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The role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis studies in *mdr1a* (−/−) and *mdr1a* (+/+) mice

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1 The aim of this study was to investigate whether blood-brain barrier transport of morphine was affected by the absence of *mdr1a*-encoded P-glycoprotein (Pgp), by comparing *mdr1a* (−/−) mice with *mdr1a* (+/+) mice.

2 *Mdr1a* (−/−) and (+/+) mice received a constant infusion of morphine for 1, 2 or 4 h (9 nmol/min/mouse). Microdialysis was used to estimate morphine unbound concentrations in brain extracellular fluid during the 4 h infusion. Two methods of estimating *in vivo* recovery were used: retrodialysis with nalorphine as a calibrator, and the dynamic-no-net-flux method.

3 Retrodialysis loss of morphine and nalorphine was similar *in vivo*. Unbound brain extracellular fluid concentration ratios of (−/−)/(+/+) were 2.7 for retrodialysis and 3.6 for the dynamic-no-net-flux at 4 h, with corresponding total brain concentration ratios of (−/−)/(+/+) being 2.3 for retrodialysis and 2.6 for the dynamic-no-net-flux. The total concentration ratios of brain/plasma were 1.1 and 0.5 for *mdr1a* (−/−) and (+/+) mice, respectively.

4 No significant differences in the pharmacokinetics of the metabolite morphine-3-glucoronide were observed between (−/−) and (+/+) mice.

5 In conclusion, comparison between *mdr1a* (−/−) and (+/+) mice indicates that Pgp participates in regulating the amount of morphine transport across the blood-brain barrier.

Keywords: Microdialysis; P-glycoprotein; *mdr1a* (−/−) mice; morphine; blood-brain barrier

Abbreviations: AUC_{ECF}, area under the unbound concentration-time curve in brain extracellular fluid; C_{in}, microdialysis perfusate concentration; C_{out}, microdialysate concentration; Log *P*, logarithmic value of the distribution of a compound in octanol over a phosphate buffer with pH of 7.4; MDR, multi-drug resistance; Pgp, P-glycoprotein

Introduction

The antinociceptive action of morphine is dependent on the rate and extent of equilibration across the blood-brain barrier. The mechanism of morphine transport into and out of the brain is still unclear. The distribution of morphine into brain has been studied using several methods: brain uptake index (Oldendorf *et al*., 1972), intravenous administration (Dahlström & Paalzow, 1975), and microdialysis (Aasmundstad *et al*., 1995). Based on the log *P* value (octanol/phosphate buffer) of −0.2 (Murphey & Olsen, 1994) one would, on the basis of passive transport, expect brain distribution of 0.61, which is higher than has been found in several studies. Brain-to-blood concentration ratios of 0.58 and 0.21 were found in rats (Xie & Hammarlund-Udenaes, 1998; Dahlström & Paalzow, 1975), and of 0.24 in the neonatal guinea-pig (Murphey & Olsen, 1994). A cerebrospinal fluid-to-blood ratio of 0.40 was reported in rabbits (Mignat *et al*., 1995). This indicates the presence of active transport out of the brain.

Multi-drug resistance (MDR) is a term that describes the cross-resistance of cells against a range of drugs with different structures and targets. MDR cells express the membrane glycoprotein (Mw 170 kDa) known as P-glycoprotein (Pgp). Pgp is an ATP-dependent active efflux pump, which leads to lower intracellular accumulation of its substrates (Gottesman & Pastan, 1993). The gene that encodes for Pgp in humans is called *MDR1*, and in rodents is called *mdr1a* and *mdr1b* (Gros *et al*., 1986). Pgp is present in tumour cells and also in normal tissues such as liver, kidneys, intestine, and brain (Cordon-Cardo *et al*., 1990; Thiebaut *et al*., 1987). In brain, Pgp is localized in the luminal membrane of the brain capillary endothelium, and contributes to the function of the blood-brain barrier (Cordon-Cardo *et al*., 1989; Schinkel *et al*., 1994; Tatsuta *et al*., 1992).

