In vivo imaging of A7 nicotinic receptors as a novel method to monitor neuroinflammation after cerebral ischemia

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Abstract
In vivo positron emission tomography (PET) imaging of nicotinic acetylcholine receptors (nAChRs) is a promising tool for the imaging evaluation of neurologic and neurodegenerative diseases. However, the role of α7 nAChRs after brain diseases such as cerebral ischemia and its involvement in inflammatory reaction is still largely unknown. In vivo and ex vivo evaluation of α7 nAChRs expression after transient middle cerebral artery occlusion (MCAO) was carried out using PET imaging with [11C]NS14492 and immunohistochemistry (IHC). Pharmacological activation of α7 receptors was evaluated with magnetic resonance imaging (MRI), [18F]DPA-714 PET, IHC, real time polymerase chain reaction (qPCR) and neurofunctional studies. In the ischemic territory, [11C]NS14492 signal and IHC showed an expression increase of α7 receptors in microglia and astrocytes after cerebral ischemia. The role played by α7 receptors on neuroinflammation was supported by the decrease of [18F]DPA-714 binding in ischemic rats treated with the α7 agonist PHA 568487 at day 7 after MCAO. Moreover, compared with non-treated MCAO rats, PHA-treated ischemic rats showed a significant reduction of the cerebral infarct volumes and an improvement of the neurologic outcome. PHA treatment significantly reduced the expression of leukocyte infiltration molecules in MCAO rats and in endothelial cells after in vitro ischemia. Despite that, the activation of α7 nAChR had no influence to the blood brain barrier (BBB) permeability measured by MRI. Taken together, these results suggest that the nicotinic α7 nAChRs play a key role in the inflammatory reaction and the leukocyte recruitment following cerebral ischemia in rats.

KEYWORDS
[11C]NS14492, [18F]DPA-714, cerebral ischemia, MRI, neuroinflammation, PET

1 | INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels widely expressed in the skeletal neuromuscular junction, and throughout both peripheral and central nervous system (Hurst, Rollema, & Bertrand, 2013). nAChRs are structurally composed of five subunits (α2–α10 and β2–β4) and the most abundant subtypes in mammalian brain are heteromeric receptors containing α4 and β2 subunits and homomeric α7 (Mukhin et al., 2000). The α7 nAChRs are expressed in a large variety of cells in the brain, including cortical neurons (Murakami, Ishikawa, & Sato, 2013), astrocytes (Sharma & Vijayaraghavan, 2001; Teaktong et al., 2003), microglia (De Simone,
Ajmone-Cat, Carnevale, & Minghetti, 2005), oligodendrocyte precursor cells (Rogers, Gregori, Carlson, Gahring, & Noble, 2001; Sharma & Vijayaraghavan, 2002) and endothelial cells (Hawkins, Egleton, & Davis, 2005), where they play a crucial role in a wide variety of physiological responses such as learning, memory, locomotion and attention (Gotti, Fornasari, & Clementi, 1997; Neumann, Shields, Balle, Chebib, & Clarkson, 2013). Furthermore, a7 receptors have been involved in some brain disorders including schizophrenia (Young & Geyer, 2013), Alzheimer's disease (Hernandez & Dineley, 2012), bipolar disorders (Thomsen, Weyn, & Mikkelsen, 2011) and traumatic brain injury (Verbois, Scheff, & Pauly, 2003). Likewise, the stimulation of a7 nAChRs protects neurons from insults associated with neurodegenerative disorders (Mudo, Belluardo, & Fuxe, 2007), ischemic damage (Shimohama et al., 1998) and intracerebral hemorrhage (Hijioka, Matsushita, Hisatsune, Isohama, & Katsuki, 2011). The protective effect of these receptors in brain pathologies has been related to the suppression of inflammation through the modulation of immune cells by the cholinergic anti-inflammatory pathway (de Jonge & Ulloa, 2007; Wang et al., 2003), however, the mechanisms involved are not well understood. Therefore, the in vivo imaging of nAChRs with positron emission tomography (PET) technique might be of paramount importance to further understanding the role of a7 receptors on inflammation underlying brain diseases (Kimes et al., 2003). In the last few years, promising radiotracers such as 4-(3-[11C]-methyl-1H-pyrrol-2-yl)-1,3,4-oxadiazol-2-yl)-1,4-diazabicyclo[2.2.2]nonane ([11C]NS14492) have been developed for in vivo imaging evaluation of cerebral a7 receptors (Chalon, Vercouille, Guilloteau, Suzenet, & Routier, 2015; Ettrup et al., 2011). [11C]NS14492 is a selective orthosteric a7 nAChR agonist PET radioligand that showed a cerebral binding accordingly to the distribution of these receptors in the healthy human brain (Breese et al., 1997; Ettrup et al., 2011). Nevertheless, although these receptors have shown major implications in brain disorders, the role of a7 receptors after brain diseases such as cerebral ischemia and its involvement on neuroinflammatory reaction with PET imaging is still largely unknown. For this reason, the purpose of this study was to investigate changes in the expression of a7 nAChRs in the rat brain after cerebral ischemia using PET with [11C]NS14492 and immunohistochemistry. In particular, we were interested in studying the role of a7 receptors on inflammation after cerebral ischemia in rats. Thus, ischemic rats treated with the a7 nAChRs agonist PHA 568487 were subjected to in vivo PET imaging of neuroinflammation with [18F]DPA-714, a specific radioligand for the translocator protein (18KDa) (TSPO), to image brain inflammation (Domercq et al., 2016; Martin et al., 2010) and to magnetic resonance imaging (MRI) for the evaluation of brain damage and blood brain barrier (BBB) permeability. Finally, real-time polymerase chain reaction (qPCR) was used to evaluate the role of a7 receptors on the expression of markers of microglia/macrophages activation and leukocyte infiltration. Hence, the results reported here provide novel information about the activity of a7 nAChRs on neuroinflammation after cerebral ischemia and might ultimately contribute to a better design of novel strategies for the treatment of neurologic diseases such as ischemic stroke.

