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# P-Glycoprotein Influence on the Brain Uptake of a 5-$HT_{2A}$ Ligand: [$^{18}$F]MH.MZ

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**Key Words**
Radiotracer · 5-$HT_{2A}$ receptor · P-glycoprotein · Pharmacokinetics · Small-animal positron emission tomography · Blood-brain barrier · Mouse

**Abstract**
***Background/Aims:*** The serotonergic system, especially the 5-$HT_{2A}$ receptor, is involved in various diseases and conditions. We have recently developed a new [$^{18}$F]-5-$HT_{2A}$ receptor ligand using an analogue, MDL 100907, as a basis for molecular imaging with positron emission tomography. This tracer, [$^{18}$F]MH.MZ, has been shown to be an adequate tool to visualize the 5-$HT_{2A}$ receptors in vivo. However, [$^{18}$F]altanserin, similar in chemical structure, is a substrate of efflux transporters, such as P-glycoprotein (P-gp), of the blood-brain barrier, thus limiting its availability in the central nervous system. The aim of this study was to determine whether transport by P-gp influences the distribution ratio of [$^{18}$F]MH.MZ in the frontal cortex. ***Methods:*** The approach was based on P-gp knockout mice which were compared with wild-type mice under several conditions. In vivo pharmacokinetic and microPET investigations were carried out. ***Results:*** All analyses showed that [$^{18}$F]MH.MZ entered the brain and was sensitive to P-gp transport. In P-gp knockout mice, brain concentrations of MH.MZ were about 5-fold higher than in wild-type animals which is reflected by a 2-fold increase in standardized uptake values of [$^{18}$F]MH.MZ in the frontal cortex of P-gp knockout mice. ***Conclusion:*** Our results give evidence for a functional role of transport mechanisms at the blood-brain barrier, specifically of P-gp, and its subregional distribution. Investigation of these mechanisms will benefit the development of more efficient radioligands and drugs for molecular imaging and pharmacotherapy of the mentally ill.


## Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is one of the evolutionary oldest neurotransmitters. It has been implicated in the etiology of numerous disease states, including depression, anxiety, social phobia, schizophrenia, obsessive-compulsive disorder, and panic disorders [1–3]. 5-HT produces its effects through various membrane-

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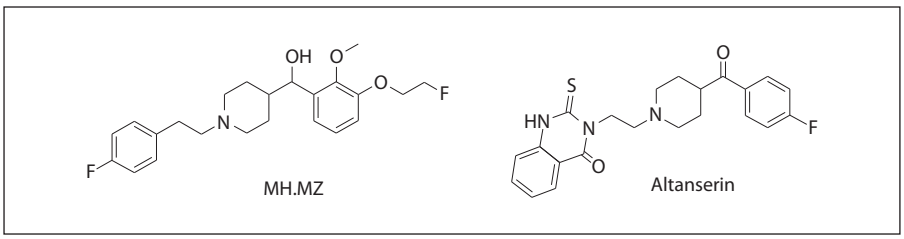


**Fig. 1.** Chemical structures of the 2 5-$HT_{2A}$ antagonists: MH.MZ and altanserin.

bound receptors. One of them, the 5-$HT_2$ receptor subtype, is widely distributed in peripheral and central tissues. Centrally, these receptors are located in the cortex, claustrum and basal ganglia. Its activation stimulates hormone secretion, for example ACTH, corticosterone, oxytocin, renin and prolactin [3, 4]. 5-HT antagonists have been implicated for treating schizophrenia [5]. Atypical antipsychotics, such as clozapine, olanzapine, quetiapine, and risperidone, are antagonists of both dopamine $D_2$ and 5-$HT_{2A}$ receptors. This combination is thought to enhance antipsychotic activity and lower extrapyramidal side effects [6, 7].

Development of specific radiotracers enabled noninvasive in vivo studies of receptor occupancy by positron emission tomography (PET). These advances improved substantially the understanding of the mechanisms underlying the antipsychotic effects of $D_2$ and 5$HT_2$ receptor antagonists [8]. However, these studies referred primarily to the dopaminergic activity [9]. Recently, several 5-HT neurotransmitter analogues were synthesized as radiopharmaceuticals to image 5-$HT_{2A}$ receptors. They permitted researchers to acquire better understanding of the serotonergic system in these conditions [10, 11].

