

# In vivo Functional Evaluation of Increased Brain Delivery of the Opioid Peptide DAMGO by Glutathione-PEGylated Liposomes

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## ABSTRACT

**Purpose** The purpose of this study was to evaluate formulation factors causing improvement in brain delivery of a small peptide after encapsulation into a targeted nanocarrier *in vivo*.

**Methods** The evaluation was performed in rats using microdialysis, which enabled continuous sampling of the released drug in both the brain (striatum) and blood. Uptake in brain could thereby be studied in terms of therapeutically active, released drug.

**Results** We found that encapsulation of the peptide DAMGO in fast-releasing polyethylene glycol (PEG)ylated liposomes, either with or without the specific brain targeting ligand glutathione (GSH), doubled the uptake of DAMGO into the rat brain. The increased brain delivery was observed only when the drug was encapsulated into the liposomes, thus excluding any effects of the liposomes themselves on the blood–brain barrier integrity as a possible mechanism. The addition of a GSH coating on the liposomes did not result in an additional increase in DAMGO concentrations in the brain, in contrast to earlier studies on GSH coating. This may be caused by differences in the characteristics of the encapsulated compounds and the composition of the liposome formulations.

**Conclusions** We were able to show that encapsulation into PEGylated liposomes of a peptide with limited brain delivery could double the drug uptake into the brain without using a specific brain targeting ligand.

**KEY WORDS** blood–brain barrier · brain delivery · liposomes · microdialysis · opioid peptide

## ABBREVIATIONS

ACN	Acetonitrile
BBB	Blood–brain barrier
BSA	Bovine serum albumin
EYPC	Egg-yolk phosphatidylcholine
GSH	Glutathione
HSPC	Hydrogenated soy phosphatidylcholine
PdI	Polydispersity index
PEG	Polyethylene glycol
$K_{p,uu}$	Unbound brain-to-plasma concentration ratio at steady state

## INTRODUCTION

The blood–brain barrier (BBB) is the key limiting factor for the delivery of drugs to the brain. Various approaches have been tried to increase the transport of drugs across this barrier (1,2). One method that has received much attention during recent years is the use of nanocarriers, such as liposomes and nanoparticles, to deliver drugs to the brain (3,4). Nanocarrier-mediated delivery is intended to direct the therapeutic agent to the target tissue and thus increase exposure of the target tissue to the drug. A purpose could also be to decrease the relative concentrations of the drug in plasma and other organs to prevent side effects. Quantitative evidence that nanocarriers actually succeed in increasing transport through the BBB *in vivo* is, however, sparse. We were able to show that encapsulation in glutathione (GSH)-coated liposomes significantly improved the brain uptake of DAMGO (H-Tyr-D-Ala-Gly-MePhe-Gly-ol), an opioid peptide with poor brain penetrating properties (5–8). The mechanism of this increase is, however, unknown. Also among other promising carrier

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candidates, the main transport mechanism has been widely debated (9,10). Receptor-mediated and adsorptive-mediated transport of the nanocarrier and its cargo, blockage of efflux transporters, or disruption of the BBB as a result of toxic effects have all been suggested as possible mechanisms of action for enhanced delivery to the brain, when using different nanocarriers. Functional studies evaluating the effects of these promising nanocarrier systems are essential in order to improve our understanding of the transport processes at the BBB and thereby to optimize the carrier properties needed for efficient CNS delivery of therapeutic drugs.

The system that successfully delivered DAMGO to the brain in our previous study consisted of fast-releasing GSH-conjugated polyethylene glycol (PEG)-coated liposomes (8). We showed that the GSH-PEG liposomal DAMGO formulation not only prolonged the exposure of DAMGO in brain, but also doubled the released, unbound, active drug in the brain interstitial fluid compared to the available, unbound drug in the blood. This proved that the GSH-PEG liposomal formulation increased the net transport of DAMGO from blood to brain across the endothelial cells of the BBB. GSH is an endogenous tripeptide with antioxidant properties for which receptors are abundant at the BBB (11,12). GSH-PEG liposomes have shown advantageous properties in both *in vitro* and *in vivo* studies in addition to ours. The uptake of GSH-PEG liposomes was significantly greater than the uptake of control liposomes in brain endothelial cells in one *in vitro* study (13). Encapsulation of methylprednisolone in the GSH-coated PEG liposomes improved its therapeutic efficacy in acute experimental autoimmune encephalomyelitis (EAE) in mice compared to that of the drug in non-coated PEG liposomes (14). GSH-PEG liposomes containing doxorubicin also appear to be effective according to preclinical and Phase I clinical cancer trials, and are currently under investigation in a Phase II study (clinicaltrials.gov NCT01386580, NCT01818713) (15,16). Similarly structured PEGylated liposomes have been shown to be a safe, effective drug delivery system in cancer treatment and have been on the market for many years, *e.g.*, containing doxorubicin as the active compound (Doxil®/Caelyx®) (17,18).

