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Crosstalk between Phosphodiesterase 7 and Glycogen Synthase Kinase-3: Two Relevant Therapeutic Targets for Neurological Disorders

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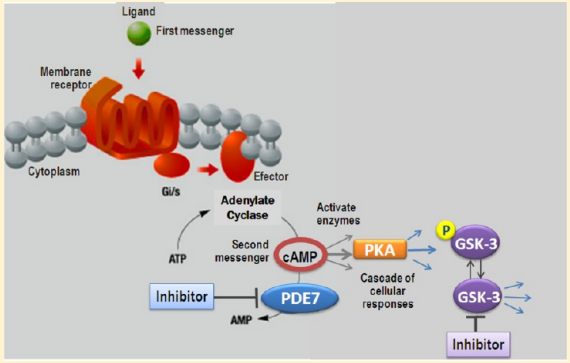
Supporting Information

ABSTRACT: Chronic neuroinflammation has been increasingly recognized as a primary mechanism underlying acute brain injury and neurodegenerative diseases. Enhanced expression of diverse pro-inflammatory agents in glial cells has been shown to contribute to the cell death that takes place in these disorders. Previous data from our group have shown that different inhibitors of the cyclic adenosine monophosphate (cAMP) specific phosphodiesterase 7 (PDE7) and glycogen synthase kinase-3 (GSK-3) enzymes are potent anti-inflammatory agents in different models of brain injury. In this study, we investigated cross-talk between PDE7 and GSK-3, two relevant therapeutic targets for neurological disorders, using a chemical approach. To this end, we compared specific inhibitors of GSK-3 and PDE7 with dual inhibitors of both enzymes with regard to anti-inflammatory effects in primary cultures of glial cells treated with lipopolysaccharide. Our results show that the GSK-3 inhibitors act exclusively by inhibition of this enzyme. By contrast, PDE7 inhibitors exert their effects via inhibition of PDE7 to increase intracellular cAMP levels but also through indirect inhibition of GSK-3. Activation of protein kinase A by cAMP results in phosphorylation of Ser9 of GSK-3 and subsequent inhibition. Our results indicate that the indirect inhibition of GSK-3 by PDE7 inhibitors is an important mechanism that should be considered in the future development of pharmacological treatments.



KEYWORDS: *GSK-3, PDE7, dual GSK-3/PDE7 inhibitors, neuroinflammation*

The increase in life expectancy observed over the last century has led to the increased incidence of a series of age-related disorders, such as neurodegenerative diseases.[1] These pathologies are debilitating and so far incurable diseases that demand intensive research for discovering new pharmacological targets and drugs involved in neuronal protection. They are characterized by the dysfunction and progressive loss of neuronal cells in susceptible areas of the nervous system and cell activation as a consequence of inflammatory processes.

Neuroinflammation has been increasingly recognized as a primary mechanism underlying acute brain injury and neurodegenerative diseases.[2−5] The activation of glial cells leads to the production of pro-inflammatory agents such as IL-1β, IL-6, TNF-α, and nitric oxide (NO), which induces neurodegeneration and neuronal cell death. Therefore, targeting the signaling pathways in glial cells responsible for neuroinflammation represents a promising therapeutic approach designed to preserve remaining neurons in those patients suffering from neurodegenerative diseases. Given the evidence of neuroinflammation in neurodegenerative disorders, agents with anti-inflammatory effects are being investigated for their neuroprotective potential. Our group has experience in the discovery and development of innovative drugs for neurodegenerative diseases exploiting chemical libraries generated as specific pharmacological probes to discover new targets for further intervention using a chemical genomic approach.[6] We have demonstrated that a number of novel phosphodiesterase 7 (PDE7) and glycogen synthase kinase 3 (GSK-3) inhibitors have potent neuroprotective and anti-inflammatory activity in different models of neurodegenerative diseases[7−11] via their specific interactions with their targets. PDE7 hydrolyzes cyclic adenosine monophosphate (cAMP), which is expressed in different brain regions and in lymphocytes and is emerging as a new target for neurological disorders.[12] We have recently shown an increase in intracellular cAMP in different brain regions of Alzheimer's and Parkinson's disease mouse models using PDE7 inhibitors.[7−13] GSK-3 is a serine/threonine kinase

