated with very low 5MTHF concentration in the CSF but normal plasma concentration of 5MTHF and since FR α is abundantly expressed in the choroid plexus, we have hypothesized that FR α provides the major route for blood-CSF transport of 5MTHF. In this study, we characterize the subcellular distribution of FR α and PCFT. We demonstrate that FR α mediates the transcellular 5MTHF transport into the CSF and that FR α -containing exosomes are targeted into the brain parenchyma.

RESULTS

Localization of FR α and PCFT in the human choroid plexus

To characterize the subcellular localization of FR α and PCFT in detail, an immunohistochemical analysis of sections of paraffin-embedded human choroid plexus specimens was performed. The detection with anti-human FR α antibody showed a polarized

distribution of FR α , with strong staining at the apical brush border membrane and a minor FR α -signal at the basolateral membrane of the choroid plexus epithelium (**Fig. 1a**). The human PCFT was mainly detected in intracellular compartments (**Fig. 1b**).

Immunofluorescence microscopy confirmed these results (**Fig. 1c**). We substantiated the reported apical localization of the RFC in the human choroids plexus (**Supplementary Fig. S1a**). The preferential apical localization of FR α seem to contradict currently held views of the role of FR α in taking up 5MTHF at the basolateral side of the choroid plexus¹². However, these findings substantiate published results obtained in mouse and rat brain sections^{13, 14} as well as in human tissues¹⁵.

FR α and PCFT localize to intracellular vesicles in Z310 cells

We transfected the Z310 cell line derived from immortalized rat choroid plexus cells¹⁶ with the human FR α , PCFT, RFC or vector, respectively, and detected their expression at high resolution. Both, FR α and PCFT were associated with intracellular vesicles that showed only marginal colocalization (**Fig. 1d**). FR α -positive vesicles colocalize with late endosomal markers that are characteristic for multivesicular bodies (**Fig. 1e**). Higher magnifications of multivesicular bodies by electron microscopy indicate the release of FR α -positive, intraluminal vesicles into the extracellular space (**Fig. f,g,h**). PCFT-positive vesicles show an electron dense lumen and are not associated with multivesicular bodies (**Fig. 1j,k**).

Basolateral to apical transport of FR α in polarized cells

We studied the transport of labelled folates in cultures of polarized cells and used the rat choroid plexus Z310 cells as a model system. Filter-grown Z310 cells, stably expressing FR α were incubated with FITC-labelled folic acid (FA-FITC). Anti-FR α antibody was added to the basolateral medium of the cells. Imaging of cells at various time points (0, 60 and 120 min) revealed FR α redistribution from the basolateral to the apical membrane (**Fig. 2a-c**). In contrast, after apical labelling, no redistribution of FR α but a loss of fluorescence signal was observed, indicating unidirectional basolateral to apical transcytosis of FR α and release of FR α from the apical membrane (**Fig. 2d and e**).

To confirm the basolateral origin of the apically detected FR α protein, we removed FR α by the addition of phosphatidylinositol-specific phospholipase C (PI-PLC) to the basolateral medium. After incubation for 0, 60 and 120 min, FR α was released from the apical membrane by the addition of PI-PLC to the apical medium. Western blot analysis revealed a gradual reduction of FR α signal by maximal 76% after 120 min incubation (**Fig. 2f and g**). This basolateral to apical cotransport of FR α together with FITC-labelled folic acid could be replicated in experiments with MDCK cells that were stably transfected with FR α (**Fig. 2h-n**). However, in case of MDCK cells no significant reduction in apical fluorescence was observed

after apical labelling and 120 min incubation (**Fig. 2k and l**). This suggests that in contrast to Z310 cells, MDCK cells do not release FR α from the apical surface.

We additionally analysed the distribution of FR α in HepG2 cells stably transfected with the human *FOLR1* cDNA. FR α accumulated at the apical bile canalicular (BC) membrane and was less intensively expressed at the basolateral membrane (**Supplementary Fig. S2a**). Blocking of vesicular membrane fusion by preincubation of HepG2 cells with N-ethylmaleimide (NEM) resulted in reduced basolateral FR α staining (**Supplementary Fig. S2b**). Preincubation with brefeldin A (BFA) to block Golgi-to-cell surface traffic resulted in preferential basolateral FR α staining, indicating a disturbed FR α trafficking even though FR α internalization was preserved (**Supplementary Fig. S2c**). These data support the notion of a vesicular transport of FR α from the basolateral to the apical membrane in polarized cells.

Exosomal FR α expression correlates with 5MTHF in human CSF

To understand the mechanism of FR α shedding into the CSF, we fractionated human CSF by sequential centrifugation with increasing centrifugal forces. Surprisingly, FR α was enriched in the P100 (100.000 x g) fraction containing small vesicles with a diameter of 40 - 100 nm and colocalizing with exosomal markers such as flotillin-2 (Flot-2) and Alix (**Fig. 3a-d**). Immunoelectron microscopy revealed the presence of FR α at the surface of exosomes, and two-color STED microscopy^{17, 18} confirmed the vesicle-specific colocalization of FR α with the exosomal marker Alix (**Fig. 3c and d**). From the same STED data, the exact vesicle size could be assessed (**Fig. 3e**) and a quantitative colocalization of FR α with Alix was determined to be in the range of 36% \pm 8%.

The importance of FR α release into the CSF was further investigated by Western blotting with human CSF from both healthy individuals and patients with cerebral folate transport deficiency. FR α could be detected in CSF of controls but was absent from patients that carry pathogenic *FOLR1* mutations (**Fig. 4a**). Since we have shown intracellular expression of the FR α missense mutants p.C65W, p.C169Y, and p.N222S⁹ their absence in CSF indicate a disturbed export of these FR α mutants. The absence of FR α was associated with a severely diminished concentration of 5MTHF in the CSF equal to or below 5 nM in untreated patients (normal range 40 – 120 nM). To test whether low CSF concentrations of 5MTHF are correlated with low or absent CSF detection of FR α in other disorders, we investigated patients with Kearns-Sayre syndrome, a mitochondrial disorder with disturbed blood-CSF transport of 5MTHF due to failure of the choroid plexus^{19, 20}. CSF samples obtained at different time points but from the same patient were compared (**Fig. 4b**). The sample with the slightly reduced 5MTHF concentration in the CSF of 39.4 nM showed a decreased but

detectable FR α signal. In contrast, CSF FR α was hardly detectable in the sample where the CSF 5MTHF was below 5 nM. The CSF samples of eight additional patients with Kearns-Sayre syndrome showed a similar correlation between 5MTHF concentration and level of FR α expression (**Fig 4c**). These findings suggest a link between CSF 5MTHF and CSF FR α and indicate a crucial role of the choroid plexus in the export and maintenance of 5MTHF and FR α in the CSF.

5MTHF transport across the choroid plexus is mediated by FR α

To reconcile our experimental data from polarized cells with the presence of FR α -expressing exosomes in the CSF we studied the transcellular transport of 5MTHF across the choroid plexus. For this purpose, we investigated the directed movements of radioactively labelled 5MTHF across Z310 cells as well as across primary porcine choroid plexus epithelial cells. FR α -transfected or vector control-transfected Z310 cells were grown in a two chamber transwell system and [3 H]5MTHF was either given into the basolateral or apical compartment. In short-term experiments over three hours [3 H]5MTHF was merely taken up by FR α -transfected Z310 cells from both compartments and vectorial transport could only be detected when PI-PLC was added to the apical compartment (**Supplementary Fig. S3**). For extended studies the radioactive medium was replaced with fresh medium after 1 h incubation to prevent transepithelial diffusion of [3 H]5MTHF. During the extended 48 h incubation [3 H]5MTHF emerged in the apical compartment of FR α -transfected but not vector-transfected cells (**Fig 5a**). Moreover, apical shedding of [3 H]5MTHF was increased upon addition of either monensin (70% increase) or transferrin (36% increase). The monensin- or transferrin-dependent increase in apical release occurred at the expense of the intracellular [3 H]5MTHF uptake. Whether Z310 cells were loaded with [3 H]5MTHF from the apical or basolateral site did not significantly alter the rate of apical shedding, but no significant basolateral release of [3 H]5MTHF could be detected under any condition. Since monensin and transferrin induce exosome release from cells²¹, these data further suggest an exosomal type of FR α -mediated transport of 5MTHF into the apical compartment.

FR α released from choroids plexus cells is bound to exosomes

The vesicular nature of the directed FR α -dependent transport was confirmed by fractionation of the apical cell culture supernatant from FR α -transfected Z310 cells. FR α was enriched in the P100 (100.000 x g pellet) fraction and was co-purified with the exosomal markers Flot-2 and Alix (**Fig. 5b**). The monensin-induced increase in FR α signal was proportional to the increase in Flot-2 and Alix release. Flot-2 and Alix release was also induced by monensin in vector-transfected cells in the absence of FR α . The vesicle-containing P100 fraction of FR α -

transfected and monensin treated Z310 cells were subjected to sucrose gradient ultracentrifugation. Very similar to human CSF (**Fig. 3b**), FR α was mainly recovered from fractions possessing the characteristic density of exosomes (between 1.11 and 1.16 g/cm³) and containing the exosomal markers Flot-2 and Alix (**Fig. 5c**). About 80% of the total FR α in the apical supernatant was present in the P100 fraction and this corresponds to the proportion of [³H]5MTHF that was recovered in the P100 fraction (Supplementary **Table S1**). The isolated vesicles showed the typical size of exosomes and could be co-stained for FR α and Alix in two-color STED microscopy (**Fig. 5d**). Quantitative analysis revealed a colocalization of 63% \pm 8% of FR α and Alix. Incubation of PKH26-labelled FR α -positive and FR α -negative exosomes with FITC-labelled folic acid revealed specific colocalization of FITC-FA with FR α -positive exosomes (**Fig. 5e**). These data demonstrate that exosomes derived from FR α -expressing Z310 cells bind folates and share major characteristics with exosomes derived from human CSF. The fact that quantitative analysis of STED images achieved a higher percentage of FR α colocalization with the exosomal marker Alix can be explained by the overexpression of FR α in Z310 cells.

