Chapter 7

Inhibition of HSV-1 induced behavioral changes and microglia cell activation by antipsychotics

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

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Abstract

Schizophrenia is a severe and chronic brain disease with unknown etiology. It is hypothesized that viral brain infections play a role in schizophrenia. In line with this hypothesis, the present study investigated whether antipsychotics can reduce virus induced activation of microglia cells (neuroinflammation) and behavioral changes in a rat model of herpes encephalitis.

Rats were intranasally inoculated with the herpes simplex virus type 1 (HSV-1). Open field behavior was assessed at day -1 pre-inoculation and on day 2 and 4 post-inoculation. To determine the effect of antipsychotics, clozapine or risperidone was administered from day 0 to day 4 post-inoculation. Positron emission tomography was used to study activated microglia cells with $[11C]-PK11195$ and dopamine D$_2$ receptors with $[11C]$-raclopride at day 5 post-inoculation.

Inoculation with HSV-1 resulted in significantly increased open field exploration on day 4 post-inoculation, but did not significantly change locomotion, anxiety and arousal. Activated microglia cells were found in the brainstem, frontal and cortical brain areas of HSV-1 infected rats. Treatment with clozapine and risperidone resulted in inhibition of microglia cell activation and inhibition of increased exploration in HSV-1 infected rats. $[11C]$-Raclopride binding was not affected by HSV-1 infection or treatment with antipsychotics.

Behavioral changes and microglia cell activation in response to HSV-1 infection of the brain were inhibited by the antipsychotics clozapine and risperidone, while no effect of D$_2$ binding was found. Additional research on the role of HSV-1 and the accompanied microglia cell activation as potential targets for the treatment of schizophrenia seems justified.
Introduction

Schizophrenia is a severe brain disease that affects approximately 1% of the world’s population [1]. The symptoms of schizophrenia usually have its onset during adolescence or early adulthood and are classified into positive symptoms, such as hallucinations and delusions, negative symptoms, such as social withdrawal and flattened emotion, cognitive symptoms and mood symptoms. To reduce symptoms, schizophrenic patients are in general treated with antipsychotic drugs. The typical (classical) antipsychotic drugs were the first drugs used in the treatment of schizophrenia and were effective in reducing the positive symptoms of schizophrenia, primarily by blocking dopamine receptors. The reduction of symptoms by typical antipsychotics has led, amongst others, to the hypothesis that dopamine is one of the major neurotransmitter systems involved in schizophrenia. Starting with the discovery of clozapine as an antipsychotic drug that not only reduced the positive symptoms, but also the negative, cognitive and mood symptoms, several new atypical antipsychotic drugs have been introduced, including olanzapine and risperidone. The increased effectiveness of the atypical antipsychotic drugs is attributed to the fact that they do not only block dopamine receptors, but also bind to a variety of other neurotransmitter receptors. Amongst others, this suggested the involvement of these neurotransmitter systems in schizophrenia.

Despite extensive research, including studies involving the variety of neurotransmitter systems, the exact etiology remains unknown. Genetic and environmental factors are thought to be responsible for the development of schizophrenia and therefore the deficits in neurotransmission. Related to the environmental factors, it has been hypothesized that herpes viruses play a role in the etiology of schizophrenia [2]. Herpes viruses are a family of large DNA viruses, of which eight types are known to cause disease in humans [3]. Schizophrenic patients were found to have an increase in the serum antibodies against the herpes simplex virus type-1 (HSV-1) and the cytomegalovirus (CMV) [2], which were associated with cognitive deficits in schizophrenic patients [4,5]. In addition, brain morphological changes were associated with exposure to HSV-1 [6,7]. In addition, treatment of CMV positive schizophrenic patients with the anti-viral drug valaciclovir resulted in a reduction in the overall schizophrenic symptoms (positive, negative and cognitive) [8]. HSV-1 may be of particular interest because either reactivation of latent HSV-1 in the trigeminal ganglion or primary infection with HSV-1 can result in herpes encephalitis, mainly involving the temporal lobe and limbic structures. Consequently, patients with herpes...
encephalitis present with changes in consciousness, confusion and can also reveal psychosis in the prodromal phase [9-11].

