Small-animal PET imaging of the type 1 and type 2 cannabinoid receptors in a photothrombotic stroke model

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Abstract

Purpose Recent ex vivo and pharmacological evidence suggests involvement of the endocannabinoid system in the pathophysiology of stroke, but conflicting roles for type 1 and 2 cannabinoid receptors (CB₁ and CB₂) have been suggested. The purpose of this study was to evaluate CB₁ and CB₂ receptor binding over time in vivo in a rat photothrombotic stroke model using PET.

Methods CB₁ and CB₂ microPET imaging was performed at regular time-points up to 2 weeks after stroke using [¹⁸F]MK-9470 and [¹¹C]NE40. Stroke size was measured using MRI at 9.4 T. Ex vivo validation was performed via immunostaining for CB₁ and CB₂. Immunofluorescent double

stainings were also performed with markers for astrocytes (GFAP) and macrophages/microglia (CD68).

Results [¹⁸F]MK-9470 PET showed a strong increase in CB₁ binding 24 h and 72 h after stroke in the cortex surrounding the lesion, extending to the insular cortex 24 h after surgery. These alterations were consistently confirmed by CB₁ immunohistochemical staining. [¹¹C]NE40 did not show any significant differences between stroke and sham-operated animals, although staining for CB₂ revealed minor immunoreactivity at 1 and 2 weeks after stroke in this model. Both CB₁⁺ and CB₂⁺ cells showed minor immunoreactivity for CD68.

Conclusion Time-dependent and regionally strongly increased CB₁, but not CB₂, binding are early consequences of photothrombotic stroke. Pharmacological interventions should primarily aim at CB₁ signalling as the role of CB₂ seems minor in the acute and subacute phases of stroke.

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Introduction

Cerebral ischaemia or stroke is one of the leading causes of complex chronic disability worldwide and has a high mortality rate [1]. The associated reduction in oxygen supply to the brain is usually the result of thrombotic or embolic middle cerebral artery occlusion (MCAO) or occlusion of one of its branches [2]. Most of the available drugs target the penumbra, which has been defined as brain tissue that is functionally impaired but potentially salvageable if treated in time [3]. Unfortunately, there is just a small time window of opportunity so that most of the drugs that work in the acute phase are ineffective if administered more than 6 h

after cerebral ischaemia [4]. Hence, elaborate research and drug development for stroke are indispensable.

There is a large amount of ex vivo evidence suggesting the involvement of the endocannabinoid system in cerebral ischaemia as it acts as a modulator of homeostasis and pathological response [5, 6]. The endocannabinoid system consists of the type 1 cannabinoid receptor (CB₁), which is predominantly expressed in the brain, and the type 2 cannabinoid receptor (CB₂), which is mainly expressed in immune cells. Other compounds are endogenous cannabinoids, such as *N*-arachidonoyl ethanolamide and 2-arachidonoyl-glycerol, and proteins responsible for their synthesis, transport and degradation [7, 8].

It has been suggested that cannabinoid agonists protect cortical neurons from ischaemic injury through interaction with CB₁, which occurs as early as 2 h and persists for at least 72 h after surgery [9]. In CB₁ knockout mice, mortality from permanent focal cerebral ischaemia is increased, infarct size and neurological deficits after transient focal cerebral ischaemia are more severe, cerebral blood flow in the ischaemic penumbra during reperfusion is reduced and NMDA neurotoxicity is increased compared to wild-type mice [10].

Although these studies support a protective effect of CB₁ activation, contradictory new data indicate neuroprotective effects of CB₁ blockade. Berger et al. have shown that administration of the CB₁ antagonist SR141716A reduces infarct size by approximately 40 % [11]. In addition, Zhang et al. have demonstrated that exogenous selective CB₂ agonists reduce infarct volume and improve motor function in a mouse cerebral ischaemia/reperfusion model. This progress is associated with decreased leucocyte/endothelial cell interactions. Moreover, the combination of a CB₁ antagonist (SR141716) and a CB₂ agonist (O-1966) provided an even higher level of protection in ischaemia by increasing the regional cerebral blood flow during ischaemia [12, 13]. Furthermore, Murikinati et al. showed that administration of the CB₂ agonist JWH-133 decreases infarct size in a mouse model of ischaemic stroke by inhibiting neutrophil recruitment to the brain [14]. CB₂ is mostly expressed in immune cells and may have an important influence on stroke-induced inflammatory reactions [12, 15].