One of the possible mechanisms behind the enhancement of drug delivery to the brain by GSH-PEG liposomes is that the ligand GSH on the surface of the liposome facilitates the transport through an active transport mechanism. It may also be possible that the liposomes themselves affect some important physiological pathways such as efflux transporters. Since the mechanism by which GSH-PEG liposomes enhance the brain delivery of compounds has not yet been fully investigated, the aim of this study was to functionally evaluate the increased uptake *in vivo*. Therefore, directly administered free DAMGO was compared to administration of three different liposomal formulations; empty GSH-PEG liposomes combined with free DAMGO, GSH-PEG liposomal DAMGO

and non-targeted PEG liposomal DAMGO. The measurements were performed using microdialysis to quantify the active, released DAMGO in brain tissue in comparison with that in blood.

## METHODS

### Liposome Preparation

Empty GSH-PEG liposomes, GSH-PEG liposomal DAMGO and PEG liposomal DAMGO were prepared as described previously (8). In short, the lipids, *i.e.*, 1000 mM egg-yolk phosphatidylcholine (EYPC, Lipoid, Cham, Switzerland), 750 mM cholesterol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 18 mM 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-conjugated PEG MW 2000 (mPEG-DSPE, 1 mol %, Lipoid, Cham, Switzerland) were dissolved in absolute ethanol and mixed with a solution of 50 mg/ml DAMGO in phosphate-buffered saline (PBS, 1:9*v/v*, Lonza Benelux BV, Breda, the Netherlands) or with PBS alone for the empty liposomes. The DAMGO acetate salt (H-Tyr-D-Ala-Gly-MePhe-Gly-ol) was purchased from Bachem (Bubendorf, Switzerland). The formed liposomes were extruded through 200 nm and 100 nm Whatman filters (InstruChemie, Delfzijl, the Netherlands) to reduce the size and obtain uniform particles. The liposomes containing DAMGO were divided into two portions; GSH-PEG-DSPE micelles were inserted into one lot of liposomes and mPEG-DSPE micelles were inserted into the other (both 72 mM, *i.e.*, 4 mol %) by incubation for 2 h at room temperature. The GSH-PEG-DSPE micelles were prepared by incubating GSH (Sigma-Aldrich, Zwijndrecht, the Netherlands) and DSPE-PEG-maleimide (NOF, Grobbendonk, Belgium) at a molar ratio of 1:1 for 2 h at room temperature. A similar amount of GSH-PEG-DSPE micelles was also inserted into the empty liposomes. After preparation of the liposomes, the excess, non-included DAMGO was removed using Zebaspin desalting columns (Thermo Scientific, Rockford, USA) equilibrated with PBS. The size of the liposomes was measured using a Malvern Zetasizer Nano ZS90 (Malvern Instruments Worcestershire, UK). The amount of encapsulated DAMGO in the liposomes was quantified using a modified Lowry protein assay (Thermo Scientific, Rockford, USA) after releasing the DAMGO from the liposomes with isopropanol. For the free DAMGO injections DAMGO was dissolved in PBS. Both the liposomes and free DAMGO solutions were sterile filtered and diluted to obtain 2.0 mg/ml DAMGO. The cholesterol levels in the liposome samples were measured using high performance liquid chromatography (HPLC). In short, a Perkin Elmer Series 200 pump with an autoinjector, an autosampler, and an evaporative light-scattering detector (ELSD) (Alltech, Ridderkerk, the Netherlands) was used. A

C18 (Kinetex, 15 cm\*4.6 mm i.d.; 2.6  $\mu$ m, Phenomenex, cat# 00 F-4496-E0) column equipped with a guard column was used for analysis. The column temperature was set at 45°C. The chromatographic conditions included a gradient elution of 0–10%, with eluent A (0.1 M ammonium acetate, pH 6.0) and eluent B (methanol) pumped at a flow rate of 1.5 ml/min. The total run time was 20 min. The nitrogen gas flow for the ELSD was set at 1.3 ml/min (5 bar) with a temperature of 38°C. The cholesterol levels were used to estimate the dilution factor for the empty GSH-PEG liposomes; they were diluted to achieve the same amount of cholesterol as obtained with the DAMGO-containing liposomes.

### Animals and Surgery

Thirty male Sprague Dawley rats with a body weight of 240–290 g were obtained from Taconic (Lille Skensved, Denmark). The rats were housed in groups and were acclimatized for 7 days before the start of the experiments in a controlled light/dark cycle (12 h/12 h) and with free access to food and water. The study was approved by the Uppsala Regional Animal Ethics Committee, Uppsala, Sweden (C328/10).

