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**PET imaging of TSPO in a rat model of local neuroinflammation induced by intracerebral injection of lipopolysaccharide.**

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1 **PET imaging of TSPO in a rat model of local neuroinflammation induced by intracerebral**  
2 **injection of lipopolysaccharide**

3

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28 **Key words:** neuroinflammation, [<sup>18</sup>F]DPA-714, PET, lipopolysaccharide

29

30 **Abbreviated title:** Characterization of LPS model

31

32 **Objective:** The goal of this study was to measure functional and structural aspects of local  
33 neuroinflammation induced by intracerebral injection of lipopolysaccharide (LPS) in rats using TSPO  
34 microPET imaging with [<sup>18</sup>F]DPA-714, magnetic resonance imaging (MRI), *in vitro* autoradiography  
35 and immunohistochemistry (IHC) in order to characterize a small animal model for screening of new  
36 PET tracers targeting neuroinflammation.

37

38 **Methods:** Rats were injected stereotactically with LPS (50 μg) in the right striatum and with saline in  
39 the left striatum. [<sup>18</sup>F]DPA-714 microPET, MRI, *in vitro* autoradiography and IHC studies were  
40 performed at different time points after LPS injection for 1 month.

41

42 **Results:** Analysis of the microPET data demonstrated high uptake of the tracer in the LPS injected site  
43 with an affected-to-non-affected side binding potential ratio (BP<sub>right-to-left</sub>) of 3.0 at 3 days after LPS  
44 injection. This BP ratio decreased gradually over time to 0.9 at 30 days after LPS injection. *in vitro*  
45 autoradiography ([<sup>18</sup>F]DPA-714) and IHC (CD68, GFAP and TSPO) confirmed local  
46 neuroinflammation in this model. Dynamic contrast enhanced (DCE) MRI demonstrated BBB  
47 breakdown near the LPS injection site at day 1, which gradually resolved over time and was absent at  
48 1 month after LPS injection.

49

50 **Conclusion:** The LPS model is useful for first screening of newly developed tracers because of the  
51 easy design and the robust, unilateral inflammatory reaction allowing the use of the contralateral  
52 region as control. Additionally, this model can be used to test and follow up the benefits of anti-  
53 inflammatory therapies by non-invasive imaging.

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58 1. Introduction

59 Neuroinflammation is the inflammation associated to with central nervous system (CNS)  
60 pathologies including Parkinson's disease (PD), Alzheimer's disease (AD), stroke, multiple sclerosis  
61 (MS), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), viral/bacterial infection,  
62 neoplasia and head trauma. These diseases trigger a cerebral immune activation which can lead to both  
63 collateral damage to brain tissue as well as neuroregeneration [1]. Microglia are resident CNS cells  
64 that have phenotypic similarities and functions to macrophages and constitute up to 10% of the total  
65 cell population of the brain. Microglia are plastic cells that show phenotypic changes in response to  
66 environmental signals, and can exert either neuroprotective or neurotoxic roles depending on the  
67 context.

68 The most studied imaging biomarker of neuroinflammation is the translocator protein (TSPO)  
69 [2] receptor or peripheral benzodiazepine receptor (PBR) that is expressed on the outer mitochondrial  
70 membrane of microglia and macrophages [3]. TSPO plays a crucial role in neurosteroidogenesis  
71 important during brain development and normal functioning during adulthood [3]. In the central  
72 nervous system, TSPO expression is low under healthy circumstances. However, in response to  
73 neuronal insults, TSPO expression increases mainly in microglia and astrocytes as detected in several  
74 neurodegenerative diseases such as PD, ALS, HD and AD [3]. The role of TSPO in regulation of  
75 neuroinflammation still remains to be elucidated. While TSPO antagonists can attenuate inflammation,  
76 a recent study of Bae et al. [4] suggested that TSPO is a negative regulator of neuroinflammation in  
77 microglia. TSPO has been identified as a valuable imaging biomarker for neuroinflammation since the  
78 degree of TSPO upregulation in response to injury is correlated with the degree of damage in  
79 neuroinflammation [5]. [<sup>18</sup>F]DPA-714 is a specific radiotracer for TSPO and has already been  
80 successfully evaluated in several animal models of neuroinflammation [6–8], in healthy humans [9]  
81 and ALS patients [10]. Recently, Owen *et al.* [11] reported a human TSPO polymorphism with a  
82 trimodal distribution in binding affinity (high-affinity, low-affinity and mixed affinity binders) for  
83 several TSPO ligands. The consequence of this polymorphism is that knowledge of binding status is  
84 needed to correctly quantify TSPO expression using these PET ligands in humans [11]. In the present

85 study, we used [<sup>18</sup>F]DPA-714 to characterize TSPO expression in a rat model of acute, local  
86 neuroinflammation induced by lipopolysaccharide (LPS). LPS is found in the outer membrane of  
87 gram-negative bacteria and activates Toll-like-receptor 4 (TLR4) mainly expressed by microglia and  
88 macrophages. TLR4 activation induces signal transduction pathways that regulate diverse  
89 transcriptional and posttranscriptional processes involved in inflammation [12]. Moon et al. [13] used  
90 the LPS model to compare [<sup>11</sup>C]PBR28 with their newly synthesized [<sup>18</sup>F]fluoromethyl-PBR28, while  
91 Dickens et al. [14] used the LPS model to compare [<sup>11</sup>C]PK11195 and [<sup>18</sup>F]GE-180. However, only  
92 one time point after LPS injection was studied to compare different TSPO tracers in these previous  
93 reports. The goal of the current study was to fully characterize the LPS rat model longitudinally with  
94 an established neuroinflammation tracer [<sup>18</sup>F]DPA-714, magnetic resonance imaging (MRI) and  
95 immunohistochemistry (IHC) to assess the dynamics of the neuroinflammatory reaction. This model  
96 will be used for screening of newly developed PET radiotracers as putative biomarkers of  
97 neuroinflammation.

98

## 99 2. Material and methods

### 100 a. LPS-induced neuroinflammation rat model

101 An acute rat model of neuroinflammation was developed using lipopolysaccharide (LPS; E.  
102 Coli 055:B5; Sigma Aldrich, St. Louis, MO, USA). Female Wistar rats (250 - 300 g; 2 months old)  
103 were kept under gas anesthesia (2.5% isoflurane in O<sub>2</sub> at a flow rate of 1 L/min) and positioned in a  
104 stereotactic head frame (Stoelting, Wood Dale, IL, USA). A small hole was drilled in the skull at the  
105 appropriate location using Bregma as reference. Neuroinflammation was induced by injecting 50 μg  
106 LPS dissolved in 4 μl of sterile NaCl 0.9% at the following coordinates: +0.5 mm antero-posterior, 3  
107 mm lateral (right hemisphere), 5.5/4.5 mm dorsoventral. After injection of 2 μl, the needle was  
108 retracted for 1 mm dorsoventral and another 2 μl was injected. The needle was left in place for an  
109 additional 10 min before being slowly withdrawn from the brain. The contralateral side was injected as  
110 control with 4 μl of sterile 0.9% NaCl solution. Animals (N=27) were housed in individually

111 ventilated cages in a thermoregulated (~22 °C), humidity-controlled facility under a 12 h/12 h  
112 light/dark cycle with access to food and water ad libitum. All animal experiments were conducted  
113 according to the Belgian code of practice for the care and use of animals, after approval from the local  
114 University Ethics Committee for Animals.