A model system has become available by the generation of *mdr1a* (−/−) mice (Schinkel *et al*., 1994; 1995). This model is useful for studying the role of Pgp on the blood-brain barrier transport of compounds *in vivo*, as the *mdr1a* gene is the most important, if not the only, gene that encodes for Pgp at the blood-brain barrier. Quite a few drugs have already been studied in this model system, comparing *mdr1a* (−/−) mice with (+/+) mice. For the drugs ivermectin and vinblastin (Schinkel *et al*., 1994), digoxin (Schinkel *et al*., 1995), dexamethasone (Meijer *et al*., 1998), rhodamine-123 (de Lange *et al*., 1998), and the inhibitor SDZ PSC 833 (Desrayaud *et al*., 1998), higher brain/plasma ratios in *mdr1a* (−/−) mice than in (+/+) mice have been reported.

It has also been demonstrated that the transport of radiolabelled morphine across the blood-brain barrier is affected by Pgp *in vitro* (Callaghan & Riordan, 1993) and *in vivo* (Schinkel *et al*., 1995). Then, antinociceptive effects of morphine were significantly elevated in rats when treated with the Pgp inhibitor GF120918 (Letrent *et al*., 1998). However,

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the influence of Pgp on the pharmacokinetics of brain distribution of unbound morphine *in vivo* is unknown. This is important to find out, since, in general, the unbound concentration of a drug is related to the effect and is the driving force for drug transport across membranes.

One technique that is particularly useful for the determination of unbound drug concentrations in the blood and tissues is microdialysis (De Lange *et al*., 1997; Elmquist & Sawchuk, 1997; Hammarlund-Udenaes *et al*., 1997). Recently the technique has been applied to *mdr1a* (−/−) and (+/+) mice, where it was shown that Pgp functionality is not affected by microdialysis studies and experimental conditions (De Lange *et al*., 1998).

The purpose of this study was to investigate whether *in vivo* blood-brain barrier transport of morphine was affected by the absence of Pgp, using microdialysis in *mdr1a* (−/−) and (+/+) mice. The concentrations of morphine in brain extracellular fluid of *mdr1a* (−/−) and (+/+) mice were estimated by correction for *in vivo* recovery using an extended retrodialysis method (Bouw & Hammarlund-Udenaes, 1998), and the dynamic-no-net-flux approach (Olson & Justice, 1993). The brain extracellular fluid and total brain and plasma concentrations of morphine were compared in *mdr1a* (−/−) and (+/+) mice. Morphine-3-glucuronide, one of the metabolites of morphine, was also studied.

Methods

Animals

The animal experiments were performed at the LACDR, Leiden University, The Netherlands. Male *mdr1a* (−/−) mice were obtained from the Netherlands Cancer Institute and were bred under SPF conditions at Instituut voor Technisch Natuurwetenschappelijk Onderzoek (TNO) (Leiden, The Netherlands). *Mdr1a* (+/+) mice were obtained from TNO. The mice were individually housed (Sylvius Laboratories, Leiden, The Netherlands) at room temperature under 12 h light-dark cycle. Food and water were available *ad libitum*. Ethical approval was obtained from the University Animal Experimental Committee (UDEC, project no. 97049), Leiden University.

Chemicals

Morphine hydrochloride and nalorphine were obtained from the hospital pharmacy (Academic Hospital, Leiden, The Netherlands). Hypnorm® was purchased from Jansen Pharmaceutics (Beerse, Belgium). Dormicum® was obtained from Roche (The Netherlands). The perfusion solution consisted of (in mM): NaCl 145, KCl 10.6, $MgCl_2$ 1.0, $CaCl_2$ 1.2, and ascorbic acid in 2 mM phosphate buffer 0.2, pH 7.4. All chemicals were of analytical grade. Solvents were of HPLC grade.