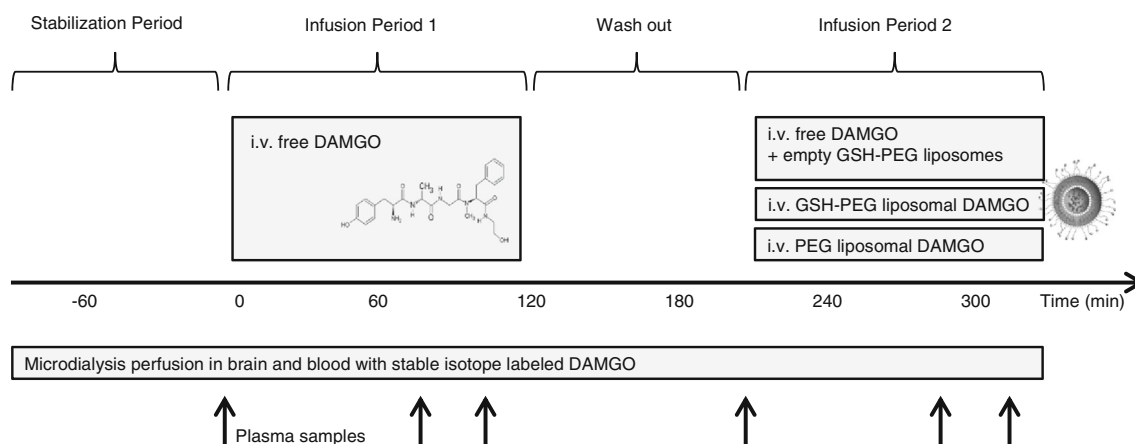
During surgery the rats were anesthetized by inhaled 2.5% isoflurane (Isoflurane Baxter®, Baxter Medical AB, Kista, Sweden) with 1.5 L/min oxygen and 1.5 L/min nitrous oxide. The body temperature was maintained at 38°C using a CMA/150 temperature controller heating pad (CMA, Stockholm, Sweden). The rats were catheterized with heparinized (100 IU/ml heparin in saline) PE-50 cannulas (MicLev, Malmö, Sweden) in the left and right femoral veins and the left femoral artery for drug administration and blood sampling, respectively. All catheters were passed subcutaneously to the posterior surface of the neck. A flexible microdialysis blood probe (CMA/20) with a 10 mm polyarylethersulfone (PAES) membrane and a 20 kD cut-off (CMA, Stockholm Sweden) was inserted into the right jugular vein for measuring unbound DAMGO concentrations in blood. The probe was fixed to the pectoral muscle by two sutures. A CMA/12 probe with a 3 mm PAES membrane (20 kD cut-off) was inserted into the striatum using a stereotaxic instrument (David Kopf instruments, Tujunga, USA) for measuring unbound DAMGO concentrations in the brain. A CMA/12 guide cannula was inserted 2.7 mm lateral and 0.8 mm anterior to the bregma and 3.8 mm ventral to the brain surface and fixed with a screw and dental cement (Dentalon® Plus Heraeus, Germany). The guide was then carefully replaced with the probe before the rats were placed in an individual CMA/120 system for freely moving animals to recover for 24 h before the start of the experiment.

### Experimental Setup

The microdialysis probes were continuously perfused with Ringer solution containing 0.5 w/w % bovine serum albumin (BSA; Sigma Aldrich, Steinheim, Germany) at a flow rate of 0.5  $\mu$ l/min, using a CMA/100 precision infusion pump and fluorinated ethylene propylene (FEP) tubings (CMA/Microdialysis AB, Stockholm, Sweden). The Ringer solution was prepared in house and consisted of NaCl (145 mM), KCl (0.6 mM), CaCl<sub>2</sub> (1.2 mM), MgCl<sub>2</sub> (1.0 mM) and ascorbic acid (0.1 mM) in 2.0 mM phosphate buffer (pH 7.4). The microdialysis probe recovery was determined for each probe by retrodialysis, assuming that transport of the solute was independent of the direction across the probe membrane, as was valid for DAMGO under *in vitro* conditions. The stable isotope-labeled DAMGO [<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N]-DAMGO (H-Tyr-D-Ala-[<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N]Gly-MePhe-Gly-ol; Bachem UK Ltd., Merseyside, UK) was used as calibrator; it was added to the perfusate at a concentration of 20 ng/ml, and was used throughout the experiment. The microdialysis samples were collected in 15  $\mu$ l fractions (at 30 min intervals) into polypropylene vials (AgnThos, Lidingö, Sweden). All samples were weighed to monitor the flow through the probe. The perfusion was started 90 min before DAMGO administration in order to equilibrate the system.

