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1 **Neuroprotective effect of the alpha 7 nicotinic receptor agonist PHA 543613 in an *in-***  
2 ***vivo* excitotoxic adult rat model**

3

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35

## 36 **Abstract**

### 37 **Background**

38 Neuroinflammation is a key-component of the pathophysiology of neurodegenerative  
39 diseases and its regulation, a major therapeutic target to reach since no efficient curative  
40 treatment is available. The link between nicotine intake and positive outcome in  
41 neurodegenerative diseases has been established suggesting a role played by nicotinic  
42 receptors especially the nicotinic acetylcholine receptors  $\alpha 7$  ( $\alpha 7nAChRs$ ). The objective of  
43 this study was to evaluate *in vivo* the potential dose effects of the PHA 543613, a high affinity  
44 agonist of  $\alpha 7nAChRs$ , on neuron survival and striatal neuroinflammation especially using a  
45 radioligand never studied in rat model of brain excitotoxic.

46

### 47 **Methods**

48 Twenty-six adult male Wistar rats were lesioned in the right striatum with quinolinic acid (QA)  
49 and received either vehicle (Sham group; n=8) or PHA 543613 at a concentration of 6 mg/kg  
50 (QA-PHA6; n=8) or 12 mg/kg (QA-PHA12; n=10) intra-peritoneally 1 h before QA lesioning  
51 and then twice a day until sacrifice at day-4 post-lesion. The measured kinetic parameters of  
52 PHA 543613 in rats were taken into account when the administration schedule was  
53 established. A first study compared TSPO quantitative autoradiography in QA-lesioned  
54 brains with [ $^3H$ ]DPA-714 and [ $^3H$ ]PK11195 (n=9). The effects of the PHA 543613 on  
55 microglia activation and neuronal survival have then been evaluated through [ $^3H$ ]DPA-714  
56 binding and immunofluorescence staining (Ox-42, NeuN) on adjacent brain sections.

57 **Results**

58 We demonstrated that [<sup>3</sup>H]DPA-714 provide a better signal-to-noise ratio than [<sup>3</sup>H]PK11195.

59 In addition, we showed that chronic PHA 543613 treatment administered at a dose of  
60 12mg/kg to QA-lesioned rats significantly protected neurons and reduced the intensity of  
61 neuroinflammation measured by TSPO and Ox-42 immunostaining.

62

63 **Conclusions**

64 This study, among the first to evaluate the effects of an  $\alpha 7$ nAChRs agonist treatment in an  
65 excitotoxic model of neuroinflammation with [<sup>3</sup>H]DPA-714, indicates that PHA 543613 exerts  
66 neuroprotective effects on the striatal neurons associated with a reduction in microglial  
67 activation. This reinforces the hypothesis that  $\alpha 7$ nAChRs agonist can provide beneficial  
68 effects in the treatment of patients with neurodegenerative diseases through modulation of  
69 neuroinflammation.

70

71 **Keywords**

72 Nicotinic acetylcholine receptors  $\alpha 7$  - PHA 543613 - TSPO – Neuroinflammation - DPA-714 -  
73 PK-11195 – autoradiography – immunofluorescence – Neurodegeneration

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## 85 **Background**

86 Neuroinflammation is a reaction of the central nervous system in response to infection, injury  
87 or trauma. This is a physiological component of innate immunity, which involves a non-  
88 specific and early response against a pathogen in order to neutralize and minimize damage  
89 at the neuronal level by repairing damaged brain tissue. Microglia-driven neuroinflammation  
90 has a beneficial effect on scavenging cell debris, tissue healing and repair. However, during  
91 chronic neuroinflammation, activated microglial cells produce pro-inflammatory cytokines,  
92 reactive oxygen species and derivatives of nitric oxide (NO) [1]. This activity is responsible  
93 for neuronal death by apoptosis which in turn stimulates microglial activation that contributes  
94 to the pathophysiology of neurodegenerative diseases [2,3]. Neurons are also able to  
95 produce proinflammatory cytokines, complement factors and derivatives of NO. These  
96 inflammatory mediators can cause neuronal dysfunction and cell death [4,5].

97 Neuroinflammation is well known as an important element of brain disorders and in particular  
98 neurodegenerative diseases [6].

99 Regulating neuroinflammation represents therefore a major challenge in the management of  
100 patients affected by neurodegenerative diseases such as Alzheimer's, Parkinson's or  
101 Huntington's diseases. Nowadays, only symptomatic treatments are available and no  
102 curative treatment has a marketing authorization for neurodegenerative diseases. Several  
103 lines of research on an anti-inflammatory therapeutic approach have been explored in animal  
104 models and in humans, but were all unsuccessful [7–11]. Epidemiological studies have  
105 shown that smokers have a lower risk of neurodegenerative diseases than the rest of the  
106 population [12–15]. These observations led to an interest in nicotine administration which, by  
107 binding to nicotinic acetylcholine receptors, may have beneficial effects on the symptoms of  
108 neurodegenerative diseases, particularly Parkinson's disease (PD) [16–20].

109 Among the different subtypes of nicotinic receptors, the  $\alpha 7$  ( $\alpha 7$ nAChRs) are highly expressed  
110 in the mammalian brain particularly by neurons and glial cells [21–23]. They belong to the  
111 family of ligand-gated ion channels and have a homopentameric structure of five  $\alpha 7$  subunits.  
112  $\alpha 7$ nAChRs are involved in the control of voluntary movement, memory and attention, sleep

113 and waking and pain and anxiety. They appear to be involved in a number of brain disorders  
114 such as drug addiction, schizophrenia and neurodegenerative diseases like Alzheimer's  
115 disease (AD) and PD [21]. It has been shown that  $\alpha 7$ nAChRs activation improved cognitive  
116 functions in several animal models that mimic AD, and the  $\alpha 7$ nAChRs agonist, EVP-6124, is  
117 currently in clinical phase III in mild to moderate AD patients [24]. Additionally, several  
118  $\alpha 7$ nAChRs agonists are being evaluated for the treatment of PD [25]. First results support  
119 the idea that drugs acting at  $\alpha 7$ nAChRs may be beneficial including one study demonstrating  
120 the role of  $\alpha 7$ nAChRs agonists for the reduction of L-dopa-induced dyskinesias [26-28]. In  
121 addition, PNU 282987 and PHA 543613  $\alpha 7$ nAChRs agonists have demonstrated  
122 neuroprotective and anti-inflammatory effects in different intracerebral haemorrhage models  
123 [29,30]. Moreover, these 2 agonists and PNU 120596, an  $\alpha 7$ nAChRs positive allosteric  
124 modulator, have proved the same effect in both *in-vitro* and *in vivo* brain ischemia models  
125 [31]. The modulation of the  $\alpha 7$ nAChRs activation in macrophages influences the synthesis of  
126 pro-inflammatory cytokines involved in the regulation of the "cholinergic anti-inflammatory  
127 pathway" [32].

128

129 PHA 543613 [N- (3R) -1-azabicyclo [2.2.2] - Oct-3- yl- furo [2,3 -c] pyridine -5-carboxamide  
130 hydrochloride] is a selective agonist of  $\alpha 7$ nAChRs, known for its high affinity for  $\alpha 7$ nAChRs  
131 [33,34]. Studies have reported a significant anti-inflammatory effect on primary cultures of  
132 neurons and astrocytes (unpublished observations, G. Page). *In vivo*, it improved recognition  
133 memory in an AD mouse model [35]. Moreover, promising effects on cognitive function were  
134 also observed in a schizophrenia' disease model [33]. Recent study has also demonstrated  
135 that PHA 543613 exerts neuroprotective effects on the striatal dopaminergic neurons with a  
136 reduction in microglial activation in a PD rat model [28]. Thus, data published in literature  
137 relate that therapies targeting glial cells, and more precisely the agonists of  $\alpha 7$ nAChRs,  
138 might provide benefit in neurodegenerative disorders [36]. However, the interactions between  
139 the neuroprotective and anti-inflammatory effects are not clearly elucidated. Regarding this  
140 question, we used a rat model of acute neuroinflammation obtained by unilateral striatal

141 injection of quinolinic acid (QA) in which we evaluated the effects of a chronic administration  
142 of PHA 543613. QA is a strong agonist of glutamate NMDA (N-methyl-D-aspartate)  
143 receptors. Overactivation of NMDA receptors causes a massive intracellular influx of calcium  
144 that leads to neuronal death by activation of various enzymes triggering different cell  
145 components then leading to neuronal death [37]. Factors released during the death of these  
146 neurons rapidly leads to significant microglial activation. This model of Huntington's disease  
147 has recently been shown to be useful in studying the overexpression of the translocator  
148 protein (TSPO), a marker of neuroinflammation [38-40]. Therefore, the main purpose of the  
149 present study was to evaluate *in vivo* the potential dose effects of the selective agonist of  
150  $\alpha 7$ nAChRs, PHA 543613, on both neuronal survival and microglial activation in a  
151 neuroinflammatory excitotoxic rat model using QA intrastriatal injection. We also interested in  
152 the effects of repeated administration of PHA 543613 on  $\alpha 7$ nAChRs density in our rat model  
153 of brain excitotoxic. As a secondary goal, the study aimed to carry out autoradiographic  
154 quantification of TSPO expression with a radioligand never studied in a rat model of  
155 neuroinflammation: the [<sup>3</sup>H]DPA-714 [41].

156

## 157 **Materials and Methods**

### 158 **Animals**

159 All procedures were conducted in accordance with the European Community Council  
160 Directive 2010/63/EU for laboratory animal care and the experimental protocol was validated  
161 by the Regional Ethical Committee (Authorization N°2015022011523044). Experiments were  
162 carried out on 10-week-old normotensive male Wistar rats (CERJ, France) weighing 290–  
163 300g at the beginning of experiments. Animals were housed in groups of 2 per cage in a  
164 temperature (21 ± 1°C) and humidity (55 ± 5%) controlled environment under a 12-h  
165 light/dark cycle, with food and water available *ad libitum*. A total of 39 animals were used for  
166 the experiments described below.

