Chapter 8

P-glycoprotein activity in the rat brain is affected by HSV-1 induced neuroinflammation and antipsychotic treatment: implication in treatment resistant schizophrenia

Janine Doorduin, Erik F.J. de Vries, Rudi A. Dierckx and Hans C. Klein

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Abstract

Schizophrenia is a chronic and disabling brain disease, with a high percentage (20-40%) of patients being resistant to antipsychotic treatment. The drug efflux transporter P-glycoprotein (P-gp) might play a role in treatment resistance. Neuroinflammation was recently shown to be present in schizophrenic patients and may influence P-gp activity or expression and thus treatment resistance. The aim of this study was to determine, in a rat model of herpes encephalitis, if neuroinflammation and antipsychotic drugs affected P-gp activity. Rats were intranasally inoculated with the herpes simplex virus type-1 (HSV-1) on day 0. Rats were treated with saline, clozapine or risperidone from day 0 until day 4 post-inoculation. Positron emission tomography with $^{11}$C-verapamil was used to study the activity of P-gp at the blood-brain barrier at day 6 post-inoculation. Plasma and tissue time-activity curves were used to calculate the $^{11}$C-verapamil distribution volume.

Inoculation with HSV-1 resulted in a decrease of the $^{11}$C-verapamil distribution volume in the brainstem and cerebellum (-22 and -21%, p<0.05), when compared to control rats. Clozapine further decreased the $^{11}$C-verapamil distribution volume in the brainstem (-6%, p<0.05), while risperidone increased the distribution volume of $^{11}$C-verapamil in the parietal/temporal/occipital cortex and in the cerebellum (15 and 16%, p<0.05), when compared to saline treatment.

HSV-1 induced neuroinflammation was found to increase the activity of P-gp at the blood-brain barrier. Clozapine further increased P-gp activity, whereas risperidone treatment counteracted the effect of HSV-1 on P-gp function. These results encourage future studies in schizophrenic patients to elucidate the role of neuroinflammation, antipsychotic treatment and P-gp in treatment resistance in schizophrenia.
Introduction

Schizophrenia is a chronic and disabling brain disease that affects about 1% of the population world-wide [1]. Although many antipsychotic drugs can be used to treat schizophrenia patients, between 20 and 40% of the patients are resistant to antipsychotic treatment [2]. Although treatment resistant patients show a considerable clinical heterogeneity and the causes of resistance are likely to be multifactorial [3], the drug efflux transporter P-glycoprotein (P-gp) might play an important role in treatment resistance in schizophrenia.

P-gp is the product of the multidrug resistance 1 (MDR1) gene and is expressed in healthy tissues, including intestine, liver, kidney and the blood-brain barrier (BBB), but also in cancer cells [4]. P-gp is an efflux pump that protects the brain and other vital tissues from harmful substances. Many pharmacological agents that are used to treat cancer and brain diseases, like epilepsy and schizophrenia, are substrates of P-gp, resulting in reduced treatment efficacy. The activity and expression of P-gp is influenced by a variety of factors, including drugs, glutamate and also gene polymorphisms [5]. In addition, neuroinflammation has been shown to regulate the activity and expression of P-gp at the BBB, which is possibly mediated by pro-inflammatory cytokines [6]. In vivo and ex vivo studies have shown conflicting results on the effect that neuroinflammation can have on activity and expression of P-gp [7-10]. Possibly, this effect depends on the time and nature of neuroinflammation (e.g. acute vs. chronic).

It has been shown that (recent-onset) schizophrenia is associated with global neuroinflammation [11-13] and that focal neuroinflammation is a feature of schizophrenia related psychosis [14]. Neuroinflammation may increase P-gp activity or expression at the BBB in a subpopulation of schizophrenic patients, which could explain treatment resistance in schizophrenia.

P-gp activity can be determined using positron emission tomography (PET) as a non-invasive imaging technique. The P-gp substrate [11C]-verapamil has frequently been used as a PET ligand for measuring P-gp activity [15]. P-gp activity can be quantified by pharmacokinetic modeling. It has recently been shown that [11C]-verapamil pharmacokinetics are well described by a two-tissue compartment model or Logan analysis without correction for radioactive metabolites [16].

