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Research Article

Induction of P-Glycoprotein Reduces the *In vivo* Activity of Risperidone in Mice

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Abstract

Objectives: P-Glycoprotein (P-gp) an efflux transporter localized in the blood-brain barrier, influences drug concentrations in the brain and thereby their clinical efficacy. P-gp knockout mice differ markedly from wild type animals with respect to pharmacokinetics and -dynamics of P-gp substrates. Using the P-gp substrate risperidone as a model drug, we studied the effects of P-gp induction on drug concentrations in blood and CNS as well as its effects on drug related behavior.

Methods: P-gp inducing drugs dexamethasone and 5-pregnene-3beta-ol-20-on-16alpha-carbonitrile (PCN) were given to FVB/N mice for 11 days. Control mice received vehicle only. On day 12, risperidone was injected i.p. For kinetic investigations, brain and serum levels of risperidone and 9-hydroxyrisperidone were measured by reversed phase-high performance liquid chromatography with spectrophotometric detection. To study pharmacodynamic effects, risperidone induced RotaRod behavior was analysed with Rota Rod.

Results: Risperidone and 9-hydroxyrisperidone concentrations were decreased in the blood serum and brain homogenate of animals treated with dexamethasone or PCN. Baseline Rota Rod behavior was only slightly affected by P-gp inducing drugs. Rota Rod deficits due to risperidone were markedly reduced after induction of P-gp by both drugs.

Conclusion: Induction of P-gp diminishes the CNS effects of drugs characterized as substrates of P-gp. Therefore, it seems likely that induction of P-gp by co-medication has the potential to minimize treatment response and increase potential side effects of CNS drugs in a clinical respect.

Introduction

Uptake and efflux transporters are the major component of biological barriers to control plasma and tissue concentrations of multiple drugs with consequences for drug efficacy at the target organ. For CNS drugs, passage of the Blood-Brain Barrier (BBB) is essential. P-Glycoprotein (P-gp) a member of the Adenosine tri phosphate Binding Cassette (ABC) super family is one efflux transporter in the BBB which impedes the entrance of its substrates in to the CNS. Due to its location at the luminal border of the BBB endothelial cells P-gp allows to prevent the entrance of substrates being a substrate of this efflux transporter. P-gp is known to hinder the entrance of several CNS active drugs such as antidepressants, antipsychotics and anti-epileptics [1-6] in humans as well as in rodents. These diminished drug concentrations at the target organ result in reduced treatment efficacy or to reach similar drug levels a higher dosing with the risk of unwanted side effects.

In mice, the expression and function of P-gp is controlled by two genes, abcb1a and abcb1b in contrast to only one ABCB1 gene in humans. By using *in vivo* models like P-gp double-knockout mice (abcb 1a/1b -/-) results indicate that P-gp has a strong impact on brain levels of different kinds of psychoactive drugs especially a number of antipsychotics and antidepressants [5,7-12]. An impact on pharmacokinetics has a likelihood of pharmacodynamics consequences as observed by differences in the drug-related behavior of P-gp deficient mice [7,9,11,12]. Knock out animals, however, do not reflect the clinical situation as patients with genetically caused absence of P-gp activity have so far not been identified. However, polymorphisms resulting in changed drug efficacy have been reported [13]. Moreover, evidence is lacking that drugs with an inhibitory potential on P-gp may give rise to clinically relevant drug-drug interactions. Up to now, it has not been reported if induction of gene expression of efflux transporters results in pharmacodynamics consequences within the CNS due to a boosted barrier function for P-gp substrates [14,15]. Enhanced expression might be one reason for treatment failure of antidepressant [14] or antipsychotic drugs [16-18]. Risperidone and its active metabolite 9-hydroxyrisperidone can be applied in mice to investigate the influence of P-gp expression-levels on pharmacokinetic [7,15,19] and pharmacodynamic [7] effects, by the impact on motor behavior of the antipsychotic.

