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

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Abstract

Background

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

Methods

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

Results

We demonstrated that [³H]DPA-714 provide a better signal-to-noise ratio than [³H]PK11195. In addition, we showed that chronic PHA 543613 treatment administered at a dose of 12mg/kg to QA-lesioned rats significantly protected neurons and reduced the intensity of neuroinflammation measured by TSPO and Ox-42 immunostaining.

Conclusions

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

Keywords

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

Background

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

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

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

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

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

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

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

Materials and Methods

Animals

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

Excitotoxic neuroinflammation model

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

Comparative evaluation of TSPO radioligands [^3H]DPA-714 and [^3H]PK-11195 by autoradiographic study in QA lesioned rats

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

Sections were rinsed twice in ice cold buffer (4°C) for 5 min, then briefly in distilled water at 4°C and dried at RT. Dry sections were made conductive by an application of metal electric tape (3M™, Euromedex, Souffelweyersheim, France) on the other side and then placed in the gas chamber of the β-imager™ 2000 (Biospace Lab, Paris, France). Acquisitions were collected over a period of 4 h. Two anatomical regions of interest (ROIs), i.e., the ipsi-lateral (IL) and contra-lateral (CL) striata were selected and identified in Paxinos and Watson atlas [42]. Using the β-vision software (Biospace Lab, Paris, France), the level of bound radioactivity was directly determined by counting the number of β-particles emitted from the delineated area. The radioligand signal in the ROIs was measured for 6 sections per brain and expressed as counts per minute per square millimeter (cpm/mm²). Specific binding was determined by subtracting non-specific binding from total binding. Radioactivity was quantified using an image analyzer (M3-vision™ Biospace Instruments, Paris, France). The percentage increase of TSPO binding in IL *versus* (vs) CL hemisphere was calculated as :
$$\frac{IL-CL}{CL} \times 100$$

Pharmacokinetics of PHA 543613

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

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

Experimental procedure and drug treatment

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

Evaluation of PHA 543613 dose-effect on neuroinflammation using TSPO

quantification by autoradiography

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

binding (expressed in cpm/mm²) from 2 ROIs, i.e., IL and CL striatum were determined.

Finally, the percentage increase of TSPO binding in IL vs CL hemisphere was calculated as :

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

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

The density of $\alpha 7$ nAChRs binding sites was measured by *in vitro* autoradiography using [¹²⁵I] α -bungarotoxin (specific activity 81.4 TBq/mmol; Perkin Elmer, Skovlunde, Denmark) on brain sections adjacent to those used in the previous study, in Sham (n=6), QA-PHA6 (n=6) and QA-PHA12 (n=6) rats. Brain sections were allowed to equilibrate at RT for 3h and then were incubated with 0.4 nmol/L of [¹²⁵I] α -bungarotoxin in 50 mmol/L Tris–HCl buffer pH 7.4 at RT for 60 min. Non-specific binding was assessed in the presence of 1 μ mol/L stable α -bungarotoxin (Tocris Bioscience, Bristol, UK). The density of $\alpha 7$ nAChRs binding sites was measured as described above (see section 3). A total of 6 sections per brain were analyzed for each rat. Data from brain sections were collected over 4h with the β -imager™ 2000. Specific binding (expressed in cpm/mm²) from 2 ROIs, i.e., IL and CL striatum was determined.

Evaluation of PHA effects on microglia activation and neuronal survival by immunofluorescence

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

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

Statistical Analysis

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

Results

Animals

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

PHA 543613 quantitative analysis with UHPLC coupled with high resolution mass spectrometry

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

Comparative evaluation of TSPO using [3 H]DPA-714 and [3 H]PK-11195

The comparison of specific binding of TSPO between [3 H]PK-11195 and [3 H]DPA-714 by autoradiography was performed on brain sections of rats sacrificed 7 days after intrastriatal injection of QA (Fig. 1). Quantification of the autoradiography revealed that the binding of TSPO either by [3 H]PK-11195 or [3 H]DPA-714 was significantly ($p < 0.05$) higher in the IL than in the CL striatum. Non-specific binding in the CL or IL striatum assessed with 1 μ mol/L PK11195, was lower with [3 H]DPA-714 than with [3 H]PK-11195, indicating a better signal-to-noise for [3 H]DPA-714 (0.06 ± 0.10 cpm/mm² vs 0.29 ± 0.02 cpm/mm² in CL respectively, $p < 0.05$; 0.14 ± 0.02 cpm/mm² vs 0.37 ± 0.02 cpm/mm² in IL respectively; $p < 0.05$) (Fig. 1A).