Animal surgery

Animals were operated on according to the procedure of de Lange *et al*. (1998). The mice were anaesthetized with Hypnorm®/Dormicum®/water (1/1/2 (v v^{-1} v^{-1}), 200 μl/mouse, intraperitonial). A polyethylene cannula (i.d. 0.28 mm, o.d. 0.61 mm) filled with saline containing 20 IE heparin was inserted into the tail vein to be used for morphine administration. The mice were then fixed on the stereotaxic frame. Incisions were made to expose the periosteum, which was locally anaesthetized with 1% lidocaine (Bufa, Castricum, The Netherlands). The periosteum was removed to expose the skull. Two holes of 1.5 mm (o.d.) were drilled in the curved skull, and the microdialysis probe (5 mm), guided by a tungsten wire (TW5-3 Clark Electro Medical Instr., U.K.), was transversally implanted through the cortex at 1.1 mm below the bregma. Thereafter, the tungsten wire was gently pulled out of the dialysis membrane, and the stainless steel needles, which were glued on both sides of the dialysis membrane, were fixed to the skull by cement (Poly F-Plus, Zinc polycarboxylate Cement, De Trey Dentsply, Dental House, Nijmegen, The Netherlands). A polyethylene cannula of 6 cm (i.d. 0.28 mm, o.d. 0.61 mm) was placed subcutaneously to warm the perfusion solution to body temperature before it entered the probe. The mice were allowed to recover on a paraffin pad (38°C) until showing movement. Then they were returned to their own cages. In total, the mice were allowed to recover for 24 h before the start of experiments.

Experimental design

Study I (retrodialysis) Five *mdr1a* (−/−) and five (+/+) mice were randomly assigned to the experiment. The microdialysis membranes used in this study were the C-DAK artificial kidney 201-800 D 135 SCE (290 μm o.d., CD Medical B.V., Rotterdam, The Netherlands). On the day of microdialysis, the probe was connected with fused silica 100 and 200 as inlet and outlet tubings, respectively. The probe was perfused with perfusion solution for 60 min to stabilize the system and to obtain blank samples. After the blank period, the perfusion solution was changed to one containing morphine (0.1 μM) and nalorphine (0.1 μM) for 80 min during a retrodialysis period. Thereafter, the perfusion solution was switched to one containing only nalorphine (0.1 μM) for the washout period of 60 min and during the experiment.

The probe was perfused at 1 μl min^{-1} with a CMA/100 microinjection pump. The microdialysis samples were collected automatically by a CMA/140 microfraction collector (Carnegie Medicine, Stockholm, Sweden) at intervals of 20 min.

Morphine was infused for 4 h at a rate of 9 nmol/min/mouse. The infusion rate was 1 μl min^{-1}. The mice were decapitated immediately after stopping the infusion, and whole brain tissue and blood were collected. The plasma was separated by centrifugation (1500 r.p.m., 10 min, 4°C). The brain tissue and plasma were frozen by liquid nitrogen and stored at −20°C until analysis.

Microdialysis *in vivo* recovery was calculated for each mouse by retrodialysis before and throughout the experiment (Bouw & Hammarlund-Udenaes, 1998). The *in vivo* recovery of morphine was determined by the loss of nalorphine during each collection interval and the ratio of retrodialysis recovery of morphine to nalorphine was calculated:

$$\text{Recovery}_{in\ vivo} = \left(1 - \frac{C_{out,\ nal}}{C_{in,\ nal}}\right) * \frac{\text{Morphine}_{\ RD\ in\ vivo}}{\text{Nalophine}_{\ RD\ in\ vivo}} \quad (1)$$

Study II (dynamic-no-net-flux, (Olson & Justice 1993)) Nine *mdr1a* (−/−) and nine (+/+) mice were used. The probe membrane was made of polyacrylonitrile sodium methallyl sulphonate (290 μm o.d., Hospal AN-69 HF). On the experiment day, the probe was treated in the same way as in Study I. After the blank period, the perfusion solution was changed to one containing morphine in concentrations of 0, 0.18 or 0.35 μM for three groups of three mice each. Morphine was intravenously infused for 4 h at a rate of 9 nmol/min/mouse. The mice were decapitated immediately after stopping

the infusion, and whole brain tissue and blood were collected as in Study I. The perfusion rate was 0.5 μl min^{-1} and microdialysis samples were collected at 30 min intervals.

Study III (infusion only) In addition to the 4 h data points of Studies I and II, mice were infused intravenously with morphine for 1 ($n=3-5$) or 2 h ($n=3$) with 9 nmol/min/mouse. This was to obtain a profile of total concentration of morphine in blood and brain. The mice were decapitated immediately after infusion. Whole brain tissue and blood were collected and treated as above.

Sample analysis

The chemical analysis was performed at Uppsala University, Sweden.