The present study aimed to extent previous investigations on P-gp induction [15], first in respect to treatment duration and second to pharmacodynamic consequences. After 11 days of sub chronic treatment with dexamethasone and 5-pregnene-3beta-ol-20-on-16alpha-carbonitrile (PCN), brain and blood levels as well as Rota Rod behavior were investigated in mice after an acute risperidone

injection. The present design models there for clinical situation with monitoring of *in vivo* outcome (therapy efficacy) and furthermore provides more clinically relevant insights compared to the use of Knock Out (k.o.) models.

Methods

Drugs

Risperidone (Risperdal®) was obtained from Janssen-Cilag GmbH (Neuss, Germany). PCN and corn oil (used as solvent for PCN) were purchased from Sigma-Aldrich (Steinheim, Germany). Physiological saline solution 0, 9% was received from Braun (Melsungen, Germany). Risperidone used for reversed phase high performance liquid chromatography was purchased from MP Biomedicals (Illkirch, France); 9-hydroxyrisperidone was kindly provided by Janssen-Cilag (Beerse, Belgium). Methanol (high performance liquid chromatography grade) and dexamethasone-21-di-sodium-dihydrogen-phosphate (Fortecortin® injects) were provided by Merck (Darmstadt, Germany). Isoflurane for anaesthesia (Forene®) was purchased from Abbott GmbH & Co. KG (Wiesbaden, Germany).

Animals

231 male FVB/N mice (25-45 g; P-glycoprotein status (abcb1a/1b +/+; FVB/N background) from the animal facility of the University Medical Center of Mainz were used. Animals were housed in groups of 2-5 with free access to food and water. A 12-h light–dark cycle (6 am to 6 pm light on) was maintained at a temperature of 22°C and a relative humidity of 60%. All experiments were conducted in accordance to the official regulations for the care and use of laboratory animals and approved by local authorities.

Study design and drug administration

For kinetic investigations 156 mice were divided into 4 groups (dexamethasone, saline control, PCN, corn oil control). Dexamethasone (50 mg/kg/d) and the known murine selective Pregnane X Receptor (PXR) activator PCN in a dose of 25 mg/kg/d were injected Intra Peritoneally (i.p.) for 11 days [15]. Control mice received vehicle only; in case of dexamethasone physiological saline, in case of PCN the vehicle was corn oil. The latter was required due to the high lipophilicity of PCN. P-gp inducing drugs were injected once daily at a 24 h interval. On day 12 risperidone was injected i.p. in a dose of 3 mg/kg [7]. 0.5, 1, 2, 3 and 6 hours after injection of risperidone, mice were anaesthetized and decapitated respectively (n=6-8 per group). Trunk blood and brain tissue samples were collected and out of it the levels of risperidone and its active metabolite 9-hydroxyrisperidone measured by high performance liquid chromatography [7]. The sum of risperidone and 9-hydroxyrisperidone levels was calculated and given as active moiety. Both mother compound and metabolite exhibit similar receptor profiles and therapeutic efficacy [20,21].

For pharmacodynamics analysis by RotaRod behavior a total of 75 mice were used. Mice were placed in a neutral position on a 3 cm diameter cylinder turning with a speed of 5 Rounds Per Minute (rpm) (RotaRod Advanced, TSE Systems, Bad Homburg, Germany). After ten seconds speed was accelerated linearly up to 27.5 rpm within 240 seconds and time was taken automatically until the mouse fell from the cylinder. Animals were trained before the start of the drug treatment for 5 days to achieve comparable performance. At day 6 behavioural training continued while treatment with P-gp inducing drugs or respective control substances started for 11 days. On the 12th day mice were injected i.p. with risperidone and tested six times 0.5, 2, 4, 6, 8, and 10 hours post injection. At the first training days without treatment three trials per day were performed. After starting with the injections, four trials per day for each mouse were conducted [22]. Thereby, baseline values were calculated of values each individual mouse achieved at days 9 to 11 (mean) on the RotaRod. This mean value served as an individual baseline score of the respective mouse (100% performance), to minimize inter-individual variation. Means of three consecutive trials at each time point of testing day (with risperidone treatment) were related to the baseline score to assess risperidone impact on individual behavior. In this way each mouse built a control to itself [7,11,12].