115

116 b. Experimental design

117

118 The experimental design is summarized in figure 1. MicroPET and MRI studies were performed on 1,  
119 3, 7 and 30 days after LPS injection. *In vitro* autoradiography and IHC were performed on 1, 4, 8 and  
120 31 days after LPS injection. There is a difference of 1 day between the *in vivo* and *in vitro* experiments  
121 because from the animals that were scanned (PET and MRI) on 3, 7 and 30 days after LPS injection,  
122 one animal was sacrificed 1 day later, respectively on day 4, 8 and 31, for *in vitro* autoradiography and  
123 IHC to verify microglial proliferation and TSPO expression in the same animal. In that manner the  
124 fluorine-18 was sufficiently decayed and brain slices could be made. On day 1 after LPS injection we  
125 did not observe significant retention of [<sup>18</sup>F]DPA-714 in the ipsilateral site in the microPET study and  
126 therefore we sacrificed the animals at day 1 after LPS injection for *in vitro* autoradiography and IHC.  
127 To increase N for *in vitro* autoradiography and IHC, additional animals were injected with LPS and  
128 sacrificed on day 1, 4, 8 and 31 after LPS injection for *in vitro* autoradiography and IHC (N≥3 for each  
129 time point and each experiment; Figure 1). IHC and *in vitro* autoradiography were performed on  
130 adjacent brain slices. Additionally, a pre-treatment study (PK11195 10 mg/kg subcutaneously 60 min  
131 before tracer injection; N=3) and a displacement study (PK11195 5 mg/kg intravenously 30 min after  
132 tracer injection; N=3) were performed at 3 days after LPS injection. All statistical studies were  
133 performed with the unpaired two-tailed t-test, a *p* value less than 0.05 was considered statistically  
134 significant. Calculations were carried out using GraphPad Prism v5.0 (San Diego, CA).

Figure 1

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136

137

138 c. Radiosynthesis

139 The radiotracer [<sup>18</sup>F]DPA-714 was synthesized as previously described [15] with some small  
140 modifications: most importantly, the semi-preparative HPLC purification was performed using an  
141 ethanol based mobile phase, EtOH:NH<sub>4</sub>OAc 10 mM pH 7 35:65 V/V. The final preparation containing  
142 less than 10% of EtOH was sterile filtered through a 0.22- $\mu$ m membrane filter (Millex<sup>®</sup>-GV,  
143 Millipore, Billerica, USA). [<sup>18</sup>F]DPA-714 (> 98% radiochemically pure) was obtained with 45-60%  
144 yield (relative to starting [<sup>18</sup>F]F<sup>-</sup> radioactivity). The specific activity at end of synthesis ranged from 56  
145 to 148 GBq/ $\mu$ mol. Precursor and reference compound were kindly provided by Prof. Michael Kassiou  
146 (University of Sydney, Australia).

147

148 d. microPET

149 Imaging experiments were performed on a Focus 220 microPET scanner (Concorde  
150 Microsystems, Knoxville, TN, USA). Rats were injected with about 74 MBq of [<sup>18</sup>F]DPA-714 via a  
151 tail vein (volume injected <1 mL; specific activity at injection: 51-130 GBq/ $\mu$ mol). During all PET  
152 sessions, animals were kept under gas anesthesia (2.5% isoflurane in O<sub>2</sub> at a flow rate of 1 L/min).  
153 List-mode 120-min microPET scans were acquired. Acquisition data were then Fourier rebinned in 27  
154 time frames (4 x 15 s, 4 x 60 s, 5 x 180 s, 8 x 300 s, 6 x 600 s) and reconstructed using maximum a  
155 posteriori iterative reconstruction. The images were spatially aligned to a rat brain [<sup>18</sup>F]FDG template  
156 in Paxinos coordinates [16] using an affine transformation, allowing the use of a predefined volumes  
157 of interest map. Time-activity curves (TAC) were generated for right and left striatum for each  
158 individual scan using PMOD software (version 3.2; PMOD technologies, Zurich, Switzerland).

159 Kinetic modeling based on simplified reference tissue model (SRTM) [17] with the  
160 contralateral (left) striatum as a reference region was performed to quantify the uptake difference  
161 between the right and the left striatum.

162

163

164 e. *In vitro* autoradiography

165 The rats were sacrificed, brain was removed, rinsed with saline to remove blood, rapidly  
166 frozen in 2-methylbutane (-40 °C) and stored at -20 °C for 24 h. Transversal sections from the brain  
167 were obtained using a cryotome (Shandon cryotome FSE; Thermo Fisher, Waltham, USA), mounted  
168 on adhesive microscope slides (Superfrost Plus; Thermo Fisher) and stored at -20 °C until  
169 autoradiography was performed. Brain slices were dried and preincubated in 50 mM tris-HCl buffer  
170 (pH 7.4) for 10 min at room temperature. Before incubation with [<sup>18</sup>F]DPA-714, the brain sections  
171 were dried. The brain sections were incubated with 590 kBq of tracer for 10 min. The brain sections  
172 were washed twice for 10 min in 50 mM tris-HCl (pH 7.4) + 0.3% BSA buffer at 4 °C. After a quick  
173 dip in water at 4 °C, the slides were dried. Autoradiograms were obtained by exposing the slides for 5  
174 min to a high performance phosphor storage screen (super-resolution screen; Perkin Elmer, Waltham,  
175 USA). The screens were read using a Cyclone Plus system (Perkin Elmer) and analyzed using  
176 Optiquant software (Perkin Elmer). The radioactivity concentration in the autoradiograms is expressed  
177 in digital light units (DLU)/mm<sup>2</sup> corrected for background. The data were processed as right striatum  
178 (LPS) to left striatum (saline) ratios (DLU/mm<sup>2</sup> in right striatum divided by DLU/mm<sup>2</sup> in left  
179 striatum).

180

181 f. Immunohistochemistry for TSPO and CD68

182 Cryostat brain sections (adjacent to the slices used for *in vitro* autoradiography) were fixed  
183 with ethanol, blocked with normal serum and incubated overnight at 4 °C with a rabbit polyclonal  
184 primary antibody against TSPO (#NBP1-45769 from Novus Biologicals, Littleton, USA) diluted  
185 1:100. Then, sections were incubated for 2 h at room temperature with a secondary anti-rabbit  
186 antibody (Alexa Fluor-488, Molecular Probes, Eugene, USA). Double immunostaining was carried out  
187 with mouse monoclonal antibodies against the microglia/macrophage marker CD68 (ED1,  
188 #MCA341R, Serotec, Kidlington, Oxford) diluted 1:100 or the astroglia marker glial fibrillary acidic  
189 protein (GFAP, #G3893, Sigma) followed by a secondary anti-mouse antibody (Alexa Fluor-546,



190 Molecular Probes). Sections were counterstained with To-Pro3 (Invitrogen, Carlsbad, USA) to  
191 visualize the cell nuclei. They were mounted in Mowiol 488 (Sigma–Aldrich) and were observed in a  
192 confocal microscope (Leica TCS, SPC, Wetzlar, Germany).