Microdialysis samples Eighteen μl microdialysis samples from Study I and 13 μl of the microdialysis samples from Study II were directly injected into the Nucleosil C_{18} HPLC column (5 μm particles, 4.6 × 150 mm, The Netherlands). A coulochem electrochemical detector (Model 5100A, ESA, Inc. MA, U.S.A.) with guard cell 5020 and analytical cell 5011 was used to analyse morphine and nalorphine. The coulochem detector potential was set at 600, 0, and 450 mV, for the guard cell, cells 1 and 2, respectively. The flow rate was 1 ml min^{-1} and the mobile phase consisted of 650 ml 0.01 M phosphate buffer (pH 2.1) containing 0.2 mM sodium dodecyl sulphate (SDS), 350 ml methanol and 20 ml tetrahydrofuran. An integrator with two channels (Shimadzu C-R5A, Japan) was used. The limit of quantification of morphine in microdialysis samples was 6.3 nM, with a coefficient of variation of 6.9% at 11.2 nM.

Plasma samples The plasma samples of morphine and its metabolite morphine-3-gluceronide were extracted using Sep-Pak C_{18} cartridges (Waters), which were first activated with 5 ml methanol, 3 ml 0.01 M phosphate buffer (pH 2.1) and 5 ml distilled water filtered through the cartridge under vacuum in order. Plasma (100 μl) was mixed with 3 ml of 0.5 M ammonium sulphate buffer (pH 9.3) in a 10 ml polystyrene tube for 5 s, and transferred to the reservoir. The plasma samples were filtered through the cartridges, which were subsequently washed with 20 ml 5 mM ammonium sulphate buffer (pH 9.3), 0.5 ml distilled water, and 0.1 ml methanol under vacuum. Lastly, 3 ml methanol was added and the eluates were collected and evaporated under a stream of nitrogen at 45°C. The dried residues were redissolved in 150 μl mobile phase, of which 50 μl was injected onto the HPLC column. The assay parameters for morphine were the same as those described for the MD samples, with the exception that the coulochem detector potential for cell 1 was 300 mV. Morphine-3-gluceronide was analysed by fluorescence detection (Jasco 821-FP, Japan) at an excitation wavelength of 212 nm and an emission wavelength of 340 nm, coupled in series with the electrochemical detector. The mobile phase consisted of 670 ml 0.01 M phosphate buffer (pH 2.1) containing 0.2 mM sodium dodecyl sulphate, 330 ml methanol and 50 ml tetrahydrofuran.

The limit of quantification of morphine and morphine-3-gluceronide in plasma were 0.01 and 0.11 μM, with coefficients of variation of 5.1 and 5.6% at 0.02 and 0.27 μM, respectively. The absolute extraction recoveries for plasma samples of morphine and morphine-3-gluceronide were 100 ± 3% and 100 ± 2%, respectively.

Brain tissue samples One half of the whole brain tissue was homogenized with a 5 fold volume of 0.1 M perchloric acid and then centrifuged for 20 min at 5000 r.p.m. at 4°C. Volumes of 200 and 400 μl of the supernatant were extracted in the same way as the plasma samples for morphine and morphine-3-gluceronide, respectively. The analytical method for morphine was the same as for plasma, with a modified mobile phase consisting of 20 ml tetrahydrofuran instead of 50 ml. For the morphine-3-gluceronide assay, the mobile phase consisted of 670 ml 0.01 M phosphate buffer (pH 2.1) containing 0.4 mM sodium dodecyl sulphate, 330 ml methanol and 20 ml tetrahydrofuran.

The limit of quantification of morphine and morphine-3-gluceronide in brain tissue samples were 0.06 and 0.16 μM, with coefficients of variation of 3.8 and 7.0% at 0.21 and 0.35 μM, respectively. The absolute recoveries for brain tissue of morphine and morphine-3-gluceronide were 102 ± 2% and 98 ± 3%, respectively.