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

Evaluation of PHA 543613 dose-effect relationship on neuroinflammation using autoradiography

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

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

The $\alpha 7\text{nAChRs}$ expression was evaluated on adjacent brain sections by [^{125}I] α -bungarotoxin binding in the IL and CL striatum from each rat in the Sham, QA-PHA6 and QA-PHA12 groups (Fig. 3A). The specific binding of [^{125}I] α -bungarotoxin (Fig. 3B) in the CL side on the one hand and in IL side in the other hand were not significantly different between the Sham, QA-PHA6 and QA-PHA12 groups (1.65 ± 0.42 , 1.60 ± 0.26 , and 1.83 ± 0.25 cpm/mm² in CL, respectively; 1.36 ± 0.42 , 1.11 ± 0.18 , and 0.83 ± 0.27 cpm/mm² in IL, respectively).

Evaluation of PHA 543613 effect on neuroinflammation by immunofluorescence

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

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

Evaluation of PHA 543613 on neuronal survival by immunofluorescence

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

541 MCAO: middle cerebral artery occlusion

542 NeuN: neuronal nuclei

543 NMDA: N-methyl-D-aspartate

544 NO: nitric oxide

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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Ethics approval

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

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

SC and DA contributed equally to this work.

Availability of data and materials

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

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Consent for publication

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Figure legends

Figure 1: Comparative evaluation of TSPO radioligands [³H]DPA-714 and [³H]PK-11195

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

Figure 2: Autoradiographic analysis of TSPO density with [³H]DPA-714 in the striatum.

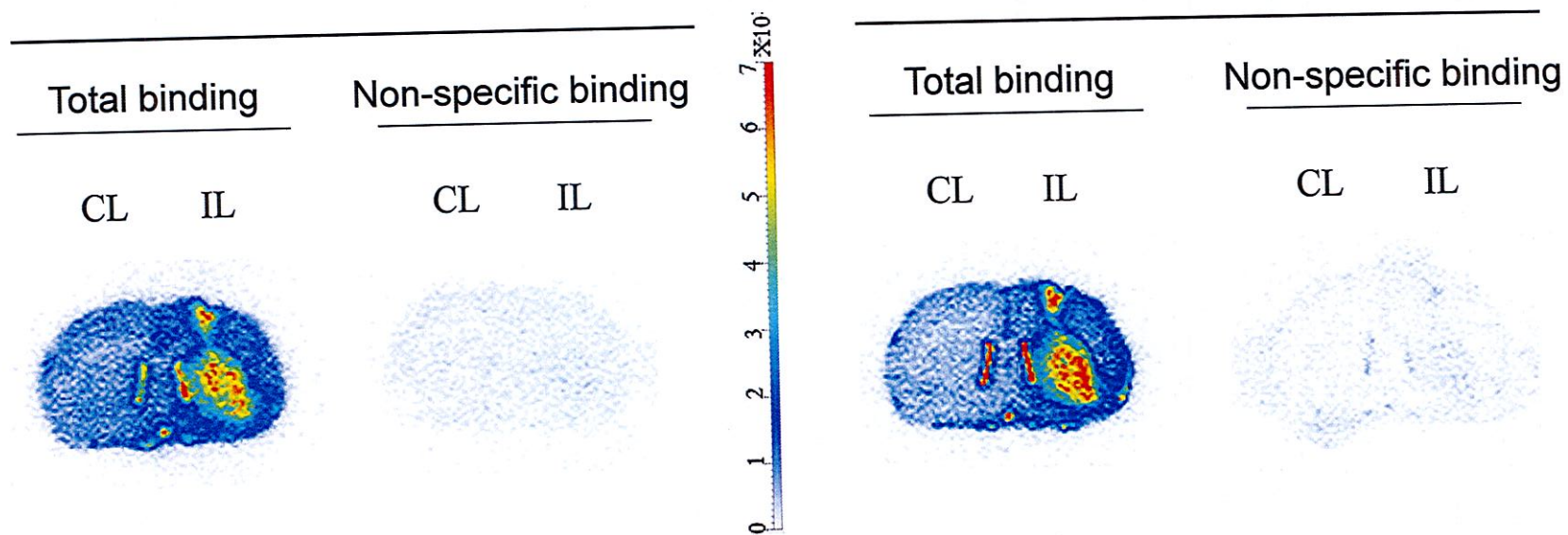
(A) Representative total (left side) and non-specific (right side) binding of [³H]DPA-714 obtained on 16 µm-thick coronal brain section in Sham rats (upper panel), QA-PHA 6 and QA-PHA12 rats (lower panel). (B) Percentage increase of TSPO binding in IL vs CL striatum (mean % ± SEM) from Sham (n= 5), QA-PHA6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