We also compared the protein composition of exosome preparations derived from Z310 cells with the one purified from human CSF by mass spectrometry (**Table 1**). Both preparations contained 84-88% commonly accepted exosomal markers²² and showed an overall similar protein profile.

To validate our findings in Z310 cells, we studied the directional 5MTHF transport in primary porcine choroid plexus epithelial cells (PCPEC). Filter-grown PCPEC were loaded with 25 nM [³H]5MTHF for 24 hours. Subsequently, medium was changed and cells were treated with 7 μ M monensin or left untreated. A significant increase of radioactivity could only be observed in the apical compartment (**Fig. 5f**). Treatment of cells with monensin further enhanced the apical accumulation of [³H]5MTHF by 33%. The rate of transport into the apical compartment corresponded to the rate obtained for FR α -transfected Z310 cells. Equivalent to Z310 cells, about 70% of the [³H]5MTHF was recovered in the P100 fraction and no significant [³H]5MTHF release was detected in the basolateral compartment.

The supernatant of FR α -transfected MDCK cells (see Fig. 2h-n) were also fractionated but did not reveal any significant amount of vesicles in the P100 fraction and did not contain detectable exosomal markers or FR α . Correspondingly, no exosomal release of 5MTHF at the apical plasma membrane could be measured.

We performed additional experiments to evaluate FR α -independent transport processes in Z310 cells at physiological 5MTHF concentration (12.5 nM). A couple of organic anion transporters are known to be expressed in Z310 cells²³. Contribution of Oat1, Oat3, Oatp2,

Oatp3, Mrp1, Mrp4, p-glycoprotein (Mdr1) and RFC to 5MTHF uptake into FR α -transfected Z310 cells was assessed by the incubation of cells with the following inhibitors of these transporters: taurocholate (TC), methotrexate (MTX), p-aminohippuric acid (PAH), probenecid and digoxin. As illustrated in **Supplementary Fig. S4**, none of the inhibitors reduced the uptake of [³H]5MTHF with the exception of MTX. However, at the 10 μ M concentration of MTX this antifolate is known to competitively block binding of 5MTHF to FR α as well.

FR α -associated 5MTHF is transported across the choroid plexus

The contribution of the PCFT and the RFC to FR α -mediated 5MTHF transport by Z310 cells was further studied by stable cotransfection of FR α with PCFT or RFC or a combination of both. Free (=cytosolic) and endosomal (= vesicular) 5MTHF was compared between FR α -transfected and FR α - plus PCFT-transfected Z310 cells (**Supplementary Fig. S5a**). While the amount of cytosolic 5MTHF was slightly higher in FR α /PCFT-double-transfected Z310 cells there was no significant differences in the endosomal 5MTHF fraction. The transfection of FR α increased the 5MTHF uptake 248-fold when compared to the vector-transfected control cells. Additional transfection with PCFT or RFC or the combination of both did not significantly alter 5MTHF uptake into Z310 cells when compared with the control cells (**Supplementary Fig. S4b**). Further, knocking-down endogenously expressed PCFT and RFC by specific siRNAs did not alter the FR α -mediated 5MTHF transport (**Fig S4c,d**). These data indicate that at least at the physiological 5MTHF concentrations in plasma (4-20 nM), the major proportion of 5MTHF that is captured by FR α at the basolateral site of the choroid plexus is transcytosed and released from the apical border of the choroid plexus.

FR α -positive exosomes penetrate into brain parenchyma *in vivo*

We have unexpectedly discovered the release of FR α as exosomal cargo into the CSF. To further understand the biological function of this transport mechanism, we injected exosomes derived from FR α -transfected or vector-transfected Z310 cells into the lateral ventricle of C57BL/6 mice. Purified exosomes were labelled with the dye PKH26 before they were applied via stereotactically implanted intraventricular cannulas. Four hours post injection, the mice were euthanized and their brains immediately frozen. Immunohistochemistry of the mouse brain sections revealed that FR α -positive exosomes penetrated into the brain parenchyma distal from the injection site and colocalized with GFAP-positive astrocytes as well as NeuN-positive neurons (**Fig. 6a and b**). In contrast, FR α -negative exosomes were mainly detected close to the periventricular ependyma. No parenchymal staining was observed after injection of 100 nM FA-FITC into the mouse brain ventricles.

FR α -positive exosomes are internalized by astrocytes *in vitro*

To further confirm these *in vivo* findings, we incubated primary mixed cortical cultures obtained from neonatal mice with either PKH26-labelled exosomes, with FA-FITC or a combination of both. After incubating the cells for 2 hours at 37 °C, cells were fixed and analyzed by immunofluorescence microscopy. FR α -positive exosomes were selectively taken up by GFAP-positive astrocytes but no significant colocalization was obtained with cells stained with markers for microglia, neurons and oligodendrocytes (**Fig. 6c**). FR α -negative exosomes did not colocalize with astrocytes but were internalized by Iba1-positive microglia. FA-FITC was also delivered to astrocytes when combined with FR α -positive exosomes. However, none of the cell types internalized 100 nM of free FA-FITC.

DISCUSSION

Several characteristics of FR α argue for its pivotal role in the 5MTHF transport across the blood-CSF barrier. Within the brain FR α is almost selectively expressed in the choroid plexus and its high binding affinity for folates (K_d 1-10 nM) perfectly matches the physiological 5MTHF concentration in human plasma (4-20 nM). Further, a hereditary FR α defect causes extremely low 5MTHF concentrations in the CSF (equal or below 5 nM) and results in a childhood onset neurodegenerative disease^{8,9}. In addition, the occurrence of FR α autoantibodies in plasma is associated with low 5MTHF concentration in the CSF as well as with a similar neurodegenerative disease²⁴. Our experiments with various polarized cells (MDCK, HepG2, Z310 cells) demonstrate a basolateral to apical sorting of FR α that provides for receptor-mediated endocytosis and transcytosis of folates (**Fig. 2 and S2**). These findings indicate that the GPI-anchored FR α promote a vesicular transport of 5MTHF across the choroid plexus.

GPI-anchored proteins are known to be associated with specific subcellular compartments. They enter the endosomal compartment by a clathrin-independent pathway to form tubular early endocytic compartments called GPI-anchored protein enriched endosomal compartments (GEECs)^{25,26}. Our data indicate FR α translocation to matured endosomes that are named multivesicular bodies and that contain intraluminal vesicles. Multivesicular bodies are known to be sites for intracellular sorting of proteins between recycling, degradation and secretion pathways²⁷. We have provided several lines of evidence that in choroid plexus cells FR α -containing multivesicular bodies fuse with the apical membrane and secrete their intraluminal vesicles as exosomes (**Fig. 1e-h, Fig. 7**). We further suggest that FR α recycles between the apical membrane and the late endosomal compartment and thus offers 5MTHF uptake from the CSF as well. The absence of effect in folate binding and

transport after transfection of human PCFT and human RFC or after siRNA-suppression of endogenous rat PCFT and rat RFC indicates the essential role of FR α -mediated transport of 5MTHF at its physiological concentration (**Supplementary Fig. S5b-d**). Further, the pharmacological inhibition of various folate transporters did not alter folate binding to Z310 choroid plexus cells (**Supplementary Fig. S4**). These results are consistent with recent studies on primary rat choroid plexus epithelial cells that show two separate folate uptake mechanisms²⁸. One FR α -dependent process mediates efficient uptake of 5MTHF at low extracellular folate concentrations. The other uptake mechanism is independent of FR α but likely depends on organic transporters such as the RFC and accounts for folate uptake at higher concentrations. It is possible that in case of abundant folate supply 5MTHF may follow other routes of internalization and transport in the choroid plexus. However, it is likely that any free 5MTHF released into the CSF will be picked up by membrane attached FR α either present on exosomes or on the apical surface of the choroid plexus. Since about 36% of all human CSF exosomes contain FR α (**Fig. 3d**) and since brain FR α exclusively originates from the choroid plexus⁸ we consider the human choroid plexus as a major source of CSF exosomes.

Our data indicate that human PCFT is localized to intracellular vesicles that are distinct from but may marginally colocalize with FR α -positive intraluminal vesicles (**Fig. 1d,j,k**). Our FR α /PCFT-cotransfection experiments in Z310 choroid plexus cells are consistent with previous reports demonstrating that PCFT and FR α colocalize to endosomal compartments and that PCFT is responsible for the 5MTHF transport out of acidified endosomal compartments into the cytoplasm^{29,30}. In case of functional loss of PCFT the cellular lack of 5MTHF will consequently compromise the function of the choroid plexus and finally also hamper the FR α -dependent 5MTHF export into the CSF. Thus, similar as in the case of Kearns-Sayre syndrome, this could explain the fact that patients suffering from hereditary folate malabsorption, the genetic defect of the PCFT, show very low 5MTHF CSF even if they are supplemented with oral folates. Further experimental studies are required to delineate the cooperative functioning of FR α together with the PCFT. PCFT-null mice³¹ as well as FR α -deficient mice³² that can both be rescued by prenatal and/or postnatal folate supplementation provide important tools to substantiate and elaborate the transmembrane transport and the intracellular routing of folates.