Despite the efforts of many research groups to characterize the expression of both CB₁ and CB₂ in stroke ex vivo, their exact and relative role in the pathophysiology of stroke remains unclear. The development of high-affinity and selective radioligands for in vivo brain CB₁ and CB₂ quantification opens new perspectives in this context.

In this study, changes in CB₁ and CB₂ after cerebral ischaemia were investigated quantitatively and longitudinally in a photothrombotic stroke model in rats. CB₁ and CB₂ expression were measured by small-animal PET with [¹⁸F]MK-9470 and [¹¹C]NE40, respectively [16–18], in

correlation with morphological measures of stroke extent and severity.

Materials and methods

Animals and surgery

Animals were housed under a 12 h light/12 h dark cycle with free access to food and water. All animal experiments were performed in accordance with national and international regulations, and approved by the local animal care and ethics commission. Animals scanned using [¹⁸F]MK-9470 (CB₁ imaging) were anaesthetized before surgery with a mixture of ketamine (Ketalar[®], 60 mg/kg; Pfizer, Brussels, Belgium) and medetomidin (Domitor[®], 0.4 mg/kg intraperitoneal; Pfizer). Anaesthesia was reversed with atipamezol (Antisedan[®], 1 mg/kg intraperitoneal; Orion Pharma, Newbury, UK) at the end of surgery. Animals scanned using [¹¹C]NE40 (CB₂ imaging) were anaesthetized with 2–2.5 % isoflurane/O₂ gas anaesthesia (Halocarbon Products Corporation, River Edge, NJ) through a face mask during surgery.

For logistic reasons in tracer production, the study design included two separate groups of animals for the CB₁ and CB₂ scans. In total, 45 male Wistar rats were investigated. The first group (CB₁ imaging) comprised 21 8-week-old rats (Janvier, Le Genest Saint Isle, France), of which 14 received a photothrombotic cortical stroke and 7 received a sham operation. The second group (CB₂ imaging) comprised 16 10-week-old rats (Janvier, and for logistic reasons concerning animal delivery, Harlan, Laboratories, Horst, The Netherlands), of which 9 received a cortical stroke and 7 received a sham operation. A final group of 8 Wistar rats (Janvier), anaesthetized with 2–2.5 % isoflurane/O₂ gas anaesthesia (Halocarbon Products Corporation), was added for histological analysis of the stroke animals. The experimental outline is given in Fig. 1. Functional imaging of brain CB₁ and CB₂ receptor availability was performed in stroke rats and sham-operated rats before surgery, and 24 h, 72 h and 1 week after surgery. For acute assessment CB₁ and CB₂ imaging was included at 3.5 h and 1–2 h, respectively. For the CB₂ imaging group, an extra time-point was added at 2 weeks after surgery as inflammatory cells participate in tissue remodelling and reconstruction following injury that takes place in the weeks after stroke [19].

Cortical ischaemia (stroke model) was induced by photothrombotic injury [20]. After intravenous injection of the photosensitizer Rose Bengal (20 mg/kg; Sigma-Aldrich, St. Louis, MO), an area of exposed intact skull was irradiated for 20 min with green light (wavelength 540 nm, bandwidth 80 nm) from a xenon lamp (model L-4887; Hamamatsu Photonics, Hamamatsu City, Japan) with heat-absorbing

Fig. 1 Experimental design for CB₁ ([¹⁸F]MK-9470) (a) and CB₂ ([¹¹C]NE40) (b) microPET imaging of photothrombotic stroke model rats

and green filters. The radiation with an intensity of 0.68 W/cm² was directed with a 3-mm optical fibre placed on the skull above the right sensorimotor cortex next to bregma. The photooxidation causes endothelial damage, platelet activation, and finally vascular occlusion [20]. The sham-operated animals underwent the same operation procedure except for irradiation with green light.