2 | MATERIALS AND METHODS

2.1 | Animal models

Adult male Sprague-Dawley rats (n = 70; 297 ± 8.3 g body weight; Janvier, France) were used for both in vivo and ex vivo studies. Animal experimental protocols and relevant details regarding welfare and drug side effects were approved by the animal ethics committee of CIC biomGUNE and were conducted in accordance with the ARRIVE guidelines and Directives of the European Union on animal ethics and welfare.

2.2 | Cerebral ischemia and treatment

Rats were anesthetized with 2.5% isoflurane in 100% O2 and transient focal ischemia was produced by a 90 min intraluminal occlusion of the middle cerebral artery (MCAO) followed by reperfusion as described previously (Justicia, Perez-Asensio, Burguete, Salom, & Planas, 2001). 8 rats were repeatedly scanned before reperfusion (day 0) and at 1, 3, 7, 14, 21, and 28 days after ischemic onset to evaluate the binding of a7 receptors by PET. During 7 consecutive days, starting at day 1 following MCAO, a group of 16 rats was treated daily with 0.1 ml N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-2,3-dihydro-1,4-benzodioxin-6-carboxamide fumarate (PHA 568487) (1.25 mg/kg, i.p.). A control ischemic group of 18 rats received the same daily volume of vehicle (normal saline) in a randomized and blinded fashion. At day 7, treated and control rats were imaged with PET and MRI to determine the effect of PHA 568487 on TSPO expression, the brain lesion volume, the BBB permeability and ex vivo studies (immunohistochemistry [IHC] and qPCR). 28 rats were used to perform IHC for a7 receptor expression at 0, 1, 3, 7, 14, 21, and 28 days after cerebral ischemia.

2.3 | Magnetic resonance imaging

T2-weighted (T2W) MRI scans were performed in all ischemic animals at 24 hr after reperfusion to select rats presenting cortico-striatal lesions for inclusion in the PET and gadolinium enhanced T1-weighted (T1W) MRI studies of BBB permeability. Furthermore, T2W-MRI scans were used to evaluate the infarction volume in treated and control rats before (day 1) and after the treatment (day 7). T1W-MRI scans were performed in ischemic rats after the treatment to determine changes in signal intensity (expressed in %) 30 min after injection of gadolinium based contrast agent (Gadovist). MRI scans were performed under 2%–3% of isoflurane in 30/70% of O2/N2. For the evaluation of BBB integrity, the tail vein was catheterized with a 24-gauge catheter for intravenous administration of Gadovist (0.2 mmol/ml, 1 ml/kg body weight). MRI experiments were performed on a 7 Tesla horizontal bore Bruker Biospec 70/30 MRI system (Bruker Biospin GmbH, Ettlingen, Germany). T2W images were acquired with a Bruker’s RARE (Rapid Acquisition with Relaxation Enhancement) sequence with the following parameters: TE = 75 ms; TR = 4,500 ms; Rarefactor = 8; Nav = 2; Matrix averages: Image matrix = 256 × 256; FOV = 25.6 × 25.6 mm (giving an isotropic in-plane resolution of 100 μm) and covering the whole brain by 18 consecutive slices of 1 mm thickness. T1W images
were acquired with same spatial location, orientation and resolution using also a RARE sequence with the following parameters: TE = 20 ms; TR = 1,000 ms; Rarefactor = 4; Nax = 2 averages.

### 2.3.1 Magnetic resonance imaging imaging analysis

The T2W MRI images were used to calculate brain infarction volumes. Regions of interest (ROIs) were manually defined using Paravision 6 software for each rat on the region of increased signal in the injured hemisphere. The total lesion volume was calculated by summing the area of the infarcted regions of all slices affected by the lesion and multiplying it by the slice thickness. The T1W-MRI images were used to calculate changes in signal intensity (in %) upon contrast agent leakage in through the affected BBB using Image J software. T1W-MRI images before and after contrast agent injection were co-registered and subtracted to measure pixel-by-pixel any signal changes (expressed in %).

Total volume as well as mean and standard deviation signal intensity changes were measured in regions of increased signal intensity (upon a threshold of 4% considered as noise level). Incidence maps of permeability were later calculated as % incidence = N° Permeable pixels / Total Cerebral Volume (in pixels).