The unusual cargo transport mechanism of 5MTHF might serve as a stabilizing and buffering system for fluctuations in folate supply and demand of the brain. Most excitingly, our results indicate that exosome targeting into the brain parenchyma crucially depends on the expression of FR α (**Fig. 6**). Though the molecular mechanism of this selective uptake is presently not clear we hypothesize that FR α induces a signal for exosome internalization. Ependyma cells and astrocytes may function as intermediates for FR α -positive exosomes on

their route to other brain cells such as neurons. This transport model would explain why normalization of 5MTHF concentration in the CSF does not necessarily correlate with the amelioration of clinical symptoms in patients suffering from a genetic FR α defect. Our investigations provide new insight into the molecular mechanism that might account for cerebral folate transport deficiency and extend this entity to other disorders that affect the choroid plexus such as Kearns-Sayre syndrome.

While exosomes have commonly regarded as vehicles for RNA, protein and lipid transfer between cells³³⁻³⁵ our studies reveal a novel function of exosome as transport medium for micronutritions.

The selective targeting of FR α -expressing exosomes to the brain parenchyma not only substantiate the biological significance of this transport shuttle but also opens up new avenues for therapeutic approaches. By designing their protein expression exosomes may serve as organ-specific delivery vehicle for therapeutic agents. This strategy has recently been exemplified with exosomes that were engineered to express a neuron-specific peptide derived from the rabies virus³⁶. Our work discloses the possibility that targeted manipulation of the choroid plexus or direct application of FR α -positive exosome-like vesicles into the CSF may be a novel strategy to deliver biological active substances into the brain. Using FR α as a natural sorting signal and taking their none-immunogenic properties FR α -positive exosomes represent a particular attractive shuttle system for a broad variety of biomolecules and organic or inorganic compounds. Further scientific exploration of the exosome biology is required to understand their intercellular trafficking and to be able to exploit their full therapeutic potential.

METHODS

Materials

Antibodies against the following antigens were used: human FR α (Mov18/ZEL; Alexis Biochemicals or NCL-L-FR α Clone BN3.2; Novocastra), flotillin-2 and CD63 (Flot-2; BD Biosciences), transferrin (Dako), ALG-2-interacting protein X (Alix; Covalab), RFC1 (Abcam), glial fibrillary acidic protein (GFAP; Dako), ionised calcium binding adaptor molecule 1 (iba1; WAKO Chemicals), neuron specific nuclear protein (NeuN; Chemicon), neurite outgrowth inhibitor protein A (Nogo-A)³⁷. An antibody against human PCFT was kindly provided by I. David Goldman (Bronx, New York, USA). Secondary antibodies were purchased from Dianova. Folic acid conjugated to fluorescein isothiocyanate (FA-FITC) was kindly provided by Philip S. Low (West Lafayette, Indiana, USA). Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* (PI-PLC) was from Sigma. The following inhibitors were used:

taurocholate (TC), methotrexate (MTX), p-aminohippuric acid (PAH), probenecid, digoxin, brefeldin A (BFA) and N-ethylmaleimide (NEM). All were purchased from Sigma.

Human samples

All CSF samples were immediately shock frozen after lumbar puncture and stored at -80°C. CSF samples recruited from patients suffering from cerebral folate transport deficiency that were recently characterized in detail⁹. CSF samples from nine patients with genetically confirmed Kearns-Sayre-syndrome (KSS) were analyzed. The KSS patients, six female and three male individuals, were aged between 11 and 25 years at the time of the lumbar puncture. CSF samples from five individuals with suspected hydrocephalus or idiopathic intracranial hypertension, three males and two females that were aged between two and 16 years were used as controls. Paraffin sections of human choroid plexus from post-mortem autopsy blocks were retrieved from the Department of Neuropathology, University of Goettingen. Informed consent was obtained from the patients or parents and the study was approved by the ethical review board of the medical faculty of the University of Goettingen.

Cell cultures and transfections

Z310 cells were grown in DMEM supplemented with 10% fetal calf serum, 10 ng/mL epidermal growth factor (Invitrogen) and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained MEM supplemented with 10% fetal calf serum and antibiotics. The human polarized hepatic cell line HepG2 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. All cells were incubated at 37°C in 5% CO₂.

Transfections were done using Effectene transfection reagent (Qiagen) and stable transfectants were selected in the presence of the appropriate antibiotics.

Porcine choroid plexus epithelial cells (PCPECs) were prepared basically as described previously³⁸. Isolated PCPECs were seeded in laminin-coated transwell culture plates (0.4 µm pore size, Costar, Cambridge, MA) with a diameter of 12 mm using a seeding density of 20 mg wet tissue/cm². At days in vitro (DIV) 10, the medium was replaced by folate-free RPMI medium (Invitrogen) without serum. The transport experiments were performed on cells at DIV 13-15 that exhibited high transepithelial electrical resistance (TEER) of about 1000 Ω x cm².

Transcytosis assays

Transcytosis assays were adapted from Polishchuk *et al.*³⁹. Before the experiments, cells were maintained in folate-free RPMI 1640 medium. Filter-grown Z310 or MDCK cells stably expressing FRα incubated on ice for 15 min with 100 nM FA-FITC together with anti-human FRα antibody (Mov18/ZEL) added to the basolateral chamber of the transwell plate. Some

filters were fixed immediately after incubation on ice, while others were shifted to 37°C for the indicated time points, fixed and prepared for confocal microscopy. Biochemical evaluation of transcytosis was based on the idea that removal of protein from the basolateral surface will result in decrease of its amount at the apical domain of the plasma membrane⁴⁰. Therefore, filter-grown Z310 or MDCK cells stable expressing FR α were incubated with PI-PLC added to the basolateral medium for the indicated time points. To detect the apical portion of FR α , cells were washed and PI-PLC was added to the apical medium 30 min on ice to harvest the receptor from the apical domain of the plasma membrane. Samples were then subjected to Western blotting.

Exosome preparation

Exosomes were prepared from human CSF or from cell culture supernatant by performing sequential centrifugation. Therefore CSF or supernatant from Z310 cells was centrifuged for 15 min at 1500 x g to remove debris. The supernatant was then subjected to ultracentrifugation at 10.000 x g for 45 min and at 100.000 x g for 150 min. The collected pellets were finally resuspended in CellLytic™M Cell Lysis Reagent (Sigma) and subjected to Western blotting. For further analysis, the exosome samples were layered on top of a sucrose gradient and centrifuged for 16 hours at 100.000 x g. Subsequently, fractions were collected, diluted 1:4 in PBS and centrifuged at 100.000 x g for 70 min. Pellets were resuspended in Laemmli sample buffer and subjected to Western blotting. For immunofluorescence analysis, the exosome pellet was resuspended in PBS and stained with PKH26 (Sigma).

Electron microscopy

Exosomes were fixed with 4% paraformaldehyde and absorbed to glow discharged formvar-carbon coated copper grids. The samples were immunolabelled with anti-human FR α antibody (Mov18/ZEL) or anti-human Flotillin-2 antibody followed by a rabbit anti-mouse antibody. The antibodies were detected with Protein A coupled to 10 nm gold particles. After contrasting by neutral uranyl acetate, samples were imaged on a Leo EM912AB-electron microscope (Zeiss) and images were acquired using on axis 2048X2048 charge-coupled device camera (Proscan, Schering). Z310 cells were treated with 7 μ M monensin for 4 hours. Cells were then fixed and prepared for postembedding immunohistochemistry according to⁴¹.

Immunofluorescence microscopy and image analysis

Confocal images were acquired on a spinning disk confocal imaging system (UltraView®, Perkin-Elmer). The system incorporated an Olympus IX81 inverted microscope using a 100X 1.4 NA lens. Excitation wavelengths and filter sets for FITC and Cy3 were as supplied by the

manufacturer. Fixed cells labelled with antibodies and/or FA-FITC were optically sectioned into z-stacks. Image processing was performed using the Zeiss LSM image browser software and NIH ImageJ (<http://rsbweb.nih.gov/ij>; Open Source).

High-resolution stimulated emission depletion microscopy

Exosomes were directly fixed on glass coverslips with 4% paraformaldehyde. Two-color stimulated emission depletion (STED) microscopy was performed as previously described¹⁸, using sheep anti-mouse polyclonal antibody labelled with Atto590 (for anti-human FR α as first antibody) and goat anti-rabbit polyclonal antibody conjugated to Atto647N (for Alix as marker for exosomes). The colocalization of FR α and Alix was analyzed by using the “colocalization threshold” algorithm from ImageJ.

Mass spectrometry analysis

Exosomes were prepared as described above and the exosome pellet was directly denatured in Laemmli sample buffer containing 50 mM dithiothreitol (DTT) for 10 min at 70°C. Mass spectrometry was then conducted as described previously⁴². Semiquantitative analysis of the relative exosomal protein abundance was determined by the ranking of unweighted spectrum counts with the program Scaffold 3.

FR α -mediated 5MTHF uptake and transport analysis

For 5MTHF uptake and transport measurements in Z310 cells, 4.2×10^4 cells were seeded in laminin coated transwell culture plates (0.3 cm² growth area, 0.4 μ m pore size) in folate-free RPMI medium. 72 hours post-confluent, cells were loaded with 12.5 or 25 nM [³H]5MTHF in folate-free RPMI medium containing 1% BSA added either to the apical or basolateral chamber of the transwell plates. [3',5',7,9-³H](6S)-5MTHF was purchased from Moravek Biochemicals (Brea, CA). Cells were then incubated at 37°C for 1 hour and were subsequently washed. Fresh RPMI medium was added containing either 7 μ M monensin (Sigma) or 0.5 mg/mL rat transferrin (Rockland, Gilbertsville, PA) and cells were then incubated at 37°C in 5% CO₂. Samples were removed from the apical and basolateral medium at the indicated time points. To stop the transport experiment, the transwell plates were placed on ice and the cells were washed three times with ice-cold hepes-buffered saline (HBS). Cells were then dissolved in 0.2 M NaOH and protein determination was done with the BCA protein assay (Pierce, Rockford, IL). Radioactivity in medium samples and cell lysates was measured in a liquid scintillation spectrometer.