Radiotracer preparation

CB₁ imaging was done using the radioligand [¹⁸F]MK-9470 (*N*-[2-(3-cyano-phenyl)-3-(4-(2-[¹⁸F]fluoroethoxy)-phenyl)-1-methylpropyl]-2-(5-methyl-2-pyridyloxy)-2-methylpropanamide), which has a high specificity and high affinity for CB₁ (*K*_i 0.7 nM) [16]. The precursor for [¹⁸F]MK-9470 was obtained from Merck Research Laboratories (MRL, West Point, PA). Radiolabelling was performed on-site by alkylation of the precursor with 2-[¹⁸F]fluoroethylbromide. CB₂ imaging was performed with the radioligand [¹¹C]NE40 (2-oxo-7-[¹¹C]methoxy-8-butyloxy-1,2-dihydroquinoline-3-carboxylic acid cyclohexylamide). Radiosynthesis of [¹¹C]NE40 (*K*_i 9.6 nM) was performed as previously described [17]. The specific activity ranges of [¹⁸F]MK-9470 and [¹¹C]NE40 were 53–320 GBq/μmol and 37–222 GBq/μmol, respectively.

Prior to small-animal PET imaging, rats were anaesthetized using 3 % isoflurane in 2.0 l/min oxygen. The effect of this anaesthetic on the uptake of [¹⁸F]MK-9470 in rat brain has been described elsewhere [21]. Tail veins were catheterized for injection of [¹⁸F]MK-9470 (22.13 ± 1.67 MBq, 6.93 × 10⁻⁵–4.24 × 10⁻⁴ μmol) or [¹¹C]NE40 (46.25 ± 9.18 MBq, 2.08 × 10⁻⁴–1.25 × 10⁻³ μmol).

PET data acquisition and reconstruction

Small-animal PET imaging was performed using a FOCUS 220 tomograph (Siemens/Concorde Microsystems, Knoxville, TN), which has a nominal transaxial resolution of 1.35 mm full-width at half-maximum. Data were acquired in a 128 × 128 × 95 matrix with pixel widths of 0.475 mm

and 1.898 mm for [¹⁸F]MK-9470 and [¹¹C]NE40 imaging, respectively, and a slice thickness of 0.796 mm. [¹⁸F]MK-9470 measurements were obtained over 20 min starting 1 h after injection after overnight fasting of the animals. Specific binding of [¹⁸F]MK-9470 to CB₁ was confirmed by a blocking imaging study in one animal. Approximately 1 h before injection of the radioligand, the animal was treated orally with SR141716A (Acomplia®, Sanofi-Aventis, Diegem, Belgium), a selective CB₁ antagonist, at a high blocking dose (10 mg/kg) [22]. [¹¹C]NE40 binding was measured via a 60-min dynamic acquisition immediately after intravenous injection of approximately 37 MBq of the tracer. Since three animals were scanned simultaneously in the CB₂ group, attenuation correction was performed using a rotating ⁵⁷Co source (10-min scan). No attenuation correction was performed for the other animals as they were scanned individually. Data were reconstructed using filtered back-projection.

MR imaging

MRI data were acquired 24 h after surgery in the CB₁ group, and 24 h and between 8 and 11 days after surgery in the CB₂ group. Rats were anaesthetized with isoflurane (Halocarbon, River Edge, NJ) in oxygen (4 % for induction, 1.5–2.5 % for maintenance). Breathing rate and body temperature were monitored and maintained at 55–80 min⁻¹ and 37 ± 1 °C, respectively. MR images were acquired using a Bruker Biospec 9.4-T small-animal MR scanner (horizontal bore 20 cm; Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradients (600 mT/m). Images were recorded using a 7-cm linear resonator (Bruker BioSpin) combined with an actively decoupled dedicated rat brain surface coil (Rapid Biomedical, Rimpf, Germany). T₂ maps were acquired using a multislice/multiecho (MSME) sequence with the following parameters: ten echo time increments of 10 ms, TR 2,252 ms, FOV 40 × 40 mm, in-plane resolution 156 μm, 20 interlaced slices of thickness 0.4 mm. High-resolution three-dimensional MR images were acquired using either a gradient-echo sequence

(FLASH; TR 100 ms, TE 12 ms, flip angle 30°, FOV 40×26×13 mm, isotropic resolution 100 µm) or a spin echo sequence (TurboRARE; TR 1,000 ms, TE 49 ms, FOV 30×30×12 mm, resolution 100×150×150 µm, 8 days after surgery). Stroke volumes were manually delineated and quantified on transverse MSME images using PMOD 3.1. Three-dimensional MRI was used for coregistration with PET images to delineate stroke lesions.