### 2.4 Radiochemistry

The radiotracer \( ^{11}C\)NS14492 was synthesized as previously described (Ettrup et al., 2011; with minor modifications) by \( ^{11}C\)-methyleneation of 4-[5-(1H-pyrrol-2-yl)-1,3,4-oxadiazol-2-yl]-1,4-diazabicyclo[3.2.2]nonane (desmethyl-NS14492, 0.3 mg, Novandi Chemistry AB, Sweden) using \( ^{11}C\)CH\(_3\)I generated via the gas-phase method as the labelling agent, acetone (80 μl) as the solvent and TBAOH (1 M in methanol, 2.5 μl) as the base (reaction time = 4 min, T = 25°C). After purification using high performance liquid chromatography (HPLC), reformulation of the collected fraction using solid phase extraction and filtration through a m sterile filter, the final injectable solution containing pure \( ^{11}C\)NS14492 was obtained. Typical radiochemical yields and specific activities were 43% ± 5% (with respect to \( ^{11}C\)CH\(_3\)I decay corrected) and 180 ± 10 GBq/μmol at injection time, respectively. Radiochemical purity was >99% in all cases.

The synthesis of \( ^{18}F\)DPA-714 (N,N-diethyl-2-(4-[(4-[(4-F)fluorooxy]phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl]acetamide was performed using a TRACERlab FXFN synthesis module (GE, Healthcare, Waukesha, WI, USA), based on a one-step procedure, as previously described (Damont et al., 2008; Kuhnast et al., 2012). Yields and specific activity values were equivalent to those previously reported. Radiochemical purity was above 95% at injection time.

### 2.5 Positron emission tomography scans and data acquisition

PET scans were performed using a General Electric eXplore Vista CT camera (GE, Healthcare, Waukesha, WI, USA). Scans were performed in mice anaesthetized with 4% isoflurane and maintained by 2-2.5% of isoflurane in 100% O\(_2\). The tail vein was catheterized with a 24-gauge catheter for intravenous administration of the radiotracer. For longitudinal assessment of α7 receptors (radiotracer: \( ^{11}C\)NS14492), animals were scanned before and during the following month after ischemia. The radioactivity (~70 MBq) was injected and dynamic brain images were acquired (30 frames: 3 × 5, 3 × 10, 3 × 15, 3 × 30, 3 × 60, 6 × 120, 8 × 300 s) in the 400–700 keV energetic window, with a total acquisition time of 60 min. For evaluation of PHA 58487 treatment efficacy after ischemia, ~70 MBq of \( ^{18}F\)DPA-714 were injected at the start of the PET acquisition and dynamic brain images were acquired for a total of 30 min (23 frames 3 × 5, 3 × 15, 4 × 30, 4 × 60, 4 × 120, 5 × 180 s). After each PET scan, CT acquisitions were also performed (140 μA intensity, 40 kV voltage), to provide anatomical information of each animal as well as the attenuation map for the later PET image reconstruction. Dynamic acquisitions were reconstructed (decay and CT-based attenuation corrected) with filtered back projection (FBP) using a Ramp filter with a cutoff frequency of 0.5 mm\(^{-1}\).

### 2.6 Positron emission tomography image analysis

PET images were analyzed using PMOD image analysis software (PMOD Technologies Ltd, Zürich, Switzerland). To verify the anatomical location of the signal, PET images were co-registered to the anatomical data of a MRI rat brain template. Two type of Volumes of Interest (VOIs) were established as follows: (1) A first set of VOIs was defined to study the whole brain \( ^{11}C\)NS14492 and \( ^{18}F\)DPA-714 PET signal. Whole brain VOIs were manually drawn in both the ipsilateral and contralateral hemispheres on slice of a MRI (T\(_2\)W) rat brain template from the PMOD software. (2) A second set of VOIs was automatically generated in the cortex and the striatum by using the regions proposed by the PMOD rat brain template, to study the evolution of \( ^{11}C\)NS14492 PET signal in these specific regions in both the ipsilateral and contralateral cerebral hemispheres. For quantification of radiotracers uptake, the last three (\( ^{11}C\)NS14492) or five (\( ^{18}F\)DPA-714) frames were used to calculate the summed PET binding during the last 15 min of acquisition. PET signal uptake was averaged in each ROI and expressed as percentage of injected dose per cubic centimetre (%ID/cc).

### 2.7 Immunohistochemistry and cell counts

Immunohistochemistry staining was carried out before ischemia (day 0), and at days 7 and 28 after cerebral ischemia as described previously (Martin et al., 2015). The first set of sections was stained for α7 with rabbit anti-rat α7 (1:300, AbCam, Cambridge, UK), for CD11b with mouse anti-rat CD11b (1:300; Serotec, Raleigh, NC, USA) and for the glial fibrillary acidic protein (GFAP) with chicken anti-rat GFAP (1:500; AbCam, Cambridge, UK). The second set of sections was stained for TSPO with a rabbit anti-rat TSPO (NP155, 1:1000) and for CD11b and for GFAP. Subsequently, sections were incubated with secondary antibodies Alexa Fluor 350 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-chicken IgG (Molecular Probes, Life Technologies, Madrid, Spain, 1:1,000) and mounted with a prolong antifade kit in slices (Molecular Probes Life Technologies, Madrid, Spain).
Standardized image acquisition was performed with an Axio Observer Z1 (Zeiss, Le Pecq, France). The number of microglia, macrophages and other infiltrated leukocytes and astrocytes immunopositive for α7 within the ischemic area was assessed at 0, 1, 3, 7, 14, 21 and 28 days after ischemia (α7/CD11b and α7/GFAP) and at day 7 to evaluate the inflammatory effect of α7 activation (TSPO/CD11b and TSPO/GFAP). Cells were manually counted in ten representative and different fields at 100x magnification by using Image J (NIH) software.