So far, in vivo studies have been performed with several 5-$HT_{2A}$ selective antagonists for PET, such as [$^{11}$C]MDL 100907 or [$^{18}$F]altanserin. Based on the advantage of the high affinity and selectivity of the compound MDL 100907, we recently developed an $^{18}$F analogue, called [$^{18}$F]MH.MZ, with a $K_d$ value of 3.0 nM. We believe this PET tracer for 5-$HT_{2A}$ receptors is superior compared with the existing ones [4, 12–14]. [$^{18}$F]altanserin is similar in structure (fig. 1) and has recently been shown to be a substrate of the efflux transporter P-glycoprotein (P-gp), located in the blood-brain barrier, thus limiting its availability in the central nervous system [15]. Rats pretreated with cyclosporin A, a P-gp inhibitor, exhibited a 52% higher total brain uptake of [$^{18}$F]altanserin and a more than 6-fold increase in the binding potential in the frontal cortex compared with untreated controls [15].

P-gp belongs to the superfamily of ATP-binding cassette transport proteins in the blood-brain barrier. Its function is to limit the access of xenobiotics into the central nervous system by efflux pumping [16]. P-gp has been shown to counteract diffusion of many drugs including antipsychotics and antidepressants [17, 18]. We recently showed that altered pharmacokinetics of risperidone and haloperidol has pharmacodynamic consequences on mice behavior [19].

PET studies on rodents also showed the influence of P-gp on the pharmacokinetics and brain uptake of 5-$HT_{1A}$ receptor ligands [$^{18}$F]MPPF and [$^{11}$C]WAY 100635 [20]. Under normal conditions, the specific brain uptake of [$^{18}$F]MPPF is 5–10 times lower than that of [$^{11}$C]WAY 100635. After cyclosporin A application, [$^{18}$F]MPPF uptake in the rat brain increased to the level of the $^{11}$C derivative. Cerebral uptake of [$^{11}$C]WAY 100635 was also increased by P-gp modulation, but was lower than that observed for [$^{18}$F]MPPF [21].

For PET imaging studies, brain delivery remains a significant challenge in the optimal design of PET ligands. Therefore, it is imperative that new radioligands for PET be evaluated for substrate affinity for carriers like P-gp.

The present study used molecular imaging with a microPET in the frontal cortex coupled with pharmacokinetic studies in wild-type and P-gp knockout mice. Cerebellum [$^{18}$F]MH.MZ uptake was used as a reference region. Our goal was to determine if [$^{18}$F]MH.MZ is a P-gp substrate by the investigation of brain uptake of the compound. In addition, we also looked at its precursor and metabolite of MDL 100907, MDL 105725, which has been characterized as an active metabolite in the rat [22].

## Methods

*Preparation of MH.MZ and [$^{18}$F]MH.MZ*

[$^{18}$F]MH.MZ as well as unlabeled MH.MZ were synthesized according to Herth et al. [13, 14].

Synthesis of MH.MZ

MH.MZ was derived from MDL 105725 by fluoroalkylation. The synthesis of MDL 105725 has already been described by Ullrich and Rice [23] and Huang et al. [24]. The synthetic route to MDL 105725 is dependent on the transformation of an ester to a ketone by an amide intermediate. Finally, MH.MZ was synthesized by fluoroalkylation of the precursor MDL 105725 in dry N,N-dimethylformamide by addition of equimolar sodium hydride and 1-bromo-2-fluoroethane in a yield of 40%.

Synthesis of [$^{18}$F]MH.MZ

[$^{18}$F]FETos diluted in 0.8 ml of dry dimethyl sulfoxide was added to a solution of 3 mg MDL 105725 (7 mmol) dissolved in 0.2 ml of dry dimethyl sulfoxide and 1.5 μl of 5 N NaOH (7 mmol). The solution remained at 100°C for 10 min and was quenched with 1 ml $H_2O$. [$^{18}$F]MH.MZ was separated from reactants and by-products by semipreparative high-performance liquid chromatography (HPLC; μBondapak $C_{18}$ 7.8 × 300 mm column, flow rate 8 ml/min, eluent: MeCN/0.05 phosphate buffer, pH 7.4 adjusted with $H_3PO_4$, 40:60). The retention times of [$^{18}$F]MH.MZ, [$^{18}$F]FETos and MDL 105725 were 9.76, 3.97 and 4.85 min, respectively. The collected product was diluted with water (4:1), passed through a conditioned Strata-X cartridge (1 ml EtOH, 1 ml $H_2O$), washed with 10 ml $H_2O$ and eluted with at least 1 ml EtOH. Finally, EtOH was removed in vacuo and [$^{18}$F]MH.MZ solved in 1 ml saline.