Image processing

Parametric images based on standardized uptake values (SUV; activity concentration in megabecquerels per millilitre×body mass in grams/injected dose in megabecquerels) were generated as a measure of CB₁ binding [16, 23–25]. Relative uptake of [¹⁸F]MK-9470 was expressed as SUV normalized on the whole-brain SUV.

Parametric CB₂ binding potential (BP) images, representing CB₂ availability, were constructed using a reference tissue model. The contralateral cortex was used as the reference tissue instead of the cerebellum, since CB₂ is expressed by a subpopulation of microglia in the cerebellum [26], and the Logan graphical method was used implemented in PMOD 3.1.

To obtain maximal use of [¹⁸F]MK-9470 image information, images were analysed on a voxel-by-voxel basis using SPM2 [27]. This methodology allows reporting results as coordinates directly corresponding to the Paxinos coordinate system of rat brain. Parametric [¹⁸F]MK-9470 images were analysed by statistical parametric mapping (SPM) both in a multisubject design using subjects (stroke vs. sham)×conditions (baseline, 3.5 h, 24 h, 72 h and 1 week after surgery; note that the 3.5-h time-point included a different set of different animals). Spatially normalized images were masked to remove extracerebral signal. All images were smoothed with an isotropic gaussian kernel of 1.6 mm.

For analysis of absolute [¹⁸F]MK-9470 receptor binding, no proportional scaling was used and an analysis threshold of 0.8 of the mean image intensity was applied. To study regional [¹⁸F]MK-9470 uptake, proportional scaling was used. To minimize false-positive findings, T-map data were interrogated at a peak level of $p_{\text{height}} < 0.001$ (uncorrected) and extent threshold $k_E > 200$ voxels (1.6 mm³), as described previously [28]. Only those clusters that were significant at the $p_{\text{cluster}} < 0.05$ (corrected) level were considered.

Histological assessment of stroke lesions

At each time-point after surgery at least two rats were killed by an intraperitoneal injection of pentobarbital followed by transcardial perfusion with 4 % paraformaldehyde in phosphate-buffered saline (PBS). The brain was removed, preserved in 4 % paraformaldehyde in PBS, then put through a dehydrating series of graded concentrations of alcohol, and finally

embedded in paraffin. Paraffin sections (5–8 µm) were made and attached to SuperFrost Plus glass slides (Menzel, Braunschweig, Germany). Sections were deparaffinized with xylol, followed by decreasing concentrations of alcohol, and then washed in PBS. Prior to immunohistochemical staining, antigens were retrieved (sections were microwaved in a 10 mM citrate buffer at pH 6.0), and endogenous peroxidase activity was quenched with 0.5 % H₂O₂.

Diaminobenzidine staining

Nonspecific binding sites were blocked with 3 % normal goat serum. Sections were then either incubated with a rabbit anti-CB₁ polyclonal antibody (Abcam, Cambridge, UK) at a dilution of 1/100 in PBS or a rabbit anti-CB₂ polyclonal antibody (Cayman Chemical, MI) at a dilution of 1/150 in PBS for 1 h. Both antibodies are immunoreactive towards rat antigens. Sections were then incubated with a peroxidase-labelled polymer conjugated to a goat antirabbit secondary antibody for 30 min (Envision System®; DakoCytomation, Glostrup, Denmark). Immunoreactivity was visualized with 3,3'-diaminobenzidine. Sections were counterstained with Mayer's haematoxylin, coverslipped, and examined under a Mirax Desk digital microscope (Zeiss, Oberkochen, Germany). Negative controls were negative and positive controls stained positive for each antibody (data not shown).