To study the role of neuroinflammation on P-gp activity at the BBB, we used a rat model of herpes encephalitis. Intranasal inoculation with HSV-1 results in invasion of
the brain by HSV-1, causing herpes encephalitis which is accompanied by severe neuroinflammation [17]. This model does not require invasive manipulations and toxic compounds to evoke neuroinflammation, which may lead to changes in the permeability of the blood brain barrier. Because P-gp might play a role in treatment resistance to antipsychotics, it is also of interest to study the effect of antipsychotics on P-gp activity. In vitro, the atypical antipsychotics clozapine and risperidone were both found to have affinity for P-gp, with risperidone having the highest affinity [18-20]. To study the indirect effect of atypical antipsychotics on P-gp activity, rats were subchronically treated with clozapine and risperidone until 48 hours before the PET study. Thus, the aim of the present study was to determine the effect of neuroinflammation and treatment with clozapine or risperidone on the activity of P-gp at the BBB.

Material and methods

Animals

Male outbred Wistar-Unilever (SPF) rats (264±26 gram) were obtained from Harlan (Lelystad, The Netherlands). The rats were individually housed in Macrolon cages (38x26x24 cm) on a layer of wood shavings in a room with constant temperature (21±2°C) and fixed, 12-hour light-dark regime (light phase from 7:00–19:00 hours). Food (standard laboratory chow, RMH-B, Hope Farms, The Netherlands) and water were available ad libitum. After arrival, the rats were randomly divided into six groups: control rats (control) treated with either saline (n=6), clozapine (n=6) or risperidone (n=5) and rats inoculated with HSV-1 (HSE) treated with either saline (n=5), clozapine (n=4) or risperidone (n=5), and allowed to acclimatize for at least seven days. All experiments were approved by the Animal Ethics Committee of the University of Groningen, The Netherlands.

HSV-1 inoculation

The HSV-1 strain was obtained from a clinical isolate, cultured in Vero-cells and assayed for plaque forming units (PFU) per milliliter. On day 0, the rats were slightly anaesthetized with 5% isoflurane (Pharmachemie BV, The Netherlands) and inoculated with HSV-1 by application of 100 μl of phosphate-buffered saline with 1x10^7 PFU of virus in the nostrils (50 μl per nostril) with a micropipette. Control rats
were treated similarly by application of 100 μl PBS without virus. Clinical symptoms in all rats were scored daily post-inoculation by the same observer.

**Drugs and treatment**

Clozapine was obtained from Sigma-Aldrich Inc (Saint Louis, Missouri, USA) and risperidone was obtained from MP Biomedicals (Irvine, California, USA). Clozapine and risperidone were dissolved in a minimal volume of 0.1 M HCl, and diluted with saline. The pH was adjusted to 6-7 with 0.1 M NaOH. The final concentration was 4.3 mg/ml for clozapine and 0.35 mg/ml for risperidone, and the total injection volume was 0.3-0.7 ml. The solutions were freshly made prior to injection.

Rats were treated with clozapine (10 mg/kg/day i.p.) or risperidone (0.5 mg/kg/day i.p.) from day 0 until day 4 post-inoculation. Control rats were treated similarly with saline. It was expected that five days of treatment in rats is sufficient to induce changes in P-gp activity.

**[11C]-Verapamil PET**

[11C]-Verapamil was synthesized as described previously [21]. After synthesis, [11C]-verapamil was formulated in ethanol/water (10/90). The radiochemical purity was always >99% and the specific activity was always > 4000 GBq/mmol.

Small animal PET scans were performed on day 6 post-inoculation, at least 48 hours after the last drug treatment. To allow for arterial blood sampling during the PET scan, a canula was inserted in the femoral artery prior to the PET scan. After canulation, the rats were positioned in the small animal PET camera (Focus 220, Siemens Medical Solutions USA, Inc.) in transaxial position with their heads in the field of view. A transmission scan of 515 seconds with a Co-57 point source was obtained for the correction of attenuation by tissue. After the transmission scan was completed, the PET tracer [11C]-verapamil (63±16 MBq) was injected via the penile vein. Simultaneously with the injection of the PET tracer a dynamic emission scan of 3600 seconds was started. Blood samples of 0.1 ml were taken at 15, 30, 45, 60, 75, 90, 120, 150, 300, 450, 600, 900, 1800 and 3600 seconds after injection. After a blood sample was taken, 0.1 ml of heparinized saline was injected into the artery to prevent large changes in blood pressure. The blood samples were centrifuged at 130,000 rpm (15,996 x g) for 5 minutes and the activity in plasma was measured using a gammacounter (LKB Wallac, Turku, Finland). The plasma-activity curve was corrected for decay.
The list-mode data of the emission scan was separated into 21 frame sinograms (8x30, 3x60, 2x120, 2x180, 3x300 and 3x600 seconds), which were iteratively reconstructed (OSEM2d, 4 iterations, 16 subsets) after being normalized and corrected for attenuation, scatter, randoms and decay.