Figure 3: Autoradiographic analysis of α7nAChRs density with [¹²⁵I]α-bungarotoxin in

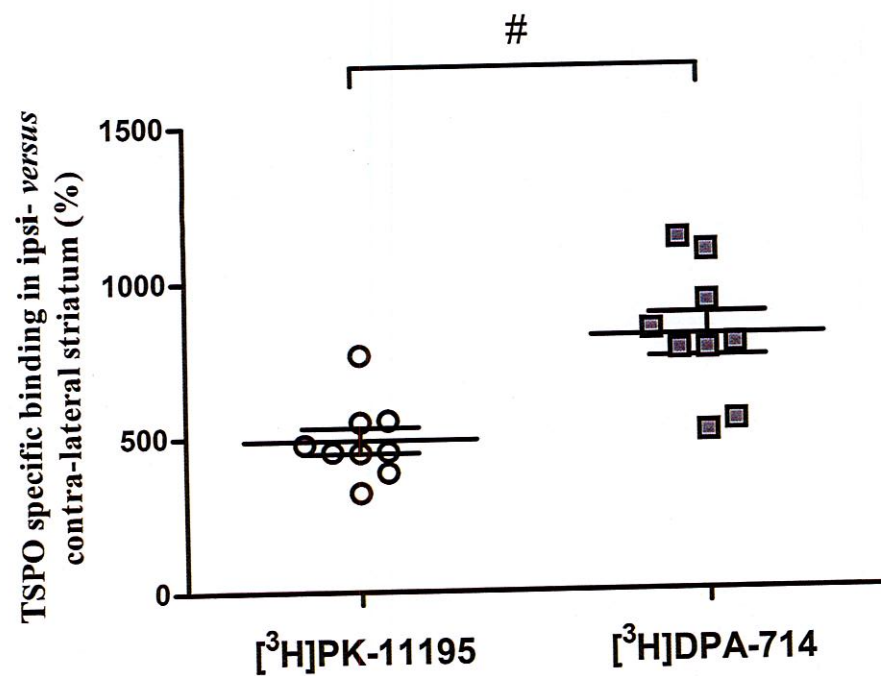
the striatum. (A) Representative total (left side) and non-specific (right side) binding of [¹²⁵I]α-bungarotoxin obtained on 16 µm-thick coronal brain section in Sham rats (upper panel), QA-PHA 6 and QA-PHA12 rats (lower panel). (B) α7nAChRs expression in IL and CL striatum from Sham (n=6), QA-PHA6 (n=6) and QA-PHA12 (n=6) rats. Data are expressed as cpm/mm². Abbreviations : CL: contralateral; IL: ipsilateral; cpm/mm²: counts per min per mm².