Immunofluorescent double staining

Nonspecific binding sites were blocked with 10 % normal donkey serum for 30 min. Sections were then incubated with a rabbit anti-CB₁ polyclonal antibody (Abcam, Cambridge, UK) at a dilution of 1/100 in PBS or a rabbit anti-CB₂ polyclonal antibody (Cayman Chemical, MI) at a dilution of 1/150 in PBS combined with a mouse anti-CD68 monoclonal antibody (Abcam) at a dilution of 1/100 in PBS or a mouse anti-GFAP monoclonal antibody (Abcam) at a dilution of 1/400 in PBS for 1.5 h. All antibodies are immunoreactive towards rat antigens. Sections were then incubated with both an Alexa Fluor 488-labelled donkey antirabbit and an Alexa Fluor 555 donkey antimouse secondary antibody for 45 min at a dilution of 1/1,000 in PBS (Invitrogen, Carlsbad, CA). Following washing with PBS, cell nuclei were visualized by staining with 4',6-diamidino-2-phenylindole dihydrochloride (Invitrogen) and again washed with normal water followed by MilliQ water. Finally, sections were coverslipped using fluorescence mounting medium (DakoCytomation, Glostrup, Denmark) and examined using AxioVision (Zeiss, Oberkochen, Germany).

Statistics

[¹¹C]NE40 BP images were analysed statistically with Statistica v. 8.0 (StatSoft, Tulsa, OK). Significance was

accepted at the 95 % probability level. Data are expressed as means $\pm$ SD.

Results

Photothrombotic stroke lesion

All animals except one in the CB₂ group survived surgery. In the CB₁ group, the average lesion volume was $12.9 \pm 6.1 \text{ mm}^3$ (range of 21.8 mm^3) as determined by high-resolution three-dimensional MR imaging. In the CB₂ group, the average lesion volume was $18.5 \pm 9.0 \text{ mm}^3$ (range of 22.9 mm^3). There was no significant difference in stroke volume between the two groups.

CB₁ binding

Absolute [¹⁸F]MK-9470 SUV values were not significantly different between stroke and sham-operated animals at all time-points measured. Regional relative analysis, however, showed that CB₁ binding was locally higher in stroke animals than in sham-operated animals around the lesion: in stroke animals CB₁ binding in the ipsilateral sensorimotor cortex was $28 \pm 9 \%$ higher 24 h ($p = 1.3 \times 10^{-7}$) and $26 \pm 8 \%$ higher 72 h

($p = 6.4 \times 10^{-6}$) after surgery than in sham-operated animals. Additionally, CB₁ binding was $17 \pm 8 \%$ higher ($p = 5.0 \times 10^{-5}$) in the ipsilateral insular cortex in stroke rats than in sham-operated rats 24 h after surgery. At 3.5 h and 1 week after surgery, no significant differences were measured between the two groups. Decreases in CB₁ binding were measured inside the necrotic lesions 24 h and 72 h after surgery compared to baseline (Fig. 2). Statistical parametric maps of [¹⁸F]MK-9470 are shown in Fig. 3 for 24 h, for 72 h and for 3.5 h/1 week after surgery. Detailed cluster peak locations, intensity differences and p values from the SPM analyses are shown in Table 1.

Since disruption of the blood–brain barrier is intrinsic to stroke and may lead to an increase in brain perfusion resulting in an aspecific increase in tracer uptake in the stroke area, specific binding of [¹⁸F]MK-9470 to CB₁ was confirmed by blocking the receptor with SR141716A in an animal 24 h after stroke [29]. After blocking with SR141716A (10 mg/kg) 1 h before injection of [¹⁸F]MK-9470, a uniform decrease of $\pm 30 \%$ in signal was observed in all brain regions including the stroke region, indicating a specific increased uptake of [¹⁸F]MK-9470 around the stroke area. Figure 4 illustrates this decrease in tracer uptake compared to the average uptake of the tracer 24 h after stroke.

Fig. 2 Representative transverse, coronal and sagittal small-animal PET images of [¹⁸F]MK-9470 tracer binding and MR images of a representative stroke animal. The images obtained at baseline, and 24 h, 72 h and 1 week after stroke are shown. The coloured bars indicate SUV values

Fig. 3 Coronal brain sections showing overlays on the regions where statistically significant increases in relative [^{18}F]MK-9470 binding were observed in animals with a photothrombotic stroke compared to sham-operated animals (**a**) 24 h after surgery ($p_{\text{height}} < 0.001$ uncorrected), (**b**) 72 h after surgery ($p_{\text{height}} < 0.001$ uncorrected) and (**c**) 3.5 h and 1 week after surgery ($p_{\text{height}} < 0.05$ uncorrected). Significance is shown with a T statistic colour scale, which corresponds to the level of significance at the voxel level. The distance between the sections is 1.00 mm with the position relative to bregma at the top left of the images. CB_1 increases are mainly located in the region around the lesions (sensorimotor cortex) 24 h and 72 h after surgery and in the ipsilateral insular cortex 24 h after surgery. No significant CB_1 changes are seen 3.5 h or 1 week after surgery

