# Supplemental information

## *In situ* brain perfusion in mice

### Materials and Methods

#### Animals

Swiss and Fvb male mice (25-35g, 7-11 weeks old) were obtained from Janvier (Genest, France). The Fvb mice triple knockout P-gp and Bcrp [Abcb1a$^{-/-}$, Abcb1b$^{-/-}$, Abcg2$^{-/-}$] mice were bred in house from progenitors obtained from the laboratory of Dr. Alfred H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The mice were housed in a controlled environment (22°C ± 2°C; 55% humidity ±10% relative humidity) and a 12-h dark-light cycle, with access to food and tap water *ad libitum*. All experiments complied with the ethical rules of the European directive (2010/63/EU) for experimentation with laboratory animals; they were approved by the ethics review committee of Paris Descartes University (study approval n°12-183/12-2012).

#### In Situ Mouse Carotid Perfusion

*Surgical Procedure and Perfusion.* The transport of $^{14}$C-diphenhydramine was measured by *in situ* carotid perfusion in mice (Dagenais et al., 2000). Mice were anesthetized with ketamine-xylazine (140-8 mg.kg$^{-1}$, intraperitoneal) and a catheter was inserted into the right carotid artery. The perfusion liquid was connected to the catheter. Before perfusion, the thorax was opened and the heart was cut. Perfusion was started immediately at a flow rate of 2.5 mL.min$^{-1}$. Each mouse was perfused with $^{14}$C-diphenhydramine (4.10$^{3}$ Bq.mL$^{-1}$; ~1.4 µmol.L$^{-1}$) and $^{3}$H-inulin (11.10$^{3}$ Bq.mL$^{-1}$). Perfusion was terminated after 60s by decapitating the mouse. The right brain was removed from the skull and dissected out on a freezer pack. Radioactivity in tissues samples and aliquots of perfusion fluid were carried out as described in Methods. The perfusion fluid composition and experiments were similar from experiments in rats (see Methods). Calculations and data analysis were done as previously described in Methods. The *in situ* brain perfusion methods used for rats and mice in this study were previously flow calibrated (Dagenais et al., 2000; Rousselle et al., 1998) which allow for the comparisons of transport parameters between species.

## Results

### Brain transport of $^{14}$C-diphenhydramine in P-gp/Bcrp-deficient and wild-type mice.

We assessed the effects of P-gp (Abcb1a) and Bcrp (Abcg2) on $^{14}$C-diphenhydramine brain transport by perfusing control Fvb mice, triple knockout [Abcb1a$^{-/-}$, Abcb1b$^{-/-}$, Abcg2$^{-/-}$] Fvb mice. The rates of $^{14}$C-diphenhydramine brain transport in control WT mice (32.4 ± 2.3 µL.s$^{-1}$.g$^{-1}$; n = 4), in triple knockout [Abcb1a$^{-/-}$, Abcb1b$^{-/-}$, Abcg2$^{-/-}$] mice (30.1 ± 3.5 µL.s$^{-1}$.g$^{-1}$; n = 4) were not statistically different (Fig. 1S).

### Passive and carrier-mediated diphenhydramine transport components at the mice brain.

The brain flux ($J_{in}$) of diphenhydramine (Figure 2S) was measured by carotid perfusion (60 s) in Krebs-carbonate buffer ($pH_e$ 7.40) at multiple concentrations. The total flux, from which the unsaturated flux was subtracted, gave the carrier-mediated flux that was

plotted against the total compound concentration. The regression plot of the carrier-mediated flux was best fitted with a Hill coefficient of 1. The brain carrier-mediated flux model gave an apparent $K_m$ of 4.46 ± 1.66 mmol.$L^{-1}$and a $V_{max}$ of 162.1 ± 44.1 nmol.$s^{-1}$.$g^{-1}$. The total brain passive diffusion component at pH 7.40 gave a $K_{passive}$ of 8.90 ± 1.40 µL.$s^{-1}$.$g^{-1}$ for diphenhydramine, equivalent to an extraction $E_{passive}$ of 21%. A comparison of the *in vivo* passive (8.9 µL.$s^{-1}$.$g^{-1}$) and carrier-mediated (~22.9 µL.$s^{-1}$.$g^{-1}$) transport rates suggests that the BBB carrier-mediated influx of diphenhydramine is 2.6-times greater than its passive diffusion when concentrations are less than the apparent carrier-mediated $K_m$.