A consequence of HSV-1 infection of the brain is the development of a neuroinflammatory response, characterized by the presence of activated microglia cells. In patients with a herpes encephalitis, caused by HSV-1, activated microglia cells were found beyond the primary focal lesion, which persisted many months after antiviral treatment [12]. In rats infected with HSV-1, we and others have showed widespread activation of microglia cells in brain areas infected with HSV-1 [13-15]. Herpes virus infection of the brain is thus accompanied by the activation of microglia cells and if herpes viruses do indeed play a role in schizophrenia, a neuroinflammatory response is expected in schizophrenic patients. Indeed, activated microglia cell were found to be present in the post-mortem schizophrenic brain [16]. In addition, functional imaging studies with positron emission tomography (PET) have shown a global activation of microglia cells in first-episodes schizophrenic patients [17] and a focal, hippocampal activation of microglia cells in psychotic schizophrenic patients [18].

Related to herpes virus infection of the brain and the accompanied activation of microglia cells, it has been suggested that neuroleptic drugs have antiviral and anti-inflammatory properties. The neuroleptic drug chlorpromazine, used to treat schizophrenia patients, was found to reduce HSV-1 activity [19]. In vitro studies on the effect of antipsychotic drugs on inflammation, showed that risperidone and haloperidol were found to inhibit the production of pro-inflammatory cytokines by microglia cells treated with interferon-γ [20]. In addition, olanzapine was also found to reduce the production of nitric oxide by lipopolysaccharide-stimulated microglia cells [21].

Based on the proposed role of herpes virus infections and neuroinflammation in schizophrenia, we hypothesized that antipsychotic drugs may in part improve schizophrenic symptoms by reducing neuroinflammatory processes. Therefore we investigated the effect of antipsychotic drugs on HSV-1 induced behavioral changes and neuroinflammation in a rat model of HSV-1 encephalitis. In this model rats are intranasally inoculated with HSV-1, resulting in transport of the virus from the olfactory epithelium towards the brain. This causes a direct and controlled dissemination of HSV-1 within the brain, involving brain areas that correspond to the frontal, orbital and temporal cortices in humans [13,22]. Behavioral studies in rats infected with HSV-1 showed the presence of learning impairment [23] and an increase
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in motor activity and aggression [24]. Inoculation of 2-day old pups with HSV-1 resulted in impairment in sensorimotor gating at adulthood [25], which has also been found to be impaired in schizophrenic patients [26].

To study behavior in HSV-1 infected rats, we exposed the rats to an open field arena to study locomotion and exploratory behavior. Activation of microglia cells was determined using PET, with the tracer $[^{11}\text{C}]$-PK11195. $[^{11}\text{C}]$-PK11195 is a ligand of the peripheral benzodiazepine receptor that is overexpressed in activated microglia cells and can therefore be used as a marker for imaging of neuroinflammation. To investigate the effect of antipsychotics on HSV-1 induced behavior and neuroinflammation, rats were treated with the atypical antipsychotics clozapine and risperidone. Clozapine and risperidone are the most commonly used antipsychotics in treatment of schizophrenia patients and were found to bind to different neurotransmitter receptors. While clozapine has moderate affinity for most neurotransmitter receptors, including dopaminergic and serotonergic receptor, risperidone has a higher affinity for mainly the dopaminergic D$_2$ and serotonergic 5HT$_2A$ receptor. With respect to the different affinity for the dopaminergic D$_2$ and the importance of dopamine in schizophrenia, the effect of HSV-1 infection of the brain and the treatment with antipsychotics on dopamine D$_2$ receptors was studied using the D$_2$ receptor ligand $[^{11}\text{C}]$-raclopride PET as well.

**Material and methods**

$[^{11}\text{C}]$-PK11195

$[^{11}\text{C}]$-PK11195 was labeled by trapping $[^{11}\text{C}]$-methyl iodide [27] in a solution of 1 mg N-desmethyl-PK11195 and 10 mg potassium hydroxide in 300 µl dimethylsulfoxide. The reaction mixture was allowed to react for 1 minute at 40 °C, neutralized with 1M HCl and passed through a 45 µm Millex HV filter. The filtrate was purified by HPLC using a µBondapak C18 column (7.8x300 mm) with acetonitrile/25 mM NaH$_2$PO$_4$ (pH 3.5) (55/45) as the eluent (flow 5 ml/min). To remove the organic solvents from the product, the collected HPLC fraction (retention time 7 min) was diluted with 100 ml of water and passed through an Oasis HLB 30 mg (1 cc) cartridge. The cartridge was washed twice with 10 ml of water and subsequently eluted with 0.7 ml of ethanol and 5 ml of water. The product was sterilized by filtration over a 0.20 µm Millex LG filter. The product was obtained in 20-70% radiochemical yield. Quality control was performed by HPLC, using a Novapak C18 column (150x3.9 mm) with
acetonitrile/25 mM NaH₂PO₄ (pH 3.5) (60/40) as the eluent at a flow of 1 ml/min. The radiochemical purity was always >95% and the specific activity was 25-100 MBq/nmol.

