New avenues in PET imaging of multiple sclerosis
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Chapter 6

PET imaging of disease progression and treatment effects in the experimental autoimmune encephalomyelitis rat model

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ABSTRACT

The experimental autoimmune encephalomyelitis (EAE) model is a model of multiple sclerosis (MS) that closely mimics the disease characteristics in humans. The main hallmarks of MS are neuroinflammation (microglia activation and monocyte invasion), demyelination and T-cell infiltration. PET imaging may be a useful non-invasive technique for monitoring disease progression and drug treatment efficacy in vivo. In this study, we evaluated PET imaging as a tool for simultaneous monitoring of neuroinflammation, demyelination and T-cell infiltration during normal disease progression and during treatment with the anti-inflammatory drug dexamethasone in the EAE rat model. Activated microglia were detected using $[^{11}\text{C}]$PK11195, showing an increased uptake in the brainstem and spinal cord during disease progression. $[^{11}\text{C}]$PK11195 uptake was also elevated in dexamethasone treated animals that showed mild clinical symptoms, which had gone at the time of imaging. Myelin was detected using $[^{11}\text{C}]$MeDAS, probably due to the small size of the lesions (average of 0.13 mm), demyelination was not detected. The $[^{18}\text{F}]$FB-IL2 binding potential was decreased at 6 and 15 days after immunization, likely due to the reduction in the number of T-cells in the blood vessels of the brain. CD3 and CD25 immunohistochemistry results were negative at all time points. We conclude that PET imaging of neuroinflammation can be used to monitor disease progression and the consequences of treatment in the EAE rat model. PET imaging was more sensitive than clinical symptoms for detecting neuroinflammatory changes in the CNS.
INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative disease characterized by inflammation (microglia activation, monocyte invasion and T-cells infiltration) and demyelination in the Central Nervous System (CNS). There are different types of MS, based on the clinical course of the disease. Relapsing-remitting MS (RRMS) is characterized by fluctuating periods of relapses and remission of clinical symptoms. In most patients, this type of MS proceeds into secondary progressive MS (SPMS), which is accompanied by an increase in neurological deficits. Primary progressive MS (PPMS) is characterized by a gradual neurodegeneration starting from disease onset, without clearly distinguishable periods of inflammatory exacerbations and remission (Luessi et al 2012).

At present, there is no cure for MS. The most common treatment of MS is immunomodulatory therapy, which focusses primarily on preventing or reducing the infiltration of aggressive immune cells into the CNS (Mulakayala et al, 2013; Thöne & Ellrichmann, 2013). Apart from the more “classical” immunomodulatory drugs, such as interferon and glucocorticoids, various novel very promising antibodies directed against molecules involved in T-cell migration and invasion (e.g. Nataluzimab) have been developed. These drugs show encouraging results, as they can significantly reduce the number, the extent and the duration of relapses in RRMS with a concomitant delay of the transition into the SP phase (Rommer et al 2013).

Severe acute relapses in MS are still treated with high doses of glucocorticoids in clinical practice. Glucocorticoids modulate the survival and migration to the CNS of inflammatory cells, resulting in a shorter duration and reduced severity of the relapse and acceleration of recovery. Methylprednisolone, intravenously injected, is the most popular glucocorticoid for treating acute relapses in MS patients, due
to its fast action and few side effects. Dexamethasone (DEX) is another example of glucocorticoid that gives a similar treatment response as methylprednisolone. However, DEX is less commonly used in MS patients, despite being a less expensive alternative for MS relapse treatment (Polman et al, 2001; Wüst et al, 2008). Prophylactic and early therapeutic administration of DEX strongly decreased the clinical symptoms and the disease duration of experimental autoimmune encephalomyelitis (EAE) in rats, and in other species, but it induced pronounced body weight loss as a clinical side effect (Donia et al, 2010).

Treatment response in MS patients is basically monitored by clinical symptom changes and by magnetic resonance imaging (MRI), which detects lesions in the CNS, but does not differentiate these lesions as inflammation, demyelination or axonal damage (Filippi and Rocca, 2011). A non-invasive imaging technique that could differentiate and quantify MS hallmarks, would be an important tool to specifically monitor therapeutic response and help to better understand drug mechanisms.

Positron Emission Tomography (PET) is a non-invasive, molecular imaging technique that has been used in multiple sclerosis for imaging activated microglia and macrophages in the EAE animal model (Mattner et al, 2013; Abourbeh et al, 2012; Xie et al, 2012; Vowinckel et al, 1997) and MS patients (Takano et al, 2013; Politis et al, 2012; Oh et al, 2011, Vas et al, 2008; Versijpt et al, 2005; Debruyne et al 2003). PET imaging of glucose metabolism has also been used in MS animal models (Buck et al, 2012) and patients (Shkil'niuk et al, 2012; Derache et al, 2006; Schiepers, 1997) and also myelin content has been imaged by PET in MS animal models (Stankoff et al, 2011, 2006; Wang et al, 2011, 2009; Wu et al, 2013, 2010; Briard et al, 2011). In two MS patients, demyelinated lesions were detected by $[^{11}\text{C}]$PIB PET in a proof of concept study (Stankoff et al, 2011). However, different
hallmarks were not longitudinally measured at the same time in these studies, while MS comprises multiple aspects that are important to monitor.