**TEA, clonidine and proton effects on the $^{14}$C-diphenhydramine transport at the mouse BBB**

Modulations of the pH alter the $^{14}$C-diphenhydramine BBB transport (Figure 1S). Increase in the $pH_i$ with $NH_4^+$ protocol induced a significant 1.6-fold reduction ($p<0.001$) in the BBB transport suggesting a role for $H^+$-antiporter function (Figure 2SA). TEA (20 mM) did not significantly affect $^{14}$C-diphenhydramine transport (Figure 1S), suggesting the lack of OCTN, MATE and OCT effects. Clonidine (10 mM), a validated $H^+$-antiporter substrate and inhibitor, induced a significant 1.5-fold reduction ($p<0.01$) in $^{14}$C-diphenhydramine BBB transport (Figure 1S).

## References

Dagenais, C., Rousselle, C., Pollack, G.M., Scherrmann, JM.Development of an in situ mouse brain perfusion model and its application to mdr1a P-glycoprotein-deficient mice. J. Cereb. Blood Flow Metab. 2000; 20: 381-386.

Rousselle CH, Lefauconnier JM, Allen DD. Evaluation of anesthetic effects on parameters for the in situ rat brain perfusion technique. Neurosci Lett. 1998; 257:139-42.

## Supplemental figures

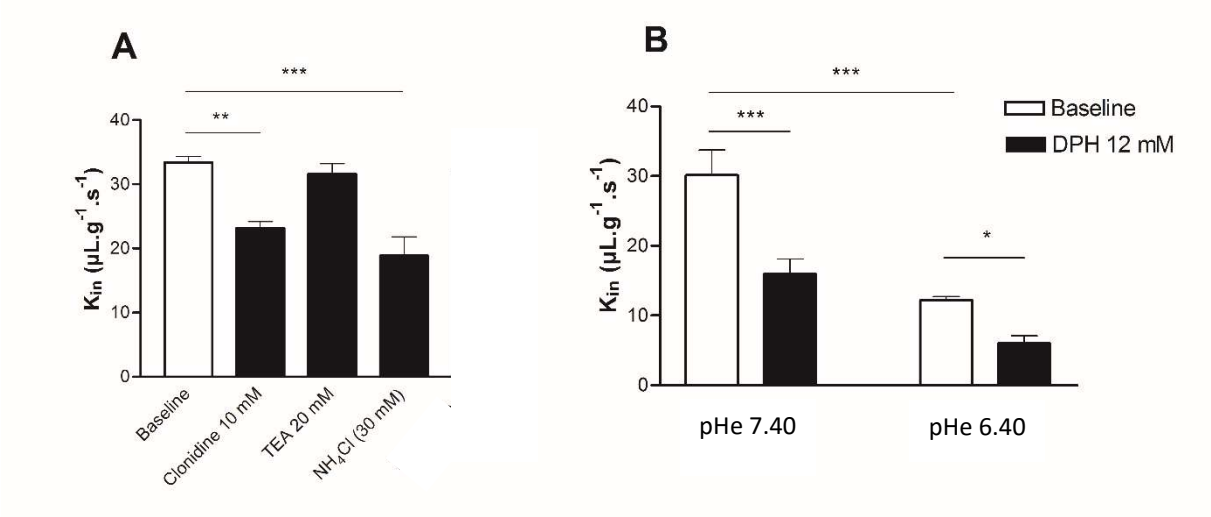


**Fig. 1S. Modulation of $^{14}$C-diphenhydramine transport across the mouse luminal blood-brain barrier (BBB). A**. Effect of cis-inhibition using clonidine 10 mM or tetraethylammonium (TEA) 20 mM, and BBB $pH_i$ increase with $NH_4Cl$ (30 mM) on $^{14}$C-diphenhydramine transport measured at pHe 7.40 ($K_{in}$; $\mu L.s^{-1}g^{-1}$) at the mouse BBB. **B.** Effect of Krebs-carbonate perfusion buffer at a pH of 6.40 or 7.40 on $^{14}$C-diphenhydramine brain transport, with (black column) or without (white column) co-perfusion with unlabeled diphenhydramine (DPH; 12 mM), measured by *in situ* mouse brain perfusion. Data represent means ± SD (n=4-5 mice). *P<0.05, **P<0.01, ***P<0.001.