[¹⁴C]-Raclopride

[¹⁴C]-Raclopride was labeled by trapping [¹⁴C]-methyl iodide [27] in a solution of 1 mg desmethylraclopride and 1.4 mg sodium hydroxide in 300 µl dimethylsulfoxide. The reaction mixture was allowed to react for 4 minute at 80 °C. After the reaction, the product was purified by HPLC using a µBondapak C18 column (7.8x300 mm) with acetonitrile/10 mM H₃PO₄ (30/70) as the eluent (flow 5 ml/min). To remove the organic solvents from the product, the collected HPLC fraction (retention time 8 min) was diluted with 100 ml of water and passed through an Oasis HLB 200 mg cartridge. The cartridge was washed twice with 8 ml of water and subsequently eluted with 0.8 ml of 1% H₃PO₄ in ethanol and 8 ml of phosphate buffer (pH 7.2). The product was sterilized by filtration over a 0.20 µm Millex LG filter. Quality control was performed by HPLC, using a µBondapak C18 column (300x3.9 mm) with acetonitrile/10 mM H₃PO₄ (30/70) as the eluent at a flow of 1 ml/min. The radiochemical purity was always >95% and the specific activity was 40-100 MBq/nmol.

Animals

Male outbred Wistar-Unilever (SPF) rats (272±30 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 allowed to acclimatize for at least seven days. During acclimatization and during the entire study all rats were handled daily. All experiments were approved by the Animal Ethics Committee of the University of Groningen, The Netherlands.

Study design

The rats were randomly divided into six groups: control rats (control) treated with either saline (n=8), clozapine (n=6) or risperidone (n=5) and rats inoculated with HSV-1 (HSE) treated with either saline (n=8), clozapine (n=6) or risperidone (n=6).
After acclimatization, the study started with an open field experiment on day -1 (figure 1). On day 0, the rats were inoculated with HSV-1 (HSE) or PBS (control) and the open field experiment was repeated on day 2 and 4 post-inoculation. On day 5 post-inoculation, a $[^{11}\text{C}]$-PK11195 and a $[^{11}\text{C}]$-raclopride PET scan were made. Treatment with saline, clozapine or risperidone started on day 0 (at 1 hour post-inoculation) and the last dose was given on day 4. The rats were sacrificed on day 6 post-inoculation to prevent severe discomfort due to HSV-1 infection of the brain. The experiments were performed between 8:00-12:00 hours, with exception of the $[^{11}\text{C}]$-raclopride PET scan that was performed between 13:00-17:00 hours.

**Figure 1** Study design. Rats were inoculated with HSV-1 or PBS at day 0. Open field (OF) experiments were carried out on day -1 pre-inoculation and on day 2 and day 4 post-inoculation. $[^{11}\text{C}]$-PK11195 and $[^{11}\text{C}]$-raclopride small animal PET scans were performed on day 5 post-inoculation. Drug treatment (saline, clozapine and risperidone) started on day 0 and finished on day 4 post-inoculation.

**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 $1\times10^7$ PFU of virus on the nostrils (50 μl per nostril) with a micro pipette. 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. The solutions were made freshly prior to injection.

Rats were treated once daily 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. On day 2 and 4, when the open field experiments were carried out, drug treatment was 1 hour after the open field experiment.

**Open field experiment**

Open field experiments on day -1 pre-inoculation and day 2 and day 4 post-inoculation, were carried out in the light phase of the daily cycle. The open field consisted of a circular black arena with a diameter of 1 m. The rats were placed into the open field near the wall and behavior was recorded for 8 minutes using a video tracking system (Ethovision 1.96, Noldus Information Technology, Wageningen, The Netherlands).