Data analysis

Determination of concentrations in the brain extracellular fluid For Study I, the unbound concentrations of morphine in the brain extracellular fluid were calculated from the dialysate concentrations corrected by the average recovery *in vivo* according to a retrodialysis method for all individual collection intervals during the experiment. The dynamic no-net flux method was used to estimate *in vivo* recoveries and unbound concentrations in the brain extracellular fluid in Study II. Data from the groups receiving perfusion concentrations of 0, 0.18 or 0.35 μM morphine (C_{in}) and dialysate concentrations in the outlet (C_{out}) were used to make a graph for each time point by plotting the difference between concentrations ($C_{in}-C_{out}$) *versus* C_{in}. The slope of the linear regression was estimated as the *in vivo* recovery, and the brain extracellular fluid concentration was equal to the value of C_{in} at the intersection of the abscissa (Olson & Justice, 1993).

Pharmacokinetic analysis The area under the concentration-time curves of unbound drug in the brain extracellular fluid ($AUC_{ECF}^{0-4\,h}$) during infusion was estimated as the sum of the product of the concentrations and the collection interval. The measured concentrations of morphine in brain extracellular fluid are the averaged concentration during the time interval.

The relationship between plasma and brain concentrations of morphine in *mdr1a* (−/−) and (+/+) mice was compared on the basis of grouped data. Groups were based on plasma concentrations of 0–600 nM, 600–1500 nM, and 1500–2500 nM. Each group had at least five data points to perform statistical testing.

Statistical analysis A non-parametric test (Mann-Whitney U) was used to compare parameter differences between *mdr1a* (−/−) and (+/+) mice. A value of $P<0.05$ was considered to be significant. The data are presented as mean ± s.e.mean.

Results

The retrodialysis loss ratios of morphine over nalorphine were 0.9 ± 0.1 and 1.1 ± 0.1 for *mdr1a* (−/−) and (+/+) mice, respectively. There was no significant difference between the retrodialysis recovery of morphine and nalorphine. The *in vivo* recoveries of morphine in *mdr1a* (+/+) and (−/−) mice were 5.6 ± 0.9 and 3.5 ± 0.7% for retrodialysis, and 21.2 and 11.4% for the dynamic-no-net-flux at 4 h, respectively. The recovery

ratios of *mdr1a* (+/+)/(−/−) mice were 1.6 (n.s.) and 1.9 for retrodialysis and dynamic-no-net-flux method, respectively.

Morphine unbound concentrations in the brain extracellular fluid of *mdr1a* (−/−) mice were significantly higher than that of *mdr1a* (+/+) mice after 4 h infusion (Figure 1). The *mdr1a* (−/−) mice had 2.7 ($P<0.05$) and 3.6 times higher concentrations at 4 h for retrodialysis and dynamic-no-net-flux method, respectively. Unbound steady-state concentrations of 247±18 nM (retrodialysis) and 361 nM (dynamic-no-net-flux) were reached for *mdr1a* (+/+) mice, but the brain concentration of morphine in *mdr1a* (−/−) mice increased throughout the 4 h infusion. The $AUC_{ECF}^{0-4\,h}$ ratios between *mdr1a* (−/−) and (+/+) mice were 4.1 ($P<0.05$) and 1.9 for retrodialysis and dynamic-no-net-flux method, respectively (Table 1).

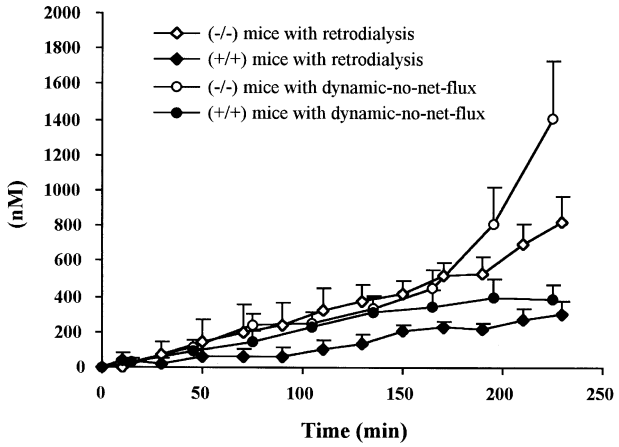

Figure 1 Unbound concentration-time profile of morphine in the brain extracellular fluid following i.v. infusion for 4 h of 9 nmol/min in *mdrla* (−/−) and (+/+) mice with *in vivo* recovery calculations. Data are presented as mean±s.e.mean.