Statistical analysis

Statistical comparisons between groups were carried out using SPSS version 21.0 (SPSS GmbH Software, Munich, Germany). Either one or 2-way analysis of variance with post hoc t-test was used to assess statistical significant group differences. Differences were considered to be statistically significant for p values less than 0.05 (Table 1).

Table 1: Calculated were ratios of levels of 9-hydroxyrisperidone divided by levels of risperidone in serum (A) and brain (B) for the different treatment conditions at various time points. Ratios of concentrations are an *in vivo* measure of 9-hydroxylation of risperidone.

A

Inducing drug	0.5h	1h	2h	3h	6h
Dexamethasone	0.83	0.43	0.25	0.09	0
Control - NaCl	3.52	1.2	0.87	0.34	0.09
PCN	1.08	0.6	0.32	0.24	0.17
Control - Cornoil	2.73	1.66	0.77	0.46	0.23

B

Inducing drug	0.5h	1h	2h	3h	6h
Dexamethasone	0.62	0.68	0	0	0
Control - NaCl	8.2	6	0.5	0.37	0
PCN	3.54	1.58	0.57	0.42	0
Control - Cornoil	12.89	4.16	1.8	0.77	0.52

Results

To evaluate the P-gp inducing effect of dexamethasone and PCN brain and serum levels of the known P-gp substrate risperidone and its major metabolite 9-hydroxyrisperidone were used (Figure: 1 and 2). In line with previously published data derived after 4 days of treatment with P-gp inducing drugs dexamethasone and PCN [15] mice treated for 11 days showed significantly decreased brain and serum risperidone levels (Figures: 1A/B and 2A/B). In some animals, risperidone and 9-hydroxyrisperidone brain levels were below the limit of quantification of the HPLC method used [7] and thus not detectable (Figures; 1A/C and 2A/C) (Figure 1).

In mice treated with dexamethasone serum levels given as active moiety of risperidone (sum of risperidone and 9-hydroxyrisperidone)