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

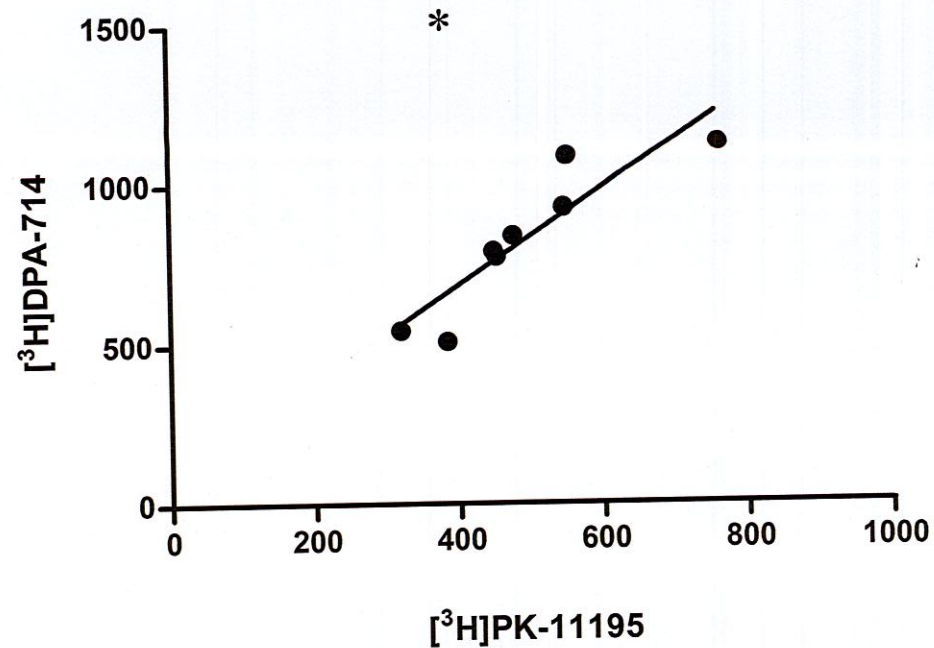
Figure 5: Immunofluorescence in rat striatum of neurons (NeuN) in Sham group and QA-PHA12 group. (A) Coronal rat brain representation. The full line and areas marked in red symbolize, respectively, the site of injury and areas where NeuN immunofluorescence was performed. (B) Representative immunofluorescence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

