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Trafficking of gold nanoparticles coated with the 8D3 anti-transferrin-receptor antibody in mouse blood-brain barrier

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Abstract

Receptor-mediated transcytosis has been widely studied as a possible strategy to transport neurotherapeutics across the blood-brain barrier (BBB). MAbs directed against the transferrin receptor (TfR) have been proposed as potential carrier candidates. A better understanding of the mechanisms involved in their cellular uptake and intracellular trafficking is required and could critically contribute to the improvement of delivery methods. Accordingly, we studied here the trafficking of gold nanoparticles (AuNPs) coated with the 8D3 anti-transferrin-receptor antibody in mouse BBB. 8D3-AuNPs were intravenously administered to mice and let recirculate for different times from 10 minutes to 24 hours before brain extraction and analysis by transmission electron microscope techniques. Results indicate a TfR-mediated and clathrin-dependent internalization process, by which 8D3-AuNPs internalize in vesicles individually. Thereafter, these vesicles may follow at least two different ways. On one hand, most of these vesicles enter intracellular processes of vesicular fusion and rearrangement in which AuNPs end up accumulated in to late endosomes, multivesicular bodies or lysosomes, which will present a high content of AuNPs. On the other hand, a small percentage of vesicles follow a different route, fusing with the abluminal membrane, opening up to the basal membrane and resulting in individual 8D3-AuNPs sited in basal membrane without detaching from the abluminal one. These 8D3-AuNPs would temporarily remain in this location until their reinternalitzation. The maximum percentage of particles that reach the basal membrane is estimated at about 4.6% of the observed particles at 2.5 h of recirculation. Altogether, although receptor mediated transport based strategy for drug delivery continues to be one of the most promising strategies to overcome the BBB, different optimization approaches should be developed to improve this strategy for crossing this barrier.

Introduction

The blood-brain barrier (BBB) is a well-coordinated and highly selective barrier which only allows lipophilic molecules under 400-600 Da of molecular mass to cross it (Pardridge, 1998; De Boer and Gaillard, 2007). Moreover, efflux transport systems present in the endothelial cells of the BBB export some of these small drugs back to the blood. Due to its restrictive properties, passage of molecules across the BBB still remains a pharmaceutical challenge. Numerous strategies have been proposed to transport therapeutic agents from the blood into the brain, like those based on receptor-mediated transcytosis across the endothelial cells of the brain capillaries (Scherrmann, 2002; de Boer and Gaillard, 2007). The transferrin receptor (TfR), abundant in brain capillaries (Scherrmann, 2002), has been extensively studied. Some monoclonal antibodies (MAbs) directed against mouse or rat TfR have been shown to be useful tools with which to study the tissue distribution of TfR and the TfR-mediated transcytosis across the BBB in these rodents, and have been proposed as possible carriers or Trojan horses to vehiculate substances across the BBB (Jefferies et al., 1984; Friden et al., 1991; Moos, 1996; Kissel et al., 1998; Lee et al., 2000; Moos and Morgan, 2001; Zhang and Pardridge, 2005). Some of these studies have concluded that these antibodies accumulate in brain capillary endothelial cells (BCECs) but do not cross the BBB (Bickel et al., 1994; Moos and Morgan, 2001; Gosk et al., 2004; Paris-Robidas et al., 2011; Manich et al., 2013) while other studies, most of them using the capillary depletion method or indirect outcome measures such as protein expression or enzymatic activity, have concluded that MAbs and/or their cargo are transported across the BBB (Friden et al., 1991; Lee et al., 2000; Shi and Pardridge, 2000; Zhang et al., 2003; Zhang and Pardridge, 2005). It has also been suggested that these MAbs can target nanoparticles to the BBB for the posterior release of their encapsulated content (Zhang and Pardridge, 2004; Pang et al., 2008; Ulbrich et al., 2009; Papademetriou et al., 2013). Recently, some studies have shown that modifying antibody's affinity in different ways enhances their transcytosis (Bien-Ly et al., 2014; Niewoehner et al., 2014). However, the intracellular mechanisms that these antibodies or constructs follow inside BCECs remain unclear.