The aim of the current study was to evaluate the potentials of PET imaging as a tool for simultaneous monitoring of neuroinflammation, demyelination and T-cell infiltration during normal disease progression and during treatment with the anti-inflammatory drug dexamethasone in the experimental allergic encephalomyelitis (EAE) rat model. To this end, we used $[^{11}\text{C}]\text{PK11195}$ for neuroinflammation, $[^{11}\text{C}]\text{MeDAS}$ for demyelination and $[^{18}\text{F}]\text{FB-IL2}$ for T cell infiltration. $[^{11}\text{C}]\text{PK11195}$ binds to the translocator protein (TSPO) which is increased in activated microglia and infiltrating monocytes and it is a well validated tracer for imaging inflammation in many neurological and psychiatric diseases, including multiple sclerosis (Doorduin et al, 2008). $[^{11}\text{C}]\text{MeDAS}$ is a novel ligand for in vivo PET imaging of myelin content which has recently shown ability to detect demyelinated lesions in the spinal cord of EAE and lysolecithin models for MS (Wu et al 2013); it also could show myelin changes in the brain of the cuprizone-induced mouse model for demyelination (de Paula Faria et al submitted data). $[^{18}\text{F}]\text{FB-IL2}$ is a new PET tracer for imaging T-cell infiltration, which was recently validated by di Gialleonardo et al. (2012a, 2012b) in models of peripheral inflammation. The tracer binds specifically to interleukin-2 receptors (IL-2R), which are overexpressed by activated T lymphocytes. The use of $[^{18}\text{F}]\text{FB-IL2}$ in CNS imaging has not been investigated yet.

In this study, the EAE rat model was used. This is an established model for MS exhibiting its major hallmarks, such as infiltration of T-cells and monocytes in the CNS, demyelination and neurodegeneration (Constantinescu et al, 2011; Stosic-Grujisic et al, 2004), resulting in MS-like neurological deficits. The exact nature of the hallmarks found in EAE models depends on the animal species, strains and sex that are used. EAE induced in female Dark Agouti (DA) rats by myelin
oligodendrocyte glycoprotein (MOG) in Incomplete Freund’s adjuvant (IFA) is an animal model of RRMS, which is characterized by multiple phases of neurological deficits, increased blood-brain barrier (BBB) permeability, multifocal infiltrations in the CNS and demyelination (Ledeboer et al, 2003). Furthermore, the use of this EAE model brings along an ethical improvement of the traditional EAE models, due to the absence of mycobacterium in emulsion used for the immunization procedure; this emulsion causes serious skin lesions and inflamed lymph nodes, being excessively painful to the animals (Storch et al, 1998; Ledeboer et al, 2003; ´t Hart et al, 2011; Billiau & Matthys, 2001).

MATERIAL AND METHODS

Animals, disease induction and scoring

EAE was induced according to the protocol described by Ledeboer et al. (2003). In order to reduce the risk of side effects, e.g. severe bladder infection, the protocol was slightly adapted. In short: adult 9-12 weeks-old 150-170 g female Dark Agouti (DA) rats (Janvier, France) were used for EAE induction. Animals were housed in groups during the entire study. DA rats were anaesthetized with isoflurane and immunized intradermally at the dorsal tail base with 25 μg of endotoxin-free rat recombinant MOG$_{1-125}$ (Tebu-bio) dissolved in 100 μl of 25 mM sodium acetate pH 4.0, and emulsified in 100 μl of incomplete Freund’s adjuvant (IFA; Difco, Detroit, MI,USA). A total volume of 200 μl (1:1) was divided in two batches of 100 μl and injected at two different locations. Rats were weighed and examined daily for neurological symptoms. Symptoms were scored on a 5-point scale: 0, no clinical symptoms; 0.5, distal limp tail; 1, complete limp tail; 2, ataxia; 3, moderate
paraparesis, i.e. the rats trips from time to time; 3.5, one hind limb paralyzed; 4, complete hind limb paralysis; 5, moribund or dead due to EAE.

Food and water were given *ad libitum*, but animals suffering from paralysis were provided water and food by hand (3-4 times a day). In case of paralysis, daily injections (1-2 times) of saline (subcutaneously) were also given. Animals reaching score 5 were terminated.

The animal experiments were performed according to the Dutch Regulations for Animal Welfare. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen (protocol: DEC 6480A).

**Experimental groups**

Animals were divided in two groups: saline treated animals and dexamethasone treated animals. The sample size was 10 animals per group at the start of the experiment: 6 animals per group were scanned longitudinally at all time points (days -2, 6, 11, 15 and 19 after immunization). The remaining 4 animals per group (1 per time) were imaged at only one time point and then terminated for immunohistochemical analysis for correlation with PET data.

At day 8 after immunization (onset of symptoms) the treatment was started: the saline treated group received daily intraperitoneal (i.p.) injections of saline, whereas dexamethasone treated animals received daily i.p. injections of dexamethasone acetate (Sigma-Aldrich) in saline at a dose of 1 mg/kg (0.4-0.5 ml of total injected volume).