Total brain and plasma concentrations of morphine were determined after 1, 2 and 4 h of morphine infusion. The *mdr1a* (−/−) mice had 2.3 ($P<0.05$) and 2.6 times ($P<0.05$) higher total brain concentration than (+/+) mice at 4 h for retrodialysis and dynamic-no-net-flux method, respectively. The brain to plasma ratio was 1.1±0.1 in *mdr1a* (−/−) mice and 0.5±0.1 in (+/+) mice (Table 2), and the brain to plasma ratio in *mdr1a* (−/−) mice was 2.2 times higher than that in (+/+) mice at 4 h ($P<0.05$). There was no significant difference in plasma levels between *mdr1a* (−/−) and (+/+) mice. *Mdr1a* (−/−) mice show a more stable brain-to-plasma ratio across the plasma concentration interval (Figure 2), with a tendency towards a higher ratio at lower plasma concentration for the (+/+) mice.

The concentration total ratios of brain extracellular fluid to plasma were 0.7 and 0.3 for *mdr1a* (−/−) and (+/+) mice at 4 h, respectively. The rat plasma protein binding is about 20% (Wang & Takemori, 1972), so unbound plasma concentrations of morphine were obtained by correcting for the protein binding. The concentration ratios of brain extracellular fluid to unbound plasma were 0.9 and 0.3 for *mdr1a* (−/−) and (+/+) mice, respectively.

Plasma and brain concentrations of morphine-3-glucuronide were detectable in both types of mice at the end of the morphine infusion. There was no significant difference between *mdr1a* (−/−) and (+/+) mice in blood-brain barrier transport of morphine-3-glucuronide (Figure 3). The morphine-3-glucuronide total concentration ratios of brain to plasma were 0.06±0.01 and 0.05±0.01, with plasma ratios of morphine-3-glucuronide/morphine of 7.3±0.5 and 9.1±1.9 for *mdr1a* (−/−) and (+/+) mice at 4 h, respectively.

Discussion

This study was designed to evaluate the role of Pgp in the transport of morphine across the blood-brain barrier using

Table 1 Parameters for blood-brain barrier distribution of unbound morphine after intravenous infusion for 4 h in *mdr1a* (−/−) and (+/+) mice (mean±s.e.mean)

	$Brain_{ECF,RD}$[a] (nM)	$Brain_{ECF,DNNF}$[b] (nM)	$AUC_{ECF,RD}^{0-4\,h}$[c] (μM *min)	$AUC_{ECF,DNNF}^{0-4\,h}$[d] (μM *min)
mdr1a (−/−)	826±140*	1411±315[e]	142±55*	109[e]
mdr1a (+/+)	304±72	389±81	35±7	58
(−/−)(+/+)	2.7	3.6	4.1	1.9

[a]Unbound brain extracellular (ECF) concentration at 4 h in the retrodialysis (RD) study ($n=5$). [b]Unbound brain extracellular (ECF) concentration at 4 h in the dynamic-no-net-flux (DNNF) study ($n=8$ and 7 for (−/−) and (+/+) mice, respectively). [c]Area under unbound concentration-time curve (AUC) from 0–4 h in the retodialysis (RD) study ($n=5$). [d]Area under unbound concentration-time curve (AUC) from 0–4 h in the dynamic-no-net-flux (DNNF) study ($n=$ [b]). [e]The values from between-group results of the dynamic-no-net-flux (DNNF) study. No statistical test possible. *Significant difference to (+/+) mice ($P<0.05$).

Table 2 Total concentrations of morphine in blood and brain at the end of the 4 h infusion in *mdr1a* (−/−) and (+/+) mice (mean±s.e.mean)

	$Brain_{tot}$ (nM)		$Brain_{tot}$[c] (nM)	$Plasma_{tot}$[d] (nM)	
	RD[a]	*DNNF*[b]			*Brain to plasma ratio*[e]
mdr1a (−/−)	968±110*	2008±333*	1637±244*	1522±168	1.1±0.1*
mdr1a (+/+)	426±40	770±130	627±91	1317±180	0.5±0.1
(−/−)(+/+)	2.3	2.6	2.6	1.2	2.2

[a]Total brain concentration (tot) in the retrodialysis (RD) study ($n=5$). [b]Total brain concentration (tot) in the dynamic-no-net-flux (DNNF) study ($n=8$ and 7 for (−/−) and (+/+) mice respectively). [c]Total brain concentration (tot, $n=$ the number of [a+b]). [d]Total plasma concentration (tot, $n=$ the number of [a+b]). [e]Total concentration ratio of brain to plasma (tot, $n=$ the number of [a+b]). *Significant difference to (+/+) mice ($P<0.05$).