In the present work we coated gold nanoparticles (AuNPs) with the 8D3 antibody, which is directed against the mouse TfR and has been proposed as a possible molecular Trojan horse to carry pharmacological substances across the BBB. AuNPs can be directly observed by TEM, avoiding immunodetection inespecificities, and be considered as the cargo to be transported by the 8D3. Using transmission electron

microscope (TEM) techniques, we studied the passage of the AuNPs across the BBB and the dynamics that AuNPs follow inside BCECs after *in vivo* administration in mice.

Materials and methods

Animals

Male ICR-CD1 mice (3-5 months-old) were used. They were kept in standard conditions of temperature (22ºC ± 2ºC) and light–dark cycles (12:12 h, 300 lx/0 lx) with access to food and water *ad libitum* until the day of the experiment. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (DAAM 7505).

Formation of the 8D3-AuNP conjugates

To form the 8D3-AuNP conjugates, the anti-TfR MAb 8D3 (AbD Serotec, UK) was covalently attached to AuNPs sized 20 nm of diameter using the InnovaCoat GOLD (20nm, 20 OD) Covalent Conjugation Kit (Innova Biosciences, Cambridge, UK). The estimated 8D3:AuNP ratio for the conjugates was 30:1.

Administration of the 8D3-AuNP conjugates and brain obtention

Mice were anaesthetized with isofluorane and a bolus of 300 µl of phosphate-buffered saline (PBS, pH 7,2) containing the 8D3-AuNP conjugate at a concentration of 430 µg 8D3/ml was intravenously injected in the caudal vein. Animals were distributed in 4 different groups, each group containing 3-4 animals. One group of animals was sacrificed after 10 min of recirculation, while the other groups after 30 min, 2'5 h or 24 h. Once the corresponding time passed, animals were anesthetized with 80 mg/Kg of sodium pentobarbital by an intraperitoneal injection, the thoracic cavity was opened and an intracardiac gravity-dependent perfusion of 50 mL of phosphate-buffered saline (PBS, pH = 7.2) followed by 50 mL of p-formaldehyde (PF, Sigma-Aldrich) at 4% and glutaraldehyde at 0'1% in phosphate buffer 0'1M was performed. Brains were then removed and collected in the same solution. Control animals were performed following the same procedures, but using an IgG antibody not directed against mouse TfR instead of the 8D3. Thus, control animals received IgG-AuNP instead of 8D3-AuNP.

Brain sample processing for TEM

Brain coronal sections of 1-mm thickness were obtained with a vibratome. Hippocampus and cortex areas were then selected and dissected, and post-fixed overnight in p-formaldehyde (PF, Sigma-Aldrich) at 4% and glutaraldehyde at 0'1% in

phosphate buffer 0'1M. Thereafter, the fixative was substituted by p-formaldehyde at 2% and tissue samples were kept in this solution for 3 days. Samples were then washed in PB 0'1M and treated with 2% osmium tetroxide (OSO_4) containing 0'8% potassium ferrocyanide (for 1 h at 4 ºC). Samples were washed and gradually dehydrated in acetone before being progressively embedded in Epon resin. Blocks polymerized at 60ºC for 48 h. Semi-thin sections (1-µm thickness) were obtained, and after methylene blue staining, the regions of interest were localized. Ultrathin sections (50-70 nm thickness) were obtained using a Leica Ultracut UC6 ultramicrotome (Leica Microsystems), and the sections were then picked up on 200 mesh copper grids and post-stained with uranyl acetate and lead citrate. Ultrathin sections were examined using a JEM-1010 transmission electron microscope (JEOL, Japan) operated at an accelerating voltage of 80 kV. Images were obtained using a CCD Megaview 1kx1k camera and the Analysis 3.2. image acquisition software.

Image analyses

The size of the 8D3-AuNPs and that of the intracellular vesicles were measured using the Image J program (National Institute of Health, USA) on TEM images. To measure the vesicle size, the contour of the vesicle was manually traced by using the "freehand selection" tool of the Image J program, and the Feret's diameter (the longest distance between any two points along the selection boundary, also known as maximum calliper) was determined.

Data analysis

3D surface plots have been performed using STATISTICA for Windows (StatSoft Inc.). The surface has been fitted to the XYZ coordinate data according to the distance-weighted least squares smoothing procedure, in which the influence of individual points decreases with the horizontal distance from the respective points on the surface.