Immunohistochemistry of choroid plexus

Immunohistochemistry was carried out as previously described⁴³. Staining on human sections was performed using mouse monoclonal FR α antibody (NCL-L-FR α) or rabbit polyclonal PCFT antibody followed by HRP-conjugated secondary antibodies. After development, the slides were counterstained with hematoxylin. For immunofluorescence, the sections were treated as described above except the use of secondary Alexa 488 donkey anti-mouse or Alexa 546 donkey anti rabbit antibodies.

Internalization assay

Mixed cortical cultures were obtained from neonatal mice and immediately plated on coverslips and incubated in the respective growth medium. Before the internalization assay, exosomes were purified from Z310 cells, stably transfected with FR α or vector and labelled with PKH26 and FA-FITC as described above. The uptake was performed by incubating the cell cultures with exosome dilutions for 2 hours at 37°C and 5% CO₂. Cells were then fixed, permeabilized and stained with the respective antibodies for immunofluorescence analysis. In vivo experiments were carried out with C57BL/6 mice at the ages between 80 days and 100 days. The mice were anesthetized with ketamine (100-125 mg/kg) and xylazine (10-12,5 mg/mL) and the intraventricular cannulas were stereotaxically implanted into the lateral ventricle as described previously⁴⁴. 10 μ L of labelled exosome solution were injected into the intraventricular cannula 4 hours before the mice were euthanized. Intact brains were then dissected, immediately frozen in liquid nitrogen pre-cooled isopentane and stored at -20°C. For fluorescence analysis, the frozen brain tissues were cryosectioned to a thickness of 10 μ m. The frozen sections were fixed in 4% paraformaldehyde for 30 min at room temperature. After washing in PBS, sections were blocked for 1 hour in PBS containing 10% (w/v) horse serum and 0.2% BSA. Antibodies against GFAP and NeuN were applied overnight in carrier containing 1% horse serum and 0.2% BSA in PBS. After washing with PBS, slides were incubated with appropriate secondary Alex488 antibodies for 2 hours. After washing in PBS sections were embedded in ProLong Gold with DAPI (Invitrogen). Images were acquired with a confocal microscope (Olympus FV1000).

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AUTHOR CONTRIBUTIONS

R.S. developed the experimental strategy and coordinated the external experiments.

M.G. and R.S. designed the experiments, interpreted data and wrote the manuscript.

A.W. and M. S. performed immunofluorescence staining and electron microscopy of human and mouse brain tissues as well as rat choroid plexus cells.

S.H. and H-J.G. performed the experiments with the primary porcine choroid plexus epithelial cells (PCPECs).

N.S. and M.S. performed the electron microscopy of exosomes and critically reviewed the manuscript.

J.B. performed the STED microscopy.

P.S.L. synthesized and provided the folate conjugated to fluorescein isothiocyanate (FA-FITC).

M.G. performed all other experiments.

All authors contributed to writing the manuscript.

STATEMENT CONFIRMING NO COMPETING FINANCIAL INTERESTS

All authors confirm that there are no competing financial interests that might influence the results or interpretation of this manuscript.

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FIGURE LEGENDS

Figure 1. Localization of FR α in human choroid plexus and in FR α -transfected rat choroid plexus Z310 cells.

(a) Immunohistochemical staining of FR α in human choroid plexus specimen shows a predominant localization at the apical brush border membrane. A minor portion of the receptor is localized at the basolateral membrane and an intracellular punctuate staining is visible too. The arrow points to the apical membrane. Scale bar: 50 μ m.

(b) Immunohistochemical staining of PCFT in human choroid plexus specimen illustrates a uniformly intracellular localization with a punctate pattern. The arrow points to the apical membrane.

(c) Immunofluorescence microscopy of human choroid plexus specimen demonstrates distinct distributions of PCFT (red) and FR α (green) as well as minor colocalization. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). The arrow points to the apical membrane. Scale bar: 50 μ m.

(d-k) Rat choroids plexus Z310 cells stably transfected with human FR α and human PCFT were fixed and prepared for immunofluorescence or electron microscopy.

Confocal fluorescent microscopy of transfected Z310 cells reveals punctate PCFT (red) and FR α (green) staining **(d)** that colocalize only marginally. However, FR α (red) significantly colocalizes with CD63 (green), an endosomal marker that is characteristic of multivesicular bodies (MVB) **(e)**, frames indicate the magnified area Scale bars: 5 μ m.

Electron microscopy after immunogold labelling of FR α demonstrates enrichment of gold particles within MVBs and close to the fusion point between MVB (black arrows) and plasma membrane (white arrows) **(f)**, scale bar: 500 nm. Higher magnification reveals that FR α is associated with small intraluminal vesicles (ILV) inside the MVB or with vesicles outside the cell (black arrowheads) **(g)**, scale bar: 250 nm, and **(h)**, scale bar 50 nm. Vector-transfected Z310 cells contain similar ILV (black arrowhead) within MVBs (black arrows) but lack FR α gold staining **(i)**, scale bar: 500 nm. PCFT staining discloses gold particles associated with medium size electron dense vesicles (black arrowhead) that are neither located within MVB nor fuse with the plasma membrane (white arrow) **(j)**, scale bar 250 nm and **(k)**, scale bar 100 nm. The specificity of the applied antibodies is illustrated in **Supplementary Fig. S1**.

Figure 2. Transcytosis of FR α in the polarized cell lines Z310 and MDCK.

(a-c) FR α -expressing Z310 cells were incubated on ice for 15 min with FA-FITC together with anti-human FR α antibody added to the basolateral chamber of the transwell plate. Filters were fixed immediately **(a)** or were shifted to 37 °C for 60 min **(b)** or 120 min **(c)** and were analyzed by confocal microscopy. xy and xz images reveal that the antibody (red) and FA-FITC (green) initially bind to FR α at the basolateral surface of the cells **(a)** while after 60 min and 120 min the antibody together with FA-FITC were transported to the apical surface of the cells **(b, c)**.

When FA-FITC and antibody were added to the apical chamber of the transwell plate no transcytosis to the basolateral membrane was observed after 120 min **(d)**. Both FA-FITC and antibody disappeared at the apical surface suggesting a release of FR α from the apical membrane **(e)**.

(f) When PI-PLC was added for 60 min or 120 min to the basolateral surface of FR α -expressing Z310 cells the amount of apically sorted FR α diminished over time as displayed by Western blotting and quantified by densitometry **(g)**. Values are means \pm s.e.m. of three independent experiments. Statistical analysis was done by Student's *t*-test (***, $p < 0.001$). **(h-j)** FR α -expressing MDCK cells were incubated with FA-FITC together with anti-human FR α antibody as described for the Z310 cells. Filters were fixed immediately **(h)** or were shifted to 37 °C for 60 min **(i)** or 120 min **(j)** and were analyzed by confocal microscopy. Antibody (red) and FA-FITC (green) initially bind to FR α at the basolateral surface of the cells **(h)** while after 60 min **(i)** and 120 min **(j)** the antibody and FA-FITC become redistributed to the apical surface of the cells.

When FA-FITC and antibody were added to the apical chamber **(k)** no transcytosis to the basolateral membrane was observed after 120 min **(l)**. In contrast to Z310 cells, no decrease of the apical fluorescent staining was observed. Scale bar for **a-e** and **h-l**: 10 μ m.

When PI-PLC was added for 60 min or 120 min to the basolateral surface of FR α expressing MDCK cells apically sorted FR α diminished over time as displayed by Western blotting **(m)** and quantified by densitometry **(n)**. Values are means \pm s.e.m. of three independent experiments (***, $p < 0.001$; Student's *t*-test). Full scan images are displayed in **Supplementary Fig. S6**.

Figure 3. Identification and characterization of FR α -positive exosomes in specimen of human CSF.

(a) Sequential centrifugation steps were conducted with human CSF specimen and the resulting pellets were analyzed by Western blotting. P10 refers to the pellet from 10.000 x g centrifugation step; P100 refers to the pellet from 100.000 x g centrifugation step. The input and the remaining supernatants after the 100.000 x g centrifugation were concentrated 20-fold prior loading on the gel. FR α and the exosomal marker flotillin-2 (Flot-2) are enriched in P100, whereas transferrin remains mainly in the supernatant. Full scan images are displayed in **Supplementary Fig. S6**.

(b) A 100.000 x g pellet was loaded on top of a discontinuous sucrose density gradient and ultracentrifuged for 16 hours. The fractions were then analyzed by Western blotting. FR α is enriched in the same fractions as Flot-2. Full scan images are displayed in **Supplementary Fig. S6**.

(c) For electron microscopy, a 100.000 x g pellet was negatively stained with uranyl acetate and immunolabeled with antibodies against FR α or Flot-2. The pellet contains mainly small membrane vesicles with a diameter of about 40 to 100 nm, with the typical cup-shaped morphology of exosomes, that are positive for FR α and Flot-2. Scale bars: 200 nm.

(d) High resolution imaging of the 100.000 x g pellet and colocalization analysis of FR α and the exosomal marker Alix by two-color STED microscopy. While standard confocal microscopy fails to display the details, STED microscopy reveals the typical size of exosomes and analysis of the images demonstrates a colocalization of FR α with Alix of 36% \pm 8% (data calculated with the NIH Image J software). Scale bar: 1 μ m.

(e) STED microscopy allows exact size determination of the vesicles positive for FR α and Alix (arrowhead in **d**).

Figure 4. Detection of FR α in CSF by Western blotting.