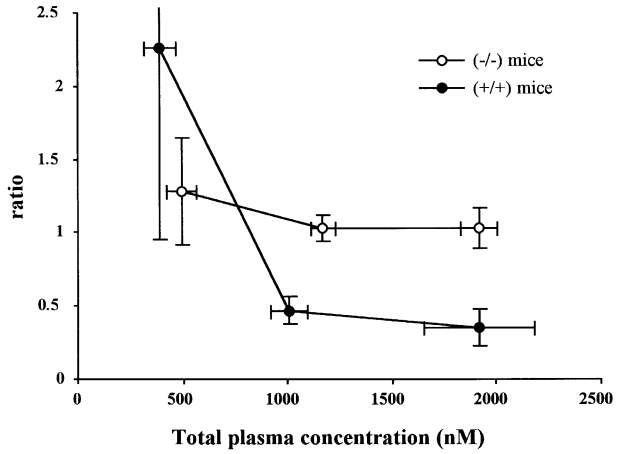

Figure 2 Total brain-to-plasma ratio *vs* plasma of morphine after 1, 2, or 4 h i.v. infusion of 9 nmol/min in *mdrla* (−/−) and (+/+) mice, grouped in plasma concentration intervals of 0–60, 600–1500 and 1500–2500 nM. Data are presented as mean ± s.e.mean.

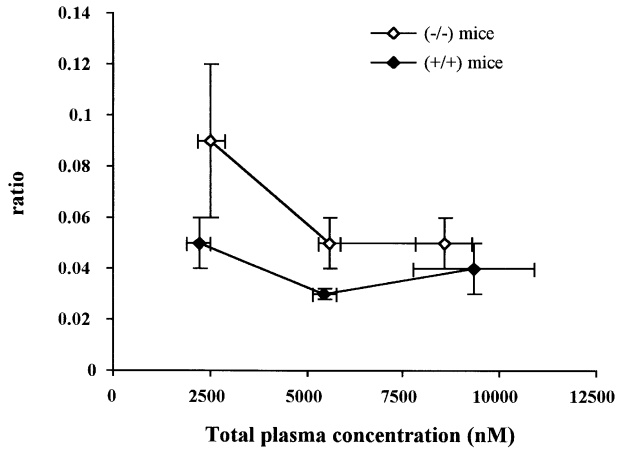

Figure 3 Total brain-to-plasma ratio *vs* plasma concentration of morphine-3-glucoronide in *mdrla* (−/−) and (+/+) mice after morphine i.v. infusion of 9 nmol/min for 1, 2, or 4 h, grouped in plasma concentrations regions of 0–4000, 4000–7000 and 7000–15 000 nM. Data are presented as mean ± s.e.mean.

intracerebral microdialysis in *mdrla* (−/−) and (+/+) mice. Differences in morphine concentrations were observed in the brain extracellular fluid and brain tissue between *mdrla* (−/−) and (+/+) mice, indicating Pgp involvement in transport of morphine across the blood-brain barrier.

Morphine is an important drug in the clinical treatment for relief of moderate to severe pain. The pharmacological responses to morphine are well related with the central nervous system disposition of morphine (Matos *et al*., 1995). Based on the log *P* value (octanol/phosphate buffer) of −0.2 one would, on the basis of passive transport, expect brain distribution of 0.61, which is higher than has been found in several studies (Xie & Hammarlund-Udenaes, 1998; Dahlström & Paalzow, 1975; Murphey & Olsen, 1994; Mignat *et al*., 1995). All these data indicate that active elimination out of the brain could restrict brain distribution. Active transport out of the brain may be caused by Pgp, which is expressed at the luminal face of the blood-brain barrier. Some studies indicate a role of Pgp in distribution of morphine into the brain (Callaghan & Riordan, 1993; Schinke *et al*., 1995).