2.7.1 | Neurological assessment

The assessment of neurological outcome induced by cerebral ischemia was based on a previously reported 9-neuroscore test (Menzies, Hoff, & Betz, 1992). Before imaging evaluations, four consecutive tests were performed at days 1 and 7 after ischemia in treated and control rats as follows: (1) spontaneous activity (moving and exploring 0, moving without exploring 1, no moving 2); (2) left drifting during displacement (none 0, drifting only when elevated by the tail and pushed or pulled 1, spontaneous drifting 2, circling without displacement or spinning 3), (3) parachute reflex (symmetrical 0, asymmetrical 1, contralateral forelimb retracted 2), and (4) resistance to left forepaw stretching (stretching not allowed 0, stretching allowed after some attempts 1, no resistance 2). Total score could range from 0 (normal) to a 9 (highest handicap) point-scale.

2.7.2 | Brain endothelial cell cultures

bEnd.3 cells are an immortalised cerebral microvascular endothelial cell line which possess key characteristics of the BBB phenotype (Brown, Morris, & O’Neill, 2007). bEnd.3 cells (ATCC CRL-2299) were grown in culture media (Dulbecco’s modified Eagle’s medium [DMEM] containing 10% fetal bovine serum) in tissue culture flasks. Cell monolayers were exposed to in vitro ischemia (1 hr) by replacing O2 with N2 and external glucose (10 mM) with sucrose (10 mM), and adding iodoacetate (50 μM) to block glycolysis in an extracellular solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 2.6 mM NaHCO3, 0.8 mM MgCl2, and 1.18 mM NaH2PO4 (pH 7.4). After ischemia, medium was replaced by culture medium and cells were cultured for 24 hr in 95:5% O2:CO2. PHA (5 μg/ml) was present during in vitro ischemia (1 hr) and during reperfusion (24 hr). Total RNA was isolated from cells 24 hr after ischemia.

2.7.3 | Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from frozen brain tissue and cultured cells using Trizol (Invitrogen). Subsequently, cDNA synthesis was conducted using SuperScript III retrotranscriptase (200 U/ml; Invitrogen) and random hexamers as primers (Promega). Primers specific for the rat pro-inflammatory and anti-inflammatory markers and for chemokines were designed using Primer Express software (Applied Biosystems) at exon junctions to avoid genomic DNA amplification (see Table 1). Data was normalized to HPRT gene (best M coefficient using GeNorm Software v3.5; Vandesompele et al., 2002). qPCR reactions were performed with SYBR-Green using a BioRad CFX96 qPCR detection system as described previously (Vallejo-Illarramendi, Domercq, Perez-Cerda, Ravid, & Matute, 2006).

2.8 | Statistical analyses

For PET, the percentage of injected dose per cubic centimeter (%ID/cc) within each region and time point following cerebral ischemia were
performed using a paired t test and the comparison within time points before and after the treatment was performed using a paired t test. Likewise, cellular expression of both microglial/α7 and astrocytic/α7 receptors before (day 0) and at days 7 and 28 after ischemia and microglial/TSP0 and astrocytic/TSP0 after treatment were compared using the same statistical analysis than that for PET imaging. For neurological outcome, results before (1 day after MCAO) and after treatment (7 days after MCAO) were averaged and compared with baseline average values using Mann-Whitney U tests. The effect of treatments in the mRNA levels for pro-inflammatory/anti-inflammatory and leukocyte infiltration markers in sham, treated and non-treated ischemic rats were compared using a one-way ANOVA followed by Bonferroni’s multiple-comparison tests for post hoc analysis. The level of significance was regularly set at P < 0.05. Statistical analyses were performed with GraphPad Prism version 6 software.

3 | RESULTS

The levels and distribution of nicotinic α7 receptors were explored by [11C]NS14492 PET imaging after 90 min MCAO and 1–28 days after reperfusion in rats. Coronal images with normalized color scale illustrate the evolution of the PET signals at control and at 1, 3, 7, 14, and 28 days after reperfusion (Figure 1). The extent of brain damage after cerebral ischemia was assessed using T2W MRI at 1 day after ischemia onset. Hyperintense regions of T2W images represented similar infarct extents as well as locations affected. All ischemic rats subjected to nuclear studies showed cortical and striatal MRI alterations (mean ± SD = 301.07 ± 52.75 mm³, n = 8).