(a) CSF of patients with cerebral folate transport deficiency and controls, respectively, were concentrated 10-fold using "Amicon Centrifugal Filter Devices". 30 μ g of protein per lane were subjected to SDS-PAGE and Western blotting. FR α is absent in the CSF of patients with cerebral folate transport deficiency while it can be clearly detected in the control samples (only one of five different shown). The patients carry the following mutations in the *FOLR1* gene: the compound heterozygous mutations p.Q118X/p.C175X and p.C169Y/p.N222S, the homozygous mutations p.C65W and p.169Y and the homozygous splice site mutation g.3576T>G. Full scan images are displayed in **Supplementary Fig. S6**. The amount of FR α in the CSF correlates with the concentration of 5MTHF in the CSF (**b** and **c**). Two CSF samples from one patient that was diagnosed with Kearns-Sayre syndrome, a mitochondrial disorder that is associated with reduced concentration of 5MTHF in the CSF, were compared. The sample with 5MTHF concentration of 39.4 nmol/L (reference value: 40 - 120 nmol/L) showed a diminished amount of FR α in the CSF while in the second sample the 5MTHF concentration in the CSF was below 5 nmol/L and FR α was not detectable (**b**). CSF samples (100 μ g each) from eight additional patients with genetically confirmed Kearns-Sayre syndrome were analyzed by Western blotting. The intensity of the FR α –signal roughly correlated with the 5MTHF concentration in the CSF (**c**). Transferrin served as loading control. Full scan images are displayed in **Supplementary Fig. S6**.

Figure 5. FR α -mediated transport of 5MTHF in Z310 cells is stimulated by monensin and transferrin.

(a) Filter-grown Z310 cells stably transfected with FR α or vector were loaded with [3 H]5MTHF for 1 hour. After three washing steps, cells were incubated with fresh RPMI medium containing either 7 μ M monensin (Mon), 0.5 mg/ml rat transferrin (Trans) or no supplementation. Samples from the apical and basolateral compartment were removed at the indicated time points. Subsequently, cells were lysed and radioactivity was measured in medium samples and cell lysates. Data are means \pm s.e.m. of three independent experiments (**, p<0.01; *, p<0.05; Student's t-test).

(b) Z310 cells stably transfected with FR α or vector were treated with 7 μ M monensin for 22 hours. The medium was collected and subjected to ultracentrifugation for exosome purification. The P100 pellets were analyzed by Western blotting and probed with antibodies against FR α , Flot-2 and Alix. Full scan images are displayed in **Supplementary Fig. S6**.

(c) To further characterize the vesicles derived from Z310 cells, the 100.000 x g pellet from monensin treated Z310 cells stably expressing FR α was subjected to sucrose gradient ultracentrifugation. FR α is mainly enriched in the fractions that contained Flot-2 and Alix. Full scan images are displayed in **Supplementary Fig. S6**.

(d) Two-colour STED microscopy of exosomes purified from Z310 cells stably expressing FR α demonstrates a colocalization of FR α with Alix of 63% \pm 13% (data calculated with the NIH Image J software). Scale bar: 1 μ m.

(e) Exosomes purified from either FR α -transfected or vector-transfected Z310 cells were stained with the dye PKH26 before they were incubated with FITC-FA for 1h at RT. Only FR α -positive exosomes colocalize with FITC-FA and thus bind folate. Scale bar: 20 μ m.

(f) Apical transport of 5MTHF in primary PCPECs. Filter-grown PCPECs were loaded with 25 nM [3 H]5MTHF for 24 hours. Cells were then incubated with 7 μ M monensin or left untreated. Radioactivity from apical medium samples was measured at the indicated time points. The increase of the apical [3 H]5MTHF concentration was further enhanced by incubating the cells with monensin. Data are means \pm s.e.m. of two independent experiments, each done in triplicate.

Figure 6. FR α -expressing exosomes penetrate into the brain parenchyma *in vivo* and are internalized by astrocytes *in vitro*

(a) Exosomes were purified from the cell culture supernatant of transfected Z310 cells and labelled with the dye PKH26 (red). 10 μ l of the exosome solutions were then injected in mice with surgical implanted intraventricular cannulas 4 hours before the mice were euthanized. Immunofluorescent staining of the mouse brain sections shows that exosomes derived from FR α -transfected Z310 cells (FR α +) cross the ependymal cell layer and are distributed deeply in the brain parenchyma distal from the injection site. In contrast, exosomes derived from vector-transfected cells (FR α -) cannot penetrate the parenchyma but are retained at the ependyma. Nuclei were counterstained with DAPI (blue). Upper scale bar: 100 μ m, lower scale bar: 20 μ m.

(b) Immunostaining of the mouse brain sections with antibodies against GFAP and NeuN reveal that exosomes (red) derived from FR α -transfected Z310 cells colocalize with GFAP-positive astrocytes and NeuN-positive neurons. Nuclei were counterstained with DAPI (blue). Scale bar 10 μ m.

(c) Purified exosomes from Z310 cells were labelled with the dye PKH26 (red), incubated with FA-FITC (green) and added to primary mixed cortical cultures on DIV 7, consisting of mouse oligodendrocytes, astrocytes, cortical neurons and microglia. After incubation with exosomes for 2 hours at 37°C, the cells were fixed and analyzed by immunofluorescence microscopy. Oligodendrocytes, astrocytes, neurons and microglia were recognized by antibodies against Nogo-A, GFAP, Iba1 and NeuN. The primary antibodies were recognized with secondary biotin-conjugated antibodies and subsequently labelled with streptavidin-coupled Pacific blue (shown in grey for better visualisation). FR α -containing exosomes (FR α +) are selectively taken up by GFAP positive astrocytes. Partial colocalization of FA-FITC and PKH26 is also observed in these cells. Exosomes purified from the culture medium of vector-transfected Z310 cells (FR α -) are not taken up by astrocytes but are internalized by Iba1-positive microglia. Note that none of the cell types was found to internalize free FA-FITC. Scale bar: 20 μ m.

Figure 7. Suggested model of human cerebral folate transport

At physiological plasma concentrations (6-40 nM) 5MTHF (F) is primarily taken up by FR α at the basolateral membrane of the human choroid plexus. The 5MTHF-FR α complex is internalized by receptor-mediated endocytosis, translocated into GPI-anchored protein-enriched early endosomal compartments (GEEC) and further transferred to multivesicular bodies (MVBs). MVBs are late endosomal compartments localized in the endocytic route. A minor proportion of internalized 5MTHF is translocated to PCFT-positive vesicles and transported into the cytoplasm. Intraluminal vesicles of MVBs containing FR α are generated by inward budding of the limiting membrane. These intraluminal vesicles are released as exosomes into the CSF after fusion of the MVB with the apical cell membrane. Fusion of the limiting membrane of MVBs with the apical membrane explains the presence of FR α molecules on the apical surface of the choroid plexus cells too. FR α -containing exosomes circulate in the CSF, cross the ependymal cell layer and are distributed in the brain parenchyma. FR α -positive exosomes might initially be taken up by astrocytes and from these further delivered to neurons. FR α -negative exosomes hardly cross the ependymal cell layer and are targeted to other brain cells, such as microglia. At higher than normal plasma concentrations of 5MTHF (> 100 nM) alternative mechanisms of uptake and transport may provide for transcellular 5MTHF delivery across the choroid plexus.

Table 1

human CSF	Z310 vector-transfected	Z310 FR α -transfected
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
ALG-2 interacting protein X (Alix)	ALG-2 interacting protein X (Alix)	ALG-2 interacting protein X (Alix)
flotillin-1	flotillin-1	flotillin-1
flotillin-2	flotillin-2	flotillin-2
folate receptor α	-	folate receptor α (human)
tumor susceptibility gene 101 (TSG101)	tumor susceptibility gene 101 (TSG101)	tumor susceptibility gene 101 (TSG101)
C-1-tetrahydrofolate synthase	C-1-tetrahydrofolate synthase	C-1-tetrahydrofolate synthase
CD63	CD63	CD63
transferrin receptor protein (CD71)	transferrin receptor protein (CD71)	transferrin receptor protein (CD71)

Table 1: Mass spectrometry analysis of exosomes purified from human CSF or from the supernatant of transfected rat choroid plexus Z310 cells.

Exosome preparations from human CSF and Z310 cells were analyzed by mass spectrometry. Each preparation contained a couple of hundred proteins with a high probability of over 95%. Among these we could identify FR α in the sample derived from human CSF as well as in FR α -transfected Z310 cells. Although expression of endogenous rat FR α in Z310 cells has previously been indicated by RT-PCR²³ the rat FR α expression might be under the mass spectrometric detection level. Exosomal markers like ALG-2 interacting protein X (Alix), flotillin-1 (Flot-1), flotillin-2 (Flot-2), CD63, tumor susceptibility gene 101 (TSG101) and transferrin receptor (CD71) could consistently be detected in all exosome preparations indicating a similar composition of exosomes derived from Z310 cells when compared with exosomes purified from human CSF. The order in Table 1 represents semiquantitative incidence of proteins determined by the ranking of unweighted spectrum counts in the program Scaffold 3. Taking the 25 most frequently identified proteins in exosomes (http://exocarta.org/exosome_markers)²² we found 22 (88%) in human CSF and 21 (84%) in the supernatant of Z310 cells, respectively.