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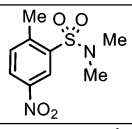
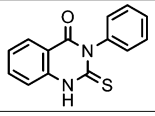
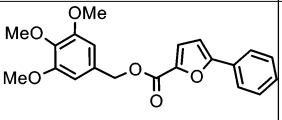
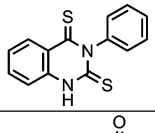
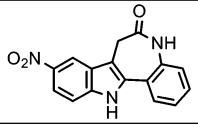
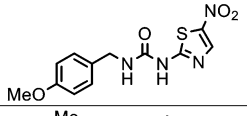
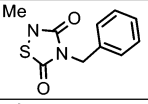
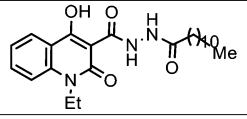
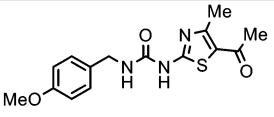
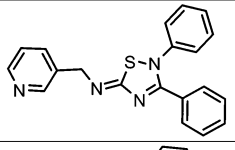
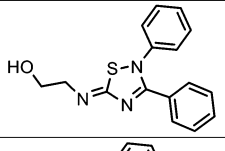
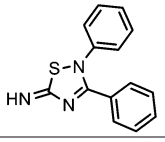
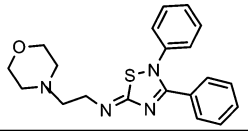
Table 1. Pharmacological Tools Used in the Study and Their Inhibition on PDE7 and GSK-3

Compound name	Chemical structure	PDE7 activity IC_{50} (μM)	GSK-3 activity IC_{50} (μM)	PDE7 Binding mode	GSK-3 Binding mode
BRL50481		0.09	<20 %Inhibition @10μM	cAMP competitive	-
S14		4.68	<20 %Inhibition @10μM	Allosteric	-
MR1.51		5.17	<20 %Inhibition @10μM	cAMP competitive	-
TC3.6		1.04	<20 %Inhibition @10μM	Allosteric	-
Alsterpaullone		<20 %Inhibition @10μM	0.005	-	ATP competitive
AR-A014418		<20 %Inhibition @10μM	0.107	-	ATP competitive
TDZD-8		41.5 %Inhibition @10μM	0.690	-	Non-ATP competitive
VP0.7		20.3 %Inhibition @10μM	2.57	-	Allosteric
VP2.51		16.4 %Inhibition @10μM	0.620	-	ATP competitive
VP1.14		0.38	1.28	Allosteric	Substrate competitive
VP1.15		1.11	1.95	Allosteric	Substrate competitive
VP1.21		1.02	0.63	Allosteric	Substrate competitive
VP3.15		1.59	0.88	Allosteric	Substrate competitive