PET image analysis

PET image analysis was performed using the Clinical Applications Packaging Program (CAPP5). Regions of interest were drawn around the bulbus olfactorius, frontal cortex, striatum, thalamus, parietal/temporal/occipital cortex, midbrain, brainstem and cerebellum in a template PET scan that was co-registered with the PET scan of interest by image fusion. The time-activity curves of these regions of interest were used for kinetic modeling using software developed in Matlab 7.1 (Mathworks, Natick, Massachusetts). The Logan analysis [22] was used to calculate the distribution volume (DV) of $[^{11}\text{C}]$-verapamil using arterial plasma as the input.

Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS for Windows, version 14.0.2. Statistical analysis on the clinical symptoms, $[^{11}\text{C}]$-verapamil DV and $[^{11}\text{C}]$-verapamil influx ($k_1$) were performed by one-way ANOVA with a LSD post hoc test. Significance was reached when the p value was ≤0.05.

Results

Clinical symptoms

Clinical symptoms (figure 1) were scored daily up to six days post inoculation and categorized into the following clinical scores: (0), no symptoms; (1), ruffled fur and irritated mouth, nose and eyes; (2), behavioral signs, like stress and lethargy, and hunched posture; (3), posterior paralysis and impairment of motor function and (4), severe paralysis, labored breathing or death. For rats inoculated with HSV-1 and treated with saline, the first clinical symptoms appeared on day 2 post-inoculation. The severity of the clinical symptoms increased gradually over time and at day 6 post-inoculation most rats (60%) had a clinical score of 3. For the HSV-1 inoculated rats that were treated with clozapine and risperidone a delay in the onset of clinical
symptoms was found. The first clinical symptoms in these rats were seen at day 5 post-inoculation, which is one day after the last treatment (at day 4). Of the clozapine treated rats, 50% did not show any clinical symptoms at day 6 post-inoculation, while 60% of the risperidone treated rats had a clinical score of 2. No statistically significant differences were found in the averages of the clinical scores in saline (2.0±1.4), clozapine (1.3±1.5) and risperidone (2.6±0.5) treated rats on day 6 post-inoculation. None of the control rats showed any clinical symptoms.

**Figure 1** Clinical scores of rats inoculated with HSV-1 on day 1 to day 6 post-inoculation that were treated with either saline (n=5), clozapine (n=4) or risperidone (n=5) from day 1 until day 4 post-inoculation. The clinical scores represent the following symptoms: (0), no symptoms; (1), ruffled fur and irritated mouth, nose and eyes; (2), behavioral signs, like stress and lethargy, and hunched posture; (3), posterior paralysis and impairment of motor function and (4), severe paralysis, labored breathing or death.

**Distribution volume of [\textsuperscript{11}C]-verapamil**

The images of the [\textsuperscript{11}C]-verapamil DV are displayed in figure 2. The images show low brain uptake, due to efflux of [\textsuperscript{11}C]-verapamil by P-gp, with no visual differences between any of the examined groups. The whole brain DV of [\textsuperscript{11}C]-verapamil is displayed in figure 3 and the DV of [\textsuperscript{11}C]-verapamil in the focal brain regions of interest is displayed in table 1.

HSV-1 infection of the brain resulted in a decrease in the whole brain [\textsuperscript{11}C]-verapamil DV (-15%, p=0.050). In addition to a global decrease in whole brain [\textsuperscript{11}C]-verapamil DV, a statistically significant focal decrease in the [\textsuperscript{11}C]-verapamil DV was found in the cerebellum (-22%, p=0.013) and in the brainstem (-21%, p=0.013).
Figure 2 Full-color in appendix. Small animal PET images of the $[^{11}C]$-verapamil distribution volume (DV) on day 6 post-inoculation in control rats (control) and rats inoculated with HSV-1 (HSE) that were treated with saline, clozapine or risperidone from day 0 until day 4 post-inoculation. The images display a coronal plane of the rat head, at the level of the brainstem.

Treatment of control rats with clozapine resulted in a statistically significant decrease in the whole brain $[^{11}C]$-verapamil DV (-15%, $p=0.036$), when compared to saline treatment. A statistically significant focal decrease in $[^{11}C]$-verapamil DV after clozapine treatment was found in the brainstem (-16%, $p=0.040$), when compared to saline treated rats. Risperidone treatment did not significantly alter whole brain $[^{11}C]$-verapamil DV ($p=0.146$). However, a statistically significant focal increase in DV was found in the parietal/temporal/occipital cortex (15%, $p=0.029$) and in the cerebellum (16%, $p=0.026$), when compared to saline treated rats.