193

194 g. Magnetic resonance imaging (MRI)

195 MR images were recorded on a 9.4T/200 Biospec small animal MR system (Bruker Biospin,  
196 Ettlingen, Germany) equipped with a 117-mm inner diameter actively shielded gradient set of 600  
197 mT/m using a 7-cm linearly polarized resonator for transmission and an actively-decoupled dedicated  
198 rat brain surface coil for receiving (Bruker Biospin). Rats were anesthetized with isoflurane in oxygen,  
199 their respiration monitored and body temperature controlled at 37 °C using a water heating circuit (SA  
200 Instruments, Stony Brook, NY, USA). The following MR images were recorded before contrast  
201 injection: (1) 3-dimensional T<sub>2</sub>-weighted (rapid acquisition with relaxation enhancement, RARE, echo  
202 time (TE) = 11.5 ms, rare factor = 16, repetition time (TR) = 1200 ms, field of view (FOV) =  
203 4x2.6x1.3 cm, matrix = 192x128x64), (2) 3-dimensional T<sub>1</sub> –weighted (fast low angle shot, FLASH,  
204 TR= 75 ms, TE= 12 ms, flip angle 15 deg, FOV = 4x2.6x1.3 cm, matrix = 400x260x130), (3) T<sub>1</sub> map  
205 (RARE with variable TR (TR=112/227/402/603/837/1118/1470/2655/ 4194/8000 ms, TE=8.7 ms ,  
206 rare factor =2, 4 axial slices, FOV= 3.5x3.5 cm, matrix 128x128) and (4) T<sub>2</sub> map (Multi slice multi-  
207 echo (MSME) sequence with array of 10 echo times starting from 12ms and equally spaced by 12 ms,  
208 TR= 2856 ms, 8 axial slices, FOV=2.5 x 2.5 cm, matrix 256 x 256). For DCE MRI a 3-dimensional  
209 T<sub>1</sub>–weighted sequence was used (FLASH, TR= 75 ms, TE= 12 ms, flip angle 15 deg, FOV =  
210 4x2.6x1.3 cm, matrix = 400x260x130) with 15 repetitions (1 min per repetition). The contrast agent  
211 (75 µl of 0.05 mmol/ml meglumine gadoterate, Gd-DOTA; Guerbet, Cadoli, Brussels, Belgium)  
212 chased with 100 µl saline, both heated to 37 °C, was manually injected via a catheter placed in a tail  
213 vein after 5 min.

214 Data acquisition and processing of T<sub>1</sub> maps was carried out using Paravision 5.1 (Bruker Biospin) and  
215 DCE data were analyzed using dedicated software (DCE@urLab v1.0,  
216 <http://www.die.upm.es/im/archives/DCEurLAB/> [18]). Average dynamic profiles for each time point

217 were determined based on manual delineation of the most apparent contrast-enhanced zone and similar  
218 contralateral region. T2 maps were generated by fitting voxelwise the 10 echoes (12, 24, 36, 48, 60,  
219 72, 84, 96, 108 and 120 ms) to a mono-exponential function (eq.1) with a fit goodness  $R^2 > 0.99$   
220 (ImageJ, <http://rsb.info.nih.gov/ij/> [19]).

221

222

223 Eq.1  $M = M_0 e^{-TE/T_2}$

224 M: voxel intensity

225 TE: echo time

226  $T_2$ : transverse relaxation time

227  $M_0$  : initial transverse magnetization

228 Each map was then smoothed by a median filter with a kernel of 136  $\mu\text{m}$ . Local T2 values were  
229 extracted by placing regions of interest of 13  $\text{mm}^2$  centered to the site of injection and on the adjacent  
230 slices.

231

### 232 3. Results

#### 233 a. Longitudinal study of LPS model: microPET, *in vitro* autoradiography and IHC

##### 234 i. Longitudinal microPET study

235 A longitudinal microPET study with [ $^{18}\text{F}$ ]DPA-714 was performed to follow up TSPO  
236 expression at 1, 3, 7 and 30 days after LPS injection. Analysis demonstrated that the right striatum in  
237 which LPS was injected showed higher uptake than the left striatum. Binding potentials calculated  
238 with SRTM are presented in table 1 ( $\text{BP}_{\text{right to left}}$  of animals from the combined MRI-PET study are  
239 included in table 1 as the PET scans were performed at the same time points). Statistical calculations  
240 proved a significant difference between 1 and 3, 1 and 7, 3 and 30, 7 and 30 days after LPS injection  
241 ( $P < 0.05$ ). A representative averaged PET image is shown in figure 6b.

242

243 ii. *In vitro* autoradiography

244 Analysis of the *in vitro* autoradiography results showed higher tracer uptake in the LPS  
245 injected striatum compared to the saline injected striatum. The signal decreased gradually in time with  
246 the highest signal at 4 days after LPS injection (figure 2a-c). The right to left ratios at 1, 4, 8 and 31  
247 days after LPS injection were  $2.1 \pm 0.6$ ,  $5.1 \pm 1.1$ ,  $4.1 \pm 0.9$  and  $3.0 \pm 1.1$ , respectively (figure 1e).  
248 Statistical calculations proved a significant difference between 1 and 4, 1 and 8, 4 and 31, 8 and 31  
249 days after LPS injection ( $P < 0.05$ ). We performed a blocking study using PK11195 ( $20 \mu\text{M}$ ) as a  
250 blocking agent on brain slices 3 days after LPS injection and PK11195 significantly reduced  
251 [ $^{18}\text{F}$ ]DPA-714 binding ( $P < 0.0001$ ; figure 2f-g).

Figure 2

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253

254 iii. Immunohistochemistry (CD68, GFAP and TSPO)

255 IHC showed immunoreactivity for TSPO and CD68 (a marker of myeloid cells expressed by reactive  
256 microglia and macrophages) at 4, 8 and 31 days after LPS injection. The intensity of CD68  
257 immunoreactivity was higher in the ipsilateral than the contralateral hemisphere indicative of  
258 microglia activation by LPS. In the ipsilateral striatum, highly CD68+ immunoreactive cells were  
259 more abundant at 4 and 8 days and decreased at day 31 (figure 3). Immunoreactivity for TSPO was not  
260 detected in the contralateral hemisphere (figure 3f) but was found in the ipsilateral hemisphere located  
261 in CD68+ cells (figure 3a-e). Notably, TSPO expression was detected at day 4 and 8 after LPS  
262 injection, but not at day 1. At day 31, TSPO staining was still detected but was less prominent than at  
263 previous time points after LPS injection. At day 4, TSPO staining was detected in ramified microglia  
264 (arrows figure 3a) and in isolated amoeboid cells with macrophage-like morphology (figure 3b) that  
265 could correspond to reactive microglia or infiltrated macrophages. TSPO+ cells with amoeboid shape  
266 predominated at day 8 (Fig. 3c, d). At day 31, faint TSPO immunoreactivity was detected in ramified  
267 CD68+ cells (figure 3e). TSPO immunoreactivity was mainly located in perivascular  
268 microglia/macrophages suggesting that the vasculature is a target of the inflammatory reaction after  
269 intracerebral LPS administration. In addition, we observed faint TSPO immunoreactivity in regions

270 surrounding the lesion site (including the corpus callosum and cortical regions) in cells with stellate  
271 morphology that could correspond to reactive astrocytes. To confirm this possibility we carried out  
272 double immunofluorescence with an antibody against the astroglia marker GFAP (figure 4). While the  
273 cells with the highest TSPO immunoreactivity were GFAP negative (figure 4a, c), GFAP positive cells  
274 located in the periphery of the LPS injection site showed low TSPO immunoreactivity at days 4, 8 and  
275 31 (figure 4b, d-f) suggesting that reactive astrocytes can express a low level of TSPO.