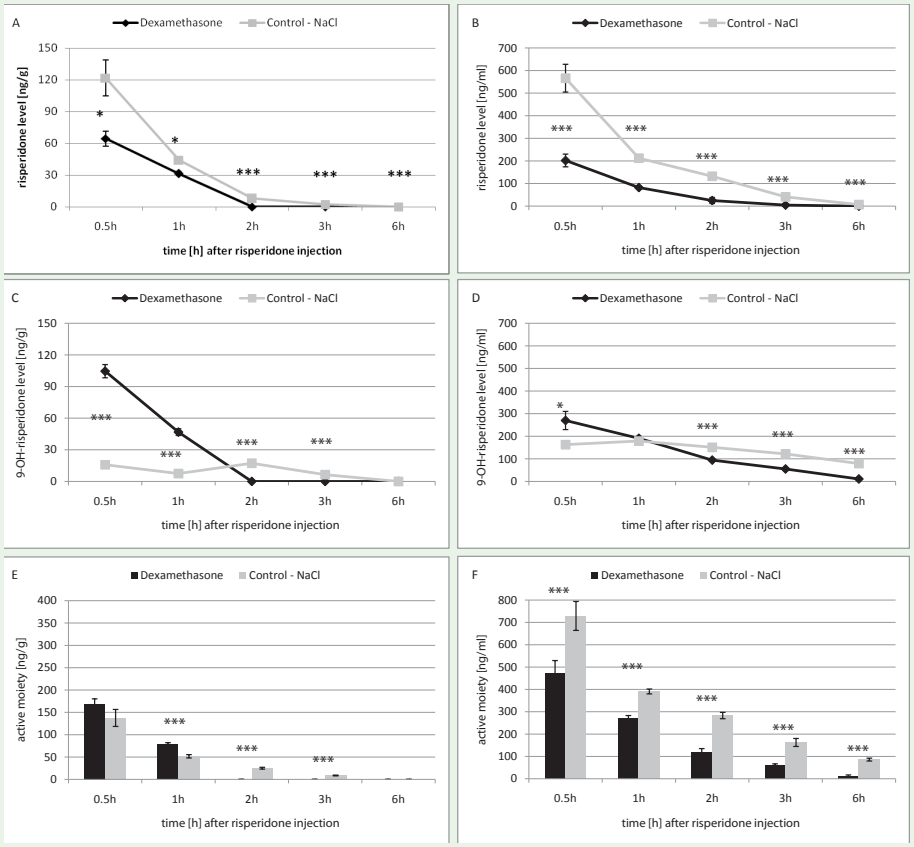

Figure 1: Concentration time profiles after induction of P-glycoprotein by 50 mg/kg/d dexamethasone for risperidone in the first row (A, B) and for its metabolite 9-hydroxyrisperidone in the second row (C,D). Brain levels were displayed in the left column (A,C) while serum levels were shown in the right column (B,D). The active moiety i.e. sum of risperidone and 9-hydroxyrisperidone is shown in the third row for brain levels on the left (E) and for serum levels on the right (F). Mice treated with dexamethasone = black lines, and controls = grey lines. Asterisks highlight significant differences indicated by post-hoc comparison of group means at various time points. Data are presented as mean +/- standard error of the mean (S.E.M.).
(* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

were significantly lower compared to NaCl injected controls at any time point (Figure: 1F). However, brain levels (active moiety), were slightly different: Within the first hour after risperidone injection brain levels of mice treated with dexamethasone were similar or higher as those of controls (Figure: 1E) whereas two and three hours after risperidone injection brain levels were lower than in control mice (Figure: 1E). Possible treatment effects on the metabolism of risperidone were reflected by the metabolic ratio (i.e. mother compound to metabolite). As the metabolic ratio is below 1.0 whenever quantifiable (Figures 1A/C, Table 1) changes in risperidone metabolism by dexamethasone have to be taken into account (Figure 2).

Brain and serum levels (active moiety) decreased significantly after 11 days of PCN pre- treatment at all 5 time points (Figure: 2 E/F). Differentiating between mother compound and metabolite, brain and serum levels of risperidone were reduced as early at 0.5h after injection while 9-hydroxyrisperidone levels were decreased starting at 2 hours after injection (Figures: 2A-D). Effects of PCN treatment on risperidone metabolism were more pronounced when compared to those of dexamethasone treatment (Table 1).

Resulting brain risperidone levels from 11 days of either dexamethasone or PCN treatment were not significantly different; however, 9-hydroxyrisperidone brain levels were markedly increased in brains of dexamethasone treated mice (Figures: 1C and 2C) and to some extent in the serum of these animals (Figure 3).

Area Under the Data (AUD) was used to elucidate effects of vehicles used for the different sub chronic treatments. AUD curves demonstrated that corn oil compared to NaCl had an impact on risperidone and 9-hydroxyrisperidone serum levels and much more pronounced on brain-levels (Figure: 3A/B). The mother compound and metabolite levels were significantly increased in brain 1h after risperidone treatment and 11 days of corn oil application (1A/C, 2A/C 1h to 6h).

Eleven days of dexamethasone and PCN treatment presumably by induction of P-gp affected risperidone-induced changes in RotaRod behavior (Figure: 4). In case of PCN treatment, behavioral performance displayed significant differences 4 hours after treatment and lasted up to 8 hours. The 2-way ANOVA revealed significant effects over time (factor A $F(5; 170) = 176.78$, $p<0.001$) over treatment (factor B $F(1; 34) = 5.34$ $p<0.05$) and for AxB interaction $F(5; 170) = 5.63$ $p<0.001$. In the case of 11 day dexamethasone treatment RotaRod behavior was statistically different already half an hour after the injection of risperidone and lasted up to 8 hours. Statistical analysis revealed significant behavioral effects over time (factor A F