167

168

169 **Excitotoxic neuroinflammation model**

170 For unilateral striatal QA lesion, rats (n=35) were anaesthetized with isoflurane (Aerrane™,  
171 Baxter, France; 4% for induction and 2% for maintenance) and placed in a stereotaxic David  
172 Kopf apparatus (tooth bar: -3.3 mm). The animals' body temperature ( $36.9 \pm 0.6^\circ\text{C}$ ) was  
173 monitored during the surgery by using a thermal probe. The animals were unilaterally  
174 injected with 150 nmol of QA (Sigma-Aldrich, Lyon, France), dissolved in 0.1 mol/L  
175 phosphate-buffered saline (PBS) pH 7.4, into the right striatum (injection rate: 0.5  $\mu\text{L}/\text{min}$ )  
176 using a 25  $\mu\text{L}$  microsyringe (Hamilton, Bonaduz, Switzerland) and a micropump (KD  
177 Scientific, Holliston, Massachusetts, USA). QA (2  $\mu\text{L}$ ) was injected at the following  
178 coordinates: AP: + 0.7 mm; ML: - 3 mm; DV: - 5.5 mm from bregma according to Paxinos  
179 and Watson [42]. The injection syringe was left in place for an additional 4 min to avoid QA  
180 back-flow, and then slowly removed. After surgery, the scalp was sutured and the rats were  
181 given buprenorphine (0.05 mg/kg sub-cutaneously) for postoperative pain.

182

183 **Comparative evaluation of TSPO radioligands [ $^3\text{H}$ ]DPA-714 and [ $^3\text{H}$ ]PK-11195 by**  
184 **autoradiographic study in QA lesioned rats**

185 We first measured the *in vitro* binding of TSPO by [ $^3\text{H}$ ]DPA-714 in comparison to [ $^3\text{H}$ ]PK-  
186 11195 in adjacent brain sections from 9 QA-lesioned rats. [ $^3\text{H}$ ]DPA-714 was prepared  
187 according to *Damont and al, 2015* [41] (specific activity 2.1 GBq/ $\mu\text{mol}$ ) and [ $^3\text{H}$ ]PK-11195  
188 was commercially purchased (specific activity 3.06 GBq/ $\mu\text{mol}$ ; Perkin Elmer, Norwalk, CT,  
189 USA). Autoradiographic studies were performed 7 days after QA lesion. After decapitation  
190 under isoflurane anesthesia, the brains were carefully removed on ice, then frozen in  
191 isopentane cooled at  $-35^\circ\text{C}$  and stored at  $-80^\circ\text{C}$  until use. Coronal brain sections 16  $\mu\text{m}$  thick  
192 were cut with a cryostat (CM 3050S™, Leica, Germany) at  $-20^\circ\text{C}$ , collected on gelatinized  
193 slides and stored at  $-80^\circ\text{C}$  for at least 4 days. Brain sections were allowed to equilibrate at  
194 room temperature (RT) for 3h, then incubated with 1 nmol/L of labelled ligand ([ $^3\text{H}$ ]DPA-714  
195 or [ $^3\text{H}$ ]PK-11195) in 50 mmol/L Tris-HCl buffer pH 7.4 at RT for 60 min. Non-specific binding  
196 was assessed in the presence of 1  $\mu\text{mol}/\text{L}$  stable PK-11195 (Sigma Aldrich, Lyon, France).

197 Sections were rinsed twice in ice cold buffer (4°C) for 5 min, then briefly in distilled water at  
198 4°C and dried at RT. Dry sections were made conductive by an application of metal electric  
199 tape (3M™, Euromedex, Souffelweyersheim, France) on the other side and then placed in  
200 the gas chamber of the β-imager™ 2000 (Biospace Lab, Paris, France). Acquisitions were  
201 collected over a period of 4 h. Two anatomical regions of interest (ROIs), i.e., the ipsi-lateral  
202 (IL) and contra-lateral (CL) striata were selected and identified in Paxinos and Watson atlas  
203 [42]. Using the β-vision software (Biospace Lab, Paris, France), the level of bound  
204 radioactivity was directly determined by counting the number of β-particles emitted from the  
205 delineated area. The radioligand signal in the ROIs was measured for 6 sections per brain  
206 and expressed as counts per minute per square millimeter (cpm/mm<sup>2</sup>). Specific binding was  
207 determined by subtracting non-specific binding from total binding. Radioactivity was  
208 quantified using an image analyzer (M3-vision™ Biospace Instruments, Paris, France).  
209 The percentage increase of TSPO binding in IL *versus* (vs) CL hemisphere was calculated  
210 as : 
$$\frac{IL-CL}{CL} \times 100$$
  
211

212

### 213 **Pharmacokinetics of PHA 543613**

214 Given the lack of data on the pharmacokinetics of PHA 543613, a study to determine the  
215 evolution of the concentration vs time in plasma and in brain was performed to choose the  
216 most suitable dosing regimen and to confirm the PHA 543613 cerebral diffusion. PHA  
217 543613 concentration was measured with ultra liquid chromatography (UHPLC UltiMate®  
218 3000 system, Thermo Scientific Dionex™, Villebon-sur-Yvette, France) coupled with high  
219 resolution mass spectrometry (Orbitrap Q exactive™, Thermo Scientific™, Villebon-sur-  
220 Yvette, France) in the serum and brain of 4 treated rats after one i.p injection of PHA 543613  
221 at a dose of 6 mg/kg. Blood samples from the jugular vein were collected at 0.5, 1, 3, 6 and  
222 24 h post injection in heparinized tubes. Blood was centrifuged at 2,000 g, for 20 min at 4°C,  
223 plasma was removed and then kept at -20°C. The rats were killed by decapitation under  
224 isoflurane anesthesia. The whole brain was quickly removed and striatum removed and kept

225 at -80°C. Striatum and plasma were homogenized respectively in 2 mL and 400 µL of ethyl  
226 acetate with internal standard (PHA 568487, Tocris Bioscience, Bristol, UK, 1 µg/mL) and  
227 centrifuged at 15,000 g, for 5 min at 4°C. Supernatants were removed; ethyl acetate was  
228 evaporated. They were filtered and injected into the liquid chromatograph (C18  
229 chromatography). A multistep gradient (followed by a 2 min equilibration time) had a mobile  
230 phase A of 0.5% formic acid in water and a mobile phase B of ACN acidified with 0.5%  
231 formic acid; the gradient operated at a flow rate of 0.3 mL/min over a run time of 7.5 min. The  
232 acquisition and data processing was performed with X-calibur 2.2 (Thermo Scientific™,  
233 Villebon-sur-Yvette, France).

234

### 235 **Experimental procedure and drug treatment**

236 PHA 543613 hydrochloride (ICOA, Orléans, France) was dissolved in sterile water and intra-  
237 peritoneally injected at a concentration of 6 or 12 mg/kg (300 µL/300 g body weight) 1 h  
238 before QA lesioning and then twice a day every day until sacrifice at day 4 post-lesion.  
239 Twenty six rats were included in this study and separated into 3 groups as follows: 10  
240 lesioned rats received the treatment at 12 mg/kg (QA-PHA12 group), 8 rats received the  
241 treatment at 6 mg/kg (QA-PHA6 group) and 8 lesioned rats received intra-peritoneal (i.p)  
242 injection of vehicle according to the same administration schedule (Sham group).

243

### 244 **Evaluation of PHA 543613 dose-effect on neuroinflammation using TSPO**

#### 245 **quantification by autoradiography**

246 First we investigated the potential dose-related effect of chronic 4-day treatment with PHA  
247 543613 on striatum expression levels of TSPO. Rodents received either vehicle (n=5) or  
248 PHA 543613 at 6 mg/kg (n=8) or 12 mg/kg (n=9) twice a day. In agreement with results  
249 previously obtained (see section 3), the density of TSPO binding sites was measured by *in*  
250 *vitro* autoradiographic experiment using [<sup>3</sup>H]DPA-714 (specific activity 2.1 TBq/mmol ; CEA ;  
251 Orsay ; France) as described above. A total of 6 sections per brain were analyzed for each  
252 rat. Data from brain sections were collected over 4h with the β-imager™ 2000. Specific

253 binding (expressed in cpm/mm<sup>2</sup>) from 2 ROIs, i.e., IL and CL striatum were determined.  
254 Finally, the percentage increase of TSPO binding in IL vs CL hemisphere was calculated as :

$$\frac{\text{IL-CL} \times 100}{\text{CL}}$$

257

### 258 **Evaluation of PHA 543613 dose-effect on $\alpha$ 7nAChRs expression by autoradiography**

259 The density of  $\alpha$ 7nAChRs binding sites was measured by *in vitro* autoradiography using  
260 [<sup>125</sup>I] $\alpha$ -bungarotoxin (specific activity 81.4 TBq/mmol; Perkin Elmer, Skovlunde, Denmark) on  
261 brain sections adjacent to those used in the previous study, in Sham (n=6), QA-PHA6 (n=6)  
262 and QA-PHA12 (n=6) rats. Brain sections were allowed to equilibrate at RT for 3h and then  
263 were incubated with 0.4 nmol/L of [<sup>125</sup>I] $\alpha$ -bungarotoxin in 50 mmol/L Tris-HCl buffer pH 7.4 at  
264 RT for 60 min. Non-specific binding was assessed in the presence of 1  $\mu$ mol/L stable  $\alpha$ -  
265 bungarotoxin (Tocris Bioscience, Bristol, UK). The density of  $\alpha$ 7nAChRs binding sites was  
266 measured as described above (see section 3). A total of 6 sections per brain were analyzed  
267 for each rat. Data from brain sections were collected over 4h with the  $\beta$ -imager™ 2000.  
268 Specific binding (expressed in cpm/mm<sup>2</sup>) from 2 ROIs, i.e., IL and CL striatum was  
269 determined.