Figure 3

Figure 4

#### 278 b. Pre-treatment and displacement microPET study

279 In order to determine whether *in vivo* binding of [<sup>18</sup>F]DPA-714 is specific for TSPO and  
280 reversible, we performed a pre-treatment study (PK11195 10 mg/kg subcutaneously 60 min before  
281 tracer injection; N=3) and a displacement study (PK11195 5 mg/kg intravenously 30 min after tracer  
282 injection; N=3) (figure 3). Figure 5a shows a microPET scan at 3 days after LPS injection. The TAC  
283 of the pre-treatment study (figure 5b) shows that binding was not blocked completely although there  
284 was a decrease in tracer uptake in the LPS injected site compared to the TAC without pre-treatment  
285 (figure 3a). IV injection of PK11195 (5 mg/kg) could fully displace the binding of [<sup>18</sup>F]DPA-714 in  
286 the LPS injected site but also in the control (saline injected) site some [<sup>18</sup>F]DPA-714 binding is  
287 displaced (figure 5c).

Figure 5

#### 290 c. MRI

291 Considering that tracer uptake may be influenced by BBB integrity, we used DCE MRI to  
292 determine the level of BBB breakdown induced by the LPS or saline injection. DCE MRI  
293 demonstrated loss of BBB integrity at the LPS injection site but not in the contralateral saline injection  
294 site (figure 6a). The contrast enhancement was most apparent at day 1, reduced at day 3 and 7 and not

295 observable 1 month after LPS injection (figure 7). Accordingly, the differences between the time  
296 points for the group averaged DCE MRI signal intensity time profiles from the striatal region with  
297 LPS injection showed a similar response. Striatal T2 values of the LPS injected side were higher  
298 compared to the contralateral side (saline injected) for each time point with a maximal T2 value  
299 reached between 3 and 7 days after LPS injection (figure 8). These data are consistent with the  
300 longitudinal PET and autoradiography data.

301

Figure 6

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Figure 7

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Figure 8

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316 4. Discussion

317 This LPS model is a very interesting animal model from a quantification point of view since it  
318 has the advantage that neuroinflammation is induced locally around the stereotactic injection site and  
319 each animal can be used as its own control using the contralateral hemisphere. *In vivo* PET imaging  
320 showed the highest tracer uptake at 3 days after LPS injection and this signal remained quite stable  
321 until 7 days after LPS injection. Afterwards, the signal gradually decreased as determined by  
322 calculating  $BP_{\text{right to left}}$  using the simplified reference tissue model with the contralateral striatum as a  
323 reference region (table 1).  $BP_{\text{right to left}}$  were also calculated in a slightly different LPS model (unilateral  
324 injection of 1-10  $\mu\text{g}$  LPS; saline injected in separate animals as control; different animal strain; LPS  
325 from different *E. coli* strains) using [ $^{18}\text{F}$ ]GE-180 and [ $^{11}\text{C}$ ]PK11195 as radioligands by Dickens et al  
326 [14]. Additionally, in this study, the animals were scanned at 16 h after LPS injection while the earliest  
327 time point in our study was 24 h after LPS injection hampering a reliable comparison of both studies.  
328 Interestingly, both studies confirmed an early (16-24 h) increase of TSPO expression after LPS  
329 injection. Boutin et al. [6] compared [ $^{18}\text{F}$ ]DPA-714 and [ $^{11}\text{C}$ ]PK11195 in a rat stroke model. Although  
330 the BP (calculated with SRTM) seemed comparable for [ $^{18}\text{F}$ ]DPA-714 in both animal models (stroke  
331 model: 3.1; LPS model 3.0), the standard deviation was higher for the stroke model (stroke model: 3.0;  
332 LPS model 0.3). This variability in animal models of neuroinflammation needs to be considered when  
333 comparing different tracers in the same model. Preferably the same animal is scanned with different  
334 tracers and the scans need to be performed within a limited time window because of the longitudinal  
335 variation in neuroinflammation as observed in this study.

336 In order to verify specific and reversible binding of [ $^{18}\text{F}$ ]DPA-714 in this specific animal  
337 model, displacement and pre-treatment studies were performed. [ $^{18}\text{F}$ ]DPA-714 binding in the lesion  
338 site could partly be blocked by pre-treatment with PK11195 (10 mg/kg subcutaneously 60 min before  
339 tracer injection). Subcutaneous administration was chosen because of the slow pharmacokinetics and  
340 thus slow but rather constant release of the blocking agent in blood. IV injection has fast kinetics but  
341 dissociation from the target at the time of tracer injection might occur. Probably the dose administered  
342 of PK11195 was not high enough for complete blocking via subcutaneous route. Interestingly, IV

343 injection (fast pharmacokinetics) of 5 mg/kg PK11195 30 min after tracer injection could fully  
344 displace the binding of [<sup>18</sup>F]DPA-714 in the LPS injected site but also in the saline injected site some  
345 [<sup>18</sup>F]DPA-714 binding is displaced which is probably due to basal TSPO expression in brain. These  
346 results are in line with the displacement study performed by Moon et al. in a more comparable LPS  
347 model than the LPS model of Dickens et al. (unilateral injection of 50 μg LPS though different rat  
348 strain; contralateral striatum as control but no saline injection) using different TSPO tracers  
349 ([<sup>18</sup>F]fluoromethyl-PBR28 and [<sup>11</sup>C]PBR28), although displacement in non-injected striatum was less  
350 than observed in our study [13,14].

351 *In vitro* autoradiography showed higher tracer uptake in the LPS injected striatum compared to  
352 the saline injected striatum. This higher uptake was already observed at 1 day after LPS injection and  
353 increased to the most intense and quite stable signal between 4 and 8 days after LPS injection after  
354 which the signal gradually decreased in time as determined by calculating the right to left ratios. The  
355 presence of PK11195 (20 μM) completely blocked [<sup>18</sup>F]DPA-714 binding, suggesting that the  
356 observed [<sup>18</sup>F]DPA-714 binding is TSPO specific. *In vitro* but not *in vivo* retention of [<sup>18</sup>F]DPA-714  
357 uptake was noticed in white matter (corpus callosum) which was also observed by Dickens et al. using  
358 [<sup>18</sup>F]GE-180 [14].

359 The *in vitro* data confirmed the *in vivo* data by a positive correlation between BP<sub>right-to-left</sub> (*in*  
360 *vivo* microPET) and right-to-left ratios (*in vitro* autoradiography). Another important validation in pre-  
361 clinical PET studies is the confirmation of expression of the target protein by IHC. The purpose of the  
362 IHC was the qualitative assessment of TSPO expression and to identify the cells expressing TSPO.  
363 TSPO expression at the cellular level was confirmed by IHC using antibodies against TSPO and  
364 CD68. CD68 positive myeloid cells were abundant at 4 and 8 days after LPS injection and this  
365 reaction was reduced at 31 days. TSPO immunoreactive cells were seen at 4 and 8 days in amoeboid  
366 CD68+ cells compatible with reactive microglia or perivascular macrophages. However, at day 4, a  
367 comparatively fainter TSPO immunoreactivity was detected in CD68+ ramified microglia cells  
368 compatible with early stages of microglia activation. Therefore, these CD68+ reactive ramified  
369 microglia cells that were more prominent at day 4 than day 8 could be responsible for the higher

370 [<sup>18</sup>F]DPA-714 binding found at day 4 compared to day 8 when most TSPO+ cells had amoeboid  
371 morphology. The [<sup>18</sup>F]DPA-714 binding site in the TSPO protein likely differs from the binding sites  
372 of the polyclonal anti-TSPO antibody. During the dynamic process of microglia activation, it is  
373 possible that the availability of those binding sites changes due to putative interactions of TSPO with  
374 other proteins that could mask certain binding sites. Further studies of protein-protein interactions  
375 along time might help to elucidate the relevant TSPO binding sites and their availability in relation to  
376 the stage of microglial activation. No CD68 or TSPO immunoreactivity was observed in the  
377 contralateral site. In addition, we detected faint TSPO immunoreactivity in astrocytes in regions  
378 surrounding the injection site, but it is currently unknown whether low TSPO expression in reactive  
379 astrocytes contributed to the PET signal.