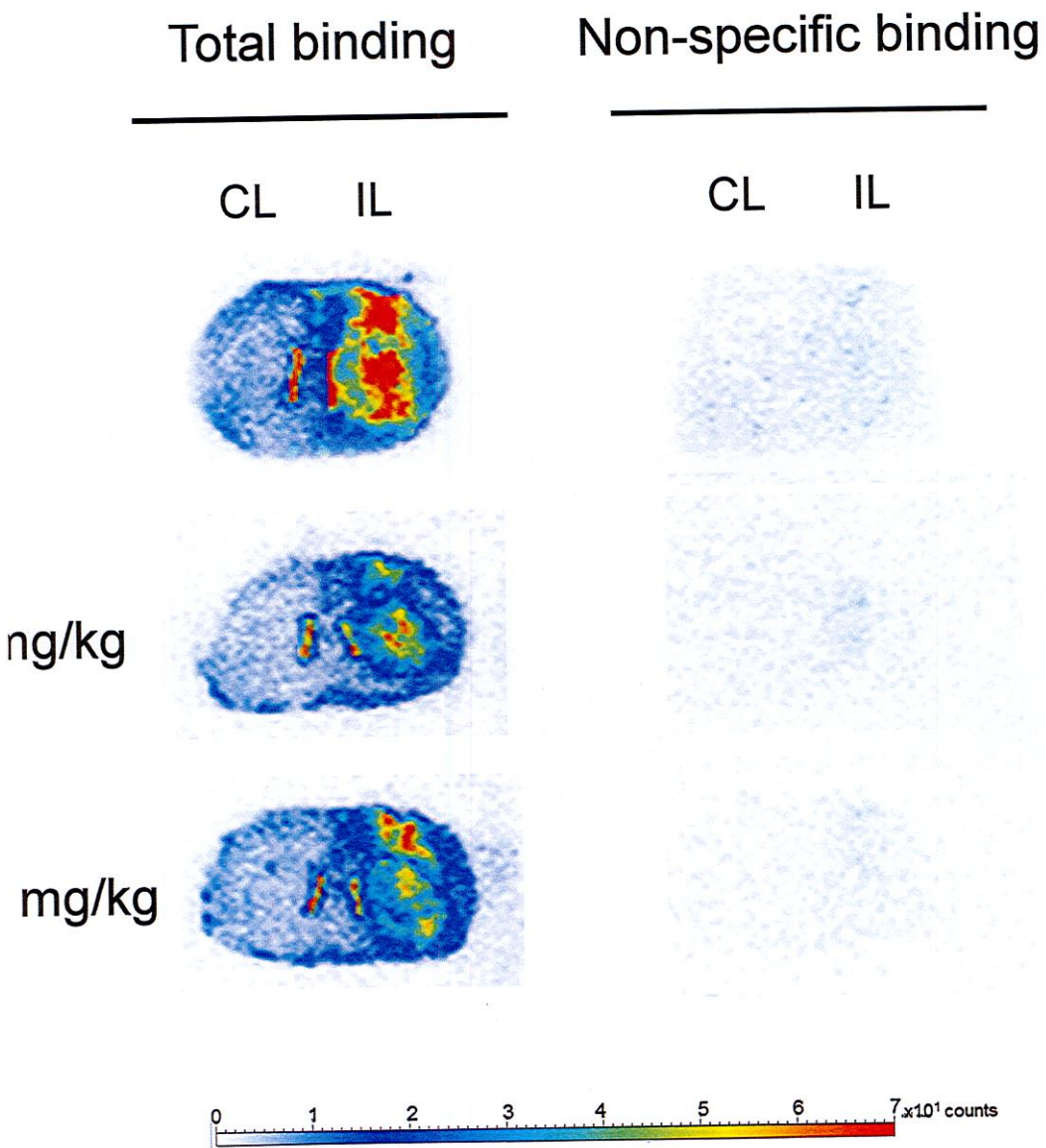


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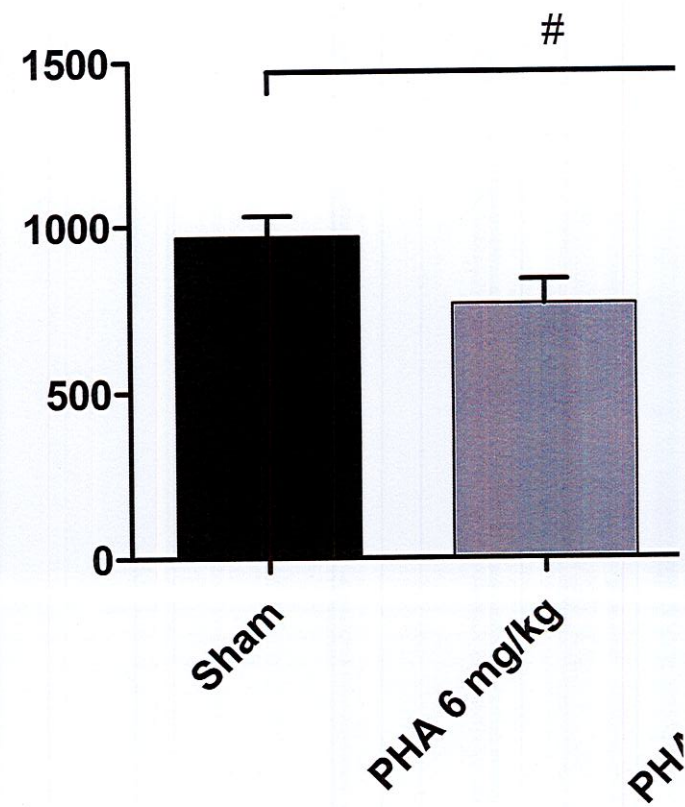