270

### 271 **Evaluation of PHA effects on microglia activation and neuronal survival by** 272 **immunofluorescence**

273 Immunofluorescence measurements were performed on rats treated by PHA 543613 12  
274 mg/kg (n=6) or vehicle (n=6). Coronal sections (16  $\mu$ m thick) of striatum adjacent to those  
275 used for the autoradiographic study were used for the immunofluorescence staining of  
276 neurons (NeuN, MAB377, Millipore, Molsheim, France) and activated microglia (Ox-42,  
277 CBL1512, Chemicon International, Temecula, CA, USA). Slices were fixed in 4%  
278 paraformaldehyde solution (Sigma, St Quentin Fallavier, France) at RT for 30 min. Tissue  
279 sections were washed 3 times in PBS 0.1 mol/L for 5 min at RT and were then incubated for  
280 3 h at RT in a buffer to enhance cell permeability and to block non-specific sites (PBS 0.1

281 mol/L/0,3%, Triton X-100/5%, normal goat serum). The sections were delineated using the  
282 Dako Pen pencil (Z0334 ; Dako, Les Ulis, France) on the glass slide before incubation  
283 overnight at 4°C with monoclonal mouse anti-NeuN (1:200) or monoclonal mouse anti-Ox-42  
284 (1:100). Primary antibodies were diluted in PBS 0.1 mol/L/0.3% Triton X-100/1% normal goat  
285 serum. After 3 washes with PBS 0.1 mol/L at RT for 5 min, sections were incubated for 1h in  
286 a dark box at RT with goat anti-mouse DyLight™ 488 (KPL, Eurobio, Courtaboeuf, France) at  
287 1:500 diluted in PBS/0.3% triton X-100/1% normal goat serum. The slices were washed twice  
288 with PBS 0.1 mol/L and twice with distilled water. Then, they were incubated with 4'-6-  
289 diamidino-2-phenylindole (DAPI) (0.1 µg/mL) for 15 min. After 3 washings in distilled water,  
290 the slices were mounted with fluorescent mounting medium (S3023, DakoCytomation,  
291 Trappes, France) and kept at 4°C until observation. Images were acquired with Morpho  
292 Strider software (Explora-Nova™, La Rochelle, France) on Leica DM 5500B microscope.  
293 Neurons and activated microglia cells were counted with Image J software (Rasband, WS,  
294 Image J, US National Institute of Health, Bethesda, Maryland, USA) in 3 areas per striatum  
295 for each section and in 2 sections per rat in order to obtain a representative sample of the  
296 whole striatum. The number of cells was determined for each rat by calculating the average  
297 obtained on the 6 analyzed areas for each hemisphere. Counting was performed by 2  
298 independent operators. The percentage of neuronal loss and of increase of activated  
299 microglia in ipsi- vs contra-lateral hemisphere were calculated as follows: 
$$\frac{IL-CL}{CL} \times 100$$
  
300

### 301 **Statistical Analysis**

302 Results were expressed as mean ± standard error of the mean (SEM). Correlation between  
303 the specific binding of TSPO by [<sup>3</sup>H]PK-11195 and by [<sup>3</sup>H]DPA-714 was estimated by a two-  
304 tailed Spearman test. To compare 2 groups of rats (PHA vs Sham), a Mann-Whitney test  
305 was used. Comparisons between the binding in the IL and CL sides were performed using  
306 the Wilcoxon one-tailed test. The level of significance was p<0.05 (GraphPad Prism software  
307 version 5, San Diego, CA, USA).

308

309 **Results**

310 **Animals**

311 No physiological issues and no difference in body weight were observed between animals in  
312 the Sham, QA-PHA6 and QA-PHA12 groups (on the day of lesion:  $300 \pm 20$  g,  $291 \pm 4$  g and  
313  $283 \pm 4$  g, respectively; at day-4 post-lesion:  $278 \pm 9$  g,  $282 \pm 2$  g and  $268 \pm 9$  g,  
314 respectively). Two rats, 1 in the Sham group and 1 in QA-PHA12 group, died on the lesion  
315 day.

316

317 **PHA 543613 quantitative analysis with UHPLC coupled with high resolution mass**  
318 **spectrometry**

319 One hour post-administration of PHA 543613 (6 mg/kg), a peak concentration was observed  
320 in serum. Then, the concentration of PHA 543613 rapidly decreased. The rate of PHA  
321 543613 in rat blood decreased by  $35 \pm 7\%$  and  $90 \pm 4\%$  at 2h and 6h post-i.p injection,  
322 respectively. At 24h post-administration, PHA 543613 was no longer detectable in serum but  
323 was present within striatum. Based on this observation, the rats were treated twice a day at a  
324 dose of 6 or 12 mg/kg during the 4 days post-lesion. The first injection was carried out 1h  
325 pre-lesion.

326

327 **Comparative evaluation of TSPO using [<sup>3</sup>H]DPA-714 and [<sup>3</sup>H]PK-11195**

328 The comparison of specific binding of TSPO between [<sup>3</sup>H]PK-11195 and [<sup>3</sup>H]DPA-714 by  
329 autoradiography was performed on brain sections of rats sacrificed 7 days after intrastratial  
330 injection of QA (Fig. 1). Quantification of the autoradiography revealed that the binding of  
331 TSPO either by [<sup>3</sup>H]PK-11195 or [<sup>3</sup>H]DPA-714 was significantly ( $p < 0.05$ ) higher in the IL than  
332 in the CL striatum. Non-specific binding in the CL or IL striatum assessed with  $1 \mu\text{mol/L}$   
333 PK11195, was lower with [<sup>3</sup>H]DPA-714 than with [<sup>3</sup>H]PK-11195, indicating a better signal-to-  
334 noise for [<sup>3</sup>H]DPA-714 ( $0.06 \pm 0.10$  cpm/mm<sup>2</sup> vs  $0.29 \pm 0.02$  cpm/mm<sup>2</sup> in CL respectively,  
335  $p < 0.05$ ;  $0.14 \pm 0.02$  cpm/mm<sup>2</sup> vs  $0.37 \pm 0.02$  cpm/mm<sup>2</sup> in IL respectively;  $p < 0.05$ ) (Fig. 1A).

336 Furthermore, the percentage increase of TSPO specific binding in IL vs CL striatum was  
337 significantly ( $p < 0.05$ ) higher for [ $^3\text{H}$ ]DPA-714 than for [ $^3\text{H}$ ]PK-11195 ( $821 \pm 70$  vs  $489 \pm 42\%$   
338 respectively) (Fig. 1B). In addition, the percentage increase of TSPO binding in IL vs CL  
339 striatum was positively correlated using [ $^3\text{H}$ ]DPA-714 and [ $^3\text{H}$ ]PK-11195 (Fig. 1B,  $\rho =$   
340  $0.9328$ ,  $p < 0.05$ ).

341

#### 342 **Evaluation of PHA 543613 dose-effect relationship on neuroinflammation using** 343 **autoradiography**

344 The TSPO density was evaluated on adjacent brain sections using [ $^3\text{H}$ ]DPA-714 binding in  
345 the IL and CL striatum from each rat in the Sham group and both PHA groups (6 mg/kg and  
346 12 mg/kg) (Fig. 2A). The percentage increase of TSPO binding in IL vs CL striatum (Figure  
347 2B) was lower in the QA-PHA6 group than in the Sham group but was not statistically  
348 different ( $764 \pm 72\%$  vs  $967 \pm 64\%$ , respectively). However, the percentage increase of TSPO  
349 binding in IL vs CL striatum was significantly lower in the QA-PHA12 group ( $731 \pm 34\%$  vs  
350  $967 \pm 64\%$ , respectively,  $p < 0.05$ ). Finally, the increased rate of TSPO was higher but not  
351 statistically different in QA-PHA6 and QA-PHA12 groups.

352

#### 353 **Evaluation of PHA dose-effect on $\alpha 7\text{nAChRs}$ expression using autoradiography**

354 The  $\alpha 7\text{nAChRs}$  expression was evaluated on adjacent brain sections by [ $^{125}\text{I}$ ] $\alpha$ -bungarotoxin  
355 binding in the IL and CL striatum from each rat in the Sham, QA-PHA6 and QA-PHA12  
356 groups (Fig. 3A). The specific binding of [ $^{125}\text{I}$ ] $\alpha$ -bungarotoxin (Fig. 3B) in the CL side on the  
357 one hand and in IL side in the other hand were not significantly different between the Sham,  
358 QA-PHA6 and QA-PHA12 groups ( $1.65 \pm 0.42$ ,  $1.60 \pm 0.26$ , and  $1.83 \pm 0.25$  cpm/mm<sup>2</sup> in CL,  
359 respectively;  $1.36 \pm 0.42$ ,  $1.11 \pm 0.18$ , and  $0.83 \pm 0.27$  cpm/mm<sup>2</sup> in IL, respectively).

360

#### 361 **Evaluation of PHA 543613 effect on neuroinflammation by immunofluorescence**

362 The activated microglia were evaluated on adjacent brain sections by Ox-42 marker in the  
363 CL and IL striatum in the Sham ( $n = 6$ ) and QA-PHA12 ( $n = 6$ ) groups (Fig. 4A and 4B). In both

364 groups, Ox-42 staining in IL striatum was significantly ( $p < 0.05$ ) increased when compared to  
365 CL striatum (Sham:  $150 \pm 13$  cells stained in the IL vs  $22 \pm 2$  in the CL side; QA-PHA12:  $112$   
366  $\pm 5$  cells stained in the IL vs  $18 \pm 1$  in the CL side,  $p < 0.05$ ) (Fig. 4C). Activated microglia (Ox-  
367 42 positive cells) in IL striatum of QA-PHA12 rats were significantly lower ( $-34\%$ ,  $p < 0.05$ )  
368 than in Sham. Moreover, activated microglia in IL vs CL were lower in the PHA12 group than  
369 in Sham ( $531 \pm 107$  vs  $609 \pm 77$ , respectively) (Fig. 4D).

370

### 371 **Evaluation of PHA 543613 on neuronal survival by immunofluorescence**

372 Neuron counting was performed on the same rats as above by using NeuN marker (Fig. 5A  
373 and 5B). In both groups, NeuN staining was significantly decreased in IL vs CL side (QA-  
374 PHA12:  $114 \pm 9$  vs  $206 \pm 11$  cells in the CL side,  $p < 0.05$ ; Sham group:  $59 \pm 13$  vs  $184 \pm 11$   
375 cells in the CL side,  $p < 0.05$ ) (Fig. 5C). The number of neurons in IL side was 93% higher in  
376 the QA-PHA12 group than in the Sham group ( $p < 0.05$ ). Moreover, the neuronal loss in IL vs  
377 CL striatum was significantly lower in the PHA12 than in the Sham group ( $44.5 \pm 4$  and  $68 \pm$   
378  $7$ , respectively,  $p < 0.05$ ) (Fig. 4D).