The total distance moved was analyzed as a measure of locomotion, the frequency of rearing as a measure of exploration and the frequency of grooming as a measure of arousal. To measure anxiety, the open field area was subdivided into three circular zones with diameters of 0.33, 0.66 and 1 meter with the centre of the zones in the centre of the open field. The total distance moved in the zone in the centre of the open field (with the diameter of 0.33 m) was used as a reciprocal measure of anxiety (i.e. a high total distance moved in the centre of the open field corresponds to low anxiety).

**PET experiment**

[$^{11}$C]-PK11195 and [$^{11}$C]-raclopride PET scans were performed on day 5 post-inoculation, between 8:00-12:00 hours and 13:00-17:00 hours, respectively. There was always more than two hours (3.5±1.2 hours) between the start of the PET scans to allow for decay of [$^{11}$C]-PK11195. For each PET scan the rats were anaesthetized by 5% isoflurane (Pharmacie BV, The Netherlands) that was mixed with medical air at a flow of 2 ml/min, after which anesthesia was maintained with 2% isoflurane. Following induction of anesthesia, 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 $^{[11]}$C-PK11195 (44±16 MBq) or $^{[11]}$C-raclopride (35±16 MBq) was injected via the penile vein. Simultaneously with the injection of the PET tracer a dynamic emission scan of 60 min was started.

The list-mode data of the emission scans was separated into 21 frames (8x30, 3x60, 2x120, 2x180, 3x300 and 3x600 seconds). Emission sinograms were iteratively reconstructed (OSEM2d, 4 iterations) after being normalized, corrected for attenuation and decay of radioactivity.

**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. For $^{[11]}$C-PK11195, the uptake in these regions of interest in the last 10 min of the PET scan was determined in Bq/cm$^3$ and converted into the standardized uptake value (SUV), which was defined as: [tissue activity concentration (MBq/cm$^3$)]/[injected dose (MBq)/body weight (g)]. It was assumed that 1 cm$^3$ of brain tissue equals 1 gram.

For $^{[11]}$C-raclopride the time-activity curves were used to calculate the binding potential (BP) with a reference tissue model, using software developed in Matlab 7.1 (Mathworks, Natick, Massachusetts). In the reference tissue model, the BP can be calculated without arterial plasma input, using the time-activity curve of a reference tissue that is devoid of the target receptor as the input. Because D$_2$-receptors are not expressed in the cerebellum, this region was chosen as a reference region to calculate the BP in the bulbus olfactorius, frontal cortex, striatum, thalamus, parietal/temporal/occipital cortex, midbrain and brainstem.

**Statistics**

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 scores was performed with a one-way ANOVA with a LSD post hoc test to assess group difference on a given day. The pre-inoculation open field data on locomotion, exploration, arousal and anxiety on day -1 was
analyzed using a one-way ANOVA with a LSD post hoc test. For locomotion on day 2 and 4 post-inoculation, analysis was performed with a univariate general linear model, with locomotion on day -1 as a covariate. Day -1 was used as a covariate to correct for individual differences in locomotion already present before inoculation. For exploration, arousal and anxiety on day 2 and day 4 post-inoculation, analysis was performed using a univariate general linear model with as covariates the exploration, arousal and anxiety on day-1 respectively, and the locomotion on the corresponding day (i.e. locomotion on day 2 was used as a covariate for analysis of behavior on day 2). Day -1 was used as a covariate for the same reason as mentioned for locomotion, being that it allows correction for individual differences. Locomotion can be a confounder in the other behavioral outcome measures and was therefore used as a covariate in the analysis of the other behavioral outcome measures. The $^{[11]}$C-PK11195 uptake and the binding potential of $^{[11]}$C-raclopride were analyzed using a one-way ANOVA with a LSD post hoc test. Significance for all tests was reached when the p value was <0.05.

**Results**

**Clinical symptoms**

Clinical symptoms (figure 2) were scored daily until day 6 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. The first clinical symptoms were seen on day 2 post-inoculation, in HSE rats treated with saline. For risperidone and clozapine treated HSE rats, the first clinical symptoms were seen on day 4 and 5 post-inoculation, respectively. At day 4 post-inoculation, a significantly lower clinical score was found in HSE rats treated with clozapine, when compared to HSE rats treated with saline (0.0 vs. 0.63±0.52, p=0.011). A lower clinical score was also found in HSE rats treated with risperidone, when compared to HSE rats treated with saline, but this was not statistically significant (0.17±0.41 vs. 0.63±0.52, p=0.099). None of the control rats showed any clinical symptoms.