*Animals*

Male mdr1a/1b (–/–, –/–) double-knockout mice (P-gp knockout; FVB/N background, Taconic, Germantown, N.Y., USA) and male wild-type controls (also FVB/N) were used. Animals had access to food and water ad libitum. Temperature and humidity were kept at 22 ± 2°C and 60%, respectively. All animals were maintained on a 12-hour light/12-hour dark cycle. Handling occurred only during the light cycle. Animal procedures were in strict accordance with the National Institutes of Health guide for the care and use of all laboratory animals and were approved by the local animal care and use committee.

*In vitro Analysis*

Serum and brain concentrations of MH.MZ were analyzed according to Kirschbaum et al. [19] and Waldschmitt et al. [25]. P-gp knockout and wild-type mice (n = 5 per group) were injected intraperitoneally with either 20 mg/kg MH.MZ or 20 mg/kg of MDL 105725. After 1 and 3 h, animals were killed by decapitation under isoflurane (Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany) anesthesia. Trunk blood was collected immediately and the brain was dissected. Serum, obtained by centrifugation of blood at 3,000 *g* for 10 min, was stored at –20°C or analyzed immediately by HPLC. One half of the brain was frozen on dry ice and stored at –20°C, the other half was weighed and homogenized in 4 volumes of methanol (HPLC grade; Merck, Darmstadt, Germany). Homogenates were centrifuged at 13,000 *g* and the supernatant was frozen at –20°C or analyzed directly by HPLC.

Serum or methanolic brain extracts were injected directly into the HPLC system with column switching and analyzed by established methods using peak heights for quantification. MH.MZ and MDL 105725 concentrations were measured using a LiChrospher column (60 RP-Select B, 125 × 4 mm, 5 μm particle size; MZ-Analysentechnik, Mainz, Germany) as analytical column. The analytical eluent contained 32.63% acetonitrile and dipotassium hydrogen phosphate (Merck, Darmstadt, Germany) adjusted to pH 3.35. Sample cleanup was done on a CN 20 μm PerfectBond precolumn (10 × 4 mm, MZ-Analysentechnik) using deionized water containing 8% (v/v) acetonitrile. Absorption was measured by spectrophotometric detection at a wavelength of 210 nm. Retention times were 8 and 11 min for MH.MZ and MDL 105725, respectively, at a flow rate of 1 ml/min.

*In vivo Imaging Studies*

MicroPET imaging was performed with a Siemens/Concorde Microsystems microPET Focus 120 small-animal PET (microPET) camera. The radiotracer [$^{18}$F]MH.MZ was applied intraperitoneally (approx. 12 MBq; specific activity A = 50 GBq/μmol) to P-gp knockout (n = 3) and wild-type (n = 3) mice [26]. Following a 45-min awake uptake period, mice were deeply anesthetized with chloral hydrate (7%) by intraperitoneal injection of 6 ml/kg and a 10-min static PET scan ensued. Dynamic PET studies showed that equilibrium is reached within this time frame and the blood pressure remained stable [14]. Images were reconstructed without scatter and attenuation correction using a 3-dimensional maximum a posteriori algorithm with 18 iterations and a regularization parameter of 0.005. Tomographic images were analyzed with pixelwise modeling computer software (PMOD; Zurich, Switzerland). Based on a digital mouse brain atlas, a region of interest (ROI) template was created including ROIs for the frontal cortex and cerebellum. MicroPET images of the P-gp knockout group were realigned and coregistered to create a mean [$^{18}$F]MH.MZ image using Statistical Parametric Mapping 2. Then, image sets of both groups were realigned to the mean image using affine transformation of Statistical Parametric Mapping 2. Statistics were derived from the ROI template applied to each transformed image set of the P-gp knockout and wild-type group. ROI data were normalized for whole-brain uptake and compared between groups (P-gp knockout vs. wild type) using a significance threshold of $p \leq 0.05$. Results were expressed as standardized uptake values, which are defined as follows: (activity concentration in Bq/ml) × (body weight in g)/(injected dose in Bq).