Brain sample processing for TEM immunostaining

For vesicular immunostaining, samples were fixed and post-fixed as those used for ultrastructure studies and described above. Thereafter, samples were washed in PB 0'1M with glycine 0'15M, gradually infused in glycerol (10%, 20%, 30%) for cryoprotection, frozen in liquid propane at -188ºC (Leica EM CPC, Leica Microsystems, Vienna) and stored in liquid nitrogen at -196°C until the freeze-substitution was done. Freeze-substitution was performed in an Automatic Freeze substitution System (AFS; Leica Microsystems), using acetone containing 0.5% of uranyl acetate, for 3 days at -90°C. On the fourth day, the temperature was slowly increased, by 5°C/ hour, to -50°C. Samples were then rinsed in acetone, *and* infiltrated and embedded in Lowicryl HM20.

Blocks polymerized for 24 h at -50ºC, and 48 h at 22ºC with ultraviolet light. Ultrathin sections were obtained using a Leica Ultracut UC6 ultramicrotome (Leica Microsystems) and placed on 200 mesh nickel grids.

Vesicular immunostaining for TEM

For the immunolabelling process, sections were incubated at room temperature on drops of glycine 50mM in PBS 10mM for 5 min, washed and blocked on drops of 5% BSA for 20 min. Incubation of the primary antibody was performed with the lysosomal marker rabbit anti-LAMP1 IgG (1/5, Abcam, UK) in 1% BSA in PBS for 2 h. After 5 washes of 5 min each on drops of 0.25% Tween 20 in PBS, sections were incubated for 1 h using a secondary antibody anti-rabbit IgG coupled to 12-nm diameter colloidal gold particles (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) using a 1:30 dilution in 1% BSA in PBS. This was followed by five washes of 5 min each on drops of PBS, a 5 min incubation on a drop with glutaraldehyde 1% in PBS, ten washes with distilled water and air-drying. As a control for non-specific binding of colloidal gold-conjugated antibody, the primary polyclonal antibody was omitted. Sections were stained with 2% uranyl acetate in methanol and lead citrate, and observed in a JEM-1010 electron microscope (Jeol, Japan) working at 80 kV. Images were obtained using a CCD Megaview 1kx1k camera and the Analysis 3.2. image acquisition software.

Results

Localization of AuNPs in brain samples

Observation by TEM of the hippocampus and cortex from animals which have previously received the administration of the 8D3-AuNP conjugate shows, in all cases, the presence of gold particles in these brain structures, regardless of whether they have been sacrificed at 10 min, 30 min, 2,5 h or 24 h after the administration. However, in the case of control animals who had received intravenous IgG-AuNP conjugate, no AuNPs were observed in their brains. This fact allows ensuring that the presence of AuNPs in the brains of the different groups of animals that received 8D3-AuNP depends on the presence of the 8D3 antibody.

The thorough analysis of samples from animals of the different 8D3-AuNP groups allowed observing different patterns of localization of AuNPs, some of which predominate in some time-points and some in others. First, these different patterns will be described and then the relationship between these patterns and the time of recirculation of the conjugate will be statistically analyzed. It should be noted that these

patterns did not show any differences regarding the cortical or hippocampal origin of the analyzed region.

Some 8D3-AuNPs are located inside the blood capillaries, attached to the luminal membrane of the endothelial cell that forms the vessel wall. In these cases, the AuNP is observed, measuring about 20 nm in diameter, and a halo of approximately 20 nm thickness is found around the gold particle, corresponding to the coating of 8D3 antibody. Consequently, the overall diameter of the 8D3-conjugated AuNP is around 60 nm in diameter. Due to the presence of the 8D3 coating, AuNPs are generally observed 20 nm far from the luminal membrane approximately. This pattern of localization is named here as LMP, acronym of Luminal Membrane Particle. In Figure 1, representative images of LMPs can be observed.

Other 8D3-AuNPs are found individually placed in clathrin-coated pits of the luminal membrane of the endothelial cells from capillary walls. No clathrin-coated pit containing two or more AuNPs has been observed. Just as it happened in the previous case, the AuNPs showed a halo of 20 nm-thickness around them and are located approximately at 20 nm from the wall of the pit. We are referring this pattern of localization as CPP, acronym of Clathrin-coated Pit Particles. Representative images from this situation can also be observed in Figure 1.