379

### 380 **Discussion**

381 Neurodegenerative diseases are a major public health problem. Available treatments are  
382 only able to improve symptoms, but are unable to slow down or stop the progression of the  
383 disease. Many efforts are being deployed for developing such disease-modifying strategies  
384 with agents able to have neuroprotective and/or neurorestorative effects. Several studies  
385 suggest that neurodegeneration occurs in part because the environment is affected cascade  
386 fashion during disease in a cell-autonomous process affecting neurons. This process, called  
387 neuroinflammation, is defined by the contributions of glial cells, elements of the blood brain  
388 barrier (BBB) or systemic inflammatory processes that can increase the severity of  
389 neurodegenerative disease [36]. Thus, these observations indicate that therapies targeting  
390 glial cells might provide beneficial effects on neurodegenerative disorders. Downregulation of  
391 the activation of microglia can be obtained through the activation of  $\alpha 7$ nAChRs localized on

392 microglial cells [43]. The agonists of  $\alpha 7$ nAChRs have been the subjects of several studies on  
393 schizophrenia but little data on the treatment of neurodegenerative diseases is published  
394 nowadays [44]. Agonists of  $\alpha 7$ nAChRs appear to be more efficient than acetylcholine at  
395 inhibiting the inflammatory signalling and production of pro-inflammatory cytokines from  
396 immune cells [45]. However, the effects induced by  $\alpha 7$ nAChRs stimulation in traumatic brain  
397 injury models appear to be either neuroprotective or toxic, highlighting the complexity of the  
398 process.

399

400 The present study aimed to improve our knowledge relating to these potential therapeutic  
401 targets by evaluating *in vivo* the effects of the selective  $\alpha 7$ nAChRs agonist PHA 543613, on  
402 both neuronal survival and microglial activation in a rodent model of excitotoxic  
403 neuroinflammation based on striatal QA injection [39]. PHA 543613 was selected because  
404 this compound has already demonstrated neuroprotective and anti-inflammatory effects in  
405 different intracerebral haemorrhage models [29,30] and a recent study demonstrated that  
406 PHA 543513 exerts neuroprotective effects on the striatal dopaminergic neurons with a  
407 reduction in microglial activation in a Parkinson's disease rat model induced by 6-  
408 hydroxydopamine (6-OHDA) lesion [28]. A preliminary study allowed us to confirm the ability  
409 of this  $\alpha 7$ nAChRs agonist to pass through the BBB and particularly to evaluate its  
410 degradation half-life in order to optimize the drug administration schedule. Therefore, PHA  
411 543613 was intra-peritoneally injected 1h before QA lesioning and then twice per day at days  
412 1, 2, 3 and 4 post-lesion. The 4-day treatment duration was motivated by recently published  
413 data reporting that quick tissue inflammation reached its highest level between day-4 to 7  
414 post-QA lesion in this model based on the studied neuroinflammation markers [40,46]. The  
415 unilateral intrastriatal injection of QA is admitted as an animal model mimicking the early  
416 stages of Huntington's disease. It reproduces some biochemical, behavioural and pathologic  
417 features of the disease in rodents and non-human primates [38,47,48]. QA is an endogenous  
418 NMDA receptor agonist with excitotoxic properties which is found in normal subjects as a by-  
419 product along the kynurenine pathway leading to the synthesis of the essential co-factors

420 nicotinic acid and nicotinamide adenine dinucleotide [49]. QA spares aspiny striatal  
421 interneurons relative to spiny projection neurons [38]. In fact, it acts as a neurotoxin and  
422 gliotoxin pro-inflammatory mediator and a pro-oxidant molecule that can alter BBB integrity  
423 [50]. Dysfunction of neuronal activity consecutive to the QA injection can induce a pro-  
424 inflammatory environment leading to the activation of surrounding microglial cells and in  
425 consequence microglial activation. In the chosen excitotoxic model of neuroinflammation, the  
426 excessive excitation of NMDA receptors leads to a massive intracellular influx of calcium.  
427 This increase is associated with a mitochondrial dysfunction characterized by a decrease of  
428 ATP levels that involves the formation and release of reactive oxygen and nitrogen species,  
429 thus inducing cell oxidative damage as part of the degenerative process [51]. Moreover, the  
430 activation of various enzymes (proteases, lipases and endonucleases) triggering different cell  
431 components is also involved in neuronal death [37].

432

433 This *in vivo* model of neuroinflammation has been shown to produce large lesions  
434 accompanied by an inflammatory response involving increased microglial activation and an  
435 axon-sparing neurodegeneration which is directly associated with the degree of brain  
436 damage [52,53]. This leads to an increase in the expression of TSPO [39,40,46]. This  
437 mitochondrial protein is considered to be a sensitive biomarker of microglial activation [54]  
438 and specific imaging tracers are currently used to explore it in preclinical and clinical studies  
439 [55-58]. We evaluated this molecular target by quantitative autoradiography. [<sup>3</sup>H]PK-11195 is  
440 a long used tracer in this type of experiment, and the tritiated homolog of the new generation  
441 tracer of TSPO, DPA-714, has been recently developed [41]. First, we compared the  
442 properties of both of these tracers in our QA rat model and demonstrated that [<sup>3</sup>H]DPA-714  
443 displayed a lower non specific binding than [<sup>3</sup>H]PK-11195. This result is in agreement with *in*  
444 *vivo* experiments which observed a higher signal-to-noise ratio using [<sup>18</sup>F]DPA-714 in  
445 comparison to [<sup>11</sup>C]PK-11195 in different rodent models such as in middle cerebral artery  
446 occlusion (MCAO) [59] and herpes encephalitis (HSE) [60]. In addition, higher inter-animal  
447 variability in MCAO and HSE models was observed than in our QA model. Thus, we used the

448 tritiated analog of a highly sensitive marker of TSPO that can also be used for *in vivo* human  
449 clinical investigations. Then, we examined whether the PHA 543613 effects on TSPO binding  
450 with [<sup>3</sup>H]DPA-714 were dose-related in our model; the compound was administered at the  
451 concentrations of 6 mg/kg [28] or 12 mg/kg. The TSPO bindings levels illustrated significant  
452 decrease of inflammation in QA-PHA12 vs sham animals while the rate of increase of TSPO  
453 binding in IL vs CL striatum did not decrease significantly between QA-PHA6 and sham  
454 group. As a result of this finding and since no difference in body weight or animal  
455 compartment were noticed between control, QA-PHA6 and QA-PHA12 animals, we decided  
456 to make further experiments with the highest dose of PHA 543613.

457

458 Effects associated to  $\alpha 7$ nAChRs agonist depend on various parameters like the levels of  
459  $\alpha 7$ nAChRs expression and function or the neuropathology stage and intrinsic properties of  
460 the  $\alpha 7$ nAChR agonists [61].  $\alpha 7$ nAChRs are known to be widely expressed throughout  
461 several brain regions in mammals by neurons and glial cells. Among them, the  $\alpha 7$ nAChRs  
462 are probably the most widespread nAChRs subtype expressed in the brain [62]. These  
463 receptors can control the release of various types of neurotransmitters, contribute to synaptic  
464 plasticity and they have been proved to participate in the regulation of inflammatory reactions  
465 [63]. However, the rapid desensitization of  $\alpha 7$ nAChRs is considered an important factor  
466 limiting its potential therapeutic use. Therefore, to approach the mechanism that could  
467 explain the beneficial effect of PHA 543613 on neuroinflammation, we quantified  $\alpha 7$ nAChRs  
468 expression in both IL and CL striata of Sham, PHA 6mg/kg and PHA 12mg/kg QA-lesioned  
469 animals. Autoradiography binding with alpha-bungarotoxin was performed at 4-days post-  
470 lesion and confirmed the expression of  $\alpha 7$ nAChRs in the striatum of all groups of rats. In  
471 addition, we observed a decreasing of binding in IL vs CL striatum in Sham, PHA6 and  
472 PHA12 rats, hypothetically reflecting the tissue destruction and thus neuron and glial cell loss  
473 induced in all animals by local QA injection. This result could also be consistent with a recent  
474 publication reporting that neuroinflammation induced by regular bacterial lipopolysaccharide  
475 injections resulted in the decrease of  $\alpha 7$ nAChRs density in the brain of mice [64]. Indeed,

476 after activation, the  $\alpha 7$ nAChRs undergo rapid desensitivation in order to limit the influx of  
477 calcium ( $\text{Ca}^{2+}$ ) into the cell which could lead to a phenomenon of excitotoxicity [65]. However,  
478 4-day chronic treatment with PHA 543613 did not lead to a significant modification of  
479  $\alpha 7$ nAChRs expression vs control which reveals no significant receptor desensitivation. This  
480 observation probably does not apply to our short-term PHA exposure suggesting no induced  
481 receptor desensitivation. Lastly, it is then noteworthy that in spite of the diminution of  
482  $\alpha 7$ nAChRs expression in lesioned striatum of PHA12-treated animals, neuroprotective and  
483 anti-inflammatory effects of the drug were observed.

484

485 The effects of the PHA 543613 treatment on microglial activation in striatum were also  
486 assessed by immunohistochemistry using Ox-42 - an antibody specific to CD11b mostly  
487 expressed in activated microglia. Measurements showed in both QA-PHA12 and Sham  
488 groups a significant massive increase of Cd11b expression in the striatum from the IL  
489 hemisphere vs CL hemisphere, confirming the inflammatory impact of QA injection on  
490 microglial cell activity. Interestingly, the number of cells expressing the CD11b was  
491 significantly (-25.5%) lower in IL striatum of QA-PHA12 rats than in IL striatum of Sham rats.  
492 To evaluate the potential effect of PHA543613 treatment on neuron survival in striatum, we  
493 used Neun marker located in the nucleus and the perinuclear cytoplasm of postmitotic  
494 neurons in mammals. We logically observed significant reduction in neuron population in IL  
495 striatum vs CL of both QA-PHA12 and Sham groups, illustrating once again the deleterious  
496 effect of QA injected locally. However, considering IL striatum counting of both QA-PHA12  
497 and Sham rats, it is noteworthy that the number of preserved neurons was approximately  
498 twice as high in the QA-PHA12 than in the Sham group, illustrating the protective effect  
499 mediated by PHA treatment in IL striatum of treated animals. Those results indicate that the  
500 neuroprotective effects on the striatal neurons are associated with a reduction in microglial  
501 activation. Although QA exerts the greatest damage to neurons where NMDA receptors are  
502 present, cholinergic and GABAergic neuronal cell types seem to be more sensitive than  
503 others especially in the striatum [49,66]. Thus, these two types of neurons do not seem to be

504 involved in the beneficial effects observed following administration of PHA 543613 in our  
505 study.