In the HSE rats treated with saline, there was a gradual increase in the clinical score from day 2 to day 6 post-inoculation. In contrast, in HSE rats treated with clozapine and risperidone, there was a delayed, but more rapid, increase in clinical symptoms.
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from day 4, the last day of treatment, to day 6 post-inoculation. No differences were found between the clinical scores of HSE rats treated with saline (2.13±1.25), clozapine (2.17±1.83) and risperidone (2.50±0.55) on day 6 post-inoculation. Thus, the antipsychotics clozapine and risperidone delayed the onset of clinical symptoms, but could not prevent them.

**Figure 2** Clinical scores of rats inoculated with HSV-1 on day 1 to day 6 post-inoculation, that were treated with either saline (n=8), clozapine (n=6) or risperidone (n=6) 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. *p<0.05 for saline treated rats, when compared to clozapine treated rats at day 4 post-inoculation.

**Open field experiment**

The results of the open field experiments, that were performed on day -1 pre-inoculation and on day 2 and 4 post-inoculation, are displayed in figure 3. The total distance moved in the open field was used as a measure of locomotion. No differences in locomotion between groups were found on day -1 pre-inoculation. Statistical analysis of day 2 and 4 post-inoculation were performed with day -1 as a covariate to correct for variance pre-inoculation. No statistically significant differences between groups were found on day 2. On day 4 post-inoculation, locomotion was statistically significantly decreased by treatment with clozapine in HSE rats, when compared to saline treatment (1250±405 vs. 2370±1085, p=0.018).

The total distance moved in the centre of the open field, the frequency of rearing and the frequency of grooming were analyzed as a measure of anxiety (i.e. a high total
distance moved in the centre of the open field corresponds to low anxiety), exploration and arousal, respectively. No group differences for anxiety, exploration and arousal were found on day -1 pre-inoculation. Because locomotion on day 2 or day 4 post-inoculation can affect anxiety, exploration and arousal on the corresponding days, locomotion was used as a covariate. In addition, anxiety, exploration and arousal on day -1 pre-inoculation, can affect the behavior on day 2 and day 4 post-inoculation. Therefore, anxiety, exploration or arousal on day -1 pre-inoculation, were also used as a covariate in the statistical analysis on day 2 and day 4.

Figure 3 Open field experiment. The total distance (A), total distance in the center of the open field (B), the frequency of grooming (C) and the frequency of rearing (D) during 8 minute exposure to the open field, on day -1 pre-inoculation and day 2 and 4 post-inoculation with PBS (control) or HSV-1 (HSE). Rats were treated with saline, clozapine or risperidone from the day of inoculation (day 0) until day 4 post-inoculation. Data are presented as mean ± standard deviation, *p<0.05 between different treatment groups on the same day and #p<0.001 for saline treated HSE rats, when compared to saline treated control rats.
HSV-1 inoculation, did not affect exploration (i.e. frequency of rearing) on day 2 post-inoculation. However, a statistically significant increase in exploration (i.e. frequency of rearing) was found for HSE rats treated with saline on day 4 post-inoculation, when compared to control rats treated with saline (18±12 vs. 8±8, p=0.005). This increased exploration in HSE rats on day 4 post-inoculation was statistically significantly reduced by treatment with clozapine (5±2 vs. 18±12, p=0.008) and risperidone (9±9 vs. 18±12, p=0.033), when compared to saline treatment. No effect of treatment on exploration was found in control rats.

In summary, HSE rats showed an increase in exploration, which was inhibited by treatment with clozapine and risperidone. Locomotion was decreased by clozapine, while anxiety and arousal were not affected by HSV-1 infection or clozapine and risperidone treatment.