3.1 | [11C]NS14492 after cerebral ischemia

The time course of nicotinic receptor α7 distribution was evaluated using [11C]NS14492 in the ipsilateral and contralateral cerebral cortex, in the striatum and the whole brain at control (day 0) and 1, 3, 7, 14, 21, and 28 days after MCAO (Figure 1, n = 8). All brain regions evaluated in the injured hemisphere showed a similar [11C]NS14492 binding evolution after focal cerebral ischemia. In the ipsilateral whole brain (cerebrum), PET signal for [11C]NS14492 showed a non-significant increase at day 1 followed by a significant PET signal increase from days 3 to 7 after ischemia in comparison to control (day 0) values (p < .05; p < .001, Figure 1f). In fact, the highest uptake was reached at day 7 following MCAO. Subsequently, the PET signal evidenced a progressive decrease from day 14 to day 28 after ischemia. In the contralateral hemisphere, [11C]NS14492 PET signal at day 1 displayed a mild increase followed by a progressive increase during the first week after ischemia onset and by a decline later on (Figure 1g). The cerebral cortex in the ipsilateral hemisphere displayed a statistically significant [11C]NS14492 PET signal progressive increase from day 3 to days 7–14 followed by a sharp decrease from 21 days after MCAO (p < .05; p < .01; p < .001, with respect to control animals, Figure 1h). In contrast, non-statistically significant differences were observed in the contralateral hemisphere despite the weak increase of PET signal observed after ischemia onset (Figure 1i). The ischemic striatum showed a similar [11C]NS14492 PET signal uptake over the first month after reperfusion than that observed in the cerebral cortex (p < .05; p < .001 with respect to control animals, Figure 1f). Finally, the contralateral striatum displayed a non-significant [11C]NS14492 binding at day 7 after MCAO (Figure 1k).

3.2 | Cellular expression and immunoreactivity of nicotinic α7 receptors in microglia, infiltrated leukocytes and astrocytes after MCAO

 Immunofluorescence staining exhibited α7 expression in a heterogeneous population of microglia/macrophages, other leukocytes and astrocytes after cerebral ischemia (Figure 2). At day 7, cells with the morphology of amoeboid reactive microglia/macrophages showed intense CD11b immunoreactivity in the lesion (in red; Figure 2b) followed by a decrease at day 28 (in red; Figure 2b). The over-activity of microglia/macrophages co-localized with the cellular expression of nicotinic α7 receptors (in blue and red; Figure 2d) at day 7 after ischemia. The number of α7+/CD11b + cells displayed a significant progressive increase from days 1–7 in relation to day 0 followed by a sharp decrease during the following weeks after reperfusion (p < .001, Figure 2e). Likewise, astrocytes displayed an increase of the GFAP immunoreactivity at day 28 (in white; Figure 2a) after ischemia, forming a thin astrocytic rim in the vicinity of the lesion. In addition, reactive astrocytes showed co-localization with α7 expression (in blue and white; Figure 2d) after MCAO. The number of α7+ /GFAP + cells showed a significant sharp increase from days 7–28 relative to day 0 (p < .001, Figure 2f). In the contralateral hemisphere both α7+/CD11b + and α7+/GFAP + cells displayed non-significant changes at different days after cerebral ischemia (Figure 2g,h).

3.3 | Effect of nicotinic α7 receptor activation on lesion outcome and neuroinflammation after ischemia

The infarct volume and the levels and distribution of TSP0 receptors were explored by MRI and PET imaging after the chronic treatment with the α7 agonist PHA 568487 (PHA) and vehicle at 7 days after MCAO (Figure 3a–f). The infarct volume measured with T2W-MRI at day 1 after ischemia showed similar values before the start of the treatment with PHA to avoid bias (Figure 3g). At day 7, the infarct volume showed a significant decrease in comparison to day 1 (before the treatment) in both non-treated and PHA treated ischemic rats (p < .05; p < .01, Figure 3g). Besides, the pharmacological activation of α7 receptors after ischemia displayed a significant reduction of the lesion volume relative to non-treated ischemic animals at day 7 after MCAO (p < .05, Figure 3g). The effect of the modulation of α7 receptors on neuroinflammation was explored with [18F]DPA-714-PET at day 7 following MCAO. All the images were quantified in standard units, that is, %ID/cc. Axial images with normalized color scale illustrate the [18F] DPA-714 PET uptake in vehicle (MCAO; Figure 3c), and PHA (MCAO + PHA; Figure 3f) ischemic rats. The treatment with PHA
showed a significant decrease of $[^{18}F]DPA$-714 binding in the ischemic cerebral hemisphere in comparison to non-treated ischemic rats ($p < .05$, Figure 3h). Neurological score at day 1 after ischemia showed similar values reflecting that the groups presented similar neurological impairment before the start of the treatments (Figure 3i). At day 7, the neurological score showed an improvement in relation to day 1 and treated ischemic rats with PHA displayed a major neurofunctional improvement in relation to that showed by non-treated ischemic rats ($p < .01$, $p < .001$, Figure 3i). Immunofluorescence staining exhibited TSPO expression in CD11b and GFAP positive cells after ischemia in vehicle and PHA-treated rats (Figure 4). At day 7, cells with the morphology of amoeboid reactive microglia/macrophages and others leukocytes showed intense CD11b immunoreactivity (in red; Figure 4a) in the lesion that co-localized with TSPO receptor expression (in green and red; Figure 4c). The number of TSPO$^+/CD11b^+$ cells displayed a significant decrease in treated ischemic rats with PHA at day 7 after ischemia in comparison with control ischemic rats ($p < .01$, Figure 4d).