506

507 We report here through the first evaluation of the  $\alpha 7$ nAChRs agonist PHA 543613 in an *in*  
508 *vivo* excitotoxic model of neuroinflammation, the strong relationship that exists between the  
509 effects of PHA 543613 on neuroinflammation and neuron integrity. We confirm that PHA  
510 543613 can reduce both neuronal loss and microglial activation in the same animals, as  
511 already observed in a rat model of Parkinson's disease [28]. The  $\alpha 7$ nAChRs are able to  
512 mediate the anti-inflammatory effect of acetylcholine by attenuating the pro-inflammatory  
513 cytokine release involved in the regulation of the cholinergic anti-inflammatory pathway in  
514 brain astrocytes or microglia [67]. However,  $\alpha 7$ nAChRs chronic stimulation has been  
515 associated with massive and persistent calcium increase inside brain cells well known to be  
516 toxic to cells expressing this receptor [68]. This statement supports the necessity to pay  
517 attention to the duration of  $\alpha 7$ nAChRs agonist regimen when used for a therapeutic purpose.  
518 Indeed, we have demonstrated the capability of a  $\alpha 7$ nAChRs-activating agent to induce fast  
519 neuroprotection facing an acute brain traumatic injury; these findings support the short-term  
520 use of  $\alpha 7$ nAChRs-activating agents as a strategy to reduce traumatic brain injury triggered  
521 BBB permeability [69]. Considering that PHA 543613 can target both of the processes  
522 involved in most neurodegenerative diseases,  $\alpha 7$ nAChRs agonists could represent a major  
523 therapeutic challenge in neurology.

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532 **List of abbreviations**

533 AD: Alzheimer disease

534 BBB: blood brain barrier

535 CL: contra lateral

536 cpm: counts per min

537 HSE: herpes encephalitis

538 IL: ipsi lateral

539 i.p: intraperitoneal

540  $\alpha$ 7nAChRs: nicotinic acetylcholine receptor  $\alpha$ 7

541 MCAO: middle cerebral artery occlusion

542 NeuN: neuronal nuclei

543 NMDA: N-methyl-D-aspartate

544 NO: nitric oxyde

545 PD: Parkinson disease

546 QA: quinolinic acid

547 ROIs: regions of interest

548 RT: room temperature

549 SEM: standard error of the mean

550 TSPO: 18kDa translocator protein

551 Vs: versus

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560 **Ethics approval**

561 All procedures were conducted in accordance with the European Community Council  
562 Directive 2010/63/EU for laboratory animal care and the experimental protocol was validated  
563 by the Regional Ethical Committee (Authorization N°2015022011523044).

564

565 **Competing interest**

566 The authors declare that they have no competing interests.

567

568 **Authors' contributions**

569 SC and DA contributed equally to this work.

570

571 **Availability of data and materials**

572 The datasets during and/or analysed during the current study available from the  
573 corresponding author on reasonable request.

574

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580

581 **Consent for publication**

582 Not applicable

583

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790

791 **Figure legends**

792 **Figure 1: Comparative evaluation of TSPO radioligands [<sup>3</sup>H]DPA-714 and [<sup>3</sup>H]PK-11195**

793 **by autoradiographic study. (A)** Representative autoradiographic images obtained on 16  
794 μm-thick coronal brain sections with [<sup>3</sup>H]PK-11195 (1 nmol/L, left panel) and [<sup>3</sup>H]DPA-714 (1  
795 nmol/L, right panel) alone or in presence of stable PK11195 (1 μmol/L, in the right of each  
796 panel) of the same animal. **(B)** Percentage of TSPO specific binding in IL vs CL striatum  
797 (mean % ± SEM) from QA lesioned rats (n= 9) with [<sup>3</sup>H]PK-11195 or [<sup>3</sup>H]DPA-714. **(C)**  
798 Correlation between [<sup>3</sup>H]PK-11195 and [<sup>3</sup>H]DPA-714 autoradiography study. The correlation  
799 is reported for the percentage of TSPO binding in IL vs CL striatum with each tracer. #p<0.05  
800 (Mann Whitney test). \*p<0.05 (two-tailed Spearman test). Abbreviations: CL:  
801 contralateral; IL: ipsilateral; SEM: standard error of the mean.

802

803 **Figure 2: Autoradiographic analysis of TSPO density with [<sup>3</sup>H]DPA-714 in the striatum.**

804 **(A)** Representative total (left side) and non-specific (right side) binding of [<sup>3</sup>H]DPA-714  
805 obtained on 16 μm-thick coronal brain section in Sham rats (upper panel), QA-PHA 6 and  
806 QA-PHA12 rats (lower panel). **(B)** Percentage increase of TSPO binding in IL vs CL striatum  
807 (mean % ± SEM) from Sham (n= 5), QA-PHA6 (n= 8) and QA-PHA12 (n= 9) rats.  
808 Abbreviations: CL: contralateral; IL: ipsilateral; SEM: standard error of the mean.

809

810 **Figure 3: Autoradiographic analysis of α7nAChRs density with [<sup>125</sup>I]α-bungarotoxin in**

811 **the striatum. (A)** Representative total (left side) and non-specific (right side) binding of  
812 [<sup>125</sup>I]α-bungarotoxin obtained on 16 μm-thick coronal brain section in Sham rats (upper  
813 panel), QA-PHA 6 and QA-PHA12 rats (lower panel). **(B)** α7nAChRs expression in IL and CL  
814 striatum from Sham (n=6), QA-PHA6 (n=6) and QA-PHA12 (n=6) rats. Data are expressed  
815 as cpm/mm<sup>2</sup>. Abbreviations : CL: contralateral; IL: ipsilateral; cpm/mm<sup>2</sup>: counts per min per  
816 mm<sup>2</sup>.

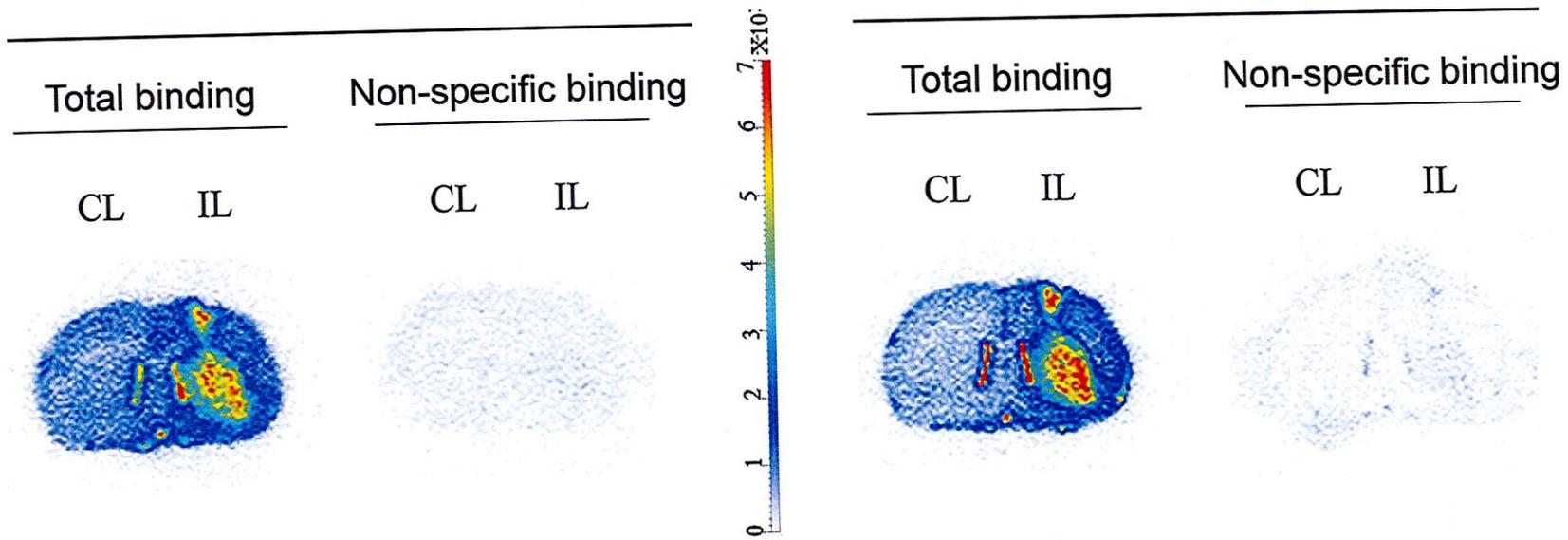
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818 **Figure 4: Immunofluorescence in rat striatum of Ox-42 in Sham group and QA-PHA12**  
819 **group. (A)** Coronal rat brain representation. The full line and areas marked in red symbolize,  
820 respectively, the site of injury and areas where Ox-42 immunofluorescence was performed.  
821 **(B)** Representative immunofluorescence images of activated microglia by Ox-42 staining  
822 (green channel) and DAPI (blue channel) in the CL (left) and IL (right) striatum of Sham  
823 (upper) (n=6) and QA-PHA12 (lower) (n=6) rats. Magnification was x 20. Scale bar, 50  $\mu$ m.  
824 **(C)** Ox-42 expression in IL and CL striatum for each group. Data are expressed as number of  
825 cells expressing Ox-42 marker. **(D)** Data are expressed as relative activated microglia in IL  
826 vs CL striatum for each group. #  $p < 0.05$  (Mann Whitney test). \*  $p < 0.05$  (Wilcoxon test).  
827 Abbreviations: CL: contralateral; IL: ipsilateral.