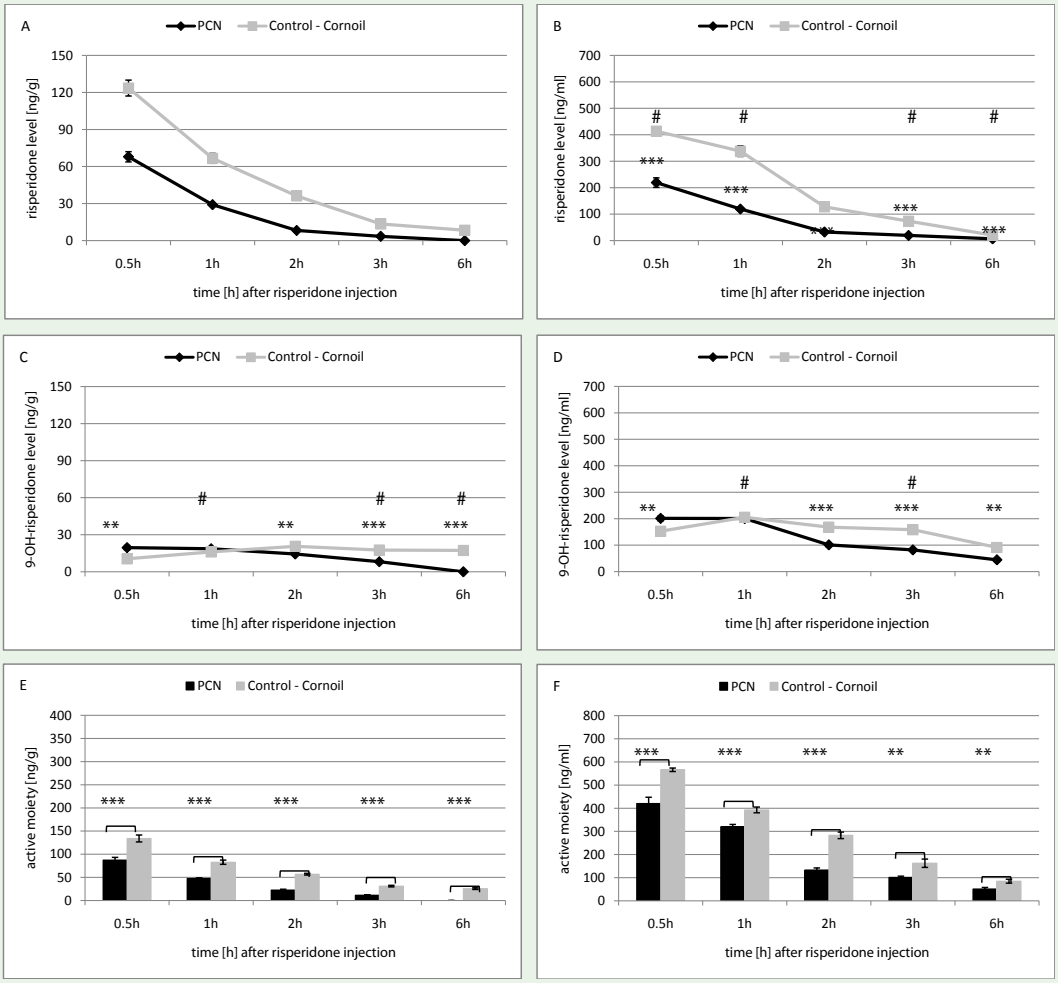

Figure 2: Concentration time profiles after induction of p-glycoprotein by 25 mg/kg/d PCN for risperidone in the upper row (A, B) and for its metabolite 9-hydroxyrisperidone in the middle row (C,D). Brain levels were displayed in the left column (A,B) while serum levels were shown in the right column (B,D). The active moiety i.e. sum of risperidone and 9-hydroxyrisperidone is shown in the lower row for brain levels on the left (E) and for serum levels on the right (F). Mice treated with PCN = black lines, and controls = grey lines. Asterisks highlight significant differences indicated by post-hoc comparison of group means at various time points. Data are presented as mean +/- standard error of the mean (S.E.M).
(* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)
in addition indicates statistical significant differences between control mice i.e. NaCl (shown in figure 1) vs. corn oil