In other cases, AuNPs are observed inside vesicles in the endothelial cells. The vesicles may have more than one AuNP, and in two cases, up to 23 AuNPs were observed in a single vesicle. These patterns of localization are named V#, # being the number of AuNPs that are inside the vesicle. Thus, the pattern V1 shows 8D3-AuNPs placed individually in vesicles inside the BCECs. When observed by TEM, these 8D3-AuNPs-containing vesicles sometimes have a circular or elliptical form and other times show irregular shapes, indicating that the vesicles could have spherical shapes in some cases but not defined in others. In some cases, some vesicles seem to merge together. Moreover, their sizes vary greatly, featuring Feret's diameter falling in a range from 80 nm up to 400 nm. Some of these vesicles can be identified as multivesicular bodies and others as lysosomes. The localization of the vesicles inside the endothelial cell may vary, sometimes being in areas near the luminal membrane but in other times near the abluminal. In some cases, the 8D3-AuNPs that are inside the vesicles appear attached to the vesicle membrane and therefore they are on the peripheral zone of the vesicle, the AuNP being located about 20 nm from the vesicular membrane. In other vesicles, however, and especially in those with a higher content of AuNPs, these particles are distributed randomly throughout the interior of the vesicle, indicating that

the interaction between 8D3-AuNPs and the vesicle membrane has been lost. All these patterns of localization are summarized in Figure 2.

Some gold particles have been found in the basal membrane of the endothelium of the capillary vessels (BMP pattern). As exemplified in Figure 3, in these cases, the particles are found in the area bordering the abluminal membrane of BCEC, indicating that the link with the abluminal membrane of the endothelial cell is maintained. These particles found in the basal membrane appear individually, never grouped, and could have arrived there due to the fusion of V1 vesicles with the abluminal membrane (representative images of this fusion can be observed in Figures 2 and 3). It has to be noted that, whatever the time of recirculation, no gold particles have been found beyond the basal membrane of capillary vessels.

Quantitative analysis of the distribution of AuNPs respect to the recirculation time

Table I shows the distribution of the observed AuNPs, depending on the location and the time of circulation. The number of analyzed capillaries and the total number of AuNPs observed is indicated for each time of recirculation. In the time of 10 minutes of recirculation, a total number of 78 capillary sections has been analysed and a total of 98 AuNPs have been observed, obtaining an average of 1.26 AuNPs by analyzed capillary. In the other recirculation times, i.e. 30 minutes, 2.5 hours and 24 hours, an average of 1.60, 3.90 and 2.09 AuNPs have been respectively observed. At the time of recirculation of 10 minutes the AuNPs are found in LMP, CPP and vesicles with a low content of AuNPs (from V1 to V4). In the recirculation time of 30 minutes, the localization of AuNPs is extended to V9, and one AuNP has been found in BMP. In the recirculation time of 2.5 hours is when there is a greater variety of locations, spanning from particles located in LMP to V23, and it is in this time of recirculation where a greater number of particles in BMP have been found, 19 in total. In the time of recirculation of 24 hours, no particles have been found in LMP or CPP, and only two particles were found in BMP. Looking at the percentage of particles in each location, at 10 minutes a 12.2% of the particles are found in LMP and a 5.1% in CPP and therefore a 17.3% of the observed particles have not yet been internalized in the endothelial cells of the capillaries. At 30 minutes these percentages have decreased to 7.4% and 2.7% respectively (10.1% in total). At 2.5 hours and 24 hours no particles are found in LMP and CPP, with the only exception of a 0.2% in LMP at 2.5 hours. Regarding particles in BMP, or particles that have reached the basal membrane, at 10 minutes of recirculation no particle is found, while at 30 minutes a 0.7% of the particles are found at this location. At 2.5 hours a 4.6% of the particles is found in BMP and at 24 hours a 1.2%. These data are represented in Figure 4.

In order to show the evolution over time of the localization of AuNPs in the different types of vesicles, a 3D graphics of the percentage of particles depending on its localization and the time of recirculation has been obtained, adjusting the data by *distance weighted least squares* (Figure 5). As can be observed, the particles in LMP and CPP and the vesicles with a low number of AuNPs dominate in the short times of recirculation, and the number of AuNPs for vesicle increases gradually with time. At 2.5 and 24 hours of recirculation, therefore, the vesicles with a high content of AuNPs predominate.