Figure 1

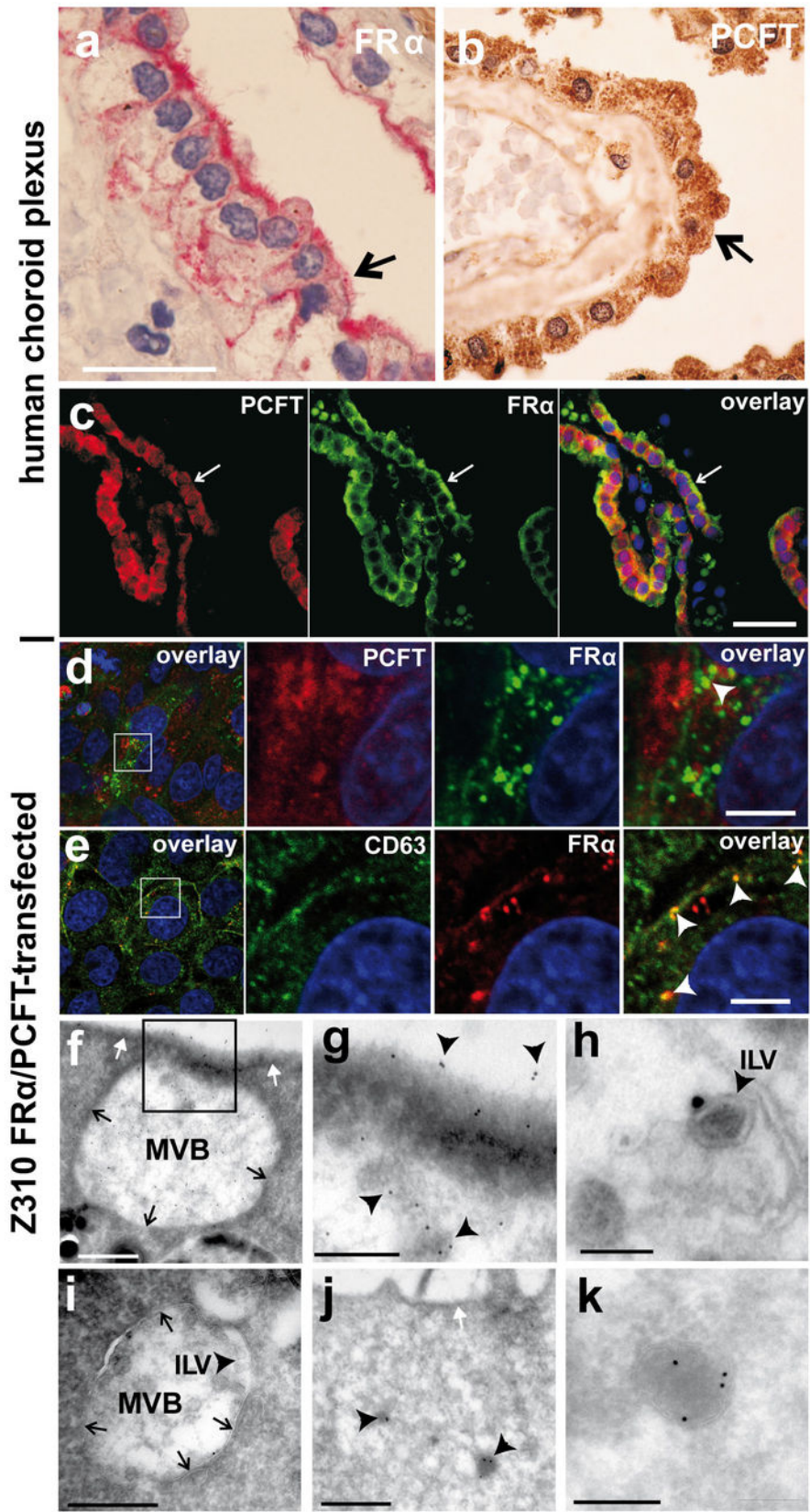


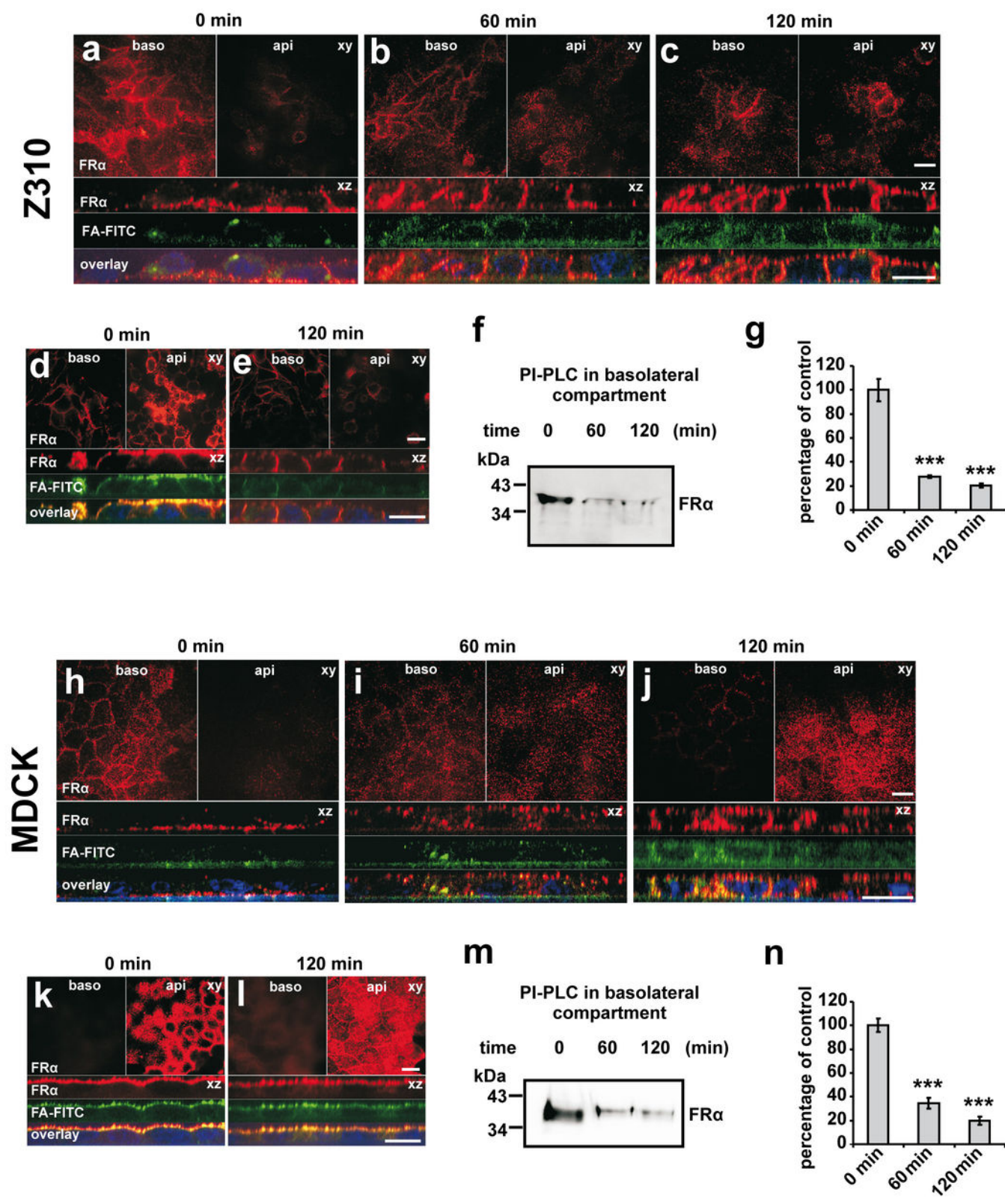
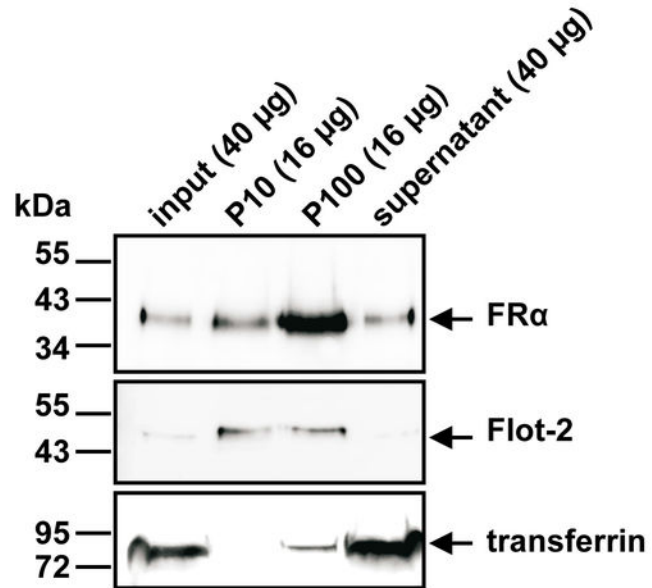
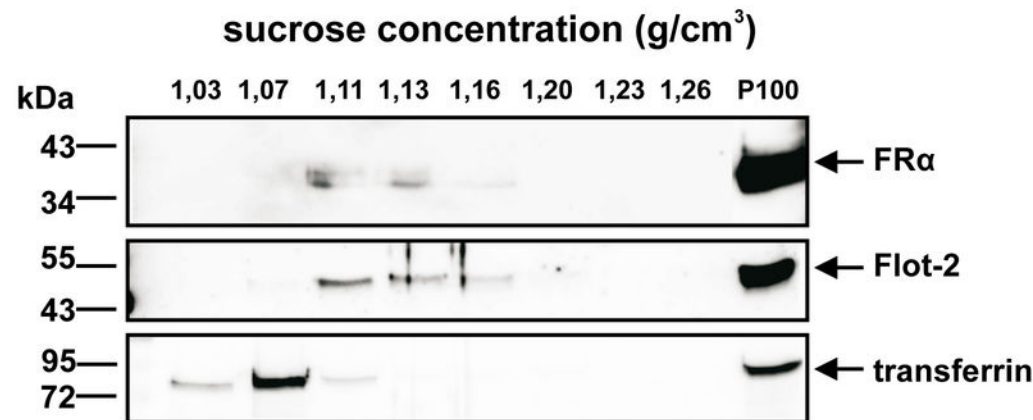
Figure 2