The rats were randomly assigned to three treatment groups with 10 rats in each group: free DAMGO + empty GSH-PEG liposomes, GSH-PEG liposomal DAMGO, and PEG liposomal DAMGO. All the rats were initially infused with free DAMGO, followed by a washout period and subsequent infusions with the liposomes, making each rat its own control (Fig. 1). In Infusion Period 1, the rats received free DAMGO (75  $\mu$ g/min/kg) as a 10-min loading infusion followed by a 2-h constant infusion of 60  $\mu$ g/min/kg through the left femoral venous catheter (Harvard 22 pump, Harvard apparatus Inc., Holliston, MA). After a washout period of 90 min, one of the three liposomal formulations was administered. The liposomal solutions were injected into the right femoral venous catheter, to obtain a steady-state, unbound DAMGO concentration in plasma similar to that obtained with free DAMGO. A 10 min loading infusion of the liposome emulsion (1250  $\mu$ g DAMGO/min/kg) was followed by constant rate infusion (60  $\mu$ g DAMGO/min/kg) for two hours. For the free DAMGO + empty GSH-PEG liposomes group, additional free DAMGO was administered into the left venous catheter simultaneously as the infusion of empty liposomes into the right venous catheter during Infusion Period 2.

For determination of the total DAMGO concentration in plasma, blood samples (~200  $\mu$ l) were taken from the arterial cannula 0, 75 and 105 min after the start of each infusion period into heparinized polypropylene tubes. The samples were centrifuged (7200 g for 5 min), and the plasma was separated and transferred



**Fig. 1** Experimental setup for the study. In Infusion Period 1, all groups received free DAMGO as a 10-min loading dose followed by a 2-h constant infusion. In Infusion Period 2, the rats were administered one of the three liposomal formulations of DAMGO in a corresponding scheme. Microdialysis samples were collected from brain and blood in fractions of 30 min throughout the experiment. Plasma was sampled at the time-points indicated by the arrows.

to clean tubes. All samples were stored at  $-20^{\circ}\text{C}$  until analysis.

### Sample Analysis

DAMGO and the microdialysis recovery calibrator [ $^{13}\text{C}_2$ ,  $^{15}\text{N}$ ]-DAMGO were quantified using liquid chromatography and electrospray ionization tandem mass spectrometry (ESI-LC-MS/MS) using a previously developed method (19). The 15  $\mu\text{l}$  microdialysis samples were precipitated with 50  $\mu\text{l}$  acetonitrile (ACN), 45  $\mu\text{l}$  of the supernatant was diluted with 150  $\mu\text{l}$  of 0.01% formic acid, and 75  $\mu\text{l}$  of the subsequent solution was injected into the ESI-LC-MS/MS system. Plasma samples (50  $\mu\text{l}$ ) were precipitated with 150  $\mu\text{l}$  ACN containing [ $^{13}\text{C}_2$ ,  $^{15}\text{N}$ ]-DAMGO as internal standard (IS), 25  $\mu\text{l}$  of the supernatant was diluted with 1000  $\mu\text{l}$  of ACN:water:formic acid (16:84:0.01;  $v/v/v$ ), and 10  $\mu\text{l}$  was injected. The LC system included a SIL-HTc autosampler (Schimadzu, Kyoto, Japan) and an LC-10ADvp pump connected to a HyPurity C18 column (50 $\times$ 4.6 mm, particle size 3  $\mu\text{m}$ ) protected by a guard column with the same properties (10 $\times$ 4.0 mm; Thermo Hypersil-Keystone, PA, USA). The mobile phase consisted of ACN, water and formic acid in the proportions 82.5:17.5:0.01. The flow rate was 0.8 ml/min, split to 0.3 ml/min before entering the detector (Quattro Ultima Triple Quadrupole Mass Spectrometer, Waters, Milford, MA, USA). The analysis was carried out in a positive ionization mode using multiple reaction monitoring with the transitions  $m/z$  514.2  $\rightarrow$  453.2 for DAMGO and  $m/z$  517.2  $\rightarrow$  456.2 for [ $^{13}\text{C}_2$ ,  $^{15}\text{N}$ ]-DAMGO. The software MassLynx 4.0 (Waters, Milford, MA, USA) was used for the spectra processing. Standard curves were prepared using concentrations in the range of 0.52–2600 ng/ml for the microdialysis samples and 11–110 000 ng/ml for the plasma samples. The coefficient of determination for the fitted curves was  $>0.993$ , and

the accuracy and inter-assay variability were within  $\pm 15\%$  coefficient of variation (CV) on all occasions.

### Data Analysis

The *in vivo* recovery for each microdialysis probe was calculated as the ratio of the difference in calibrator concentration between the dialysate ( $C_{\text{calibrator,out}}$ ) and perfusion fluid ( $C_{\text{calibrator,in}}$ ) to the concentration in the perfusion fluid (Eq. 1).

$$\text{Recovery} = \frac{(C_{\text{calibrator,in}} - C_{\text{calibrator,out}})}{C_{\text{calibrator,in}}} \quad (1)$$

$C_{\text{calibrator,in}}$  was determined from triplicate samples from the ingoing microdialysis perfusion fluid before and after the experiment.  $C_{\text{calibrator,out}}$  is the average concentration of [ $^{13}\text{C}_2$ ,  $^{15}\text{N}$ ]-DAMGO in the collected dialysate during the experiment. The concentration of unbound DAMGO ( $C_u$ ) in the blood and brain was then calculated from Eq. 2

$$C_u = \frac{C_{\text{dialysate}}}{\text{Recovery}} \quad (2)$$

where  $C_{\text{dialysate}}$  is the concentration of DAMGO in the collected microdialysis samples and the recovery is calculated based on Eq. 1.