### 3.4 Effect of the PHA treatment on the expression of pro-inflammatory and anti-inflammatory microglia/macrophages and leukocyte infiltration markers

We next investigated the impact of $\alpha_7$ receptors activation on microglia and infiltrated macrophages phenotypes after stroke. Polarized
FIGURE 2  Immunofluorescent labeling of GFAP (white), CD11b (red) and α7 (blue) in the ischemic area, shown as three channels. The data show temporal evolution of α7 receptor in microglia/macrophages, other infiltrated leukocytes and astrocytic cells at day 0 (control; left column, n = 4), day 7 (middle left column, n = 4), and day 28 (right column, n = 4) after ischemia. GFAP-positive astrocytes increase in the ischemic area over time (a). CD11b-reactive microglia and infiltrated leukocytes at day 7 (b) corresponds to the temporal α7 immunoreactivity (c). (d) Merged images of three antibodies at different time points. The number of CD11b-reactive microglia/macrophages and GFAP-reactive astrocytes were measured at different time points (e and f). Scale bars, 5 μm [Color figure can be viewed at wileyonlinelibrary.com]
microglia/macrophages after cerebral ischemia are commonly distinguished by their expression of signature genes for surface markers and cytokines/chemokines (Hu et al., 2012). qPCR was used to measure the effect of the α7 receptors agonist PHA in the expression of pro-inflammatory (iNOS, TNF, and CCL2) and anti-inflammatory (arginase, mannose, and TGF β) markers in the region of the infarction and the contralateral brain hemisphere at sham control and at day 7 after MCAO in vehicle and PHA-treated rats (Figure 5). Both pro-inflammatory (iNOS, TNF, and CCL2) and anti-inflammatory (arginase, mannose, and TGF β) gene markers showed a significant overexpression following cerebral ischemia in vehicle-treated rats (MCAO) in comparison with sham control rats (p < .01; p < .001, Figure 5). Following treatment with PHA, there was no significant change in most of the pro-inflammatory and anti-inflammatory markers with respect to non-treated ischemic rats (Figure 5). However, the expression of CCL2, a monocyte chemoattractant protein that mediates macrophage recruitment and migration to the peripheral and central nervous system (Selenica et al., 2013), displayed a massive decrease following treatment with PHA. Because of this, we have assessed in parallel the evaluation of leukocyte infiltration markers (E-selectin, P-selectin, ICAM, VCAM, and CD3) after in vivo (MCAO) and in vitro ischemia (brain endothelial cell cultures, bEnd.3) in the presence and absence of PHA (Figure 6). Leukocyte infiltration markers were evaluated in the region of the infarction and the contralateral brain hemisphere at sham control and at day 7 after MCAO in vehicle and PHA-treated rats (Figure 6a–d). After MCAO, leukocyte infiltration gene markers displayed a significant overexpression for P-selectin and VCAM (p < .01; p < .001, Figure 6b,d) and non-significant increase for E-selectin and ICAM in relation to control sham rats. The activation of α7 receptors with PHA induced the significant depletion of mRNA expression for P-selectin,
induced the significant increase of CD3 marker expression with respect to control. The contralateral hemisphere showed the infiltration of T lymphocytes in the region of the infarction ($p < .01$, Figure 6i). The activation of $\alpha_7$ receptors with the treatment displayed a significant reduction of $T$ cell infiltration compared with non-treated ischemic rats ($p < .05$, Figure 6i).

### 3.5 Permeability of the blood brain barrier after the activation of $\alpha_7$ receptor in ischemic rats

Finally, the BBB permeability was explored by gadolinium contrast enhanced T1W-MRI at day 7 after MCAO following the daily treatment with PHA and vehicle (Figure 7). Averaged MRI images of permeability maps for all the rats included in the study (MCAO, $n = 10$ and MCAO + PHA, $n = 9$) showed that brain regions irrigated by the MCA and affected by the brain infarction become permeable to the contrast agent to some extent (Figure 7a,b). The infarct volume measured with T2W-MRI at day 1 after ischemia showed similar values before the start of the treatment with PHA to avoid bias (Figure 7c). The % of permeability day 7 after reperfusion showed non-significant changes between PHA-treated and control (non-treated) ischemic rats (Figure 7d). Overall, these results showed that the activation of the $\alpha_7$ nAChRs did not significantly reduce the BBB disruption after cerebral ischemia.

### 4 DISCUSSION

The physiological mechanism termed the “cholinergic anti-inflammatory pathway” described the attenuation of the systemic inflammatory response through the stimulation of the vagus nerve (Borovikova et al., 2000). Indeed, the activation of $\alpha_7$ nAChRs following brain injury has displayed the reduction of inflammatory cytokines release by macrophages inhibiting the inflammatory process (Wang et al., 2003; Han et al., 2014). Therefore, the in vivo imaging PET of these receptors is of vital importance since they have shown major potential implications in therapeutics of inflammation of cerebral diseases such as cerebral ischemia (Han, Li, et al., 2014; Han, Shen, et al., 2014; Kalappa, Sun, Johnson, Jin, & Uteshev, 2013; Sun, Jin, & Uteshev, 2013). Because of this, we have assessed the in vivo expression of $\alpha_7$ receptors and TSPO in reactive glial cells using PET imaging procedures, in combination with MRI, immunohistochemistry, qPCR and neurofunctional evaluation after cerebral ischemia in rats.