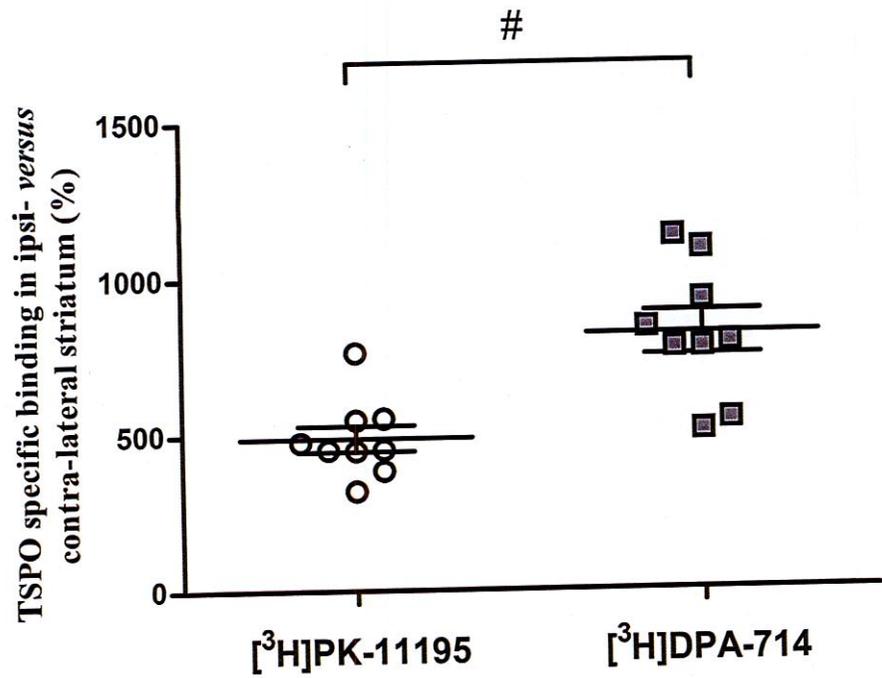
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829 **Figure 5: Immunofluorescence in rat striatum of neurons (NeuN) in Sham group and**  
830 **QA-PHA12 group. (A)** Coronal rat brain representation. The full line and areas marked in  
831 red symbolize, respectively, the site of injury and areas where NeuN immunofluorescence  
832 was performed. **(B)** Representative immunofluorescence images of neurons by NeuN  
833 staining (green channel) and DAPI (blue channel) in the CL (left) and IL (right) striatum of  
834 Sham (upper) (n=6) and QA-PHA12 (lower) (n=6) rats. **(C)** Neuronal expression in CL and IL  
835 striatum for each group. Data are expressed as number of cells expressing NeuN marker.  
836 **(D)** Data are expressed as relative neuronal loss in IL vs CL striatum for each group #  
837  $p < 0.05$  (Mann Whitney test). \*  $p < 0.05$  (Wilcoxon test). Abbreviations: CL: contralateral; IL:  
838 ipsilateral.

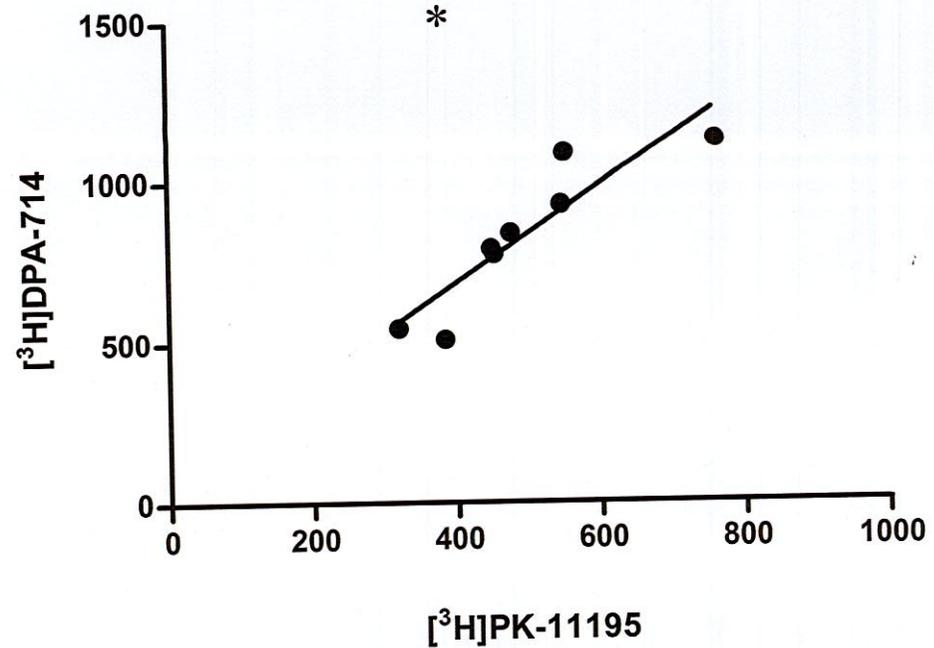
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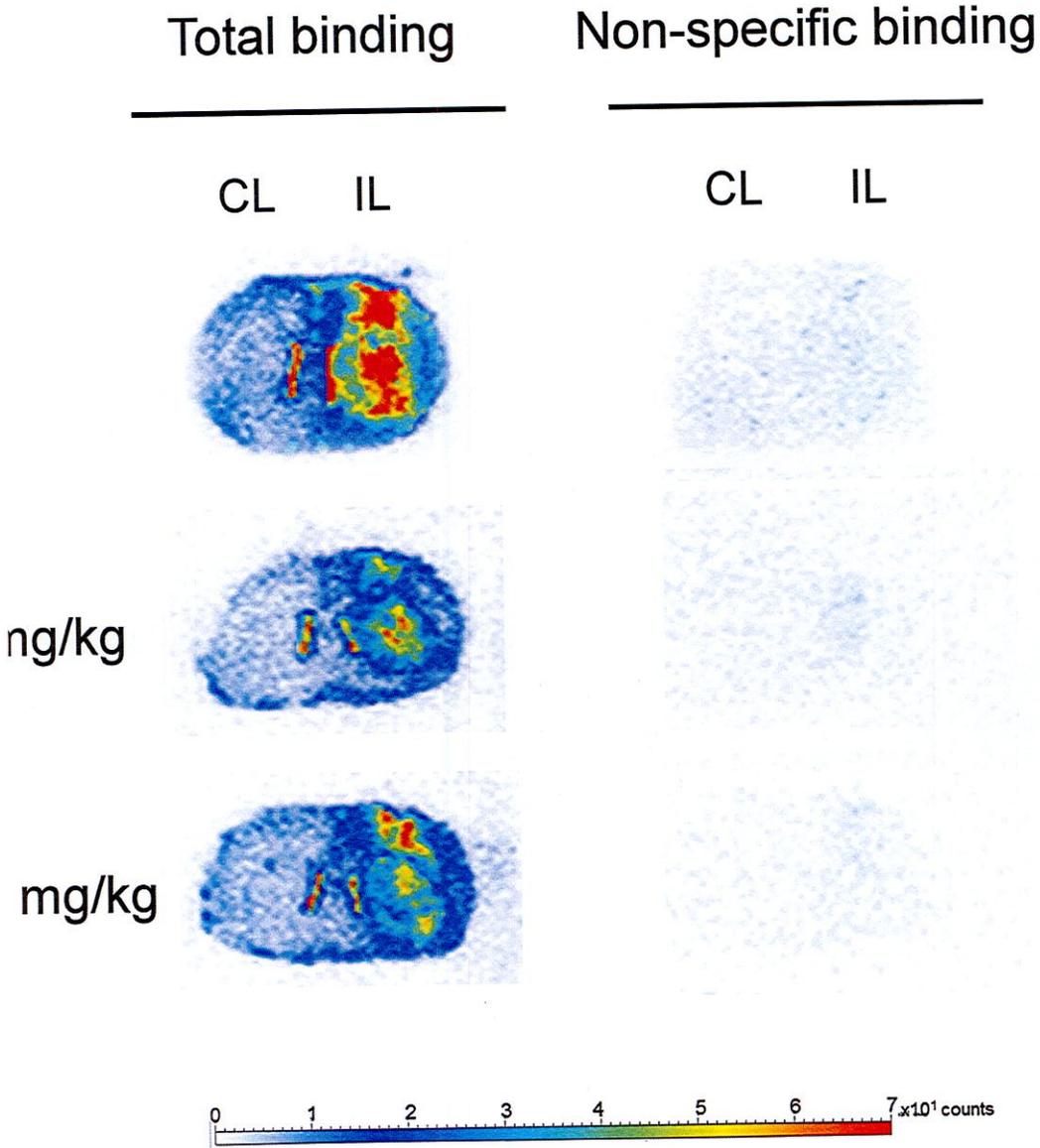


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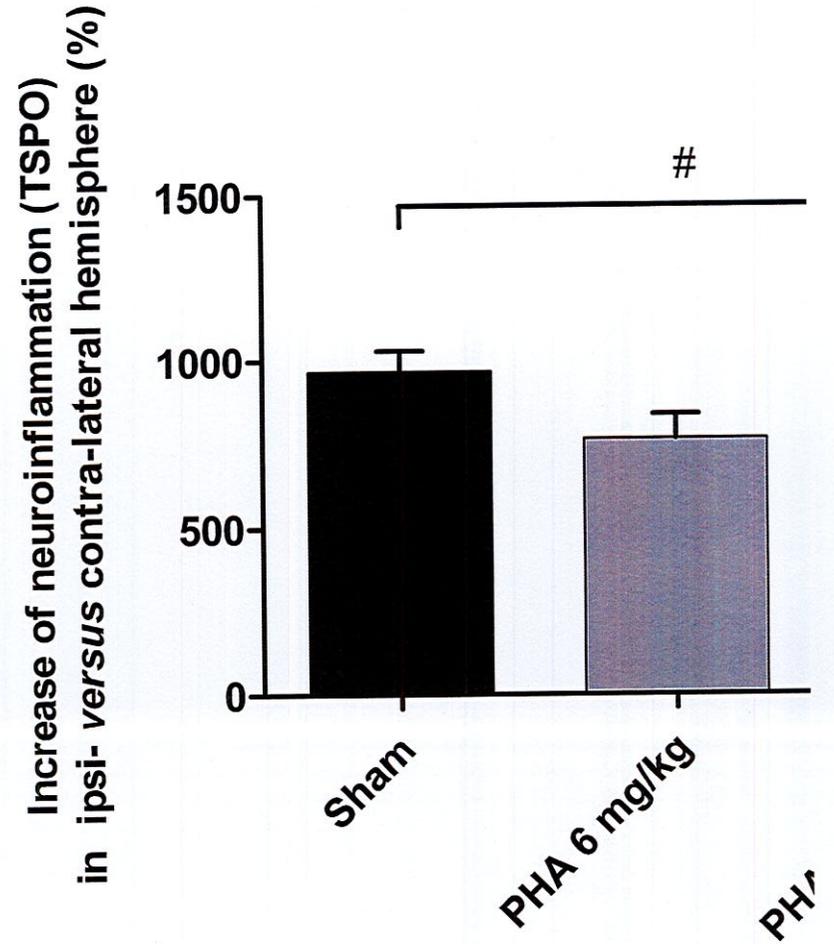