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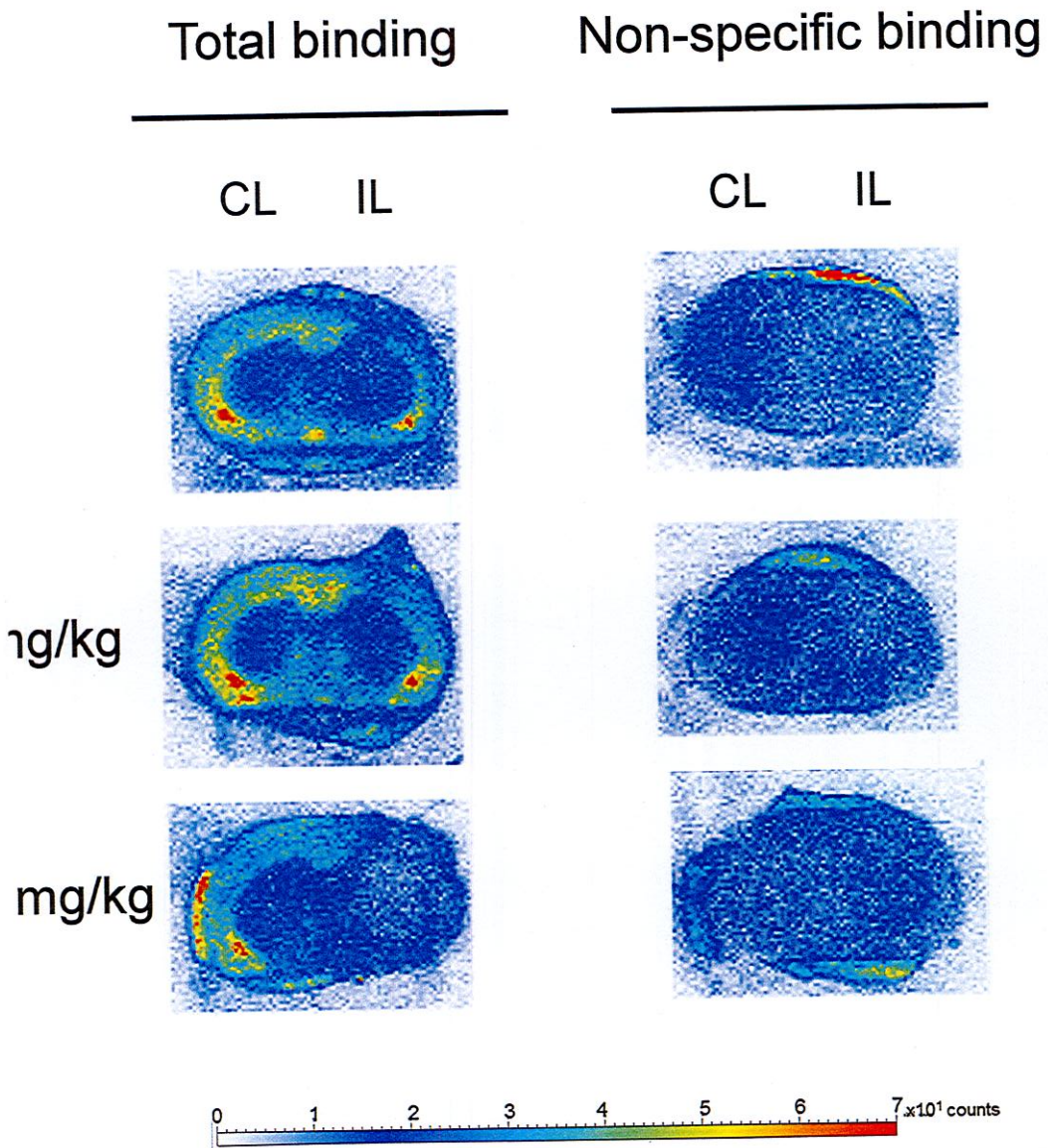