380 We performed MRI studies to be certain that tracer uptake was not due to BBB disruption but  
381 to upregulation of TSPO expression induced by the LPS injection. DCE MRI indicated maximal BBB  
382 breakdown at day 1 after LPS injection with progressive restoration and full recovery at 1 month after  
383 LPS injection. These data suggest that tracer uptake was due to TSPO binding as no tracer uptake was  
384 seen at day 1 after LPS injection when BBB breakdown was at maximum. The fact that *in vivo*  
385 microPET data (possible influence of BBB integrity on tracer uptake) and *in vitro* autoradiography  
386 data (no influence of BBB on tracer uptake) were positively correlated, suggested tracer uptake during  
387 *in vivo* PET imaging was not due to BBB disruption. Conversely, BBB breakdown will favour  
388 vascular and perivascular inflammation and might contribute to microglia/macrophage activation  
389 around the vasculature. The quantification of the striatal transverse relaxation (T2) showed a nice  
390 correlation with the PET and autoradiography imaging suggesting that T2 in this case may reflect the  
391 underlying inflammation process.

392 The characterization of the LPS model with an established neuroinflammation tracer is of great  
393 importance as we are developing and evaluating new radiotracers for potential new targets upregulated  
394 during neuroinflammation. The longitudinal variation in neuroinflammation (as measured in this  
395 study) has to be taken into account when comparing different tracers. The LPS model is useful for first  
396 screening of newly developed tracers because of the easy design and the robust, unilateral



397 inflammatory reaction allowing the use of the contralateral region as control. The radioligands with the  
398 most potential can be selected for further screening in more clinical relevant disease models  
399 accelerating the translation to human PET studies. Additionally, this animal model is suitable to  
400 evaluate (new) anti-inflammatory therapy in an initial preclinical setting. This preliminary screening  
401 can select the most potential compounds for further evaluation and accelerate the translation of the  
402 therapy to humans.

403           This model will now be used to evaluate radioligands for other targets upregulated during  
404 neuroinflammation such as the CB<sub>2</sub> and P2X<sub>7</sub> receptor thereby accelerating the PET radioligand  
405 development for *in vivo* imaging of neuroinflammation [3].

406

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488 **TABLES**

489

490 **TABLE 1: Kinetic modeling.** Binding potential  $\pm$  SD in right striatum obtained by SRTM with left  
491 striatum as reference tissue

<b>Left striatum as reference tissue</b>				
Days after LPS injection	1 (n=3)	3 (n=5)	7 (n=4)	30 (n=3)
Binding potential	0.4 $\pm$ 0.1	3.0 $\pm$ 0.3	2.5 $\pm$ 0.6	0.9 $\pm$ 0.2

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511 **FIGURE CAPTIONS**

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513 **FIGURE 2. Experimental design.** Following experiments were performed: 1 day after LPS injection,  
514 microPET (n=3), MRI (n=6), *in vitro* autoradiography and IHC (n=3); 3 days after LPS injection  
515 microPET (n=5) and MRI (n=5); 4 days after LPS injection, *in vitro* autoradiography and IHC (n=3); 7  
516 days after LPS injection, microPET (n=4) and MRI (n=6); 8 days after LPS injection, *in vitro*  
517 autoradiography and IHC (n=4); 30 days after LPS injection, microPET (n=3) and MRI (n=4); 31 days  
518 after LPS injection, *in vitro* autoradiography and IHC (n=4). ARX: *in vitro* autoradiography; IHC:  
519 immunohistochemistry

520

521

522 **FIGURE 2. *In vitro* autoradiography of brain slices of rat LPS model using [<sup>18</sup>F]DPA-714.**  
523 Transversal brain sections made of rats, respectively 4 days (a), 8 days (b) and 31 days (c) after LPS  
524 injection, incubated with [<sup>18</sup>F]DPA-714. LPS was injected in the right striatum and the LPS-induced  
525 TSPO expression is clearly visualized by [<sup>18</sup>F]DPA-714. [<sup>18</sup>F]DPA-714 binding could be blocked with  
526 20 μM PK11195 3 days after LPS injection (> 96%) (d-e). The signal is the highest in the LPS  
527 injected striatum 4 days after LPS injection and the right to left (R/L) ratio decreases gradually in time  
528 (f).

529

530 **FIGURE 3. IHC on LPS model: TSPO and CD68.** TSPO is expressed in CD68+ reactive  
531 microglia/macrophages. Images show TSPO (green), CD68 (red), the nuclei labelled with TO-PRO3  
532 (blue), and the merged staining in sections taken from the ipsilateral hemisphere at day 4 (a, b), 8 (c,d)  
533 and 31 (e) after LPS administration. TSPO is mostly expressed in CD68+ cells in the ipsilateral  
534 hemisphere (arrows in a-e) but is not detected in the contralateral hemisphere (f). Bar scale: 10 μm.

535

536 **FIGURE 4. IHC on LPS model: TSPO and GFAP.** Faint TSPO expression is observed in reactive  
537 astrocytes. Images show TSPO (green), GFAO (red), the nuclei labelled with TO-PRO3 (blue), and  
538 the merged staining in sections taken from the ipsilateral hemisphere at day 4 (a, b), 8 (c-e) and 31 (f)  
539 after LPS administration. Cells strongly immunoreactive for TSPO are not GFPA positive (arrows in  
540 a-c). However, reactive astrocytes located at the periphery of the injection site in the ipsilateral  
541 hemisphere show a faint TSPO immunoreactivity 8 and 31 days after LPS administration (arrows in d-  
542 f). Bar scale: 10 μm.

543

544 **FIGURE 5. MicroPET studies with [<sup>18</sup>F]DPA-714 in a LPS rat model.** a: TAC of a microPET scan  
545 at 3 days after LPS injection; b: TAC of a microPET study after pre-treatment with PK11195  
546 (subcutaneously 10 mg/kg 60 min before tracer injection) at 3 days after LPS injection; c: TAC of a  
547 displacement microPET study with PK11195 (intravenously 5 mg/kg 30 min after tracer injection) at 3  
548 days after LPS injection. Animals were kept under gas anesthesia (2.5% isoflurane in O<sub>2</sub> at a flow rate  
549 of 1 L/min). Rats were injected with about 74 MBq of [<sup>18</sup>F]DPA-714 via a tail vein.

550 **FIGURE 6. *In vivo* DCE MRI and microPET study of one representative animal in time.** a:  
551 dynamic signal intensity (SI) changes from the LPS or saline injection zones. BBB disruption near the  
552 LPS injection site (right hemisphere) is evident at 1 day and reduced at 3 and 7 days. No contrast  
553 enhancement is seen 1 month after the LPS injection or near the saline injection site (left hemisphere;  
554 all time points). b: averaged microPET images of the whole scan (120 min) after [<sup>18</sup>F]DPA-714  
555 injection were acquired. Data were normalized for injected activity and body weight of the animal  
556 (SUV). Animals were kept under gas anesthesia (2.5% isoflurane in O<sub>2</sub> at a flow rate of 1 L/min). Rats  
557 were injected with about 74 MBq of [<sup>18</sup>F]DPA-714 via a tail vein.