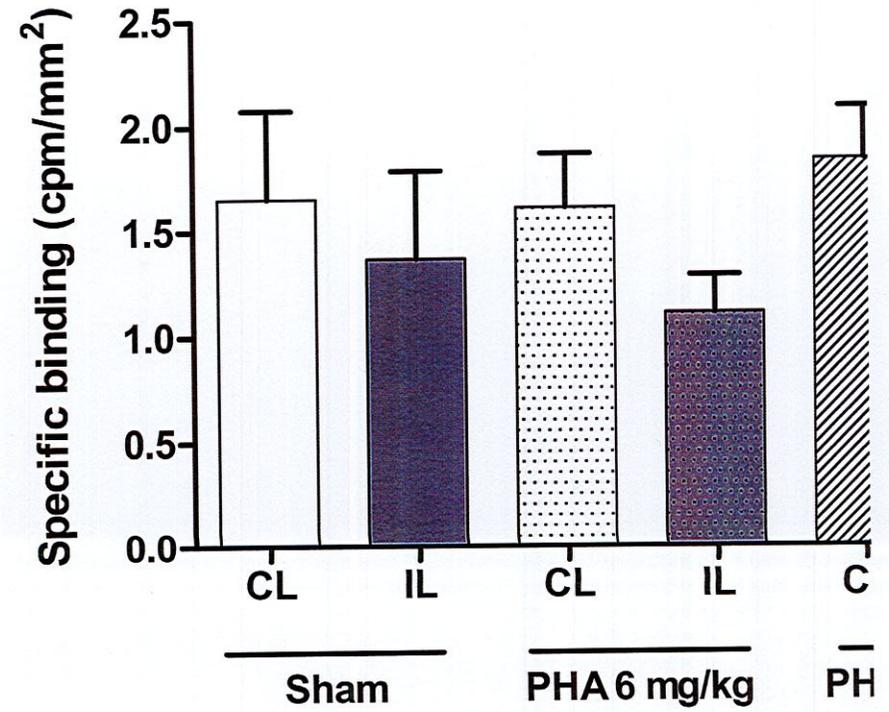
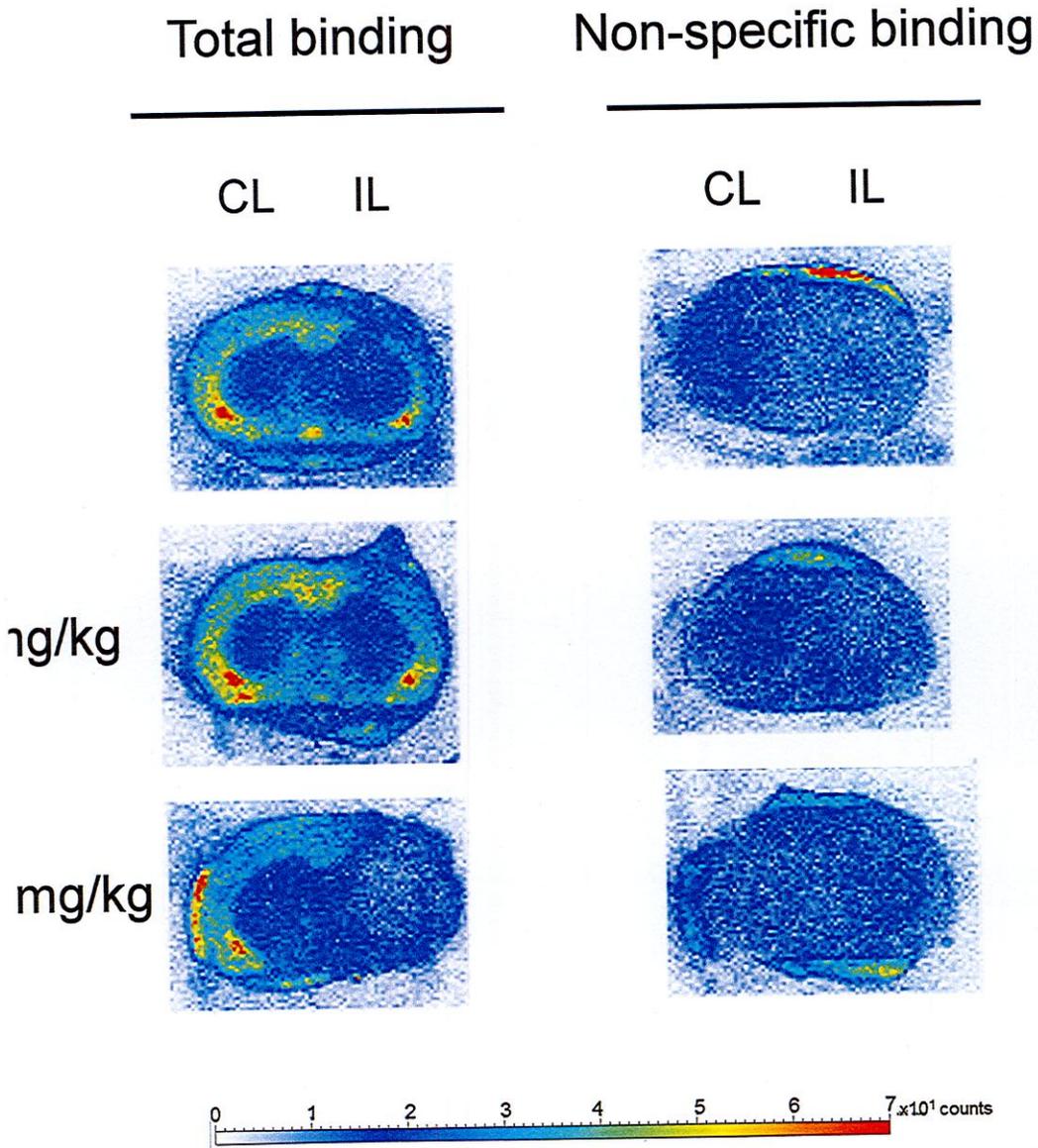
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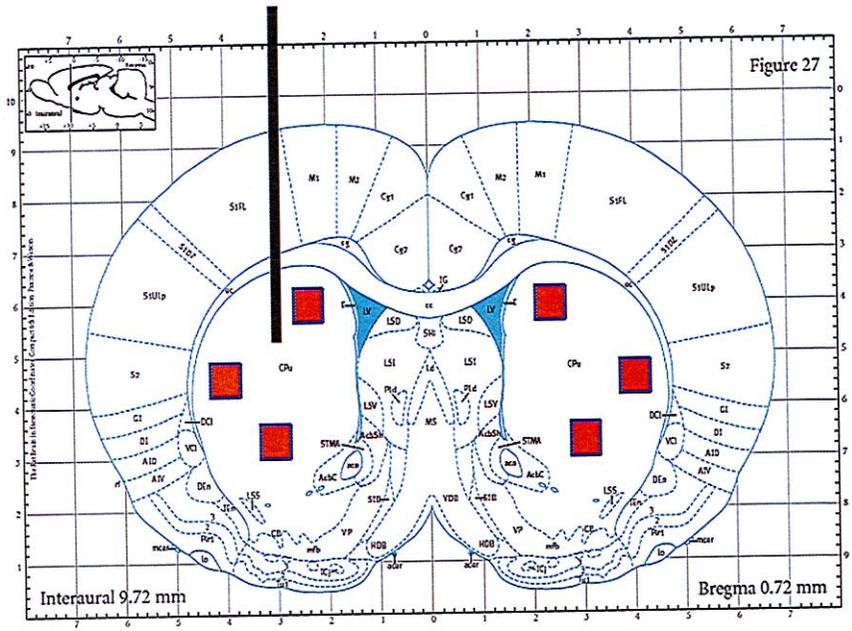




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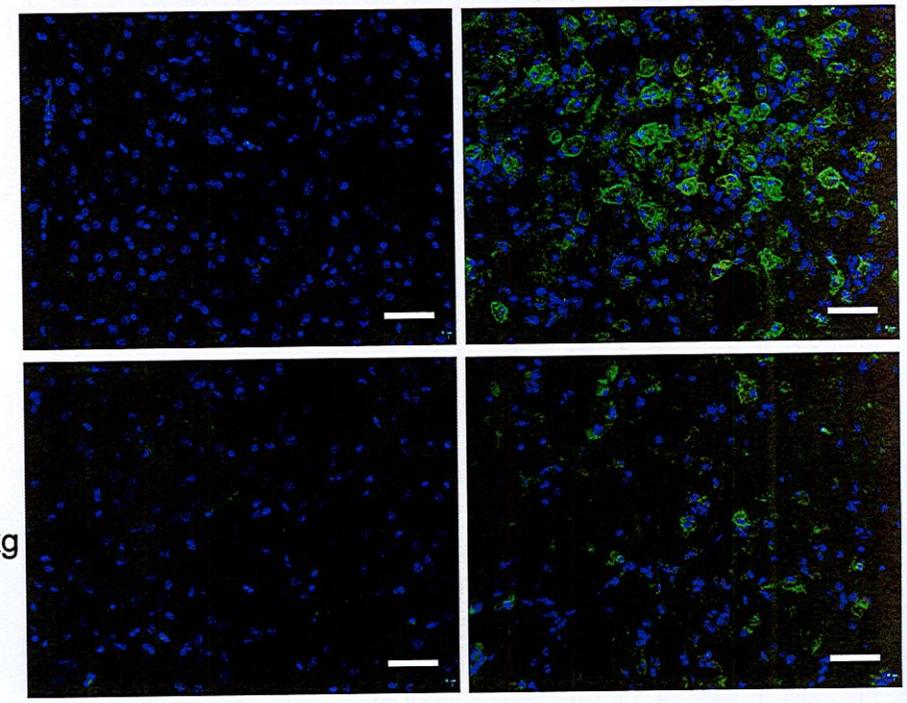