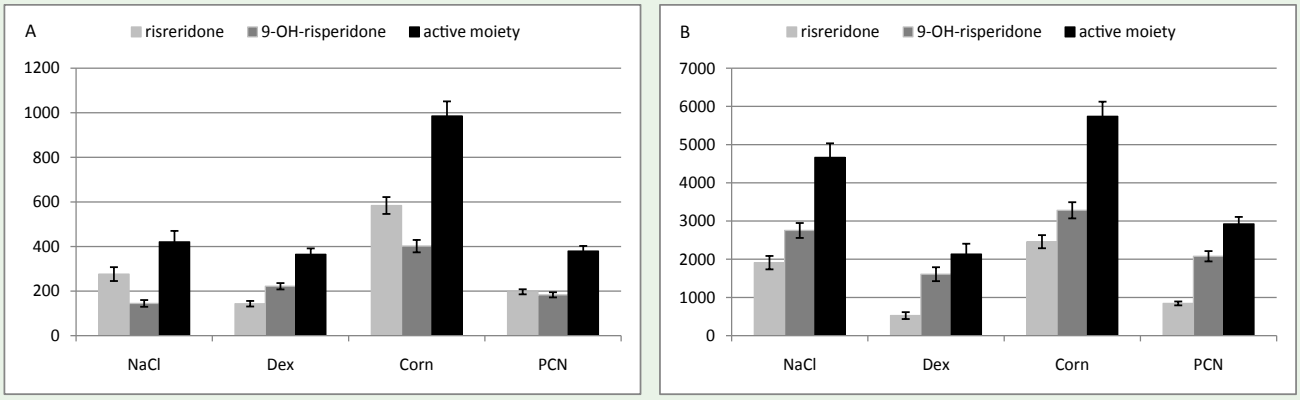

Figure 3: AUD (Area Under the Data) values for brain levels (A) and serum levels (B) for the different treatment conditions:
NaCl = saline injected control group for dexamethasone treated mice; Dex = dexamethasone treated mice; Corn = corn oil injected control group for PCN treated mice; PCN = PCN treated mice. Data are presented as means +/- Standard Error of The Mean (SEM).

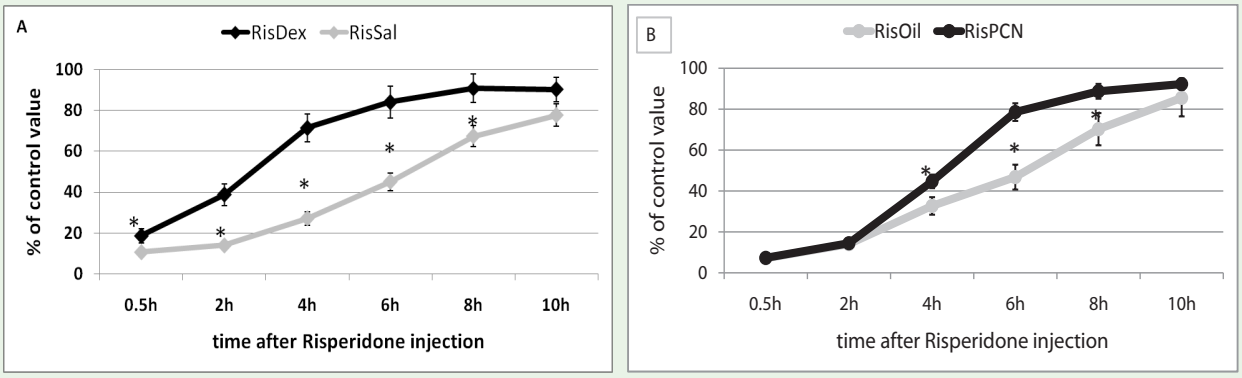

Figure 4: Pharmacodynamic consequences displayed by RotaRod performance after risperidone injection of mice treated either with the P-glycoprotein inducer dexamethasone (A) or PCN (B) Data points represent means +/- standard error of the mean values (SEM). Asterisks (* $p < 0.05$) highlight significant differences indicated by post-hoc comparison of group means at various time points.The dark line represents valuesof inducer-treated mice compared to the respective control mice (grey line).
RisSal =control condition Risperidone and saline, RisDex = Risperidoneafter 11 days of dexamethasone treatment, RisOil =control condition Risperidone and corn oil, RisPCN = Risperidone after 11 days of PCN treatment.