558

559 **FIGURE 7. *In vivo* DCE MRI.** Dynamic signal intensity (SI) changes from the LPS or saline  
560 injection zones resulting from the Gd-DOTA injection at the different time points 1 day, 3 days, 1  
561 week and 1 month. a: BBB disruption near the LPS injection site (right hemisphere) is present at 1, 3  
562 and 7 days. b: No contrast enhancement is seen at 1 month after the LPS injection or near the saline  
563 injection site (left hemisphere; all time points; CA: contrast agent).

564 **FIGURE 8. Parametric T2 maps.** a: Parametric T2 maps at day 1, 3, 7 and 30 of a representative  
565 animal with injection of 50 µg LPS in the right striatum (white arrow) and saline on the left side. T2  
566 maps are all scaled from 30 to 70 ms. b: Striatal T2 values, data are expressed as mean ± SD. Day 1: N  
567 = 3, Day 3: N = 5, Day 7: N = 6, Day 30: N = 5.

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Figure 1

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Figure 2

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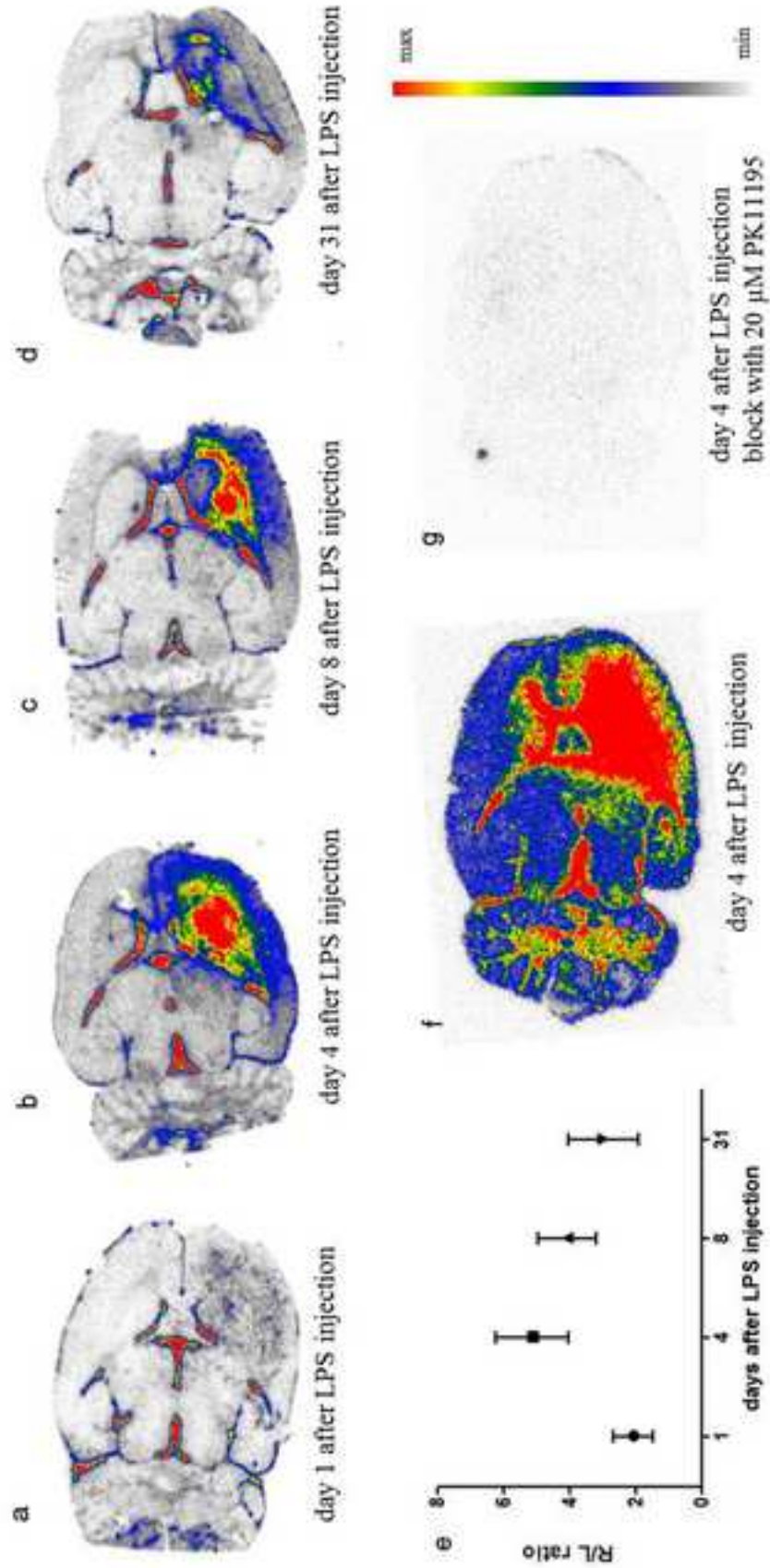




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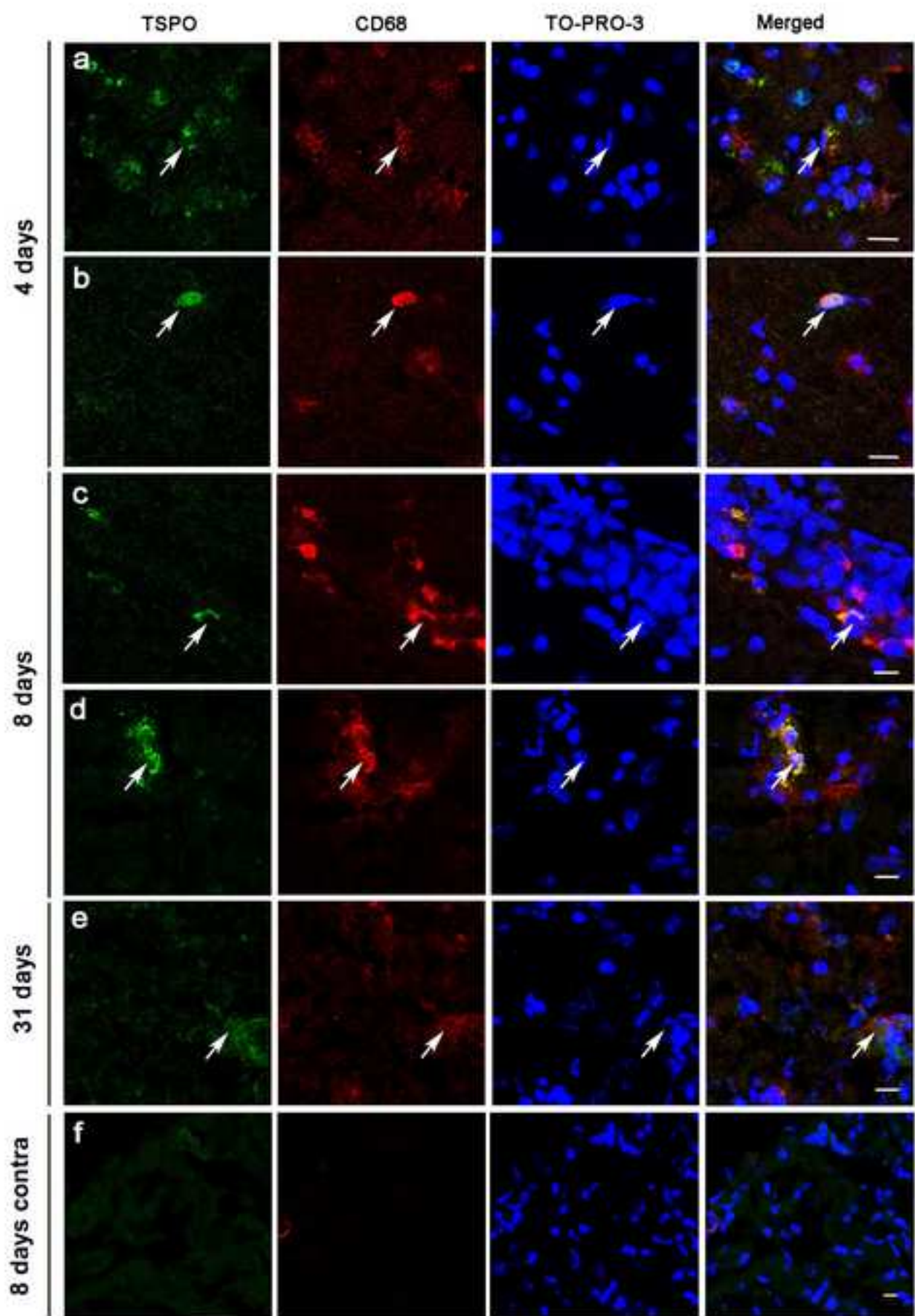