Morphine was constantly infused and total brain concentrations and corresponding blood concentrations were measured at different times. It was found that the total brain-to-blood concentration ratio in *mdrla* (−/−) mice was about 1, while in (+/+) mice this ratio was 0.5 at 4 h, clearly indicating the effect of Pgp functionality at the blood-brain barrier level.

Two methods were used to correct microdialysate concentrations for the *in vivo* recovery (retrodialysis and the dynamic-no-net-flux method). Different membranes and flow rates were used in the retrodialysis as compared with the dynamic-no-net-flux method. For both methods the *in vivo* recovery of morphine was higher in the (+/+) mice. *In vivo* recovery may be higher in (+/+) mice in comparison with *mdrla* (−/−) mice, as predicted by theory (Bungay *et al*., 1990) and experimental finding (de Lange *et al*., 1998); and is fully discussed by de Lange *et al*. (1998). The estimated concentration-time profiles of unbound morphine in brain extracellular fluid were similar in the two approaches, being significantly higher in the *mdrla* (−/−) mice. The brain extracellular fluid concentrations of morphine increased slowly during the experimental period for both the retrodialysis and dynamic-no-net-flux methods, and it took longer to reach the steady-state level than was expected from plasma parameters (Gårdmark *et al*., 1993). This could be due to the longer half-life of morphine in the brain extracellular fluid in comparison to serum (Aasmundstad *et al*., 1995).

The unbound concentration of morphine in brain extracellular fluid and in total brain show the same pattern in *mdrla* (−/−) and (+/+) mice. As the total morphine concentration in brain homogenates was 1.3 and 1.7 fold higher than in brain extracellular fluid of *mdrla* (−/−) and (+/+) mice, this indicates that the brain affinity to morphine is not influenced by the absence of Pgp. Here it is assumed that plasma protein binding of morphine in mice is the same as in rats (20%; Wang & Takemori, 1972). If so, brain extracellular fluid concentrations would be 34 and 92% of the unbound plasma concentration for *mdrla* (−/−) and (+/+) mice, respectively. This suggests that Pgp contributed to the overall active efflux transport of morphine in the blood-brain barrier.

The total brain concentration ratios of *mdrla* (−/−)/(+/+) at 4 h was 2.6 in our result, while Schinkel *et al*. (1995) reported that total brain distribution of [^{3}H]-morphine in *mdrla* (−/−) mice was 1.7 fold of that in (+/+) mice 4 h after an i.v. bolus injection. The reason for the lower ratio in their results compared with those presented in this study could be due to morphine and its metabolite being analysed together by measuring total radioactivity, while also the administration (bolus *vs* infusion) cannot be compared directly. This might result in underestimating the effect of the absence of *mdrla* Pgp, in case the metabolites are no substrates for Pgp.

There was a tendency towards a higher brain/plasma ratio at lower plasma concentrations of morphine (0–700 nM). This may indicate that besides passive transport not only Pgp governs the blood-brain barrier transport of morphine across the blood-brain barrier. More studies are needed to elucidate the mechanism(s) behind this finding.

Morphine is mainly metabolized to morphine-3-glucoronide in rodents (Kuo *et al*., 1991). Morphine-3-glucoronide is a hydrophilic substance, although possible penetration across the blood-brain barrier is suggested (Carrupt *et al*., 1991; Gaillard *et al*., 1994). Our observations showed that morphine-3-glucoronide was transported into the brain in both *mdrla* (−/−) and (+/+) mice to a low extent (ratio brain to plasma 0.05), as has been found in other studies (Mignat *et al*., 1995; Murphey & Olsen, 1994). There were no significant differences

in brain concentration or brain-to-plasma concentration ratios of morphine-3-glucoronide between the two types of mice, and the metabolism of morphine to morphine-3-glucoronide was not affected by the absence of *mdrla* Pgp. This suggests that morphine-3-glucoronide is not a Pgp substrate.

Conclusion

Using microdialysis to study morphine blood-brain barrier transport in *mdrla* (−/−) and (+/+) mice, it is shown that Pgp is involved in regulating the extent of morphine transport across the blood-brain barrier.

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