**[^11]C]PK11195 synthesis**

[^11]C]-(R)-PK11195 was synthesized by trapping[^11]C]methyl iodide in a solution of 1 mg (R)-N-desmethyl-PK11195 (ABX) and 10 mg potassium hydroxide in 300 μl dry dimethylsulfoxide (Sigma-Aldrich). The reaction mixture was allowed to react for 1
minute at 40°C. The mixture was neutralized with 1M HCl and passed through a 45µm Millex HV filter. The filtrate was purified by HPLC using a μBondapak C18 column (7.8x300 mm) with acetonitrile / 25 mM phosphate buffer pH 3.5 (55/45) as the mobile phase (flow 5 ml/min). To remove 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 cartridge (Waters). The cartridge was washed twice with 10 ml water, eluted with 8 ml ethanol and diluted in 9 ml saline. Quality control was performed by HPLC, using a Novapak C18 column (150x3.9 mm) with acetonitrile / 25 mM phosphate buffer pH 3.5 (60/40) as mobile phase at a flow of 1 ml/min. The radiochemical purity was always higher than 95% and the specific activity >30 GBq/μmol.

\[^{11}\text{C}]\text{MeDAS synthesis}\n
\[^{11}\text{C}]\text{MeOTf} was produced as previously described (Elsinga, 2002; Li & Conti, 2010) and transported with a stream of helium gas (30 ml/min) into the reaction vessel containing 0.7-1 mg of N-desmethyl-MeDAS precursor (Sigma-Aldrich) in 0.5 ml dry acetone (Merck). After the \[^{11}\text{C}]\text{MeOTf} was trapped in the reaction vial, the mixture was heated at 90 °C for 3 min. The acetone was evaporated and the residue dissolved in 0.5 ml of acetonitrile and diluted with 0.5 ml of water and then purified by HPLC using a reverse-phase C18 platinum column (Alltech) and acetonitrile / 25 mM phosphate buffer pH 7.2 (35/65) as mobile phase (flow 5 ml/min).

The radioactive peak with a retention time of 8 min consisting of purified \[^{11}\text{C}]\text{MeDAS} was collected from the HPLC and formulated as isotonic solution before injection \textit{in vivo}. To this purpose, the collected HPLC fraction was diluted in 100 ml of distilled water and passed through a SepPak® C18 light cartridge.
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(Waters) that was pre-conditioned with ethanol (8 ml) and water (12 ml). The product was eluted from the cartridge with ethanol (1ml) and diluted with 0.9% sodium chloride (9 ml). The synthesis of $[^{11}\text{C}]$MeDAS was fully automated using a Zymark robotic system.

$[^{11}\text{C}]$MeDAS quality control was performed using an analytical C18 X-Terra reversed phase HPLC column (Waters) and acetonitrile/30 mM ammonium acetate pH 10.0 (15/85) as mobile phase (flow 1.5 ml/min). UV absorption was measured at a wavelength of 254 nm. The $[^{11}\text{C}]$MeDAS peak appeared at a retention time of 14 minutes. Radiochemical purity was always >95% and the specific activity >80 GBq/μmol.

$[^{18}\text{F}]FB$-IL$_2$ synthesis

$[^{18}\text{F}]$SFB (N-succinimidyl 4-[$^{18}\text{F}$]fluorobenzoate) was prepared according to a method described by Wester et al (1996) and the conjugation procedure with IL2 (Proleukin, Novartis) was described by di Giolleonardo et al (2012). Purification was done by HPLC using C18 Econosphere column (Alltech) and ethanol:water containing 0.1% trifluoactic acid (25:75) as mobile phase (flow 1 ml/min). The tracer was diluted in PBS (1:1) before administration to animals. The total injected volume was 0.4 ml (20-30 MBq) per animal.

PET imaging

Animals were longitudinally imaged with different PET tracers in a dedicated small animal PET scanner (Focus 220, Siemens Medical Solutions USA, Inc.) at five different time points: baseline (day -2) and day 6, day 11, day 15 and day 19 after immunization. On each scan day, animals were anesthetized with isoflurane 5% in medical air, and then kept under isoflurane (1.0-2.0%) anesthesia during tracer
injection, tracer distribution and image acquisition. In each animal, $[^{11}\text{C}]$PK11195 PET was performed in the morning, followed by $[^{11}\text{C}]$MeDAS PET in the afternoon:

1. For imaging of microglia activation/monocyte invasion, 30-60 MBq of $[^{11}\text{C}]$PK11195 (0.5 ml) was injected into the tail vein. After 40 minutes, the animals were positioned on their belly in the PET camera with the head and upper part of the spinal cord (cervical + half of the thoracic part) in the field of view. Forty-five minutes after tracer injection a 30 min static emission scan was acquired.

2. PET imaging of demyelination was performed in a similar manner, with the exception that the emission scan was started 30 min after injection of $[^{11}\text{C}]$MeDAS (40-60 MBq; 0.5ml).

In addition, a pilot PET imaging study with $[^{18}\text{F}]$FB-IL2 was performed in EAE animals that did not receive any treatment (n=4), because brain imaging with this tracer has never been performed before. The purpose of this study was to investigate the feasibility of the imaging technique, rather than to investigate treatment efficacy.

3. For imaging of T-cell infiltration, $[^{18}\text{F}]$FB-IL2 (20-30 MBq; 0.4 ml) was injected into the tail vein and a 60 min dynamic emission scan started immediately after injection.