The brain distribution of DAMGO after administration of the different formulations was described by the ratio of unbound drug in brain to that in blood at equilibrium ( $K_{p,uu}$ ) (20,21), calculated as:

$$K_{p,uu} = \frac{C_{u,ss,\text{brain}}}{C_{u,ss,\text{blood}}} \quad (3)$$

where  $C_{u,ss,\text{brain}}$  and  $C_{u,ss,\text{blood}}$  represent the concentrations of unbound drug in brain and blood at steady state, as obtained

from the microdialysis samples collected 90–120 min after the start of each administration period.

Clearance (CL) of DAMGO was estimated by non-compartmental analysis according to:

$$CL = \frac{R_0}{C_{ss,plasma}} \quad (4)$$

where  $R_0$  is the infusion rate of DAMGO and  $C_{ss,plasma}$  is the total (released and encapsulated) concentration of DAMGO in plasma 105 min after the start of the infusion. The fraction of unbound vs total DAMGO in plasma when liposomes were not present, and the ratio of unbound to total DAMGO in plasma including liposomal DAMGO were calculated as  $C_{u,ss, blood} / C_{ss,plasma}$ .

Data are presented as mean values  $\pm$  standard deviation (SD). XLstat (MS Excel 2007, Microsoft Corp., Seattle, USA) was used for the statistical analysis. To explore differences in the brain distribution of DAMGO among the three groups (free DAMGO+empty GSH-PEG liposomes, GSH-PEG liposomal DAMGO and PEG liposomal DAMGO), a one-way analysis of variance (ANOVA) was performed on the  $K_{p,uu}$  from Infusion Period 2, individually normalized for the  $K_{p,uu}$  of free DAMGO from Infusion Period 1. Tukey-Kramer tests were used for comparisons between individual groups. A  $p$ -value  $<0.05$  was considered statistically significant. The Student  $t$ -test (paired) was used for comparison with the reference period within each group.

## RESULTS

The liposomes in all liposomal formulations were comparable in size, DAMGO content and cholesterol content. The average size of GSH-PEG liposomal DAMGO liposomes was 112 nm and the polydispersity index (PdI) was 0.046. PEG liposomal DAMGO and empty GSH-PEG liposomes were 109 nm (PdI 0.055) and 121 nm (PdI 0.064), respectively. The loading efficiency was 17% for GSH-PEG liposomal DAMGO (4.6 mg/ml) and 19% for PEG liposomal DAMGO (5 mg/ml). After dilution, both formulations contained 2 mg/ml DAMGO and 8 mg/ml cholesterol. Empty GSH-PEG liposomes were diluted to 8 mg/ml cholesterol before infusion.

The recovery of the microdialysis probes was stable throughout the experiment. The average recovery of the brain probes was  $0.17 \pm 0.05$  and of the blood probes was  $0.79 \pm 0.07$ . Sticking to the probe or tubing material was not observed when 0.5% BSA was included in the dialysate.

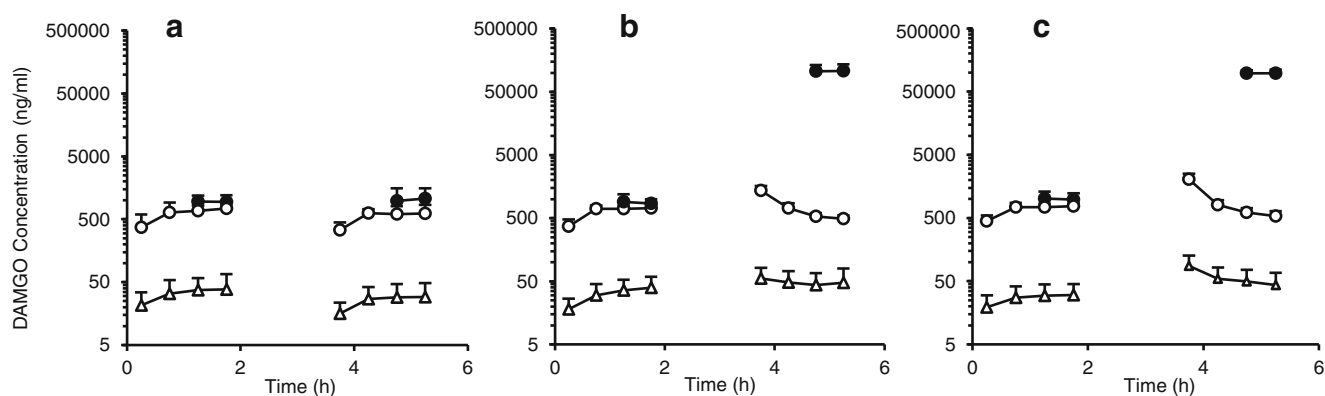
Administration of free DAMGO during Infusion Period 1 resulted in fast attainment of equilibrium in both blood and brain (Fig. 2,  $n=29$ ). One rat in the free DAMGO+empty GSH-PEG liposomes group was excluded because of