(5; 185) =115.30, p<0.001) over treatment (factor B F (1; 37) =21.39 p<0.001) and for AxB interaction F (5; 185) =7.38 p<0.001. While statistical analysis revealed no effect of the solvent corn oil (factor A time F (5; 185) =129.14 p<0.001, factor B solvent F(1;37)=0.235 n.s), AxB F(5;185) =0.60 n.s.), direct comparison of the two P-gp-inducing drugs revealed stronger effects of dexamethasone than PCN (factor A time F(5;170)=167.52 p<0.001, factor B P-gp related treatment F(1;34)=4.30 p<0.05, AxB F(5;170) =5.17 p<0.001) i.e. risperidone affected mouse behavior to a lesser extent when they received dexamethasone treatment before. Statistical post-hoc results were displayed in figures 4A and 4B. All together the result presented here indicated drug-drug interactions with a clear influence on drug distribution and there for effectiveness of treatment exemplified by *in vivo* behavioral differences (Figure 4).

Discussion

The efflux transporter P-glycoprotein regulates at the BBB the efficacy of several psychotropic drugs such as antiepileptic, antidepressant and antipsychotic drugs [6,7,9-12,23]. P-gp mediated transport is subject to modulation by either inhibition or induction, which can affect pharmacokinetics, pharmacodynamics, efficacy or safety of its substrates [24]. Our animal study using risperidone as model substance for P-gp-substrates indicates that induction of P-gp affects the disposition of such drugs substantially increasing blood levels and decreasing brain concentrations. The supposed increase of P-gp expression was indicated by decreased brain levels of risperidone and 9-hydroxyrisperidone as a surrogate parameter [7,25]. Decreased brain levels after induction of P-gp were already reported [15]. In present investigation modulation of the BBB transporter was expanded to a sub chronic treatment regime. The longer treatment resulted in comparable effect sizes indicating no habituation but maybe a ceiling effect. This, however, is important in clinical settings when drugs are given chronically. In addition clear evidence was given that P-gp induction affects not only drug levels but thereby has also functional consequences. While the ability to balance on a rotating rod is decreased in P-gp k.o. mice [7], present results demonstrated an extenuated effect after treatment with dexamethasone or PCN. The effect of an improved ability to stay on the rod besides similar risperidone treatment as reported here is in line with the reduction of risperidone brain levels responsible for the motor impairment. Both P-glycoprotein inducers decreased brain concentrations of risperidone active moiety by approximately 15 percent after 11 days of dexamethasone, and 60 percent after PCN treatment respectively. Thus the results indicated that PCN had the strongest effect on brain levels of risperidone active moiety. This again highlights the clinical relevance of such mechanisms, as relevant brain levels are important for any antipsychotic effect.

The influence of P-gp expression levels on risperidone and 9-hydroxyrisperidone brain levels has been reported repeatedly in mice [5,7,15] and in humans in the context of P-gp polymorphisms [16-18,25-30]. Although in humans P-gp deficiency has not been reported so far, however, a recent investigation was able to demonstrate that polymorphisms in the human ABCB1 gene influenced the pharmacokinetics of risperidone and 9-hydroxyrisperidone [17]. This indicates that modulation of P-gp expression could affect clinical outcomes. The results of our *in vivo* investigation in mice supported the relationship between supposed up-regulation of P-gp by treatment with dexamethasone or PCN and resulting brain levels of P-gp substrates. The mechanism by which dexamethasone and PCN modulate P-gp is possibly via transcription factors regulating P-gp expression [31]. Expression at the blood-brain barrier is linked to PXR activation and species differences in substrate affinities of this transcription factor have been described [32,33]. PCN selectively activates murine PXR, whereas dexamethasone activates both murine and human PXR [32]. P-gp expression can also be modulated by the Constitutively Expressed Androstane Receptor (CAR) and receptor crosstalk between PXR and CAR is evidenced [34]. Both receptors act as heterodimers with the retinoid X receptor for example and bind to common response elements [34]. There are data demonstrating that nuclear receptor (PXR; CAR) crosstalk can lead to modulation of the expression of multiple cytochrome enzymes and transporter proteins in humans [35,36]. Dexamethasone may also influence gene transcription pathway through the glucocorticoids receptor. Pregnane X receptor also regulates the expression of the multidrug resistance- associated proteins isoform 2 and 3, which are two other important transporters at the blood-brain barrier [37,38]. It has also previously been shown, that the expression of the efflux transporters