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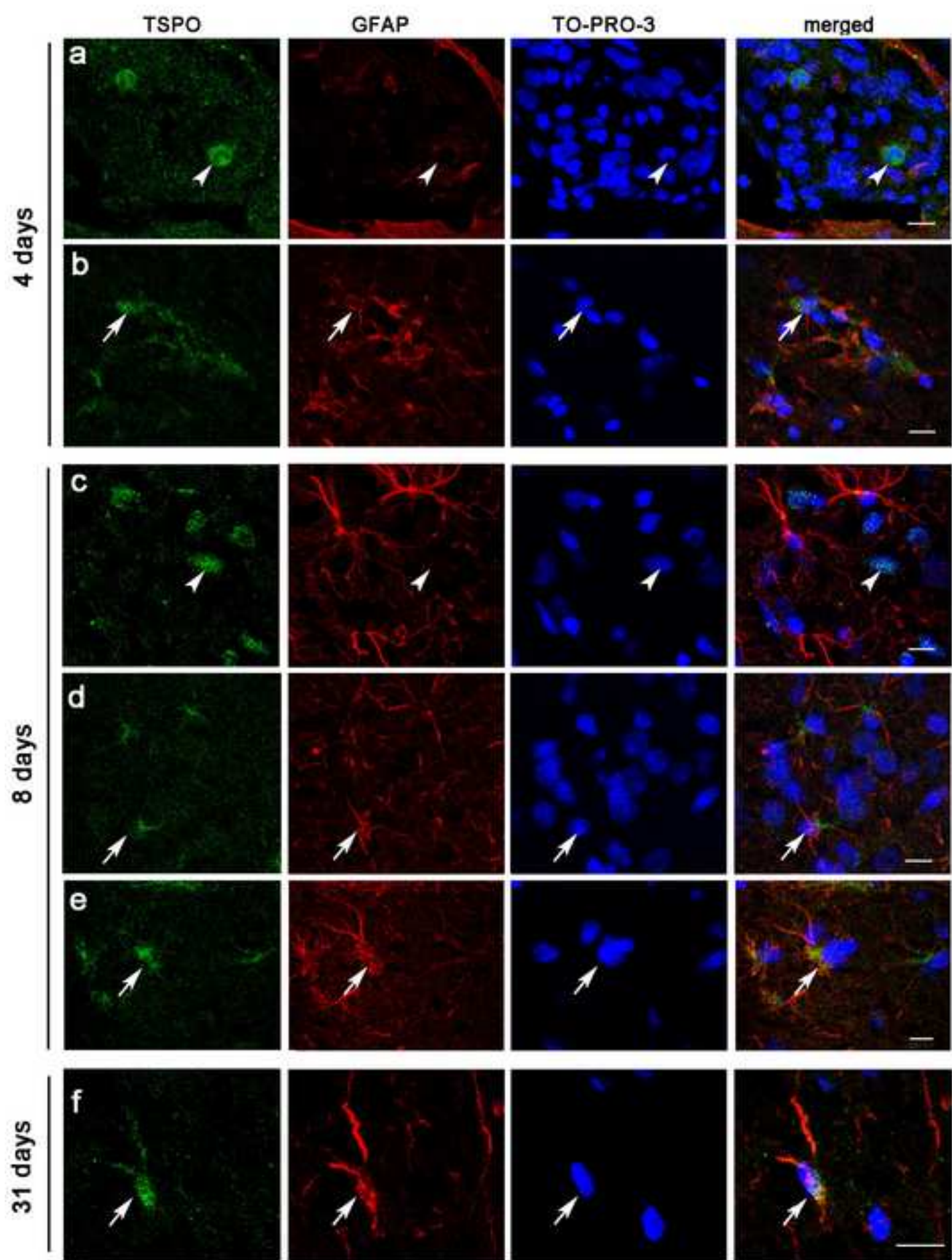


Figure 5

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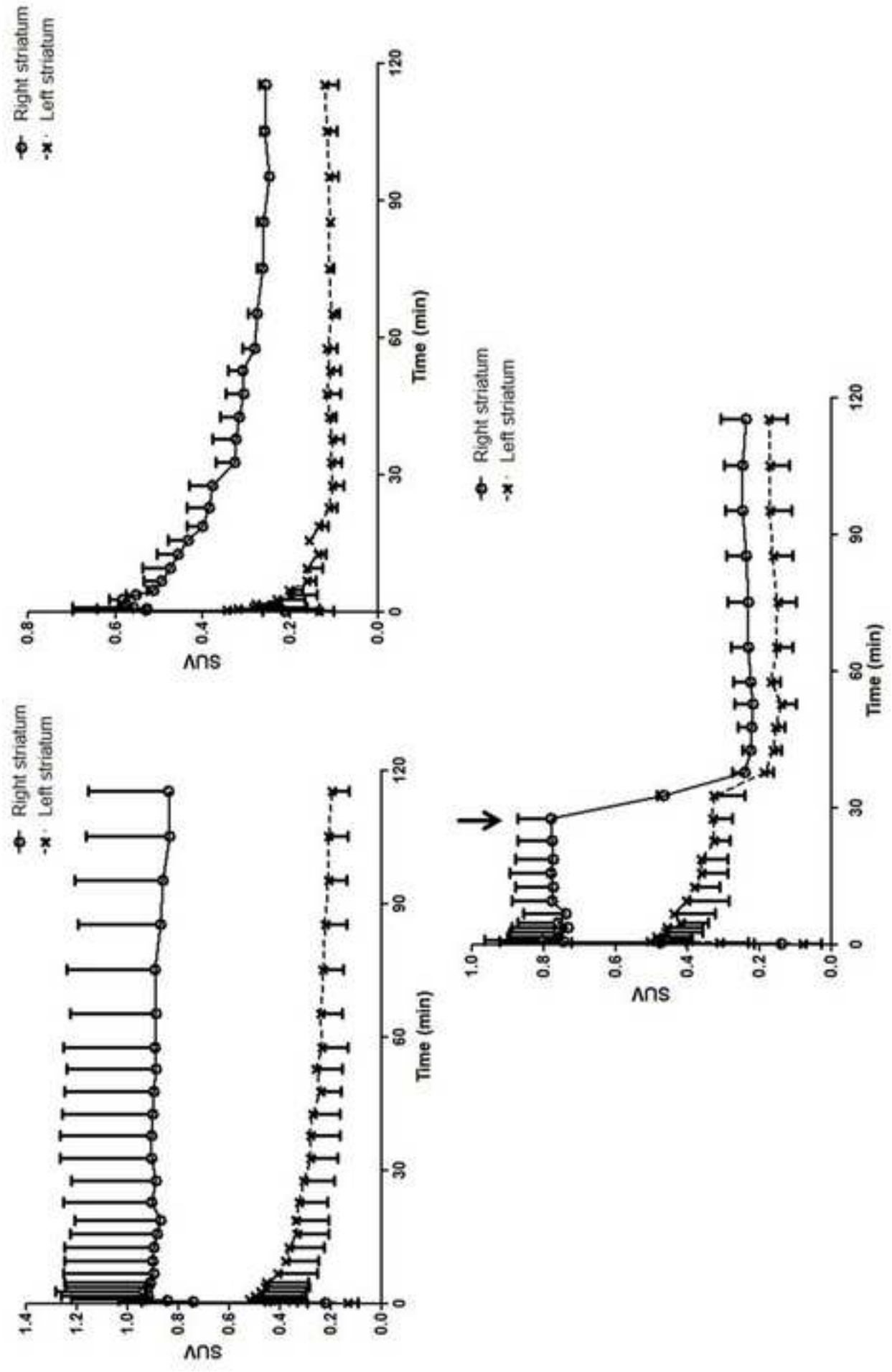