### 4.1 PET imaging of $\alpha_7$ nAChRs with $^{[11]}$CNS14492

A previous study has shown the high BBB penetration of the $\alpha_7$ receptor PET radioligand $^{[11]}$CNS14492, an indispensable requirement for an useful PET brain tracer that is influenced by its lipophilicity (Ettrup et al., 2011). Such study also showed that the highest $^{[11]}$CNS14492 binding in the pig brain was in the cortical areas and thalamus, followed by a moderate binding in striatum and low binding in the cerebellum (Ettrup et al., 2011). Indeed, the biodistribution of $^{[11]}$CNS14492 in the pig brain was in accordance with the ex vivo distribution of $\alpha_7$ nAChRs in the human brain as measured with $^{[12]}$I-α-bungarotoxin, a selective
antagonist for these receptors (Breese et al., 1997). Hence, all these findings are consistent with the $[^{11}C]NS14492$ distribution in the healthy rat brain before the induction of the cerebral ischemia showed in the present study (Figure 1).

4.2 | In vivo and ex vivo overexpression of $\alpha 7$ receptors after experimental stroke

Recently, we have demonstrated the role played by $\alpha 4\beta 2$ nAChRs on neuroinflammation after cerebral ischemia in rats using in vivo PET imaging. Indeed, this study provided novel information that might contribute to a better design of anti-inflammatory strategies (Martin et al., 2015). Similarly, $\alpha 7$ nAChRs have been proposed as pharmacological targets with major clinical implications in the control of the inflammatory response (de Jonge & Ulloa, 2007). In the present study, $\alpha 7$ receptor expression was evaluated with $[^{11}C]NS14492$ from day 1–28 after MCAO in rats, to show its relationship to the neuroinflammatory reaction after cerebral ischemia. In the infarcted brain hemisphere, $[^{11}C]NS14492$ binding experienced a progressive increase at days 1–3 followed by a maximum increase at day 7 and a progressive decline from days 14–28 after reperfusion (Figure 1). These results stand in agreement with the PET imaging distribution of TSPO, a well-known marker for neuroinflammation following cerebral ischemia (Domercq et al., 2016; Martin et al., 2010; Martin et al., 2015). Hence, taking into

**FIGURE 5** $\alpha 7$ receptors activation after cerebral ischemia induces changes in mRNA expression of pro-inflammatory and anti-inflammatory microglial markers. qPCR was performed using RNA extracted from the region of the infarction at sham (control; $n = 4$) and at day 7 after MCAO in vehicle ($n = 8$) and PHA-treated rats ($n = 7$). Expression of mRNA for pro-inflammatory markers iNOS (a), TNF (b) and CCL2 (c) and for anti-inflammatory markers Arginase (d), Mannose receptor (e), and TGFβ (f) were performed after ischemia. **$p < .01$ and ***$p < .001$ compared with sham.**
account that the TSPO overexpression is principally due to the activation of microglia/macrophages and astrocytes (Martin et al., 2010), our results could suggest that $[^{11}C]$NS14492 binding increase is linked to the inflammatory response. The first step to verify this hypothesis was the characterization of the immunohistochemical characterization at days 7–28 after cerebral ischemia (Figure 2). These results confirmed the overexpression of $\alpha_7$ receptors in microglia/macrophages and infiltrated leukocytes at day 7 and in astrocytes at day 28 after MCAO. Therefore, these findings supported the evidence that the increase of $[^{11}C]$NS14492 uptake after cerebral ischemia was mainly promoted by the $\alpha_7$ nAChRs expression on CD11b positive cells.

4.3 $\alpha_7$ receptor activation reduces tissue damage effects and attenuates inflammation after ischemia
Pharmacological activation of $\alpha_7$ nAChRs with the agonist PHA 568487 has previously shown the reduction of brain edema, ischemic stroke injury, neuroinflammation and oxidative stress following ischemic stroke in mice (Han, Li, et al., 2014; Han, Shen, et al., 2014; Zou et al., 2016). In our study, we explored the effect of PHA on brain edema with T2W-MRI and neuroinflammation by using in vivo $[^{18}F]$DPA-714 PET binding following cerebral ischemia (Figure 3). The treatment with PHA after MCAO in rats induced a significant decrease of brain edema that is commonly used to measure the extent of the infarct volume (Figure 3g). The activation of $\alpha_7$ receptors also showed a significant decrease of TSPO expression with $[^{18}F]$DPA-714 PET, supporting the role of these receptors on neuroinflammation after cerebral ischemia (Figure 3h). In addition, the treatment with PHA improved the neurological function following seven days after reperfusion (Figure 3i). These findings support those showed by Sun and colleagues who observed an improvement of neurological function after MCAO using $\alpha_7$ nAChRs modulators (Sun et al., 2013). Besides, ischemic rats treated with PHA displayed a significant decrease of CD11b positive cells expressing TSPO (Figure 4), confirming the results obtained with $[^{18}F]$DPA-714 PET and the role of $\alpha_7$ receptors on microglia/macrophages and other leukocyte reactivity after cerebral ischemia in rats. Recently, Schmidt and colleagues described that the depletion of both selective and unselective monocyte/macrophage did not affect functional outcome after cerebral ischemia in mice (Schmidt et al., 2017). Therefore, the improvement of the neurological outcome showed by ischemic rats treated with PHA could be due to reduction of microglial activation and/or the infiltration of other immune cells to the infarction.