Figure 3

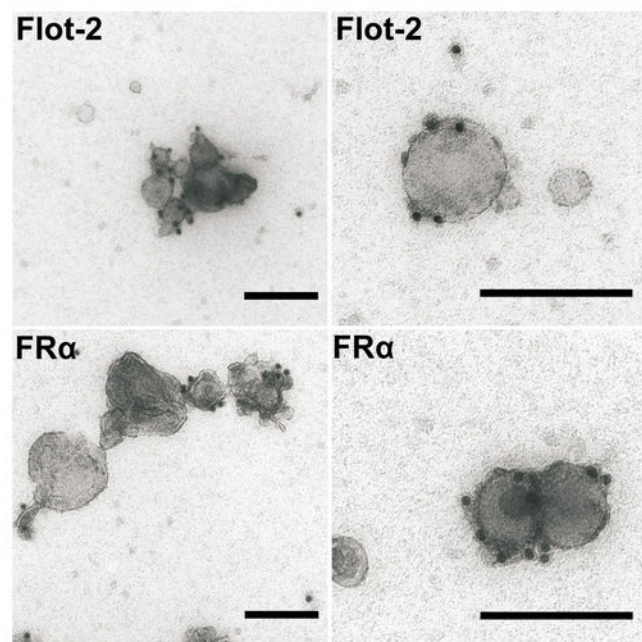
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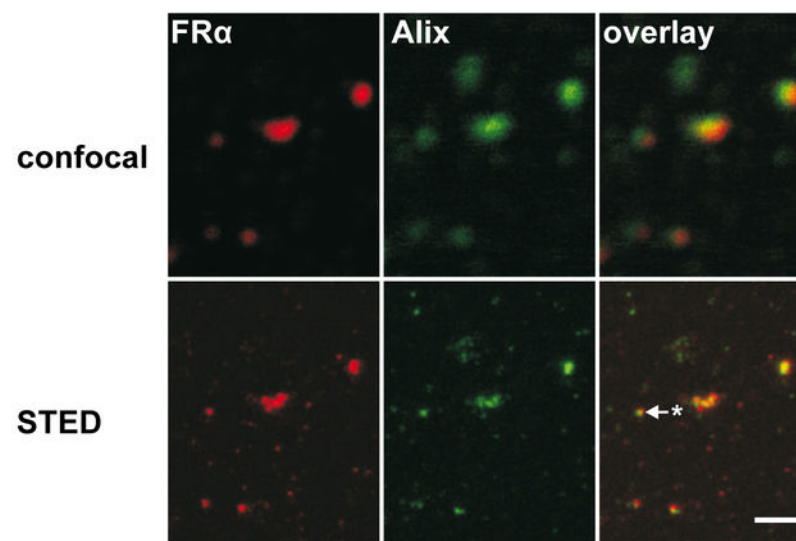
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d



e

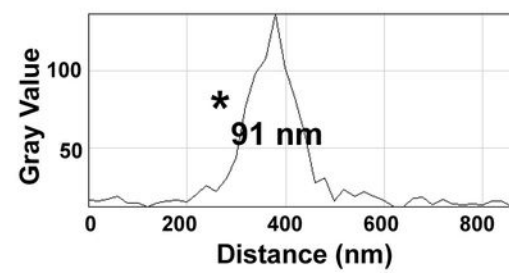
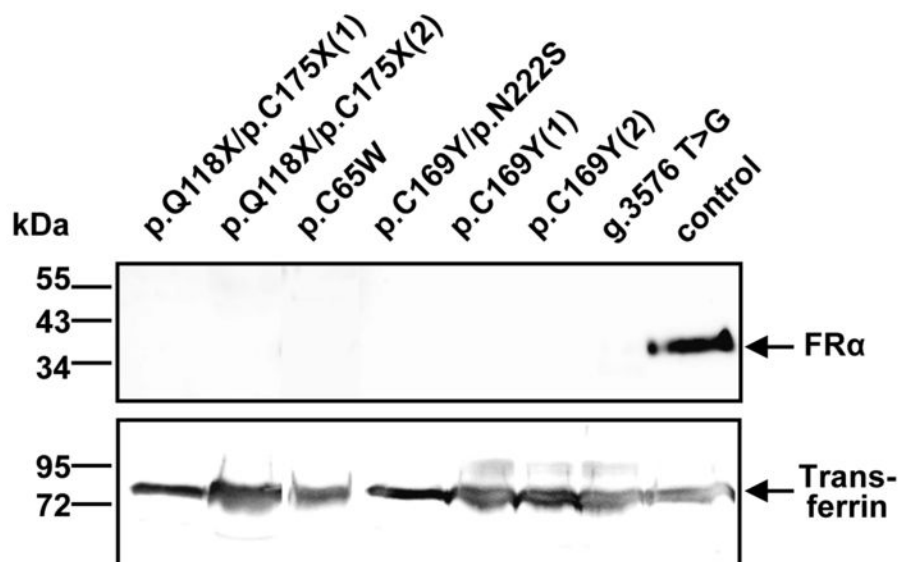
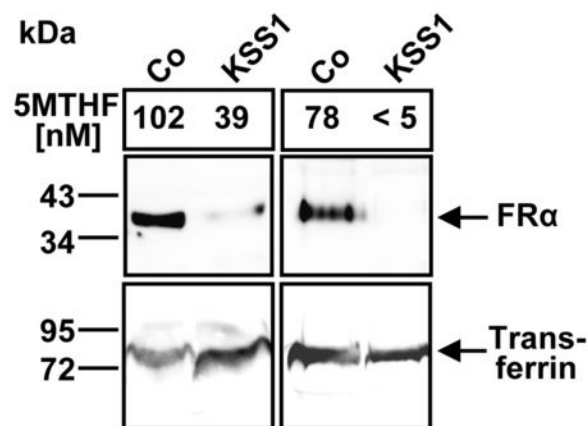


Figure 4

a



b



c

KSS-Patient	2	3	4	5	6	7	8	9	Co
5MTHF [nM]	17	20	37	24	26	15	< 10	12	84
FRα									
Transferrin									