CB_2 binding

At none of the time-points measured was a significant difference in [^{11}C]NE40 BP detected between the stroke and sham-operated animals, either in the stroke area or in other cortical sides of the animals. Figure 5a shows the [^{11}C]NE40 binding before surgery and 24 h, 72 h, 1 week and 2 weeks after surgery in a representative stroke model. No significant uptake could be distinguished at either time-point. Figure 5b shows the average time-activity curves for both the ipsilateral and the contralateral region of the stroke animals 1 week after

surgery. Data were similar at all other time-points measured in both stroke and sham-operated animals.

Histological analysis

Animals were killed at different time-points (1–2 h, 3.5 h, 24 h, 72 h, 1 week and 2 weeks after stroke; two or three per time-point). Brain slices were stained for both CB_1 and CB_2 and counterstained with haematoxylin (Fig. 6). Between 24 h and 72 h after surgery, CB_1^+ cells accumulated around the border of the photothrombotic lesion corresponding to

Table 1 Peak locations for the clusters in the group comparisons at $p_{\text{height}} \leq 0.001$ uncorrected, $k_{\text{ext}} > 200$ (categorical analysis, [^{18}F]MK-9470, stroke > sham)

Time after surgery (h)	Cluster level		Voxel level		Structure				Location
	p_{corr}	k_{E}	T	p_{uncorr}	Difference (%)	x	y	z	
24	<0.001	6,325	5.94	≤ 0.001	+28.2	-5.2	-2.4	-4.0	Around the lesion, i.e. sensorimotor cortex
			5.05			-4.4	1.2	-3.2	
			3.40			-1.8	3.8	-2.6	
	<0.001	1,037	4.22	<0.001	+17.5	-6.0	-1.8	-7.6	Insular cortex
			4.01			-4.0	0.2	-8.0	
72	<0.001	1,398	4.83	<0.001	+26.2	-5.0	-2.4	-2.0	Around the lesion, i.e. sensorimotor cortex

Fig. 4 In vivo [^{18}F]MK-9470 microPET images in rats 24 h after stroke. **a** Average image of all stroke rats scanned 24 h after surgery. **b** [^{18}F]MK-9470 image acquired after blocking with SR141716A (10 mg/kg orally) approximately 1 h before injection of the radioligand. Blocking with SR141716A resulted in a decrease of $\pm 30\%$ in all brain regions. The colour scale indicates SUV units. A predefined VOI map is overlain on the images

the uptake of [^{18}F]MK-9470 (Fig. 6a). No CB_1^+ cells were seen in the lesion itself, nor in the insular cortex at these time-points. From 1 week after stroke, CB_1^+ cells infiltrated into the inner part of the lesion resulting in abundant cells inside the lesion at 2 weeks (Fig. 6a). At 1–2 h, 24 h and 72 h after stroke, no CB_2^+ cells could be seen beside the lesions, in accordance with the lack of specific [^{11}C]NE40 uptake at these time-points (Fig. 6b). A small amount of CB_2^+ cells was seen 1 and 2 weeks after surgery in the border zone of the lesions (Fig. 6b).

Next, we characterized the CB_1^+ and CB_2^+ cells in more detail by double immunofluorescent staining with antibodies against CB_1/CB_2 and GFAP (marker for astrocytes) or CD68 (marker for activated macrophages and microglia). In the border of the lesions, none of the CB_1^+ or CB_2^+ cells were GFAP $^+$, demonstrating that the CB_1^+ or CB_2^+ cells accumulating around the stroke region were not astrocytes (data not shown). A minority of the CB_1^+ and CB_2^+ cells stained positive for CD68 (Fig. 7).