*Statistics*

Student's t test was used to analyze the effects of genotype on the pharmacokinetics for each time point and substance. p values <0.05 were considered statistically significant. Area under the data, which is the area under the curve between 0 and 3 h after injection, was calculated using the trapezoid rule. Statistical analysis of microPET data was done by multivariate analysis of variance (MANOVA) with post hoc t test. All statistical analyses were performed using SPSS version 12.0G for Windows (SPSS Inc., Chicago, Ill., USA).

## Results

*In vivo Studies*

Serum and brain levels of MH.MZ and MDL 105725 were measured 1 and 3 h after drug administration to study P-gp expression-dependent differences. In vivo distribution data revealed significantly different brain concentrations of MH.MZ between wild-type and P-gp

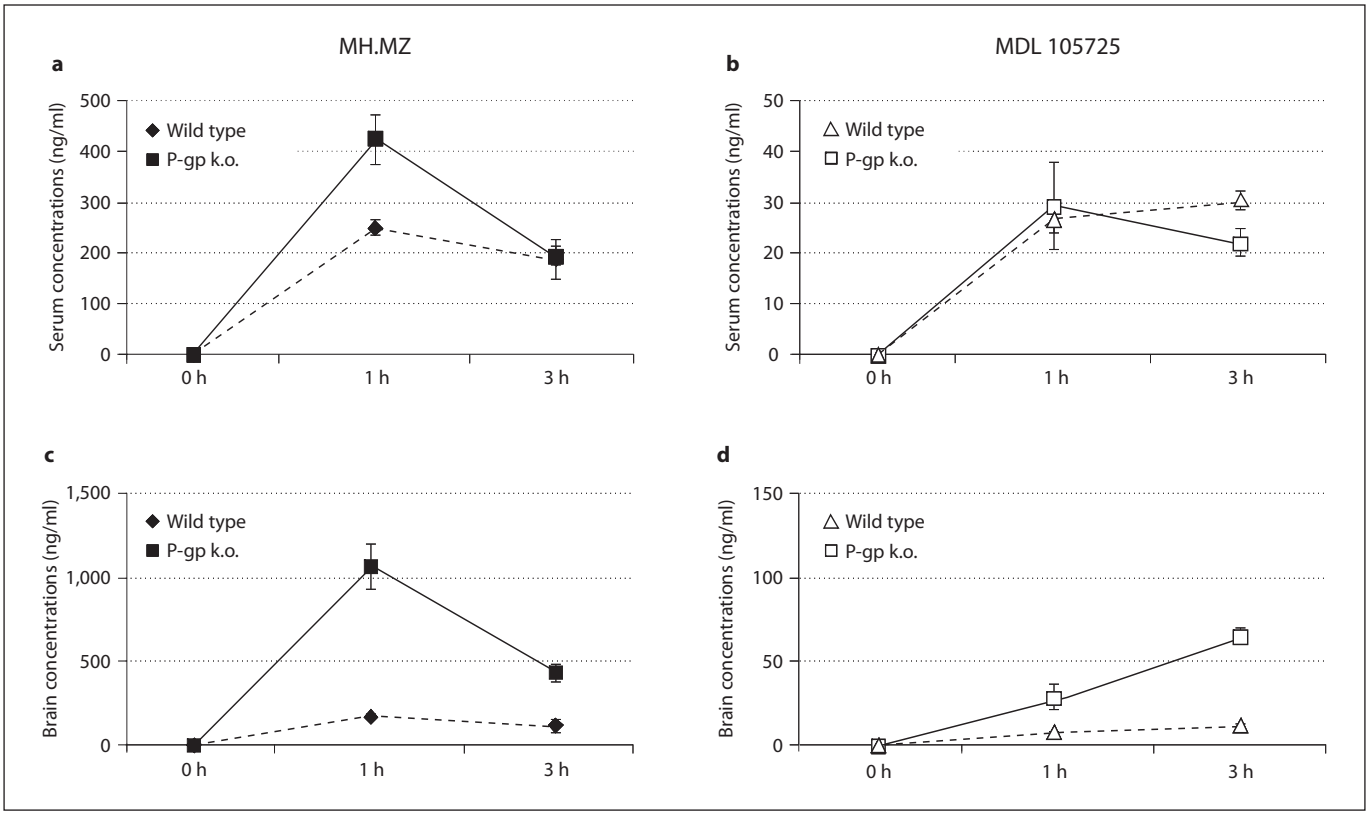


**Fig. 2.** Serum (**a**, **b**) and brain (**c**, **d**) concentration time profiles for MH.MZ (**a**, **c**) and MDL 105725 (**b**, **d**) in FVB wild-type (dashed line) and P-gp knockout (k.o.) mice (solid line) following intraperitoneal injection. Data points represent mean ± SEM values.