After each emission scan, a transmission scan of 515 seconds with a $^{57}\text{Co}$ point source was performed for attenuation and scatter correction. Animals were allowed to wake up in a pre-warmed cage after imaging acquisition and they were visually monitored until complete recovery from anesthesia. Immediately after the last PET scan in the study was completed, animals were
perfused with saline under deep anesthesia and the brain and spinal cord were dissected for (immuno)histochemical analysis.

**PET image reconstruction and analysis**

Emission sinograms were iteratively reconstructed (OSEM2d, 4 iterations) after being normalized, corrected for attenuation and decay of radioactivity. The list-mode data of the dynamic $^{18}$FFB-IL2 emission scans were separated into 20 frames (8x30, 2x60, 2x120, 2x150, 3x300 and 3x600). The images were analyzed by INVEON research workstation software (Siemens), by drawing regions of interest (ROI) in different parts of the brain (cerebellum, brainstem, striatum and whole brain) using a rat brain MRI image as the template and the spinal cord using the $^{11}$CMeDAS image of the same animal and the same time point as the template.

Radioactivity concentration, obtained from the ROIs, were corrected for injected dose and presented as percentage of injected dose per gram of tissue (%ID/g) for $^{11}$CPK11195 and $^{11}$CMeDAS PET.

For $^{18}$FFB-IL2, the apparent binding potential in the whole brain was measured with a two tissue compartment model (2TCM) using the time-activity curve (TAC) of the heart as input curve.

**Luxol Fast Blue histochemistry**

The changes in the level of myelination of the brain and spinal cord were analyzed by Luxol Fast Blue (LFB) histochemistry. The LFB working solution was prepared by dissolving 0.5 g of Solvent Blue 38 (Sigma) in 500 ml 96% ethanol supplemented with 10% acetic acid. Sections were dehydrated in an ascending ethanol series (50%, 70%, 80% and 96%) and incubated in the LFB working solution at 58°C for 14-16 hours. After incubation, sections were washed with 96% ethanol and
distilled water. Differentiation was performed with a 0.125% lithium carbonate solution, after which the sections were rinsed with 70% ethanol and distilled water, counterstained with cresyl violet and coverslipped with DePeX (Merck).

**Iba1 immunohistochemistry and scoring**

Brain and spinal cord sections for immunohistochemistry were blocked with 5% normal goat serum (NGS) and 3% fetal calf serum (FCS) in phosphate buffered saline (PBS) containing 0.1% Triton-X (Fluka) for 1 hour at room temperature. The primary antibody, rabbit anti-Iba1 (Wako), was applied in PBS-Triton-X containing 1% of NGS and 1% FCS overnight at 4°C (dilution 1:1000). On the next day, the sections were washed three times with PBS at room temperature and incubated in the dark with the secondary antibody (anti rabbit Cy3 1:400 in 1% FCS and 1% NGS in PBS-Triton-X) for 90 minutes at room temperature. After that, the sections were washed 3 times with PBS and then counterstained with Hoechst 1:1000 (Fluka) and coverslipped with Mowiol (Calbiochem). The slides were analyzed using a Zeiss fluorescent microscope.

Scoring of neuroinflammation was performed by an independent researcher with expertise in Iba1 immunohistochemistry, who received the slides coded by numbers without any indication of the identity of the rat or study group. The inflammation score was given as (0) no inflammation; (1) Sporadic inflammation in 1-5 small areas; (2) Multifocal inflammation in 5-10 small areas; (3) Multifocal inflammation in 5-10 large areas; (4) Multifocal inflammation in large parts of the tissue.
**CD3/CD25 immunohistochemistry**

Cryostat sections of brain and spinal cord were blocked with 5% bovine serum albumin (BSA) in PBS for 15 minutes at room temperature. Subsequently, sections were incubated overnight at 4°C with biotin labeled mouse anti rat CD3 (1:250, BD Biosciences cat.no. 554831) or with biotin labeled mouse anti rat CD25 (1:100, eBiosciences, San Diego, USA, cat. no. 13-0390). Biotin labeled mouse IgG (1:250, BD Biosciences cat.no. 559805) and biotin labeled mouse IgG1 (1:100, BD Biosciences cat.no. 550615) served as isotype controls, respectively. All antibodies were diluted in PBS containing 1% BSA. Sections were washed for 5 minutes in PBS and then incubated for 5 minutes with Dako Real peroxidase block (Dako Netherlands BV, Belgium, cat.no. S2023). After washing two times with PBS for 5 minutes, the sections were incubated with Avidin horseradish peroxidase according to the manufacturers’ instructions (Vectastain® ABC kit, PK-4000, Vector Laboratories, Burlingame, USA). Sections were rinsed three times with PBS and then incubated with Novared (Vector® Novared substrate kit for peroxidase, SK-4800, Vector Laboratories) for 10 minutes. Sections were counterstained with hematoxilin. Rat spleen was used as a positive control for staining. Per rat three non-serial sections were analyzed for positive staining in a blind fashion.