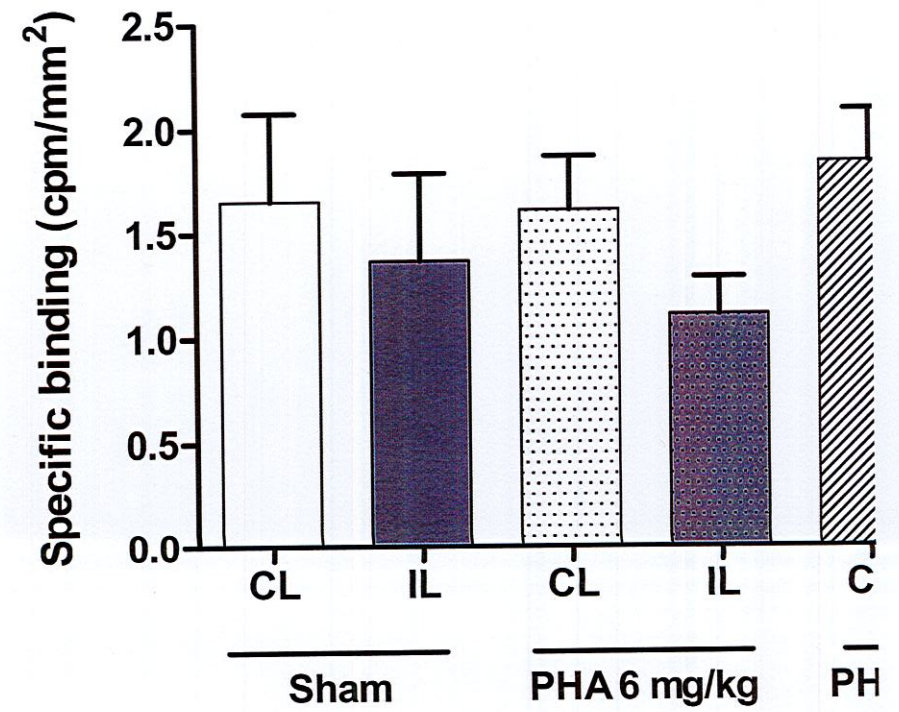
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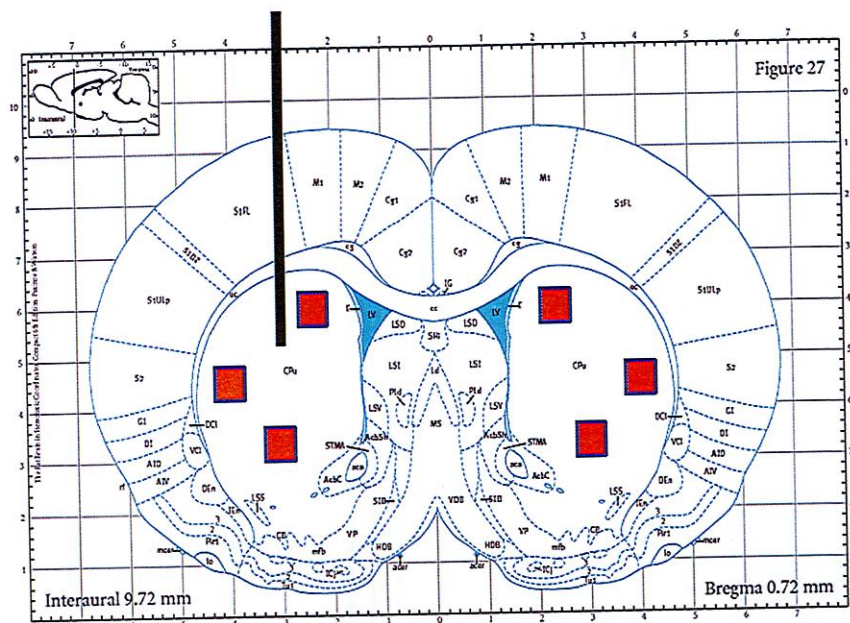
Increase of neuroinflammation (TSPO)
in ipsi- versus contra-lateral hemisphere (%)