Characterization of 8D3-AuNPs-containing vesicles

To further characterize the intracellular trafficking of 8D3-AuNPs in BCECs, and in order to elucidate these particles' fate inside these cells, a vesicular immunostaining was performed using the lysosomal marker anti-LAMP1 IgG antibody (Figure 1O and 1P) in 24 h brain samples. In these brain samples, 32 capillary sections were analysed and we observed 21 vesicles containing 8D3-AuNPs. Only 3 of these vesicles (14'3%) colocalized with LAMP1 immunostaining, being considered then lysosomes. Therefore, most AuNPs found in vesicles seem to be localized in non-lysosomal vesicles like multivesicular bodies or late endosomes.

Discussion

The results obtained from the analysis of the localization of AuNPs at the different times of recirculation allowed establishing a time-dependent trafficking pattern of the AuNPs coated with the 8D3 anti-TfR antibody on mouse BBB. This trafficking, which is summarized in the diagram of Figure 6 and will be justified below, includes a TfR-mediated and clathrin-dependent endocytosis process of 8D3-AuNPs. Through this process of endocytosis, 8D3-AuNPs internalize individually and lead to V1 vesicles. These V1 vesicles may follow thereafter at least two different endocytic routes. On one hand, most of these vesicles enter an intracellular process of vesicular fusion and rearrangement that end up moving the AuNPs to late endosomes, multivesicular bodies or lysosomes, which will present a high content of AuNPs. On the other hand, a small percentage of V1 vesicles follow a different route, fusing with the abluminal membrane, opening up to the basal membrane and resulting in BMP particles, which would temporarily remain attached in this position until their possible reinternalitzation.

The first point of this trafficking, the TfR-mediated and clathrin-dependent endocytosis, is justified by the lack of AuNP internalization when the complex is made with IgG-AuNP instead of 8D3-AuNP, which indicates that the 8D3 antibody directed against the

TfR is necessary for the internalization. Moreover, the images corresponding to LMP and CPP show that the 8D3 coating of the 8D3-AuNPs is in contact with the luminal membrane of the BCECs, probably indicating the link between 8D3 and the TfR. The fact that the internalization is performed by clathrin-coated pits is completely consistent with previous studies about TfR and iron transport across the endothelial cells of the BBB (ref ....). Such internalization by clathrin-coated pits explains why the entry of 8D3-AuNPs is individualized: the size of endocytic clathrin-coated vesicles, which is remarkably uniform, is about 100 nm of diameter (Hillarieau and Couvreur, 2009; Traub, 2011), while the 8D3-AuNPs measure about 60 nm in diameter. Then, the vesicles resulting from the entry of AuNPs will be of V1 type.

The vesicles with more content of AuNPs might be formed, therefore, from the fusion and resorting of V1 vesicles with other vesicles, and the temporary adjustment of the location of the AuNPs in the different types of vesicles supports this idea. As has been shown, the number of AuNPs per vesicle increases gradually with time. Moreover, some of these vesicles that contain more than one AuNP are multivesicular bodies and lysosomes, which indicates that they have been formed from the resorting and fusion of different vesicular structures. The low percentage of colocalization between LAMP1-positive vesicles and AuNPs-containing vesicles suggest that AuNPs are collected in late endosomes or multivesicular bodies devoid of any degradative capability.

On the other hand, the consideration that the BMP particles come from type V1 particles that have fused with the abluminal membrane is based on some images that suggest it, and on the fact that these BMP particles are found in the basal membrane always individually, never grouped among them, and always linked to the abluminal membrane of the endothelial cell. This suggests that the 8D3-AuNP complex is still attached to the TfR and that the vesicle has not undergone the other endosomal maturation and sorting process before reaching the abluminal membrane. If BMP particles would have come from vesicles with a high content of AuNPs, some of them would have been found grouped and, moreover, as in some cases these AuNPs lose the 8D3 coating and therefore are not linked to the vesicular membrane, we could have found them far from the abluminal membrane and/or even exceeding the limits of the basal membrane. As mentioned, in no case AuNPs have been found beyond the basal membrane.

The fact that the presence of AuNPs is not observed beyond the basal membrane and that at 24 hours of recirculation the percentage of AuNPs in basal membrane is lower than that observed at 2.5 hours, could indicate that these AuNPs, which achieve the basal membrane attached to the abluminal membrane, are not released to the

parenchyma but return to the inside of the endothelial cells, possibly in vesicles that join internal processes of vesicular fusion and rearrangement.