**Statistical analysis**

The results are presented as mean ± standard deviation. Differences between groups were analyzed by 1 way ANOVA, 2 way ANOVA, or t-test, whenever applicable, (GraphPad Prism) using a Bonferroni post hoc test to correct for multiple comparisons. Differences were considered statistically significant when p<0.05.
RESULTS

EAE disease progression

EAE incidence was 100% in the animals treated with saline, of which 92% became paralyzed. Of the animals treated with dexamethasone only 50% showed minor symptoms of disease, but never became paralyzed, confirming the positive effect of dexamethasone treatment on disease development. Weight loss and disease score during the disease progression in both study groups are shown in Figure 1. The disease onset was between 7 and 13 days after immunization. Relapses of this Relapsing-Remitting EAE rat model took place at 14-15 days (first relapse) and at day 19 after immunization (second relapse). The condition of the animals improved in the recovering phase (day 16-18), but the disease regression was not complete.

The weight loss in the dexamethasone treated EAE group (maximum 20%) was significantly higher than in the saline treated EAE group (maximum 12%), except during the two relapses (14-15 days and 19 days after immunization) when the paralyzed animals in the saline group obviously ate less.
Figure 1: EAE disease progression in saline treated rats and rats treated with dexamethasone (1 mg/kg daily, starting at day 8). All animals were included, independent if imaging was performed or not at that specific time point (per group: n = 10 from day 1 to 6; n= 8 from 7 to 11; n=6 from day 12 to 14; n= 4 from 15 to 19). The bar graph shows disease scores and the line graph shows the percentage of weight loss relative to the day of the immunization procedure (day 0). Black line and bars represent the saline treated EAE group and the interrupted line and stripped bars represent the dexamethasone treated EAE group. Statistical differences in weight loss between control and dexamethasone groups are represented by *(p<0.05), **(p<0.01) and ***(p<0.001). Disease scores are significantly different between groups for day 11 onward.

PET imaging of microglia activation and monocyte invasion

$[^{11}\text{C}]$PK11195 PET images (Figure 2) and quantitative analysis of tracer uptake in the brain (Figure 3) show an increase in neuroinflammation over time. The saline treated group showed a significant increase in $[^{11}\text{C}]$PK11195 uptake in the brainstem at day 15 (+74%, p<0.001) and day 19 (+55%, p<0.001) after immunization as compared to baseline scan. Increased $[^{11}\text{C}]$PK11195 uptake was also observed in the spinal cord at day 11 (+38%, p<0.001), day 15 (+45%,
p<0.001) and day 19 (+38%, p<0.001) after immunization, as compared to the baseline scan. Other regions of the brain did not show any significant increase in tracer uptake.

In the dexamethasone treated group, uptake of $[^{11}\text{C}]$PK11195 was significantly increased only in the brainstem (+26%, p<0.05) at day 19 after immunization.

Figure 2: Illustrative $[^{11}\text{C}]$PK11195 PET images at baseline and at different time points after immunization. Top: animals treated with saline, Bottom: animals treated with dexamethasone. White interrupted lines show whole brain area in the coronal view; solid arrows show brainstem and dashed arrows depict the spinal cord in the sagittal view.
Figure 3: Quantification of $[^{11}C]PK11195$ uptake at different time points showing progression of neuroinflammation in the saline treated EAE group (left) and in the dexamethasone treated (DEX) EAE group (right). Significant differences, as compared to baseline, are illustrated by *($p<0.05$) and ***($p<0.001$) ($n= 6$ for the first 3 time points and $n=4$ for the last 2 time points).

Figure 4 shows the correlation between $[^{11}C]PK11195$ uptake and the disease score at the day of the scan. The saline treated group was divided in 3 groups: animals without symptoms (score 0), animals with symptoms, but without paralysis (score 0.5-3.0) and animals with paralysis (score 3.5-4.0). The dexamethasone treated group was divided in two groups: animals that never presented any symptoms (DEX never symptoms) and animals that presented symptoms at an early time point during the study (DEX previous symptoms), even though symptoms had resolved at the day of imaging (excepted for 1 animal scanned at day 11 that presented distal limp tail, score 0.5).

When $[^{11}C]PK11195$ uptake was correlated with the disease score in the saline treated EAE group, an increase in tracer uptake was detected in the brainstem (+30%, $p<0.05$ and +76%, $p<0.001$) and in the spinal cord (+24%, $p<0.01$ and +65%, $p<0.001$) in moderately (score 0.5-3.0) and severely affected animals (score...
3.5-4.0), respectively. The dexamethasone treated EAE animals showed increased uptake in the brainstem (+25%, p<0.05) and spinal cord (+22%, p<0.01) in the animals that presented symptoms at an earlier time point during the study, but not in the animals that never presented symptoms during the entire course of the study. These results suggest, that $[^{11}\text{C}]$PK11195 can be used to detect neuroinflammation even when clinical symptoms of the disease are not present (anymore).

**Figure 4:** $[^{11}\text{C}]$PK11195 uptake related to disease score, presented as percentage of the injected dose per gram of tissue, classified according to the disease score at the day of imaging. Scans were grouped by the disease score, independent of the time point, at which the image was acquired. Black bars represent the saline treated group and white bars represent dexamethasone treated group. Statistically significant differences, as compared to score 0 of the respective group, are illustrated by *(p<0.05), **(p<0.01) and ***(p<0.001).
The $^{[11]}\text{C}\text{PK11195}$ uptake results were confirmed by Iba1 immunohistochemistry (Figure 5). Immunohistochemistry showed the presence of activated microglia/macrophages only in the brainstem and spinal cord. The intensity of this inflammation correlated well with PET imaging in both regions (Figure 6). The correlation of inflammation intensity was made between ex vivo tissue and the respective $^{[11]}\text{C}\text{PK11195}$ image at the termination day.