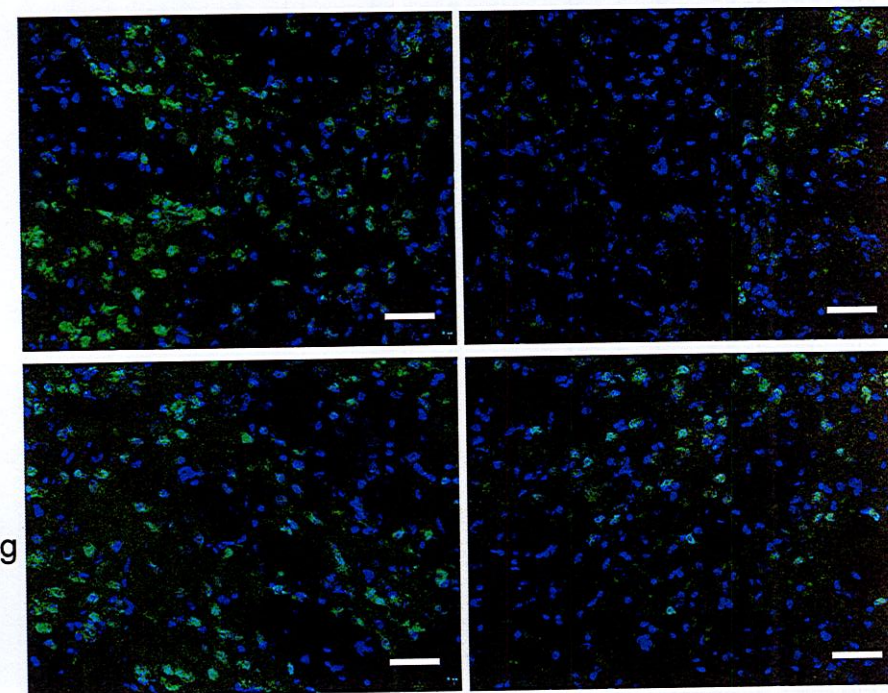
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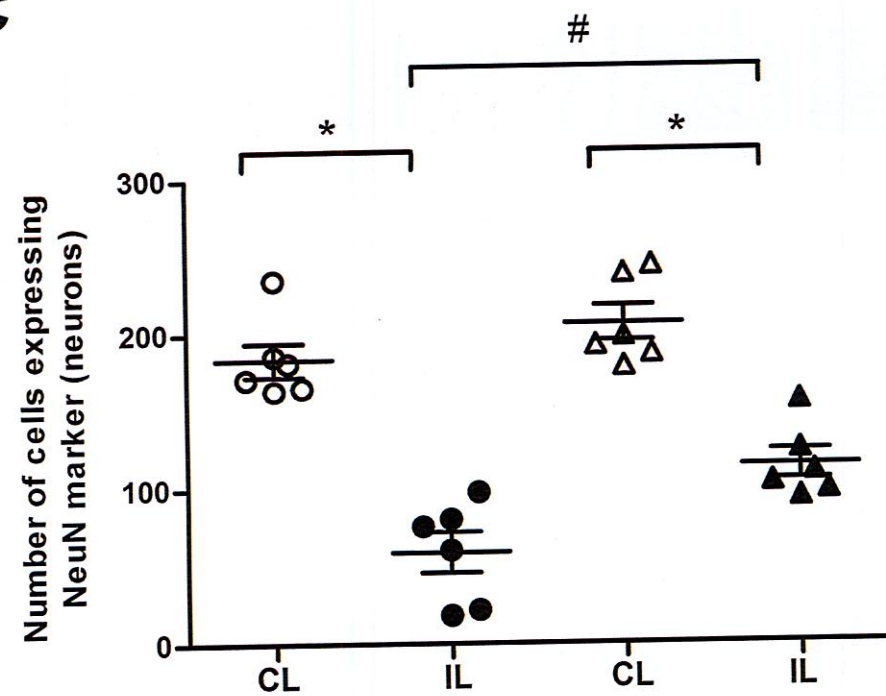


Sham

PHA 12 mg/kg



C



D