knockout mice (fig. 2). MH.MZ levels were 6.2-fold (1 h) and 3.7-fold (3 h) higher in brains of P-gp knockout mice (both $p < 0.05$). MDL 105725 levels in these mice exceeded brain concentrations in wild-type mice by 3.7-fold (1 h) and 5.5-fold (3 h) (both $p < 0.05$; fig. 2). Serum levels of MH.MZ showed a 1.7-fold (1 h, $p < 0.05$) increase and no difference after 3 h (n.s.) between these 2 mouse lines. MDL 105725 concentration was only slightly increased after 1 h (1.1-fold; n.s.) and decreased after 3 h (0.7-fold; n.s.) for wild-type versus knockout mice (fig. 2; table 1). Area under the data calculations revealed that MH.MZ brain concentrations in P-gp knockout mice attained 512% of the level measured in wild-type mice, while MDL 105725 brain levels in P-gp knockout mice represented 483% of wild-type mice brain concentrations. Further, serum levels of P-gp knockout mice attained 138% of the level measured in wild-type mice, while the MDL 105725 serum levels in P-gp knockout mice represented only 89% of wild-type mice serum concentrations (table 1). Amounts of MH.MZ and MDL 105725 in the brains of P-gp knockout mice were 3-fold higher after 1 h compared with brains of wild-type mice. After 3 h, MH.MZ was still 3-fold higher, while the metabolite MDL 105725 was 7.5-fold higher in P-gp knockout mice (fig. 3).

**Table 1.** Area under the data values (ng/ml/3 h) in P-gp knockout (k.o.) and FVB wild-type mice for the time period 0–3 h after intraperitoneal injection

| Drug | Brain | | Serum | |
|---|---|---|---|---|
| | P-gp k.o. | wild type | P-gp k.o. | wild type |
| MH.MZ | 3,952.40 | 771.90 | 1,637.50 | 1,186.99 |
| MDL 105725 | 268.30 | 55.50 | 139.00 | 156.99 |

*Imaging*

With respect to the afore-reported globally increased uptake of [$^{18}$F]MH.MZ in the brains of P-gp knockout

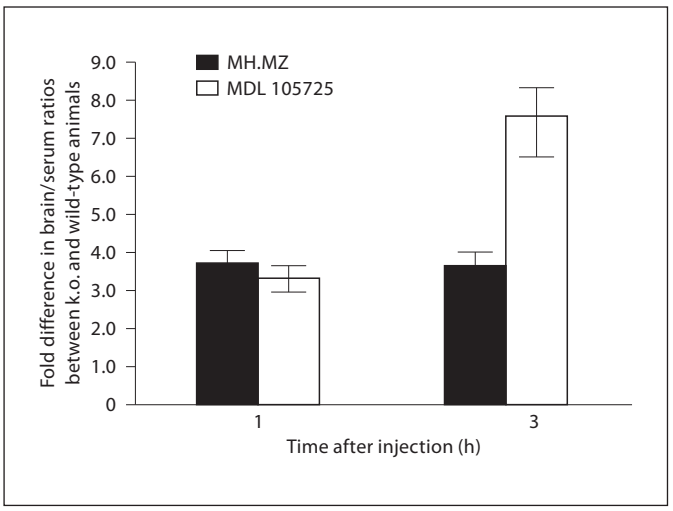


**Fig. 3.** Bars display the x-fold increase in brain/serum ratio seen in P-gp knockout (k.o.) mice compared with wild-type animals. Data points represent mean ± SEM values.