**[11C]-PK11195 PET**

The [11C]-PK11195 PET images of control and HSE rats, treated with saline, clozapine or risperidone, are displayed in figure 4. The PET images showed low [11C]-PK11195 brain uptake in control rats, which was not affected by treatment with clozapine and risperidone. In HSE rats treated with saline, visual analysis of the PET images showed an increased [11C]-PK11195 uptake when compared to control rats treated with saline, mainly in the bulbus olfactorius and brainstem. This increased uptake was inhibited by treatment with clozapine or risperidone. To quantify the uptake of [11C]-PK11195, the SUV from the last 10 minutes of the PET scan was calculated for all examined brain areas (table 1). In saline treated rats, a statistically significant increased [11C]-PK11195 uptake was found in the bulbus olfactorius (p<0.001), frontal cortex (p=0.001), striatum (p=0.013), parietal/occipital/temporal cortex (p=0.020) and brainstem (p=0.019) of HSE rats, when compared to control rats. Treatment with clozapine or risperidone prevented the increase in [11C]-PK11195 uptake in HSE rats and consequently the brain uptake in these groups resembled the uptake in saline treated control rats. In HSE rats treated with clozapine, a statistically significant increased [11C]-PK11195 uptake only remained in the brainstem (p=0.013), when compared to control rats treated with clozapine. In addition, pre-treatment of HSE rats with clozapine statistically significantly reduced the [11C]-PK11195 uptake in the bulbus olfactorius (p=0.012), when compared to saline treated HSE rats. For risperidone treated rats, a statistically significant higher [11C]-PK11195 uptake in HSE rats only remained in the striatum (p=0.011), when compared to control rats.
Treatment with clozapine and risperidone did not affect the $[^{11}C]$-PK11195 uptake in sham inoculated rats.

**Figure 4** Full-color in appendix. $[^{11}C]$-PK11195 small animal PET images of control rats (control) and rats inoculated with HSV-1 (HSE), treated with saline, clozapine or risperidone, on day 5 after inoculation. The images display a coronal plane of the rat head at the level of the brainstem, in which the brain is delineated by a dashed line. The images represent brain uptake between 15 and 60 minutes after injection.

**Table 1** $[^{11}C]$-PK11195 brain uptake in control rats (control) and rats inoculated with HSV-1 (HSE), treated with either saline, clozapine or risperidone, on day 5 after inoculation. The values represent the standardized uptake value (SUV, mean ± standard deviation) from the last 10 minutes of the 60 minute small animal PET scan. *p<0.05 or **p<0.005 as compared to control

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Ctrl, control; P/T/O, Parietal/Temporal/Occipital
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**Figure 5** Full-color in appendix. Small animal PET images of the $[^{11}\text{C}]$-raclopride binding potential (BP) of control rats (control) and rats inoculated with HSV-1 (HSE), treated with saline, clozapine or risperidone, on day 5 after inoculation. The images display a coronal plane of the rat head at the level of the brainstem, in which the brain is delineated by a dashed line.

**Table 2** $[^{11}\text{C}]$-Raclopride brain uptake in control rats (control) and rats inoculated with HSV-1 (HSE), treated with either saline, clozapine or risperidone, on day 5 after inoculation. The values represent the binding potential (BP, mean ± standard deviation) of $[^{11}\text{C}]$-raclopride.

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<tr>
<td>Brainstem</td>
<td>0.30±0.20</td>
<td>0.39±0.08</td>
<td>0.41±0.07</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.44±0.17</td>
<td>0.50±0.17</td>
<td>0.51±0.07</td>
</tr>
</tbody>
</table>

Cntrl, control, P/T/O; Parietal/Temporal/Occipital cortex

**[11C]-Raclopride PET**

The $[^{11}\text{C}]$-raclopride BP images (figure 5) showed the highest BP in the striatum, consistent with the distribution of dopamine D$_2$-receptors in the brain. No differences
between control and HSE rats, treated with either saline, clozapine or risperidone could be demonstrated.

The values of the BP were statistically significantly higher in the striatum, when compared to all other brain areas (p<0.005), but no differences in BP were found between control and HSE rats, treated with saline, clozapine or risperidone. In addition, no differences between any of the groups were found for the influx of $[^{11}\text{C}]-\text{raclopride}$ ($K_{i}/k_{i}$) into or the efflux of $[^{11}\text{C}]-\text{raclopride}$ ($k_{2}$) out of brain tissue.

**Discussion**

The present study showed that HSV-1 infection of the brain induced activation of microglia cells and specific behavioral changes in the rat. Treatment with the atypical antipsychotics clozapine and risperidone inhibited both the behavioral changes and the activation of microglia cells. To the best of our knowledge, this is the first study that showed that atypical antipsychotics can inhibit activation of microglia cells and HSV-1 induced behavioral changes, *in vivo*.