Discussion

We characterized changes in both CB_1 and CB_2 for the first time in vivo in a rat model of stroke by small-animal PET using [^{18}F]MK-9470 and [^{11}C]NE40, respectively. [^{18}F]MK-9470 and [^{11}C]NE40 small-animal PET imaging is a unique noninvasive method for the in vivo evaluation of pathological changes in the endocannabinoid system in animal models of

focal cerebral ischaemia, with potential translational value. Whole-brain characterization of CB_1/CB_2 in stroke could be used in the proof-of-principle mechanistic investigation of potential pharmacological treatment approaches [13].

[^{18}F]MK-9470 binding was subacutely increased in the region around the strokes by on average 28 % at 24 h, and remained increased by an average of 26 % 72 h after surgery. No signal changes were observed at early time-points (4 h after stroke). [^{18}F]MK-9470 PET is thus a promising in vivo tool for the study of the effects of new drugs that target the endocannabinoid system in the subacute phase after stroke [13]. The increased CB_1 binding at these time-points was confirmed by immunohistochemical analysis. Histologically, at both time-points a large number of CB_1^+ cells were detected around the border zone of stroke lesions. Jin et al. found induction of CB_1 mRNA in the boundary zone surrounding focal ischaemic brain lesions beginning 2 h and persisting for 72 h or more after ischaemia using western blot and immunohistochemistry [9]. Our findings are thus in line with those of this previous ex vivo study, although no increased CB_1 binding was detected 3.5 h after stroke induction. Zhang et al. evaluated the expression of CB_1 by real-time PCR in the ischaemic hemisphere in a mouse model with transient MCAO from 1 h after ischaemia [13]. CB_1 mRNA levels were measured at different time-points up to 24 h after MCAO, and showed a maximal increase at 6 h. There seems to be a delay between mRNA expression and increased binding observed in this model.

Fig. 5 [^{11}C]NE40 PET imaging in stroke rats. **a** Images in a representative animal obtained at baseline and 24 h, 72 h, 1 week and 2 weeks after stroke (columns 1–5, respectively) coregistered with the corresponding MRI images (last column individual MR images of the same animal with VOIs delineated; top row, middle row and bottom

row transverse, sagittal and coronal views, respectively). **b** Average [^{11}C]NE40 uptake in rats 1 week after surgery. There is no significant difference in tracer uptake between the ipsilateral region around the stroke and the contralateral region. Data are shown as means $\pm$ SD

It has been suggested that an increase in CB_1 has neuroprotective effects in several neuropathologies that could be mediated by CB_1 -induced presynaptic inhibition of glutamate release [30]. More specifically, there is a well-established association between CB_1 and inhibition of Ca^{2+} channels that are linked to the release of the excitotoxic neurotransmitter glutamate [31]. Excitotoxicity is also one of the most important pathophysiological events in stroke, occurring very early after onset triggering acute tissue damage and a

number of more delayed events such as postischaemic inflammation [19]. The results of our study suggest that CB_1 is involved in the inflammatory reaction rather than having an immediate effect on the inhibition of glutamate release, since no increase in tracer binding or in CB_1 expression was detected by immunohistochemistry at the early time-point of 3.5 h after stroke.

Another striking finding of the current study was the *ex vivo* detection of CB_1^+ cells inside the lesion 1 and 2 weeks

Fig. 6 Representative immunohistochemical staining for CB₁ (a) and CB₂ (b) in stroke lesions 1–3.5 h, 24 h, 72 h, and 1 and 2 weeks after surgery. **a** At 24 h until 72 h increased CB₁ expression is seen around the stroke lesion. At 1 week CB₁⁺ cells are detected inside the lesion rim. At 2 weeks after surgery the lesion is fully covered with CB₁⁺

cells. No increased CB₁ expression is seen at 3.5 h after stroke. **b** Low levels of CB₂⁺ cells are seen starting 1 week after surgery. No CB₂⁺ cells are seen at 1–3.5 h, 24 h and 72 h after surgery. *Inset* areas shown at higher magnification in the images below