**Figure 5**: Iba1 immunohistochemistry illustrating differences in microglia activation in brainstem (top) and spinal cord (bottom) in saline treated animals without symptoms (left) and animals presenting paralysis (right). Scale bar = 100 μm.
**Figure 6**: Correlation between $[^{11}\text{C}]$PK11195 uptake at the termination day (last scan) and Iba1 immunohistochemistry score in the same animal. Each dot corresponds to an individual animal: brainstem (left) and spinal cord (right). Both groups (saline and dexamethasone treated) were included in the correlation.

**PET imaging of demyelination**

$[^{11}\text{C}]$MeDAS PET was used for imaging of demyelination in the EAE rat model. No significant differences were found at any time point of the disease progression, as compared to baseline (Figure 7 and 8). When tracer uptake was compared to disease score, no correlation was found either (Figure 9), suggesting that $[^{11}\text{C}]$MeDAS PET imaging was not sensitive enough to detect demyelination in the current set up.
Figure 7: Illustrative $[^{11}\text{C}]$MeDAS PET images at the different stages of EAE progression in the saline treated EAE group. Solid arrows indicate the brain and dashed arrows indicate the spinal cord in the sagittal view.

Figure 8: $[^{11}\text{C}]$MeDAS uptake at different time points after immunization in the saline group (left) and in the dexamethasone (DEX) group (right). No significant differences compared to baseline were found at any time point (n= 6 for the first 3 time points and n=4 for the last 2 time points).
Figure 9: $^{11}$CMeDAS uptake related to disease score, presented as percentage of the injected dose per gram of tissue, classified according to the disease score at the day of imaging. Scans were grouped by the disease score, independent of the time point, at which the image was acquired. No significant differences were found.

To confirm the presence of lesions, which was not detected by the PET scan, histochemistry was performed. Tissues were stained for myelin (Luxol Fast Blue) showing small demyelinated lesions in the brainstem and upper part of the spinal cord (figure 10). The average of lesion diameters of 8 rats was $0.13\pm0.06$ mm. Together these results suggest that demyelination could not be detected by PET scanning due to the relatively small lesion sizes in this animal model.
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Figure 10: Luxol Fast Blue staining image illustrating small demyelinated lesions in the spinal cord. Scale bar = 500 µm.

**PET imaging of T-cell infiltration**

The third hallmark of MS to be measured by PET was T-cell infiltration in the brain using $[^{18}\text{F}]$FB-IL2. In all animals brain uptake of $[^{18}\text{F}]$FB-IL2 was low. It was impossible to detect any temporal changes in tracer uptake by visual analysis (Figure 11). However, the whole brain binding potential of this tracer (Figure 12) was significantly decreased at day 6 (0.29±0.56, p<0.01) and at day 15 (0.83±1.17, p<0.05) after immunization, as compared to baseline scan (4.66±1.38). In contrast, the binding potential of $[^{18}\text{F}]$FB-IL2 had returned to baseline levels at day 11 (4.34±0.83) and 19 (3.58±2.62) after immunization.

Figure 11: Illustrative $[^{18}\text{F}]$FB-IL2 PET images of EAE rats at different time points after immunization. Brain is indicated by white interrupted lines in the coronal view.
Figure 12: $[^{18}\text{F}]$FB-IL2 PET showing a significant decrease in whole brain binding potential at day 6 (** p<0.01) and day 15 (* p<0.05) after immunization, as compared to baseline (n= 4 for the first 3 time points and n=2 for the last 2 time points).

**Immunohistochemistry CD3 and CD25**

To confirm the $[^{18}\text{F}]$FB-IL2 PET results immunohistochemistry was performed at brain sections using antibodies against CD3 and CD25. However, T-cell infiltration was not detected in the brain and spinal cord at any time point. Therefore, with the temporal changes in $[^{18}\text{F}]$FB-IL2 PET could not be correlated to immunohistochemistry of T cells in the brain parenchyma.

**DISCUSSION**

This study showed that $[^{11}\text{C}]$PK11195 uptake was increased in the brainstem and spinal cord during disease progression of the saline treated EAE animals. $[^{11}\text{C}]$PK11195 uptake was also elevated in dexamethasone treated EAE animals that showed mild clinical symptoms at disease onset, which resolved after the
start of treatment. $[^{11}\text{C}]$MeDAS PET could not detect demyelination, probably due to the small size of the CNS lesions, being smaller than the resolution of the PET scanner. $[^{18}\text{F}]$FB-IL2 binding potential decreased at day 6 and 15 after immunization, likely due to the absence of T-cells in the blood vessels of the brain and not due to decreased infiltration of these cells into the CNS parenchyma.