problems with drug administration. The total plasma clearance of DAMGO was  $67.8 \pm 13.1$  ml/min/kg and the fraction unbound was  $0.8 \pm 0.2$ . Complete washout was obtained before the second infusion period (at 205 min) as the total DAMGO concentration in plasma was below the limit of quantification ( $<11$  ng/ml).

As designed, the level of unbound DAMGO in blood was similar during both infusion periods and between all groups (Fig. 2). Also, no differences in the pharmacokinetic profiles of total DAMGO were observed after administration of free DAMGO during Infusion Period 1 or free DAMGO with empty GSH-PEG liposomes during Infusion Period 2 (Fig. 2). For the GSH-PEG liposomal DAMGO and the PEG liposomal DAMGO, steady state was reached at the last two time points (75 and 105 min after infusion start). The total plasma concentration of DAMGO (including both released DAMGO and DAMGO remaining in the liposomes) was much higher than the unbound DAMGO concentration in plasma for the two liposomal formulations containing DAMGO (GSH-PEG liposomal DAMGO:  $123 \pm 32$  times higher; PEG liposomal DAMGO:  $105 \pm 31$  times higher). The DAMGO plasma clearance for the GSH-PEG liposomal DAMGO and the PEG liposomal DAMGO was thus much smaller than the plasma clearance for free DAMGO:  $0.54 \pm 0.36$  and  $0.52 \pm 0.10$  ml/min/kg, respectively.

No differences in concentrations in plasma or brain were observed when DAMGO was administered as the free peptide during Infusion Period 1 between the three groups. During Infusion Period 1 the brain distribution of DAMGO was limited with an average unbound brain to blood ratio,  $K_{p,uu}$ , of  $0.05 \pm 0.03$  ( $n=29$ ). When calculating the  $K_{p,uu}$  for the 3 separate groups,  $K_{p,uu}$  was  $0.05 \pm 0.04$  for the free DAMGO+empty GSH-PEG liposomes group,  $0.05 \pm 0.03$  for the GSH-PEG liposomal DAMGO group and  $0.04 \pm 0.02$  for the PEG liposomal DAMGO group (Fig. 3).

The co-administration of empty GSH-PEG liposomes together with free DAMGO did not change the  $K_{p,uu}$  ( $0.05 \pm 0.03$ ) compared to the administration of free DAMGO in Infusion Period 1 ( $t$ -test within groups). However, the administration of GSH-PEG liposomal DAMGO significantly increased the  $K_{p,uu}$  to  $0.10 \pm 0.06$  ( $p=0.019$ ). A significant increase in  $K_{p,uu}$  was also observed after PEG liposomal DAMGO administration to a similar magnitude ( $K_{p,uu}$   $0.08 \pm 0.04$ ,  $p=0.001$ ). The multiple comparison analysis (ANOVA, Tukey-Kramer), based on the individual change in  $K_{p,uu}$  from Infusion Period 1 (free DAMGO) to Infusion Period 2 (liposomal formulation), confirmed the increase in brain distribution of DAMGO when encapsulated into GSH-PEG liposomes ( $p=0.013$ ) and PEG liposomes ( $p=0.012$ ) compared to the free DAMGO+empty GSH-PEG liposomal group. No significant difference was observed between the two liposome-encapsulated DAMGO groups (Fig. 3).



**Fig. 2** Observed concentration-time profiles for unbound DAMGO in brain interstitial fluid (*triangles*), blood (*circles*) and total drug in plasma (*filled circles*) after i.v. administration of free DAMGO (0–2 h) or liposomal DAMGO (3.5–5.5 h), when administered as a 10-min loading dose followed by a constant-rate infusion of 60  $\mu\text{g}/\text{min}/\text{kg}$ . A) Free DAMGO + empty GSH-PEG liposomes group ( $n=9$ ), B) GSH-PEG liposomal group ( $n=10$ ) and C) PEG liposomal DAMGO group ( $n=10$ ).