Finally, the fact that at 24 hours of recirculation the total number of AuNPs respect the number of capillaries analysed seem to decrease suggests that some of these vesicles with high levels of AuNPs, which lose 8D3 coating and are no longer linked to the vesicular membrane, could open up again to the capillary bloodstream and release there the contained AuNPs.

Given the controversy about the possible use of TfR for the vehiculation of substances across the BBB, the present study provides new information that can clarify some yet to be answered questions, concerning the dynamics and intracellular mechanisms that AuNPs follow when decorated with 8D3 antibody. The results suggest that the 8D3 antibody may be useful for the vehiculation of substances (AuNPs in this case) towards the endothelial cells that constitute the wall of brain capillaries. At the times of recirculation tested, we found a maximum of AuNPs inside the endothelial cells at 2.5 hours, although the levels are elevated even after 24 hours. Only a small percentage of particles can access the basal membrane (the maximum value observed is 4.6% of particles at 2.5 hours of recirculation) but they do not enter the brain parenchyma.

These results contradict previous studies where a high passage eficiency of the anti-TfR MAb and/or their cargo across the BBB is concluded (Penichet et al., 1999; Lee et al., 2000; Papademetriou et al., 2013). Despite differences in the experimental design or construct architecture, the disagreeing conclusions can be probably due to the indirect detection techniques that are used in those studies. Radioactivity measurements to calculate both the construct clearance from blood and the construct entry in brain parenchyma could wrongly suggest that the construct have been successfully transcytosed: decreasing radioactivity in blood and increasing radioactivity levels in brain homogenates may also be produced when the construct is internalized inside the endothelial cells of brain blood vessels, without reaching brain parenchyma. This fact might also explain results obtained from enzimatic activity measurements (Zhang and Pardridge, 2005). Capillary depletion method may also lead to misleading interpretations, as it is very difficult to achieve a careful and accurate separation and depletion of brain capillaries (Lee et al., 2000; Moss and Morgan, 2001).

In other studies, the pharmacological effects of the attached cargo have been used to test if a substance improves its effect when conjugated to the MAb (Bickel et al., 1993; Wu and Pardridge, 1996). Although promising results have been long ago presented, this is also an indirect technique and the intracellular mechanisms by which the

pharmacological substance reach the brain parenchyma remain unclear. In other cases where targeted nanocarriers have been used to encapsulate the pharmacological substance, it can be possible that after the internalization of the construct the nanocarrier releases the encapsulated cargo to the brain parenchyma, (Zhang and Pardridge, 2004; Pang et al., 2008; Ulbrich et al., 2009).

All these indirect techniques are not fully reliable to study the passage of molecules across the BBB, and the use of direct visual techniques are being necessary to ensure molecular Trojan Horses' efficiency, as well as to fully understand the intracellular mechanisms they use to vehiculate substances across the BBB.

Gold nanoparticles have been used in different studies to monitor different brain targeting ligands and study their ability to cross the BBB (Harding et al., 1983; Bickel et al., 1994; van Gelder et al., 1997; Candela et al., 2008; Prades et al., 2012; Wiley et al., 2013). Moreover, AuNPs have been proposed as useful tools for diagnosis and drug delivery, by improving their size, shape and physico-chemical properties (Weintraub, 2013; Cheng et al. 2014; Shilo et al., 2014). Electron microscopy techniques allow us to accurately monitor the way these AuNPs behave intracellularly at the endothelial cells of brain blood vessels, when decorated with different ligands, such as endogenous ligands, peptides or MAbs. To date, few *in vivo* studies exist in which AuNPs decorated with compounds that recognize the TfR are monitorized (Prades et al., 2012; Wiley et al., 2013), and none of the studies use MAbs as the targeting ligand. Prades et al. used a TfR targeting peptide to vehiculate the AuNPs across the BBB, whereas Wiley et al. used the transferrin itself as the TfR targeting ligand. In both cases AuNPs succeeded in crossing the BBB and reaching the brain parenchyma. However, the percentage of AuNPs found in brain parenchyma by Prades et al. only accounted for 0.07% of AuNPs of the injected doses, and an exhaustive observational analysis should be necessary to elucidate the mechanisms by which these AuNPs have transcytosed to the brain parenchyma, and if it is due to a receptor-mediated transport. In the case of Wiley et al., a quantitative analysis of the obtained images was performed. Varying the AuNP particle size and the Tf amount per AuNP (avidity) of the conjugated AuNPs, significative differences could be observed in the amount of AuNPs that reached the brain parenchyma. AuNPs with the highest avidities were bound to the BBB, but demonstrated reduced accumulation in brain parenchyma relative to AuNPs with reduced avidity.