Fig. 7 Double immunofluorescence staining of the stroke regions with anti-CB₂ (green) and anti-CD68 (red) antibodies 1 week after stroke. Only a minority of CB₂⁺ cells are positive for CD68 (white arrows CB₂⁺ cells, yellow arrows CB₂⁺ cells costained for CD68)

after stroke, filling the necrotic tissue. Indirectly, this process was also seen by [^{18}F]MK-9470 PET as the decrease in tracer binding present within the stroke lesion 24 h after surgery had almost disappeared 1 week after surgery (Fig. 2). This finding supports those of studies that have addressed the expression profile of CB₁ in inflammatory cells such as lymphocytes, microglia/macrophages and astrocytes, which form a so-called glial scar and/or accumulate inside the lesion over time [32–34]. However, surprisingly, none of the CB₁⁺ or CB₂⁺ cells were GFAP⁺ (GFAP is a cell-specific marker that distinguishes astrocytes from other glial cells) at any of the time-points. As only a proportion of these cells were CD68⁺ and as both CB₁ and CB₂ are expressed on T lymphocytes, where they mediate the immunomodulatory effects of cannabinoids on T-cell function [33], and based on the morphology of the cells, it is possible that the CB₁⁺ or CB₂⁺ cells accumulating inside and around the stroke region 1 week after stroke are T lymphocytes.

We also observed a 17 % increase in CB₁ binding in the ipsilateral insular cortex 24 h after stroke. Involvement of CB₁ in the insular cortex in vivo in a rat model of stroke has not been found before. Nevertheless, this finding may support those of studies indicating that the insula plays putative role in motor recovery in stroke patients. Loubinoux et al. found better motor recovery, more specifically in handgrip strength and finger-tapping speed, 1 year after stroke in patients with a higher early activation in the ipsilateral M1, S1, premotor cortex and insula using fMRI [35]. One caveat, however, is that no correction for partial volume effects was performed in our study.

Regarding the visualization of CB₂ in the rats with photothrombotic stroke, no significant differences in uptake between the ipsilateral and contralateral hemispheres nor between the stroke and sham-operated animals were measured, even though some CB₂⁺ cells were detected ex vivo in the border zone of the lesion starting 1 week after surgery. An increase in CB₂ expression has previously been exclusively described in models involving MCAO [4, 13, 15]. Ashton et al. showed clusters of CB₂ and CD45⁺ microglia/macrophages via immunohistochemistry and Western blot in the ischaemic penumbra at 3 days after MCAO until at least 7 days after surgery [15]. In addition, Zhang et al. measured a small decrease in CB₂ mRNA expression during the first 3 h after ischaemia, but then a continued increase up to 24 h, which was the latest time-point measured. An ex vivo increase in CB₂ expression in the photothrombotic rat model of stroke has not been described before. Although the MCAO model is the model most used preclinically, we opted for the photothrombotic stroke model because an ischaemic lesion can be induced in any desired cortical area, with good reproducibility in lesion size and a high survival rate in comparison to the MCAO model. Since CB₂ expression in activated microglia depends on the combination of cytokines and molecules encountered by these immune cells, the stroke model that is used may have a major

influence on the results [36]. A photothrombotic stroke is followed by an almost immediate disruption of the blood–brain barrier, vasogenic oedema, microvascular occlusions and a peripheral inflammatory response as early as 3 days after the insult [37]. In the MCAO model, macrophage activity starts after a delay of 1–5 days and persists for a longer time [38]. However, our findings may in part also be explained by the low nanomolar affinity of [^{11}C]NE40 for human CB₂ (K_i 9.6 nM). As the expression of CB₂ in our model is rather limited, it is possible that CB₂ radioligands with subnanomolar affinity are needed to be able to visualize the neuroinflammation. As a result [^{11}C]NE40 may not be sensitive enough to detect low concentrations of CB₂⁺ cells in pathological conditions.

Although not shown by small-animal PET imaging, the presence of CB₂⁺ cells shown by immunostaining—albeit modest—in this stroke model, does not refute a potential role of CB₂ in inflammatory processes inherent to the pathophysiology of stroke.

Conclusion

A time-dependent increase in CB₁ binding was present in the region surrounding photothrombotic stroke lesions 24 h and 72 h after the stroke event, extending to the surrounding insular cortex at 24 h after surgery. Normalization occurred within 1 week. No significant changes were detected in CB₂ expression in vivo, although some CB₂⁺ cells were visible ex vivo. These results support pharmacological interventions targeting the subacute phase after stroke to further study the role of CB₁ in the inflammatory response.

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Conflicts of interest None.

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