The EAE rat model for RRMS is characterized by periods of relapse and remission of the clinical symptoms, which is related to the waves of inflammatory cell infiltration. We planned to image three different types of temporal changes in the model during periods of relapse and remission. By using three different PET tracers within one study, a very complete view of disease progression in individual animals over time could be obtained. This led to a dramatic decrease of the number of animals required for this type of longitudinal studies. For this study, we designed our set-up based on literature (Ledeboer et al, 2003) and yet unpublished studies from our groups, which have shown that the first neurological symptoms are usually observed at 8-9 days after immunization, with the first disease peak at 11-12 days, the remission at 15-17 and the beginning of the second relapse at 18 days after immunization. However, in the present study the disease progression was somewhat different with regard to the peak of the first relapse (appeared at day 15) and the remission phase (at 16-17 days after immunization), resulting in a longer period of clinical symptoms and a shorter period of remission. This difference compared to previous studies may be caused by the use of anesthesia at multiple occasions during this study (for immunization and multiple PET scans); in previous studies anesthesia was only used for immunization. Our animals had higher maximum disease scores (3.5±1.3) than previously published (2.4±0.1) (Ledeboer et al, 2003) and a relatively high death incidence, which led to a decrease in group size from 6 to 4 animals for the last 2 time points (days 15 and 19 after immunization). This could be caused by the use...
of anesthesia in this study, but also to some variation in disease progression in animal models between different laboratories or other factors. Even though the intent to image animals during the remission phase was unfortunately compromised, we could nicely show that $^{11}$C]PK11195 uptake, corresponding to microglia activation and monocyte invasion, correlated well to the clinical progression of the rats. The maximum tracer uptake was found during the clinical peak of the disease (15 days after immunization). When tracer uptake was compared to the disease score (Figure 5), $^{11}$C]PK11195 uptake was maximum in animals with the highest disease scores (paralysis). The increase in tracer uptake was significant only in the brainstem and spinal cord. The presence of activated microglia in these regions was confirmed by immunohistochemistry and in agreement with previous findings by Storch et al (1998). These results show that the brainstem and spinal cord are among the predilection sites for inflammatory lesions.

Using $^{11}$C]PK11195 PET, we could also monitor the treatment effects on the inflammatory response in the animals treated with the anti-inflammatory drug dexamethasone. The treatment started at the disease onset (day 8) and it was responsible for preventing clinical symptoms in 50% of the animals. Animals that did not show any clinical symptoms also did not show any changes in $^{11}$C]PK11195 uptake. This likely means that the glucocorticoid treatment prevented the auto-inflammatory response in these animals. Glucocorticoids were shown to moderate the initial inflammatory response and decrease leukocyte infiltration to the inflammation site (Coutinho & Chapman, 2011). Furthermore, glucocorticoids have shown to induce weight loss and decrease food intake in rodents. Although the mechanism is not confirmed, it is suggested that orexigenic neuropeptides are down-regulated in hypothalamus of rats during glucocorticoid
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treatment, inducing metabolic changes (Liu et al, 2011). Weight loss in EAE animals after dexamethasone treatment has been reported previously (Kiefer & Kreutzberg, 1991; Donia et al, 2010) and it was confirmed in our study that dexamethasone treated animals lost more body weight than the saline treated animals (about 10% more). Glucocorticoids act on T-cells, macrophages and microglia, but T-cells are the most important target for treatment of EAE. Glucocorticoids induce apoptosis of peripheral lymphocytes (Wüst et al, 2008). Consequently, T-cell can no longer infiltrate in the CNS, which explains the lack of inflammation after treatment, as was observed by $^{[11]}$C]PK11195 and immunohistochemistry.

The other 50% of the animals treated with dexamethasone presented only mild symptoms and never became paralyzed. Interestingly, the $^{[11]}$C]PK11195 uptake in these animals was increased to the same level as that in the saline treated animals with score 0.5-3.0 (no paralysis). In both groups, the same regions of the CNS (brainstem and spinal cord) were affected. These results suggest that dexamethasone treatment in the animals that did show some clinical symptoms was started after the initiation of T-cell infiltration, whereas T-cell infiltration had not started at the initiation of treatment in the animals that never showed any symptoms. Treatment with dexamethasone was started at day 8 after immunization, when disease onset was expected. Apparently, treatment was started too late to inhibit cellular infiltration in half of the animals, but treatment was still able to reduce the inflammatory response.

Our results show the ability of $^{[11]}$C]PK11195 PET to show temporal inflammatory changes during disease progression, as well as the effects of treatment on the inflammation. PET imaging with this tracer is clearly more sensitive for the detection of changes in disease status than clinical evaluation, since PET showed increased tracer uptake even in animals on dexamethasone treatment that
presented no symptoms anymore at the time of the scan. These results were confirmed by immunohistochemistry. $[^{11}\text{C}]$PK11195 PET may therefore be an important imaging tool not only for monitoring disease progression and drug efficacy in the clinic, but also to facilitate drug development, where different molecules, different doses, different administration routes, etcetera can be evaluated by a non-invasive in vivo technique, that is faster, more reliable, more sensitive and using less animals than conventional neuropathological methods.