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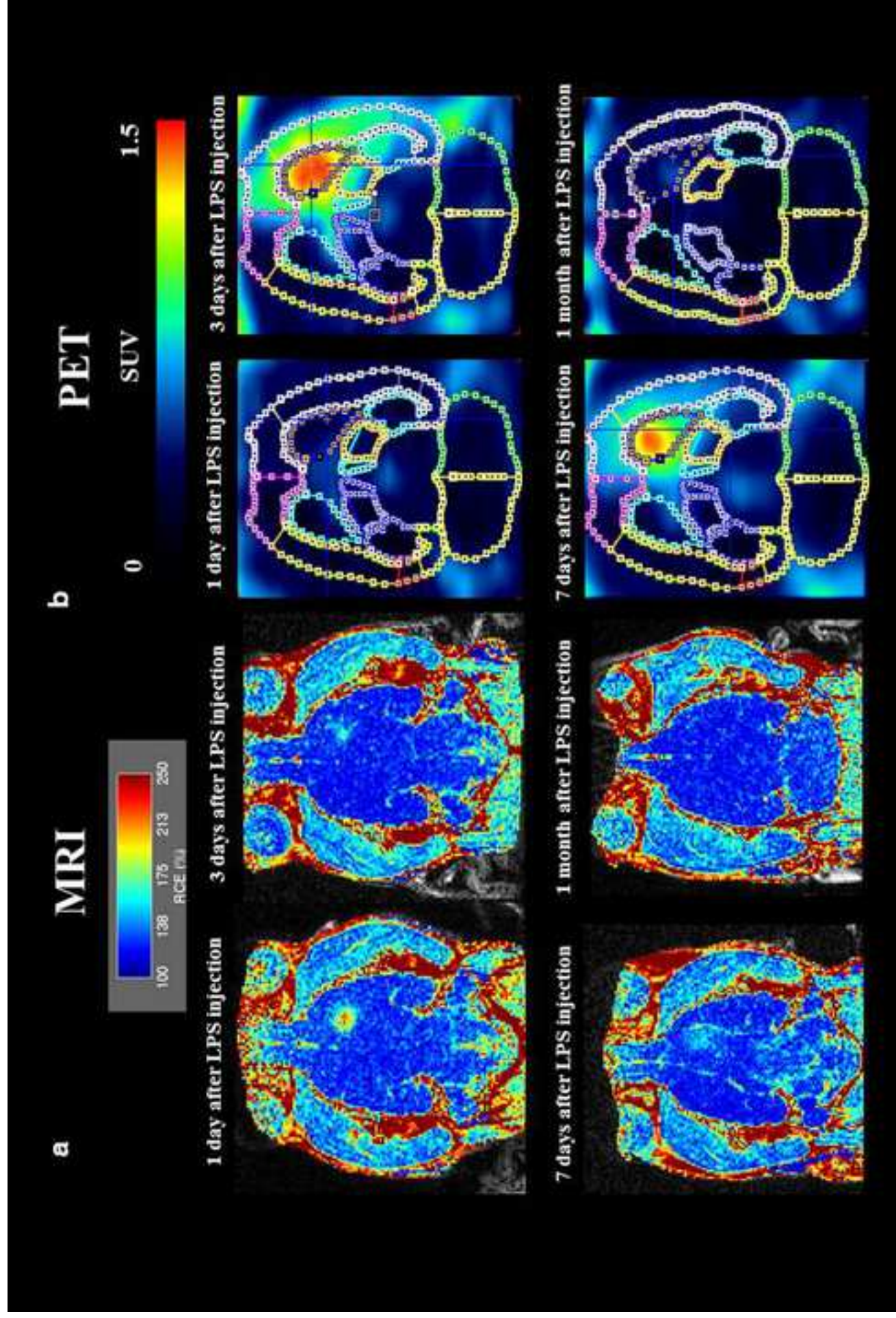


Figure 7

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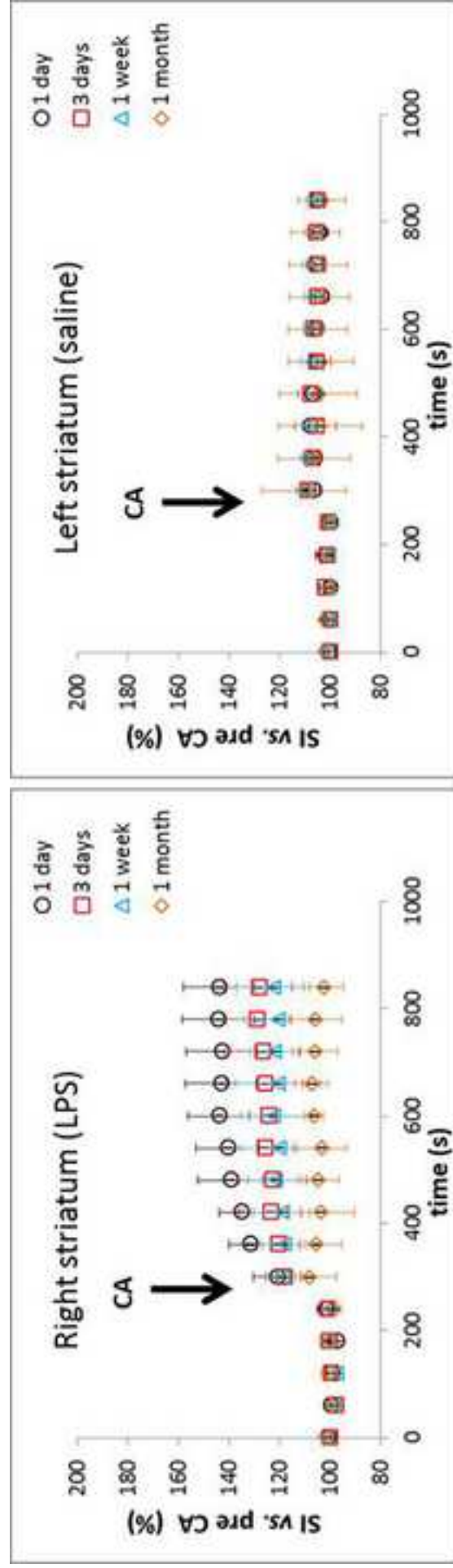


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