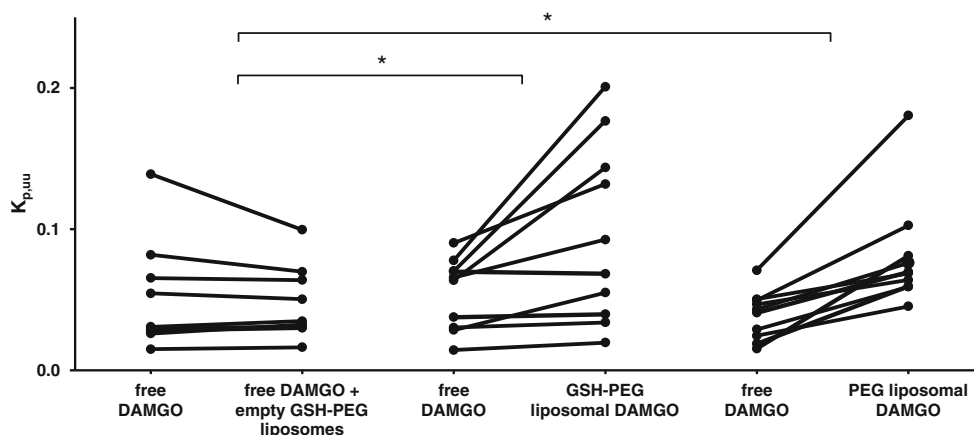
## DISCUSSION

We have previously shown that GSH-PEG liposomes improve the brain uptake of the opioid peptide DAMGO (8). In this paper, we further examined the characteristics responsible for the increased uptake transport *in vivo*. By measuring the released DAMGO with microdialysis in the brain, as well as in blood, we were able to obtain a measurement of the pharmacologically relevant uptake into brain tissue. The purpose of the study was not to investigate the transport of the liposomal carriers themselves, but to follow the peptide cargo.

The influence of the liposomes was functionally evaluated by co-administering empty liposomes with the free drug. No difference in brain uptake was observed between co-administration and administration of free DAMGO by itself. This excludes any possible influence of the GSH-PEG liposomes themselves on BBB function, as has earlier been proposed for nanocarrier delivery using other systems (22,23).

Thus, the GSH-PEG liposomes do not increase transport across the BBB by increasing the permeability of the capillaries, *e.g.*, by solubilization of membrane lipids, opening of tight junctions or inhibition of efflux transporters. The absence of a direct effect of the liposomes on the BBB is in line with earlier safety studies with GSH-PEG liposomes. A modified Irwin test in male Wistar rats ( $n=8$  per group) showed no neurobehavioral effects of GSH-PEG liposomal doxorubicin and empty GSH-PEG liposomes (P. Gaillard, unpublished observations). This indicates that the GSH-PEG liposomes can be safely used and do not influence the BBB integrity.

An increased brain delivery of DAMGO was only observed when the drug was encapsulated into the liposomes, indicating a clear liposomal delivery contribution to the increased concentration of DAMGO in the brain. A proposed mechanism of action for the increased brain delivery using GSH-PEG liposomes involves targeting of GSH to transporters at the endothelial cells of the BBB. Sodium-dependent active



**Fig. 3** The ratio of unbound DAMGO concentration in brain to unbound DAMGO concentration in blood ( $K_{p,uu}$ ) 105 min after the start of infusion of free DAMGO, free DAMGO + empty GSH-PEG liposomes, GSH-PEG liposomal DAMGO or PEG liposomal DAMGO.  $K_{p,uu}$  from the different infusion periods in the same individual are connected. \*indicates significantly ( $p < 0.05$ ) higher increase in brain exposure to DAMGO for GSH-PEG liposomal DAMGO and PEG liposomal DAMGO relative to free DAMGO + empty GSH-PEG liposomes using ANOVA followed by a Tukey-Kramer multiple comparison test;  $n=9-10$ .

transport of GSH has been characterized by Kannan *et al.* in both rodents (11,24) and human cerebrovascular endothelial cells (HCEC) (12). Evidence of increased brain uptake of other drugs using GSH-PEG liposomal preparations has recently been published. The use of the GSH-PEG liposomal system safely increased the brain delivery of doxorubicin in mice, resulting in inhibition of brain tumor growth and improved survival (15). Also, the efficacy of GSH-PEGylated liposomal methylprednisolone was enhanced compared to the use of non-targeted liposomes in a mice EAE model of neuroinflammation (14). In the present study, we confirmed increased brain delivery of DAMGO when it was administered in GSH-PEG liposomes compared to administration of free DAMGO.