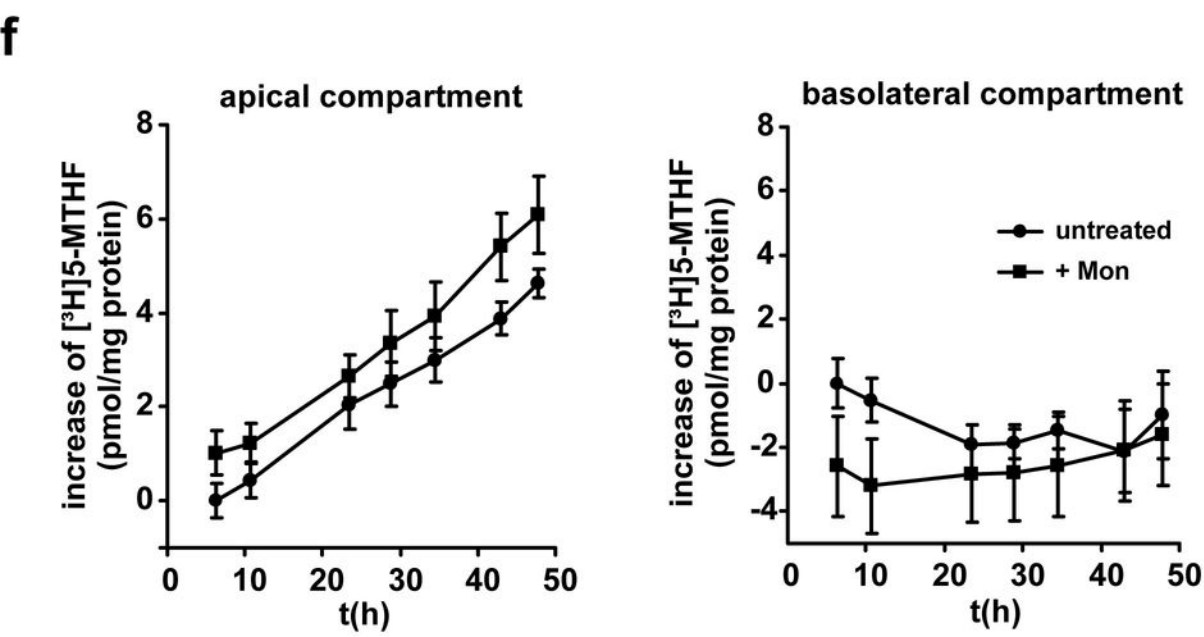
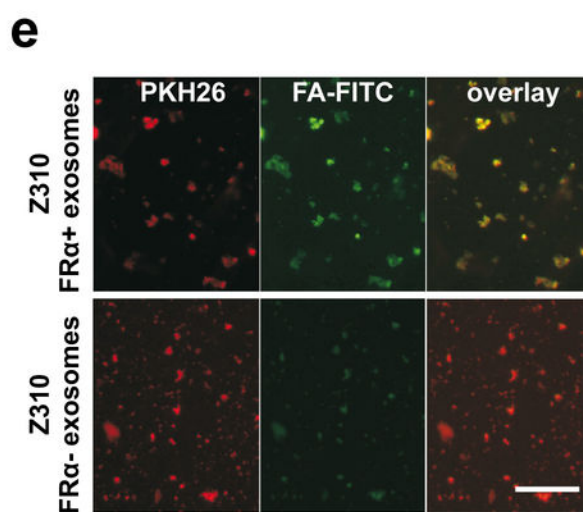
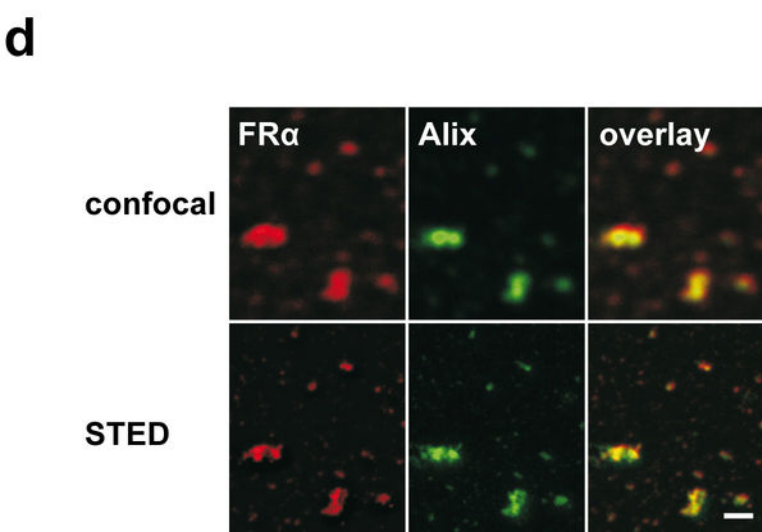
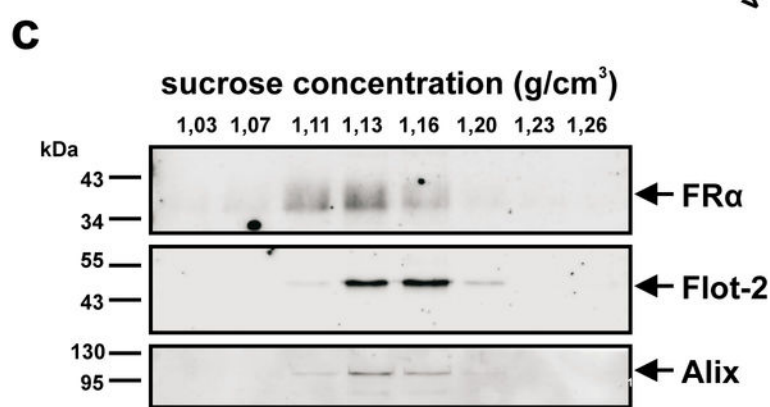
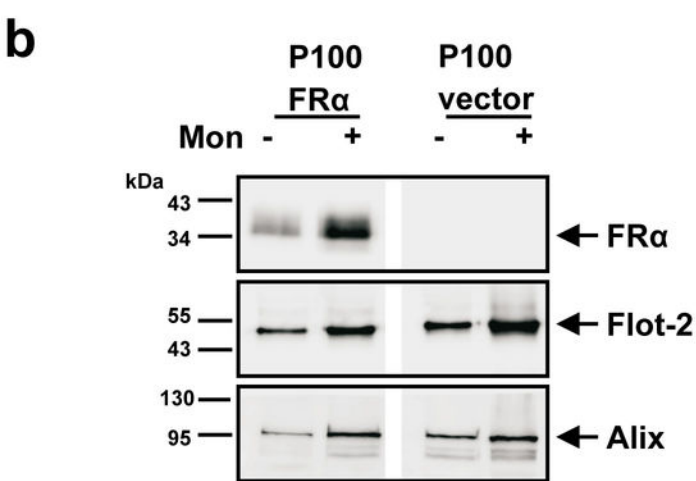
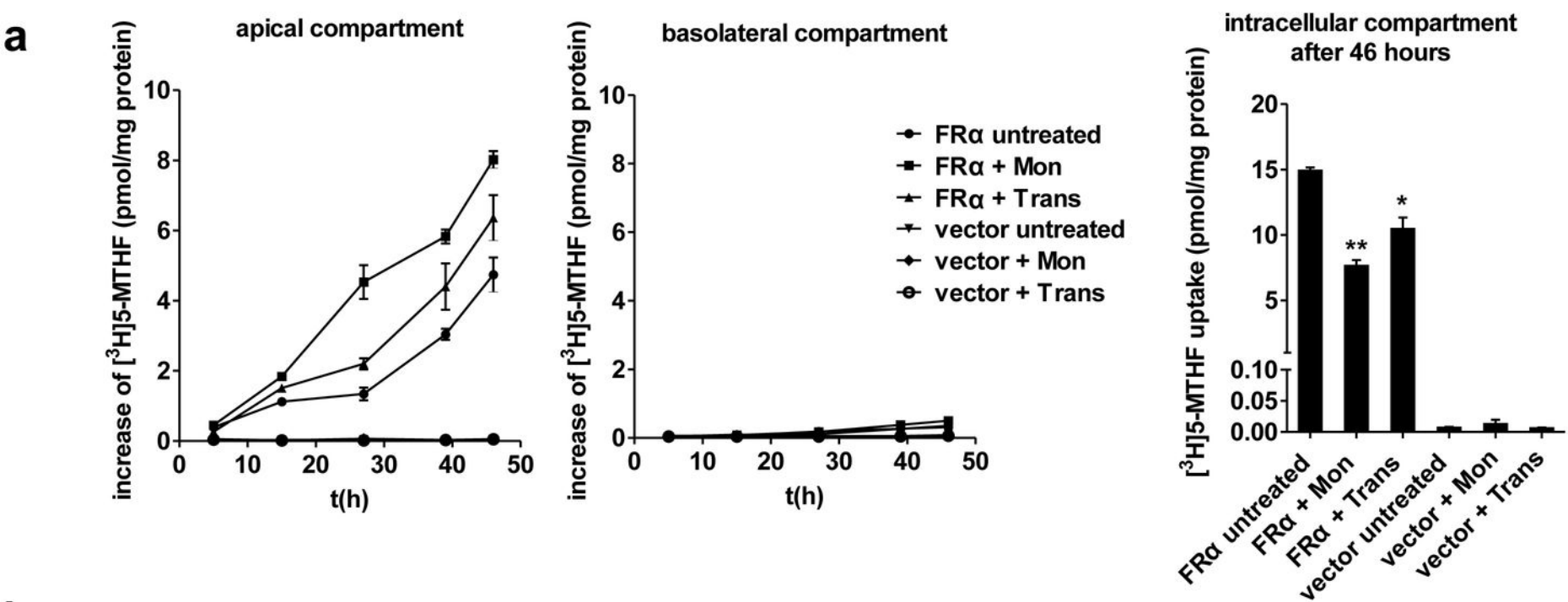
Figure 5

Figure 6

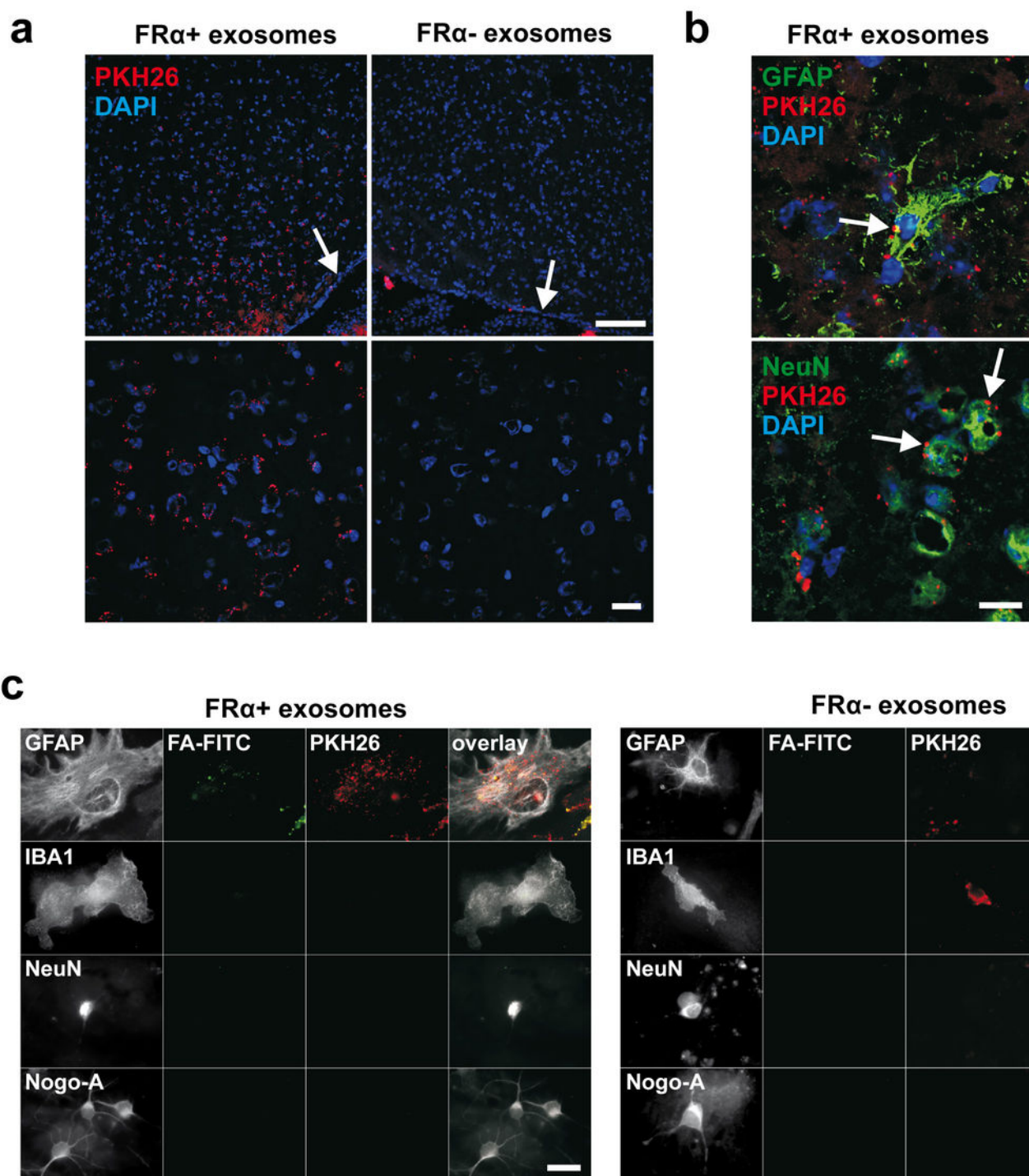


Figure 7