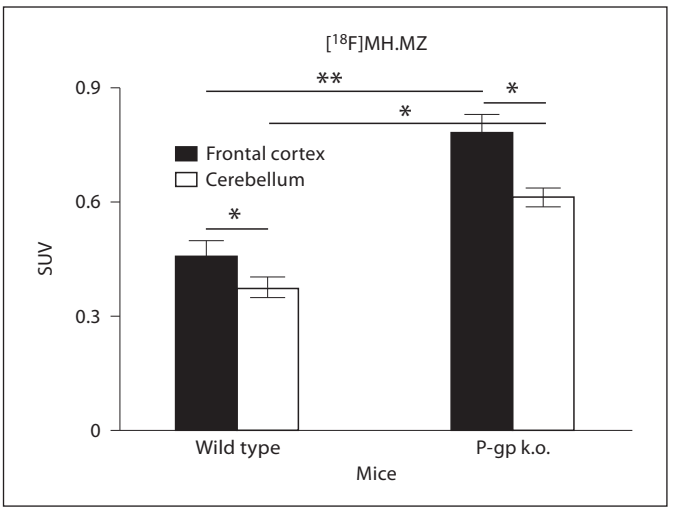


**Fig. 4.** Comparison of brain accumulation expressed as standardized uptake values (SUV) of [$^{18}$F]MH.MZ in P-gp knockout (k.o.) and FVB wild-type mice in the frontal cortex and the cerebellum. Data points represent mean ± SEM values. Asterisks indicate significant SUV differences with * $p < 0.05$ and ** $p < 0.01$ (post hoc t test following MANOVA).

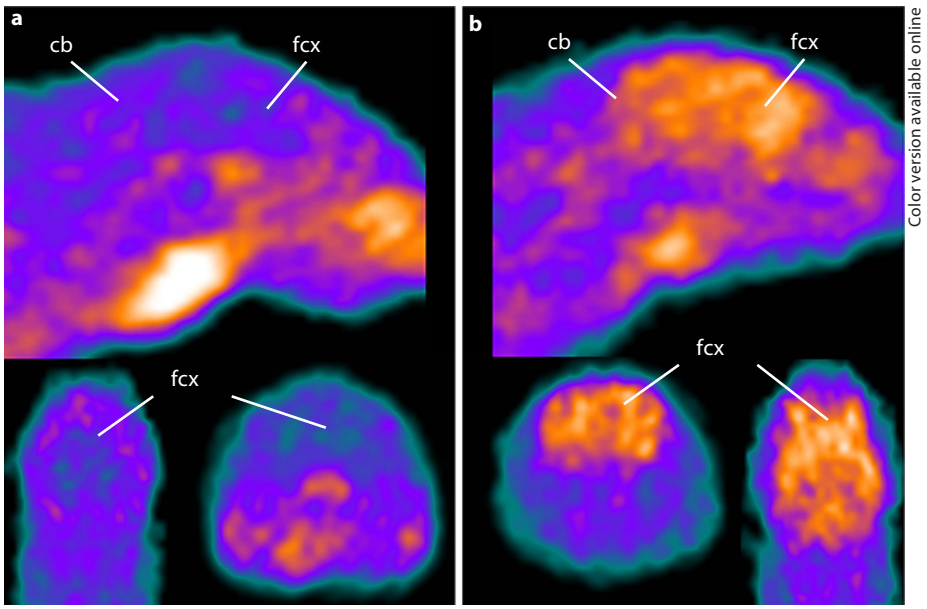


**Fig. 5.** [$^{18}$F]MH.MZ microPET scan of an FVB wild-type (**a**) and P-gp knockout (**b**) mouse brain. Images show sagittal as well as coronal and horizontal views. Images derive from a 10-min static scan 45 min after intraperitoneal injection of approximately 12 MBq. The imaging was performed with the Focus 120 microPET scanner. Resolution for mouse brain is limited in this scanner; relevant brain regions are indicated. fcx = Frontal cortex; cb = cerebellum.

versus wild-type mice, the in vivo studies of [$^{18}$F]MH.MZ by microPET were consistent with the pharmacokinetic differences (fig. 4). [$^{18}$F]MH.MZ entered the brains of the mice with a significant difference ($p < 0.05$) between brain regions and the 2 genotypes (fig. 4, 5). ROI analysis showed a higher accumulation in the frontal cortex compared with the cerebellum, both in wild-type and P-gp knockout animals (fig. 5). MANOVA indicated significant main effects on factor A genotype ($F_{1,4} = 32.403$, $p \leq 0.01$) and factor B brain region (treated as repeated measure; $F_{1,4} = 84.794$, $p \leq 0.001$) as well as a significant A × B interaction ($F_{1,4} = 9.621$, $p \leq 0.05$) (fig. 4). How-

ever, frontal cortex to cerebellum ratios of wild-type and P-gp knockout animals did not differ significantly (cortex/cerebellum in wild-type 1.221 ± 0.029, in P-gp knockout 1.274 ± 0.028).