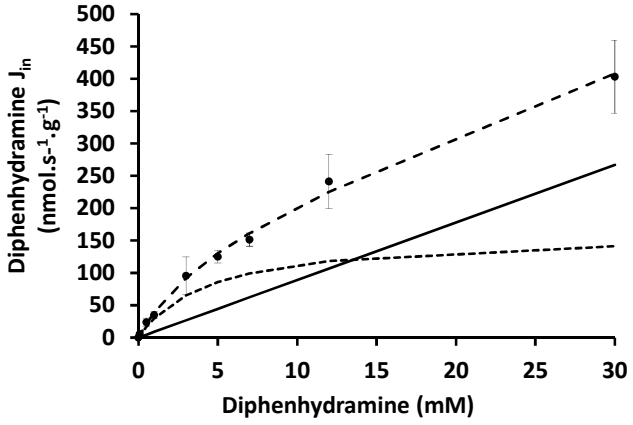


**Fig. 2S. Passive and carrier-mediated flux of diphenhydramine at the mouse luminal BBB.** Total flux ($J_{in}$; $nmol.sec^{-1}.g^{-1}$; dashed line) reported as mean ± SD measured in the Swiss mouse brain (n = 4-6 per concentration) and fitted to total diphenhydramine concentrations in Krebs-carbonate perfusion fluid at $pH_e$ 7.40. The straight dotted line represents the passive diffusion flux for diphenhydramine. The solid line represents data fitted to the carrier-mediated Michaelis-Menten equation by nonlinear least-squares regression obtained by subtracting the passive flux from the total flux. The estimated parameters for diphenhydramine transport are: $K_m$ of 4.46 ± 1.66 $mmol.L^{-1}$, $V_{max}$ of 162.1 ± 44.1 $nmol.s^{-1}.g^{-1}$, $K_{passive}$ of 8.90 ± 1.40 $\mu L.s^{-1}.g^{-1}$.

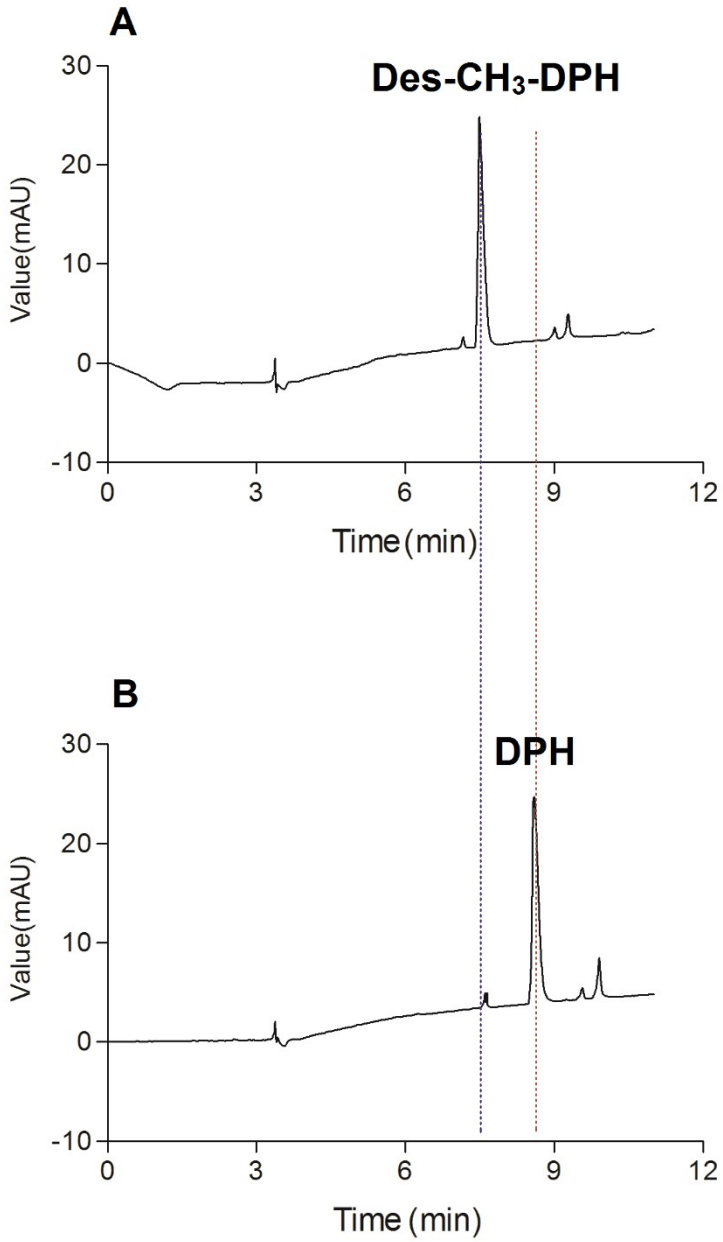


**Fig. 3S. Representative HPLC-UV chromatograms of diphenhydramine and *N*-desmethyl-diphenhydramine.** Unlabeled *N*-desmethyl-diphenhydramine hydrochloride (Des-$CH_3$-DPH; A) and diphenhydramine hydrochloride (DPH; B) were dissolved at 100 mg/L in acetonitrile/water (50/50; v/v). Hundred µL of each solution were injected into the HPLC system. The mobile phases consisted in 10 mM ammonium acetate in water (A) and acetonitrile (B). A linear gradient from 30% to 75% of B in 9 min was applied to the column at a flow rate of 5 $mL.min^{-1}$. Compounds were separated using an Atlantis preparative T3 10 µm, 10 × 250 mm at 25 °C (Waters, France). Detection wavelength was set at 254 nm.