P-glycoprotein and Breast Cancer Resistance Protein (BCRP) is partly mediated by glucocorticoid receptor activation of dexamethasone, while multidrug resistance- associated protein 2 expressions showed a glucocorticoid receptor independent effect [39]. Multidrug resistance- associated protein 2 is highly co-expressed with P-gp [38] and is also, like P-gp and BCRP, located at the apical membrane of the blood-brain barrier [40,41]. Multidrug resistance- associated protein 2 is possibly able to take over P-gp or enhance pharmacokinetic effects if a substance is a substrate of both efflux transporters. Up to now potential drug-drug interactions by induction of P-pg or other transporters have so far not attracted much attention in psychiatry. However, evidence for clinical relevance is growing [42,43,44]. Which of those mechanisms is responsible in present investigation has to be determined in future investigations. Nevertheless, drug-drug interaction effects demonstrated indicate the risk for diminished treatment response due to a lack of active compound at the target organ. Or the respective risk of increased unwanted effects due to higher dosing to achieve appropriate treatment.

Besides induction of P-gp expression effects of dexamethasone and PCN on drug metabolizing cyp450 enzymes must also be considered [45]. The present investigation also revealed an increased metabolism of risperidone in dexamethasone treated mice as indicated by a metabolic ratio (i.e. mother compound to metabolite) of below 1.0 whenever quantifiable (Figures 1A/C, Table 1). Significantly increased 9-hydroxyrisperidone brain levels especially after dexamethasone treatment (Figure 1B) indicated the impact on drug metabolizing enzymes as well and account for higher active moiety at early time points. As a result of increased metabolism in dexamethasone treated mice 9-hydroxyrisperidone brain levels were markedly increased compared to PCN treated mice. This however, might account for the weaker impairment of dexamethasone treated mice in the rotarod test at the early time point (Figure 4), an indication for a weaker impact of 9-hydroxyrisperidone on mouse behavior compared to risperidone itself. This is supported by clinical investigations reporting less side effects of paliperidone (9-hydroxyrisperidone) compared to risperidone [46].

Previous studies demonstrated that PCN and dexamethasone induce CYP3A isoenzymes in rodents [45,47-51]were able to show that both P-gp inducers have the ability to induce cyp3a11, the mouse equivalent of cyp3a4 which is involved in the metabolism of risperidone. Enhanced biotransformation by hepatic cytochrome enzymes can also decrease brain and serum levels of risperidone and 9-hydroxyrisperidone by reinforced excretion of the drugs. Subsequently, the results of the present study indicate a third contributing factor: the use of corn oil as solvent for PCN. The comparison of AUD values (area under the data) showed a difference between both control groups. Indicating particular higher brain levels of risperidone and 9-hydroxyrisperidone (Figure 3) an effect driven by a decreased elimination (Figures 1/ 2 B/D). However, one has to bear in mind that corn oil was given for 11 days but the last time 24h before the risperidone.

Conclusion

In conclusion, by using risperidone as model compound, we were able to show a relevant pharmacological effect of increased P-glycoprotein transport activity. This is clearly evidenced by the fact that brain and serum levels of risperidone and its active metabolite can be influenced by co-administered drugs. This resulted in different in effect sizes of the antipsychotic-treatment measured by behavioral impairment. Further studies are needed to clarify underlying mechanisms and the involvement of other transporters should also be considered, since the observed effects may be relevant in the clinic in case of treatment failure.

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