4.4 Effect of PHA on pro-inflammatory/anti-inflammatory microglial/macrophages
The activation of $\alpha_7$ receptors with PHA following cerebral ischemia in mice has been previously related to the reduction of pro-inflammatory and the increase of anti-inflammatory markers gene expression suggesting the direct influence of these receptors on the microglial function through the control of microglial polarization state following stroke (Han, Shen, et al., 2014). For this reason, we evaluated the mRNA expression of typical microglial markers in ischemic rats after treatment with PHA. In our study, the expression
of known pro-inflammatory markers (iNOS, TNF and CCL2) together with anti-inflammatory markers (arginase, mannose and TFGβ) showed a significant increase in relation to sham-operated rats (Figure 5). After treatment with PHA, both pro-inflammatory and anti-inflammatory marker levels experienced a similar non-significant decrease in relation to non-treated ischemic rats with the exception of arginase that displayed similar gene expression after α7 receptor activation (Figure 5D). Our results stand in contrast with the alteration of microglia/macrophage polarization by the administration of PHA immediately after permanent MCAO observed by Han and collaborators (Han, Shen, et al., 2014) that might be argued by the use of a different experimental design, MCAO model and rodent specie. Despite this, it must be pointed out that the chemokine (C-C motif) ligand 2 (CCL2) displayed the most noticeable expression decrease after α7 nAChRs activation. CCL2 is a monocyte chemoattractant protein that mediates macrophage recruitment and migration during peripheral and central nervous system inflammation (Selenica et al., 2013). Therefore, these results motivated the evaluation of the potential role of α7 receptor on the expression of leukocyte infiltration markers in the injured brain after cerebral ischemia.

4.5 | Effect of α7 receptor activation on leukocyte infiltration markers and permeability of the BBB

To tackle this concern we studied the effect of PHA on the mRNA expression of cell adhesion molecules as E-selectin, P-selectin, ICAM and VCAM following in vivo and in vitro cerebral ischemia (Figure 6). Following MCAO, all cell adhesion molecules showed an expression increase that was severely reduced after the treatment with PHA for all the receptors studied. Similarly, in vitro ischemia on bEnd.3 cells, a cerebral endothelial cell line with characteristics of the BBB (Brown et al., 2007), showed an increase of selectines and CAMs after the induction of in vitro ischemia that was reverted after the activation of α7 receptors (Figure 6). In addition, the activation of α7 receptors with PHA promoted the decrease of the expression of CD3 marker, a component of the T lymphocyte co-receptor commonly expressed in all T cells (Figure 6). These results stand in agreement with the control of α7 nAChRs over T cells activation described by De Rosa and colleagues (De Rosa, Dionisio, Agriello, Bouzat, & Esandi Mdel, 2009). Indeed, the infiltration of immune cells might be involved in the pathogenesis of the secondary neurodegeneration after ischemic stroke and the control of the T cell recruitment could provide promising therapeutic
properties (Jones et al., 2018). Therefore, the reduction of infiltrated T cells after treatment with PHA could promote the improvement of the neurological outcome after cerebral ischemia. Likewise, TSPO expression has been described in other inflammatory cells such as T lymphocytes (Zheng et al., 2011), hence, the decrease of TSPO levels after PHA treatment observed in this study could also be influenced by the decrease of T cell infiltration. Furthermore, the reduction of the expression of different leukocyte infiltration markers might also promote the decrease of both macrophage infiltration and activation of microglia as previously showed with in vivo and ex vivo studies (Figures 4 and 5). Despite this, other mechanisms such as the leakage of the BBB can enhance the infiltration of leukocytes into the brain parenchyma following stroke. Indeed, after ischemic stroke BBB is usually damaged due to the complex process driven by the cascade of mediating factors (Merali, Huang, Mikulis, Silver, & Kassner, 2017). For this reason, we evaluated the effect of PHA on BBB integrity by MRI and the contrast agent Gadovist at day 7 after cerebral ischemia (Figure 7). MRI images showed similar percentage of permeability values in both non-treated and PHA-treated ischemic rats (Figure 7d). Therefore, these results confirmed that the activation of the α7 receptors did not reduce the BBB disruption after cerebral ischemia evidencing the role of these receptors on the recruitment of leukocytes through the control on adhesion molecules expression of the cerebrovascular endothelium.

In summary, PET imaging with [11C]NS14492 was carried out to evaluate the role of α7 nAChRs on neuroinflammatory response after experimental ischemic stroke in rats. The present findings showed [11C]NS14492 binding increase in the injured hemisphere during the first week after MCAO followed by a progressive decrease later on. α7 receptor overexpression was identified in microglia and infiltrated macrophages at day 7 after ischemia. Besides, the activation of α7 nAChRs was able to promote a decrease of inflammation, volume of infarction, expression of cell adhesion molecules, decrease of T cell infiltration and the improvement of the neurological outcome after MCAO in rats. These results provide valuable knowledge regarding the role of nicotinic α7 receptors on inflammatory reaction after brain ischemia that might contribute to the discovery of a novel target for theranostics of ischemic stroke.

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CONFLICT OF INTERESTS
The authors declare no competing financial interests.

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