In the present study, most of the AuNPs remain inside the BCECs and only a small percentage of AuNPs reach the basal lamina, probably due to a different endosomal sorting. Moreover, this small percentage has not reached the brain parenchyma,

probably due to the strong union between the 8D3 and the TfR. This result is in agreement with other studies in which the 8D3 and RI7 anti-TfR antibodies can colocalized with basal membrane markers (Paris-Robidas et al., 2011; Alata et al., 2013).

Nevertheless, it should not be discarded that once arrived to the basal membrane, if the linkage between the Trojan Horse and the transported cargo possessed such properties that permitted the cargo to dissociate from the carrier, this cargo could reach the brain parenchyma.

At this point of our knowledge, different optimization studies should be developed to improve this receptor mediated transport (RMT)-based strategy for crossing the BBB. As mentioned before, targeted nanocarriers with encapsulated drug are a promising strategy that needs to be further developed, as the encapsulated drug can be released from the internalized complex and transcytosis of the drug might be completed on its own. Secondly, as mentioned above, different MAb-cargo linkage strategies should be also studied with the aim of finding a linkage that once inside the endothelial cells, let the cargo dissociate from the MAb, avoiding MAb's endosomal sorting and promoting endosomal escape. The acid-sensitive hydrazone bond could be postulated as a candidate linkage, as the endosomal acidification could contribute to the linkage breakdown (Kratz et al., 1997). However, endosomal escape does not indicate escape to the brain parenchyma, and further studies are necessaries. Another idea in which several research groups are working on and promising results are being achieved relies on the hypothesis of decreasing MAb's affinity for the receptor to facilitate transcellular transport and avoid sequestration by BCECs. Different approaches have been proposed to achieve this purpose; Bien-Ly et al. (2014) have recently designed low-affinity bispecific antibodies, whereas Niewoehner et al. (2014) have proposed a monovalent shuttle format (single Fab fragment of an anti-TfR MAb) as a more efficient tool to complete transcytosis of the cargo.

Thus, RMT-based drug delivery continues to be one of the most promising strategies to overcome the BBB, although further studies in the intracellular mechanisms, as well as improvements in construct design are needed.

Acknowledgments

Figure legends

Figure 1. Representative images of luminal membrane particles (LMP) and clathrin-coated pits particles (CPP). L: lumen of the brain capillary. BM: basal membrane. Insets: magnifications of the regions containing the 8D3-AuNP. Scale bar: 200 nm. Note that in all cases there is a gap between the AuNP and the luminal membrane of the endothelial cell. This gap corresponds to the coating of 8D3, that can be slightly appreciated.

Figure 2. Representative images of AuNPs incorporated to the endothelial cells of the brain capillaries. V#: vesicle containing # AuNPs. From A to D and from G to I: 8D3-AuNP attached to the vesicular membrane. E, F and from J to L: AuNPs los the connectivity with the vesicular membrane. M: resorting of vesicules containing AuNPs. N and O: fusion of V1 particles with abluminal membrane. V1 in D: lysosome; V1 in F: multivesicular body. L: lumen of the brain capillary. BM: basal membrane. Scale bars: 200 nm.

Figure 3. Representative images of basal membrane particles (BMP, arrows). L: lumen of the brain capillary. BM: basal membrane. In A, the origin of BMP from the fusion of V1 vesicle with abluminal membrane is suggested. Scale bar: 300 nm.

Figure 4. Percentage of AuNPs on luminal surface (LMP+CPP) and basal membrane (BMP).

Figure 5. 3D graphic of the percentage of particles in function of their localization and the time of recirculation. Data adjusted by distance weighted least squares.

Figure 6. Deduced trafficking of the 8D3-AuNPs on mouse BBB.