A similar increase was, however, also seen with the PEG liposomal DAMGO, which implies that GSH was not solely responsible for the increased brain delivery of DAMGO in this study. The increased net transport of DAMGO for the (non-GSH) PEG liposome group is in itself an intriguing finding, showing that PEGylated liposomes could increase the brain delivery of a compound. This implies a more nonspecific mechanism at the BBB. As discussed above, this effect is however not mediated by the empty liposomes themselves, since the compound has to be encapsulated into the liposomes to increase brain uptake. Mechanisms of uptake may include transcytosis of the liposomes across the BBB, endocytosis partly into the capillaries, or release of DAMGO before it actually crosses the barrier. The increased brain delivery of DAMGO by the liposomes may be explained by prolonged retention of the substance in the capillaries. If DAMGO is concentrated in the area close to the capillary walls, more substance would be available for crossing the BBB and more substance would reach the parenchyma without actually affecting the transport route. The glycocalyx present on the luminal membrane of the endothelial cells has however been shown to attract and bind particularly cationic nanocarriers and GSH-PEG liposomes have according to previous studies a slightly negative charge (15,25,26). Another mechanism of action could be endocytosis of the whole liposome. By shielding the substance from degrading enzymes or efflux pumps in the endothelial cells, the liposomes could ensure that a larger net portion of DAMGO reaches the brain parenchyma.

These results are not in line with previous studies where a benefit of GSH coating was shown using the brain microdialysis technique studying ribavirin (J. Rip, unpublished observations) and carboxyfluorescein (13). A beneficial influence from the GSH-PEG liposomes was also indicated when a different approach with cerebral open-flow microperfusion (cOFM) was used to study doxorubicin (16). Reasons for the different results between studies may be caused by differences with regard to the (drug) molecules and the liposomal composition. The DAMGO peptide is a more hydrophilic compound than doxorubicin, carboxyfluorescein or methylprednisolone,

which may have influenced the processes involved in the BBB transport of the drugs, and thereby the differing effects of GSH-PEG *vs* only PEG on this transport. Maybe even more importantly, the hydrophilic nature of DAMGO affected the choice of liposomal composition. The liposomal formulation of DAMGO contained a different phospholipid from that used in the formulations of the other molecules (EYPC *vs* hydrogenated soy phosphatidylcholine; HSPC) (13–15). EYPC results in a less stable liposomal formulation, which is required to enable *in vivo* release of the more hydrophilic DAMGO from the liposomes. This release is based on breakdown of the lipid layers, using a different mechanism from that for the other, previously used formulations containing small molecular compounds. The doxorubicin and methylprednisolone liposomes were made using an active loading approach resulting in an internal precipitate of the compound (27,28). The stability of the precipitate determines the release of the compound from the liposomes, since the dissolved small molecules can cross the liposomal lipid layer. Differences in the composition of the liposomes may lead to different *in vivo* pharmacokinetics and distribution of the intact liposomes and, even more likely, of the released compound.

The properties of the compound encapsulated in the liposomes may also play an important role for the success rate of the formulation. In the current study we aimed to deliver a peptide analog while we used small molecular weight compounds in the other studies. Nonetheless, DAMGO is of similar size to the other molecules (MW of 514 g/mol). With a  $K_{p,uu}$  of 0.05, DAMGO is distributed to the brain to a very limited extent. It translates to an efflux clearance from the brain being approximately 20 times higher than the influx clearance (20,21). This would in itself indicate a good opportunity for improvement by administering the compound with a nanocarrier. However, in order to increase the  $K_{p,uu}$  of the compound, the carrier has to be able to influence either the uptake or the efflux. It has previously been proposed that DAMGO is a substrate for P-glycoprotein, based on an APTase activity assay (29), which may cause the higher efflux than influx. In addition, the permeability of DAMGO is very low (on the same level as mannitol; Caco-2 assay, unpublished observations). Metabolism in the brain tissue or in the capillary wall can result in peptide breakdown and may contribute to a higher clearance of DAMGO. Independent of the reason for the higher efflux out of the brain, efficient efflux of the compound could counteract the chance of any brain delivery benefits obtained from the use of a nanocarrier. If the efflux of the compound is efficient or if the compound is highly permeable, a low-capacity pathway into the brain like the GSH pathway would probably struggle to influence the net transport across the barrier.

In the present study we were able to show that encapsulation of a peptide with limited brain delivery properties formulated in PEGylated liposomes, either with or without the

specific brain targeting ligand GSH, could double the uptake of the drug into the brain. Since co-administration of empty GSH-PEG liposomes with the drug had no effect on uptake, the liposomes as such did not cause this increase. The strength of the study is that both the released active compound and the total carrier-compound exposure were monitored simultaneously in the systemic circulation and in brain parenchymal tissue. Administration of both the liposomes and the free DAMGO as constant infusions allowed for any possible influence of the liposomal preparation on the increased half-life to be disregarded. This gives us a better understanding of substance-carrier interactions. The mechanisms behind the ligand not being critical for brain delivery of DAMGO, but being essential for the increased brain uptake of other substances are interesting and need to be studied further in order to learn more about the interplay between carrier and drug properties and to optimize nanocarriers in the future.

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**Compliance with Ethical Standards** All procedures involving animals performed in the study were in accordance with the ethical standards of the institution and approved by the Uppsala Regional Animal Ethics Committee, Uppsala, Sweden (C328/10).

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