In clozapine treated rats, no significant differences in the $[^{11}C]$-verapamil DV were found between control rats and rats inoculated with HSV-1, either in the whole brain
or in individual brain regions. In the risperidone treated rats, whole brain $^{[11]}\text{C}$-verapamil DV was not different between the rats inoculated with HSV-1 and control rats ($p=0.062$). However, a statistically significant focal decrease in DV was observed in the parietal/temporal/occipital cortex ($-18\%$, $p=0.013$) and the cerebellum ($-28\%$, $p=0.001$) of HSV-1 infected rats, as compared to control rats.

**Figure 3** The whole brain distribution volume of $^{[11]}\text{C}$-verapamil on day 6 post-inoculation in control rats (control) and rats inoculated with HSV-1 (HSE), that were treated with saline, clozapine or risperidone from day 0 up until day 4 post-inoculation. Data are presented as mean ± standard deviation. *$p<0.05$ when compared to control rats.

**Table 1** Distribution volume of $^{[11]}\text{C}$-verapamil on day 6 post-inoculation in control rats (control) and rats inoculated with HSV-1 (HSE), that were treated with saline, clozapine or risperidone from day 0 up until day 4 post-inoculation. Data are presented as mean ± standard deviation. *$p<0.05$ when compared to control rats, **$p<0.005$ when compared to control rats and # $p<0.05$ when compared to saline treated rats.

<table>
<thead>
<tr>
<th></th>
<th>Saline (n=6)</th>
<th>HSE (n=5)</th>
<th>Clozapine (n=6)</th>
<th>HSE (n=4)</th>
<th>Risperidone (n=5)</th>
<th>HSE (n=5)</th>
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<tr>
<td>Bulbus olf</td>
<td>1.14±0.12</td>
<td>1.00±0.34</td>
<td>0.96±0.12</td>
<td>0.79±0.20</td>
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<td>Frontal ctx</td>
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<td>0.91±0.24</td>
<td>0.78±0.12</td>
<td>0.68±0.24</td>
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<td>Striatum</td>
<td>0.78±0.22</td>
<td>0.68±0.15</td>
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<td>0.60±0.09</td>
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<td>Cerebellum</td>
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<td>0.74±0.11*</td>
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<td>0.67±0.13</td>
<td>1.12±0.10*</td>
<td>0.81±0.11**</td>
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<td>Brainstem</td>
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<td>0.70±0.13</td>
<td>1.06±0.10</td>
<td>0.92±0.14*</td>
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*Ctrl, control; P/T/O, Parietal/Temporal/Occipital
Influx ($k_1$) of [$^{11}$C]-verapamil

The DV of [$^{11}$C]-verapamil is dependent on both the influx ($k_1$) of [$^{11}$C]-verapamil into brain tissue and the P-gp related efflux ($k_2$) from the brain. Changes in the influx of [$^{11}$C]-verapamil, due to for example changes in the permeability of the BBB, could explain the change in [$^{11}$C]-verapamil DV. In the present study, however, no statistically significant differences in the whole brain $k_1$ were found between control rats and rats infected with HSV-1 treated with saline (0.26±0.12 vs. 0.19±0.11, $p=0.490$). When compared to saline treatment, clozapine treatment did not affect the $k_1$ of [$^{11}$C]-verapamil in control (0.26±0.12 vs. 0.22±0.02, $p=0.676$) or HSE rats (0.19±0.11 vs. 0.14±0.07, $p=0.611$). Risperidone treatment, when compared to saline treatment did not change the $k_1$ of [$^{11}$C]-verapamil in control (0.32±0.16 vs. 0.26±0.12, $p=0.586$) or HSE rats (0.34±0.36 vs. 0.19±0.11, $p=0.211$). In addition to the findings in whole brain $k_1$ of [$^{11}$C]-verapamil, no statistically significant differences were found in the $k_1$ of [$^{11}$C]-verapamil in the focal regions of interest, in saline and clozapine treated rats. In contrast, risperidone treatment caused a statistically significant increase in $k_1$ in the parietal/temporal/occipital cortex ($p=0.011$) of rats infected with HSV-1, when compared to saline treated rats.