**B**

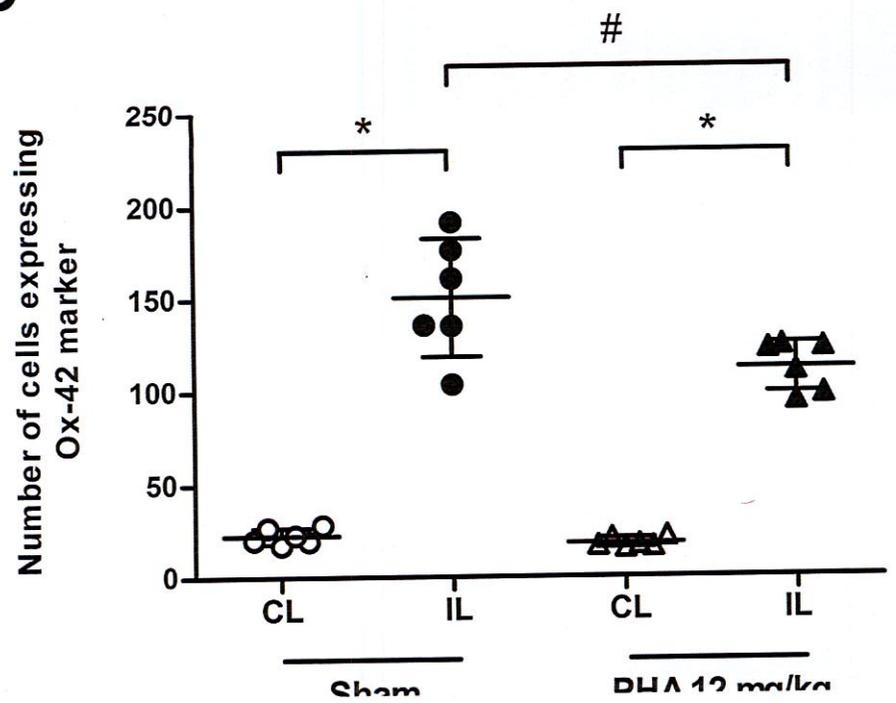


Sham

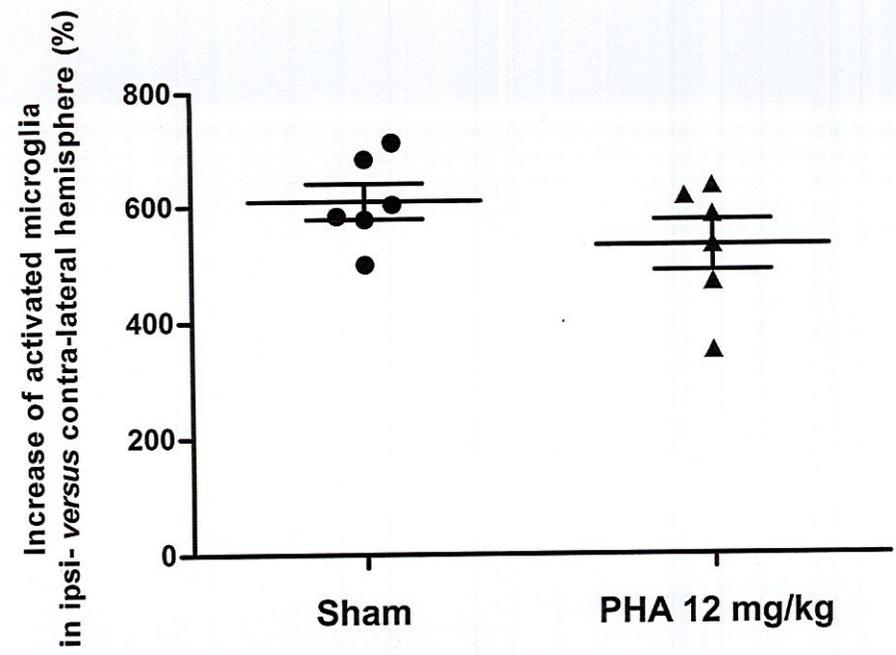
PHA 12 mg/kg

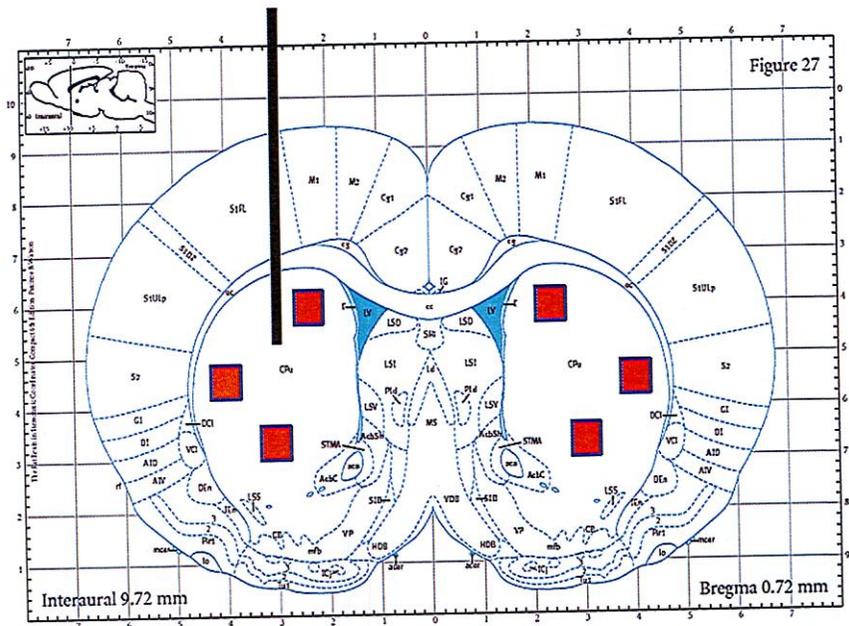


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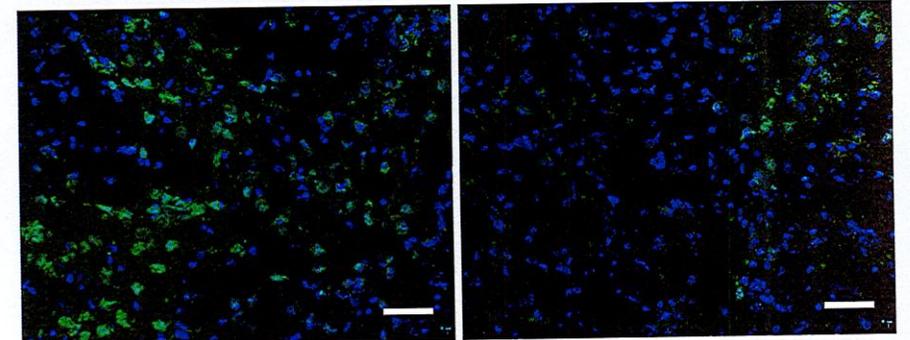


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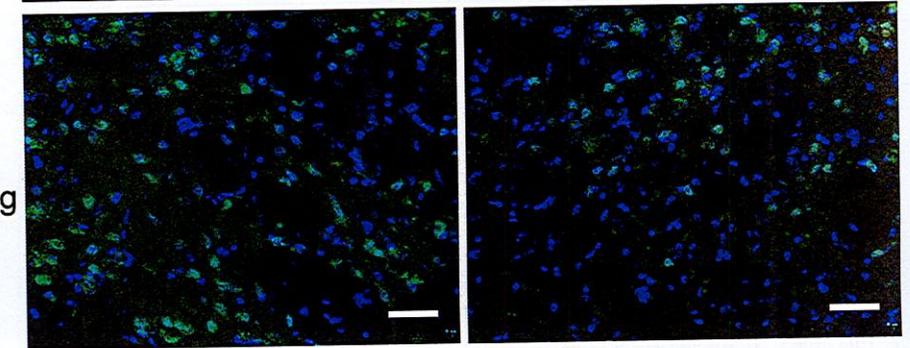




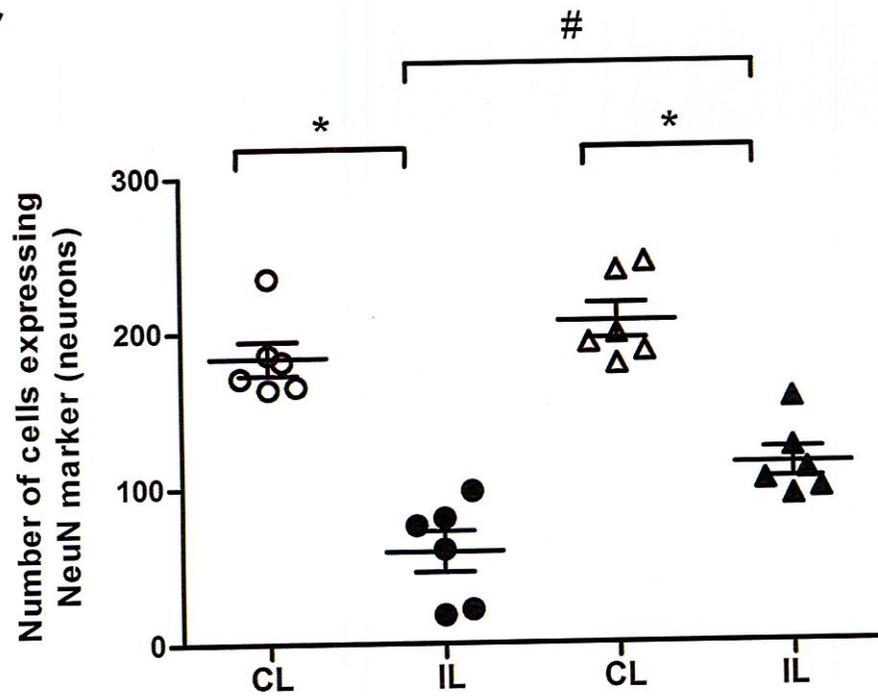
Sham



PHA 12 mg/kg



C



D