The presence of activated microglia cells, as measured with $[^{11}\text{C}]-\text{PK11195}$ PET, that was found in the frontal brain areas, cortical brain areas and in the brainstem at day 5 post-inoculation, was consistent with the presence of infectious virus and activation of microglia cells in these areas, as reported in literature [22,28,29]. The inhibition of HSV-1 induced activation of microglia cells by clozapine and risperidone implies a novel mechanism of action of these drugs. It has been shown that global activation of microglia cells is present in the grey matter in recent-onset schizophrenic patients [17] and that focal activation of microglia cells appeared to be a feature of schizophrenia-related psychosis [18]. Thus, efficacy of antipsychotic treatment may in part be explained by the anti-inflammatory properties of these drugs. Indeed, it has been shown *in vitro* that typical and atypical antipsychotic drugs inhibited the expression of pro-inflammatory cytokines by LPS and IFN-γ induced activation of microglia cells [20,21,30,31].

The outcome measure of the effect of antipsychotic treatment in HSV-1 infected rats was the activation of microglia cells, which was inhibited by clozapine and risperidone. However, one could argue that antipsychotic treatment inhibited viral replication and thus HSV-1 infection of the brain, resulting in a prevention of microglia cell activation. However, the appearance of clinical symptoms after clozapine and risperidone treatment was terminated, suggested that viral infection of the brain was
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only temporarily suppressed, if not at all. Antipsychotic treatment was effective in suppressing the clinical symptoms in HSV-1 infected rats up to day 4 post-inoculation, but was not effective in preventing the symptoms at day 5 and 6 post-inoculation. If treatment with clozapine and risperidone would have prevented viral replication, the severe clinical symptoms at day 6 post-inoculation would most likely not have occurred, because the viral entry of the brain would have been prevented. Thus, clozapine and risperidone appear to exert their protective effect predominantly by the suppression of the activation of microglia cells.

Important questions that rise from the findings of the present study are how HSV-1 induced activation of microglia cells caused increased exploratory behavior and what the mechanism is behind inhibition of microglia cell activation by clozapine and risperidone. The occurrence of behavioral changes could be related to dopaminergic neurotransmission. The increased exploratory behavior was consistent with the increased locomotor activity that was observed in HSV-1 infected rats in their home cage, especially between day 4 and 5 after inoculation [24]. Changes in locomotor activity are often used to assess rodent models for schizophrenia and the effect of treatment with antipsychotics, and can be attributed to an increase in the action of dopamine at both the D₁ and D₂ receptors [32]. In psychostimulant rodent models, the amphetamine induced increase in dopamine caused an increase in locomotion and rearing in the open field [33,34]. Based on the behavioral data of the present study and the resemblance of this behavior with amphetamine induced behavioral changes, dopamine seems to be involved. Indeed, it has been shown that acute HSV-1 infection of the brain caused an increase in dopamine release [35,36], while chronic HSV-1 infection may cause a decrease in dopaminergic activation [37]. Although dopamine may play a role in HSV-1 induced behavioral changes, it was shown in the present study that there were no changes in the binding potential of [¹¹C]-raclopride in HSV-1 infected rats, suggesting there are no changes in the expression of D₂ receptors or release of dopamine. However, it should be noted that [¹¹C]-raclopride PET was performed one day after the last behavioral test and termination of drug treatment. Consequently, any changes in dopaminergic activity could have been normalized in this period of time.

The inhibition of the activation of microglia cells may be due to binding of clozapine and risperidone to the variety of neurotransmitter receptors that are expressed on microglia cells [38] or to inhibition of pro-inflammatory cytokines that are involved in progression of the activation of microglia cells. Clozapine and risperidone were found
to bind with different affinity to different neurotransmitter receptors, which could explain why clozapine was found to be more effective in inhibiting HSV-1 induced behavioral changes and activation of microglia cells than risperidone. However, to give a definite answer to the question how HSV-1 infection of the brain and the accompanied microglia cell activation affects behavior and what the mechanism is behind inhibition by antipsychotics, as well as the difference between antipsychotics, further research is necessary.

**Conclusion**

The increase in exploratory behavior and the microglia cell activation in response to HSV-1 infection of the brain are inhibited by the atypical antipsychotics clozapine and risperidone. The present study implies a novel mechanism of action of clozapine and risperidone, which is to inhibit HSV-1 induced behavioral changes by reducing microglia cell activation in the brain. Additional research on the role of HSV-1 and the accompanied microglia cell activation as a potential target for the treatment of schizophrenia is warranted.

**Acknowledgement**

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