Discussion

Since 20 to 40% of the schizophrenic patients were found to be resistant to antipsychotic treatment, it is of importance to gain more insight into the mechanism behind treatment resistance in order to improve treatment strategies. We hypothesized that neuroinflammation, which is proposed to play a role in schizophrenia, has a role in regulating P-gp activity.

In the present study, we have demonstrated that HSV-1 infection caused increased P-gp activity at the BBB, in particular in the brainstem and cerebellum. Recently, we have shown that neuroinflammation in the rat model of herpes encephalitis is most pronounced in these brain areas [23]. The change in P-gp activity in these brain areas is therefore most likely caused by the presence of HSV-1 induced neuroinflammation. Because it has been shown that schizophrenia is associated with neuroinflammation, treatment resistance may thus be caused by neuroinflammation induced changes in P-gp activity. The increased P-gp activity due to neuroinflammation is consistent with previous findings related to the effect of pro-inflammatory cytokines on P-gp expression. It has recently been shown *in vitro* that the pro-inflammatory cytokine
tumor-necrosis factor alpha increased P-gp activity and expression [7,10]. In contrast, it has also been reported that the intraventricular injection of lipopolysaccharide resulted in a decrease in \textit{in vivo} P-gp expression [8]. As already mentioned by Miller \textit{et al.} [9], it is plausible that the first response to pro-inflammatory cytokines is a decrease in P-gp activity, whereas the delayed response could cause an increase in P-gp activity and expression. Although a dynamic regulation of the P-gp expression by pro-inflammatory cytokines in time seems likely, the time span may vary between different \textit{in vitro} and \textit{in vivo} models.

Because P-gp is suggested to play a role in resistance to antipsychotic treatment, it is of interest to study the effect of antipsychotics on P-gp activity. It has been shown that antipsychotics, including clozapine and risperidone, have affinity for P-gp [18-20], resulting in a decreased treatment efficacy. However, to our knowledge, it has not been studied if chronic treatment with antipsychotics influences P-gp activity. In the present study, we have shown that clozapine treatment of healthy control rats increased P-gp activity in some brain areas. This suggests that clozapine can eventually induce resistance in the normal brain. In treatment resistant schizophrenic patients, however, the atypical antipsychotic drug clozapine was found to be most effective. In contrast to clozapine, risperidone decreased the activity of P-gp, suggesting that risperidone could increase treatment efficacy. It remains to be elucidated how clozapine and risperidone affect P-gp activity, however, increased P-gp activity may be due to increased P-gp expression. The expression of P-gp is under regulation of nuclear receptors (e.g. steroid and xenobiotic receptors) that can bind many different ligands, thereby affecting P-gp activity [5]. Binding of antipsychotics to these nuclear receptors may thus explain the antipsychotic induced changes in P-gp activity.

Taken that a neuroinflammatory response in schizophrenic patients is in part responsible for treatment resistance, it is especially of interest to know the effects of antipsychotics on P-gp activity or expression during a neuroinflammation. Both neuroinflammation and clozapine increased the activity of P-gp at the BBB and these effects were shown to be additive. Treatment of HSV-1 infected rats with risperidone resulted in no net change in P-gp activity, when compared to saline treated control rats, showing that risperidone was effective in inhibiting the increase in P-gp activity induced by neuroinflammation.

Although we have found an effect of neuroinflammation and antipsychotics on the P-gp activity at the BBB, one could argue that the found change in $[^{11}\text{C}]$-verapamil DV is related to changes in $[^{11}\text{C}]$-verapamil metabolism. It was, however, shown by
Lubberink et al. [16] that the $^{11}$C-verapamil time-activity curve was well described by a two-tissue compartment model or Logan analysis without metabolite correction. In addition, the last treatment with clozapine and risperidone was 48 hours before the $^{11}$C-verapamil PET scan, and given the half-life of clozapine and risperidone of 2 to 4 hours in rats, it seems unlikely that the metabolism of clozapine and risperidone affected the formation of $^{11}$C-verapamil metabolites.

**Conclusion**

HSV-1 induced neuroinflammation was found to increase P-gp activity at the BBB. Because neuroinflammation is suggested to play a role in schizophrenia, this is of great interest with respect to treatment resistance in schizophrenic patients. The HSV-1 induced increase in P-gp activity was augmented by the atypical antipsychotic clozapine, but was antagonized by risperidone. Increasing knowledge on treatment resistance in schizophrenia is of importance for improving treatment strategies. These findings may therefore promote further research on the role of P-gp and neuroinflammation in treatment resistant schizophrenia in a clinical setting.

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