Another inflammatory process evaluated in this study was T-cell infiltration. It is known (Ledeboer et al, 2003; Coutinho & Chapman, 2011) that T-cell infiltration is responsible for EAE development, but in vivo imaging techniques are not yet available for evaluation of this process in living animals. In addition to the evaluation of monocyte invasion and microglia activation by $[^{11}\text{C}]$PK11195 PET, the in vivo evaluation of T-cell infiltration would be highly relevant for monitoring disease mechanisms, disease progression and therapeutic efficacy. In the attempt to achieve this goal, we evaluated a PET tracer that was recently validated by our group, $[^{18}\text{F}]$FB-IL2, for imaging T-cell infiltration in the CNS. $[^{18}\text{F}]$FB-IL2 has been validated in peripheral inflammatory models (di Gialleonardo et al 2012a, 2012b), but it has not been used for CNS imaging yet. Since BBB permeability is increased in the EAE model (Ledeboer et al, 2003), the hydrophilic properties of the tracer may in this disease model be less limiting for its use. Therefore, we decided to perform a pilot study to evaluate the perspectives of this tracer in CNS imaging of RRMS.

The best way to evaluate $[^{18}\text{F}]$FB-IL2 PET data is to use the binding potential for quantification of tracer uptake (di Gialleonardo et al, 2012b). This normally requests arterial plasma sampling during the dynamic scan to obtain a plasma input curve. Since arterial sampling is not possible in longitudinal studies such as
ours, and since the heart was visible in the images (within the field of view), we used the blood pool of the heart as input curve (Koeppe, 2007). $[^{18}\text{F}]$FB-IL2 does not show any metabolites in plasma and therefore metabolite analysis in plasma was not required.

Our results did not show an increase in tracer uptake at any time point, but unexpectedly, showed significant decreases in the apparent binding potential at day 6 and day 15 after immunization, as compared to baseline scan. It should be noted that despite the small sample sizes ($n = 2-4$) used in this pilot study, the decrease in the apparent binding potential was statistically highly significant. It is known (Ledeboer et al, 2003) that T-cell infiltration occurs in the CNS tissue during EAE, but it was not detected by either $[^{18}\text{F}]$FB-IL2 PET or immunohistochemical analysis, at the times points evaluated. This is likely due to the fact that T-cell infiltration is an early event in the EAE progression and was not present anymore at the time of investigation. On the other hand, peripheral blood abnormalities in EAE model have been studied by Rose et al (Rose et al, 1989), who showed differences in peripheral T-cell levels during EAE progression. The peripheral T-cell concentration in blood was decreased before disease onset, returned to normal when clinical symptoms appeared, decreased again at the peak of the first relapse, and increased to the normal values during the remission phase and following relapses. This description of peripheral T-cell alterations fits perfectly with the profile found in apparent binding potentials of $[^{18}\text{F}]$FB-IL2 in our study: periods of low levels of peripheral T-cell corresponded to reduced tracer binding. We propose that $[^{18}\text{F}]$FB-IL2 uptake reflects the presence of T-cells in the brain blood vessels, rather than T-cells infiltrated in the brain parenchyma, which could be explained by the inability of the tracer to pass the BBB and/or by the absence of significant levels of T-cells in the CNS. Consequently, the only accessible binding sites remaining were the peripheral T-cells in the brain vasculature. Binding of the
tracer to peripheral T-cells would also explain the discrepancy between PET imaging and immunohistochemistry, which indicated an absence of significant amounts T-cells in the CNS tissue in all time points.

Demyelination was another hallmark evaluated by PET imaging in our study. The tracer $^{[11]}C$MeDAS was used to image myelin content. Brain and part of the spinal cord (cervical + half of thoracic) were visualized in the images. It was not possible to image the lower spinal cord due to the dimensions of the field of view of the microPET scanner. No changes in myelin content were observed over time in this study. Our results differ from the recently published study by Wu et al (2013), who showed differences in myelin content in the spinal cord in EAE and lysolecithin models. However, the significant demyelination found by Wu et al. (2013) was located in the bottom part of the thoracic spinal cord, which was not included in our images. Demyelination was detected in brainstem and spinal cord of our control animals by the Luxol Fast Blue staining, but the lesions were too small (average 0.13 mm) to be detected with the resolution of our small animal PET scanner (approximately 1.5 mm). Therefore, we cannot conclude, that $^{[11]}C$MeDAS PET is unable to show demyelination. We can only speculate that technical limitations were the reason for the inability to detect the lesions.

In conclusion, disease progression and therapeutic effects of dexamethasone on microglia activation and monocyte invasion in the EAE rat model for MS could be longitudinally monitored by $^{[11]}C$PK11195 PET. Using this tool, we could still detect activated microglia/monocyte in the brainstem and spinal cord of dexamethasone treated EAE rats, even when clinical symptoms were no longer present, suggesting that PET can detect even very subtle changes in disease status. $^{[18]}F$FB-IL2 PET showed a strong reduction in the apparent binding potential of the whole brain during disease progression (day 6 and 15), but this is likely related to changes the
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in T-cell levels in the brain blood vessels, rather than changes in T-cell numbers in
the brain parenchyma. Changes in myelin content could not be detected during
disease progression by $^{11}$CMeDAS PET, likely because the resolution of the PET
camera was insufficient to detect the small lesions. Overall, this study shows that
PET imaging with multiple tracers, in combination with relevant disease models,
provides a highly useful approach to longitudinally study disease progression of
MS at a molecular level.

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