## Discussion

In summary, all analyses showed that [$^{18}$F]MH.MZ entered the brain and was sensitive to P-gp efflux transport. In P-gp knockout mice, brain concentrations were about 5-fold higher than in wild-type animals.

Pharmacokinetic peculiarities of individual patients due to variations in absorption, distribution, metabolism or elimination of drugs alter the efficacy of drug treatment [27]. One contributing factor is the passage through the blood-brain barrier, which is regulated by various efflux transport proteins. P-gp, a member of the ATP-binding cassette superfamily, plays an important role as an efflux pump at the blood-brain barrier for many drugs [17–19, 28]. The passage through the blood-brain barrier is important not only with respect to drug treatment but also with respect to the use of PET tracers as a diagnostic tool. Poor penetration of radiolabeled compounds limits their effectiveness [15, 21, 29, 30]. Ex vivo studies, in support of advancing in vivo P-gp evaluation, have already demonstrated that PET tracer uptake in the brain can be quantified after modulation of P-gp. For example, brain uptake of the P-gp substrates [$^{11}$C]verapamil or [$^{11}$C]carvedilol increased in the presence of P-gp inhibitors [31–33]. Laćan et al. [34] recently brought an additional fact into consideration, which is the heterogeneous brain distribution of P-gp that might influence quantification by the simplified reference tissue method using the cerebellum as a nonspecific reference region for modeling. Although the distribution of the P-gp content throughout the rat or mouse brain has not been comprehensively analyzed, brain regional differences are supported by several studies including PET analysis [35, 36].

We recently developed an $^{18}$F analog of MDL 100907, [$^{18}$F]MH.MZ, as a superior PET tracer of the 5-HT$_{2A}$ receptor, in rats [13, 14]. Its similarity in structure (fig. 1) to [$^{18}$F]altanserin raised the suspicion that MH.MZ might be a P-gp substrate like [$^{18}$F]altanserin recently shown to be a substrate of P-gp [15, 36]. The present study was carried out to evaluate whether MH.MZ is a substrate of the efflux transporter P-gp.

Our results showed that the absence or presence of P-gp alters the uptake of MH.MZ and [$^{18}$F]MH.MZ into the brain. In P-gp knockout mice treated with 20 mg/kg of MH.MZ, a time-dependent increase of up to 6-fold in the brain to serum concentration ratio was observed compared with wild-type mice. A similar increase was found for the metabolite MDL 105725. Similarly, our PET results revealed a significantly higher uptake of [$^{18}$F]MH.MZ in P-gp knockout mice compared with wild-type mice (fig. 4, 5). The uptake in both mouse lines was higher within the frontal cortex known to possess greater amounts of 5-HT$_{2A}$ receptors compared to the cerebellum, supposed to be devoid of 5-HT$_{2A}$ receptors [37]. However, the increase seen in knockout mice did not reflect an increase in specific binding as the cortex/cerebellum ratio was not statistically different in post hoc analysis between genotypes (fig. 4). There are 2 reasons to explain this fact. First, the suitability of the cerebellum as a reference region is still controversial. Eastwood et al. [38] reported some 5-HT$_{2A}$ receptor binding in the cerebellum of humans. Laćan et al. [34] reported a P-gp-dependent uptake in the cerebellum of rats. In fact the cerebellum as an adequate reference region because of its lack of 5-HT$_{2A}$ receptors remains questionable. Second, a region-specific distribution of P-gp which might change the regional ratios depends on the different expression levels in both mouse lines [34]. Specificity of binding in the cortex might be masked by a higher influx of the drug in the cerebellum due to the lack of P-gp in knockout mice. However, region-specific distribution of P-gp has to be investigated specifically in future studies. Collectively, our results suggest a prominent role of P-gp with respect to PET tracer uptake and interpretation of receptor densities dependent on kinetic models.

In summary, we demonstrated by different methods that MH.MZ enters the brain and that its uptake is dependent on P-gp-mediated efflux. Our results show that brain to serum concentration ratios and brain uptake in small-animal PET were higher in P-gp knockout mice compared with wild-type mice. We anticipate that the enhanced brain levels of [$^{18}$F]MH.MZ are a direct measure of the parent radioligand and inactivity of radiometabolites.

Our results give evidence for a functional role of transport mechanisms at the blood-brain barrier, specifically of P-gp, and its subregional distribution. Investigation of these mechanisms will benefit the development of more efficient radioligands and drugs for molecular imaging and pharmacotherapy of the mentally ill.

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