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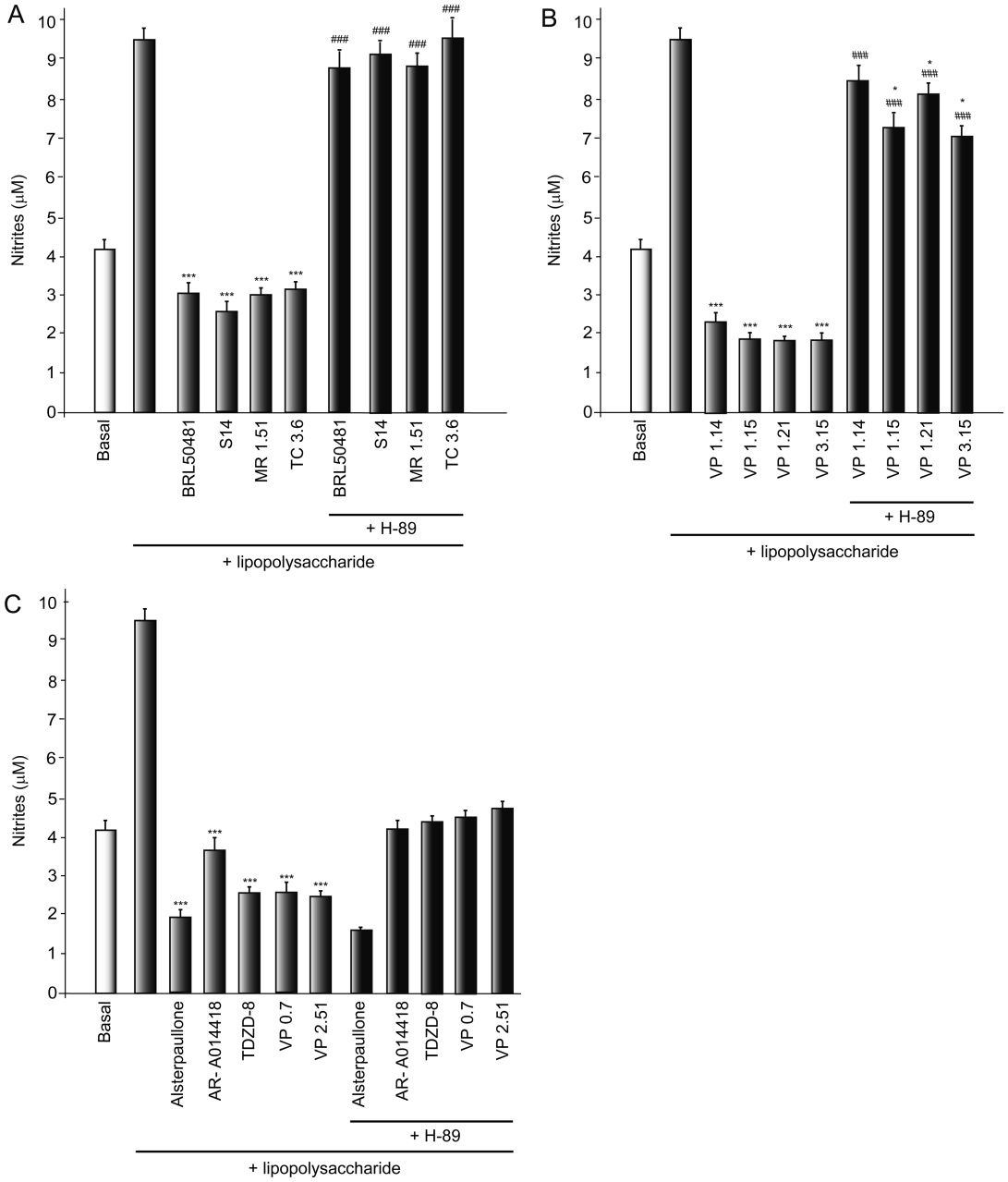

Figure 1. Anti-inflammatory effect of PDE7, PDE7/GSK-3, or GSK-3 inhibition. Nitrite production was measured by the Griess reaction in the supernatant of astrocytes primary cultures that were treated for 24 h with lipopolysaccharide (10 μg/mL) in the presence of the different compounds (10 μM). (A) PDE7 inhibitors. (B) PDE7/GSK-3 dual inhibitors. (C) GSK-3 inhibitors. Some cultures were preincubated with the PKA inhibitor, H-89. Values represent the mean ± SEM from six replications in at least three different experiments. *$p \leq 0.05$, ***$p \leq 0.001$ versus LPS-treated cells; $^{\#\#\#}p \leq 0.001$ versus the values obtained in the absence of H-89-treated cultures.

ubiquitously expressed in mammals that regulates many cellular pathways and plays key roles in several pathologies associated with neurodegenerative diseases.[14] In fact, some GSK-3 inhibitors are in clinical development for the treatment of Alzheimer's disease.

The mechanisms of action of the PDE7 and GSK-3 enzymes could be related, although so far this connection has not been clearly identified. Among the 11 families of PDEs, PDE7 specifically hydrolyzes the second messenger cAMP,[15,16] which is hypothesized to play an important role in neuroprotection and neuroinflammatory responses.[17,18] Among the diverse actions in which cAMP is involved, it activates the protein kinase A (PKA) signaling pathway that could lead to the inactivation of GSK-3 by its phosphorylation at Ser9, which also results in neuroprotection.[19,20] In this regard, Lipina et al.[21] have shown a synergy between increases in cAMP levels and inhibition of GSK-3 in animal models of schizophrenia. The aim of the present study was to investigate the connection between the inhibition of PDE7 and GSK-3 and to explore the actions of several novel PDE7 and GSK-3 inhibitors on inflammation induced by lipopolysaccharide (LPS) in primary cultures of astrocytes and microglia.

RESULTS AND DISCUSSION

Pharmacological Tools Selection. We studied three different groups of chemical probes previously reported as

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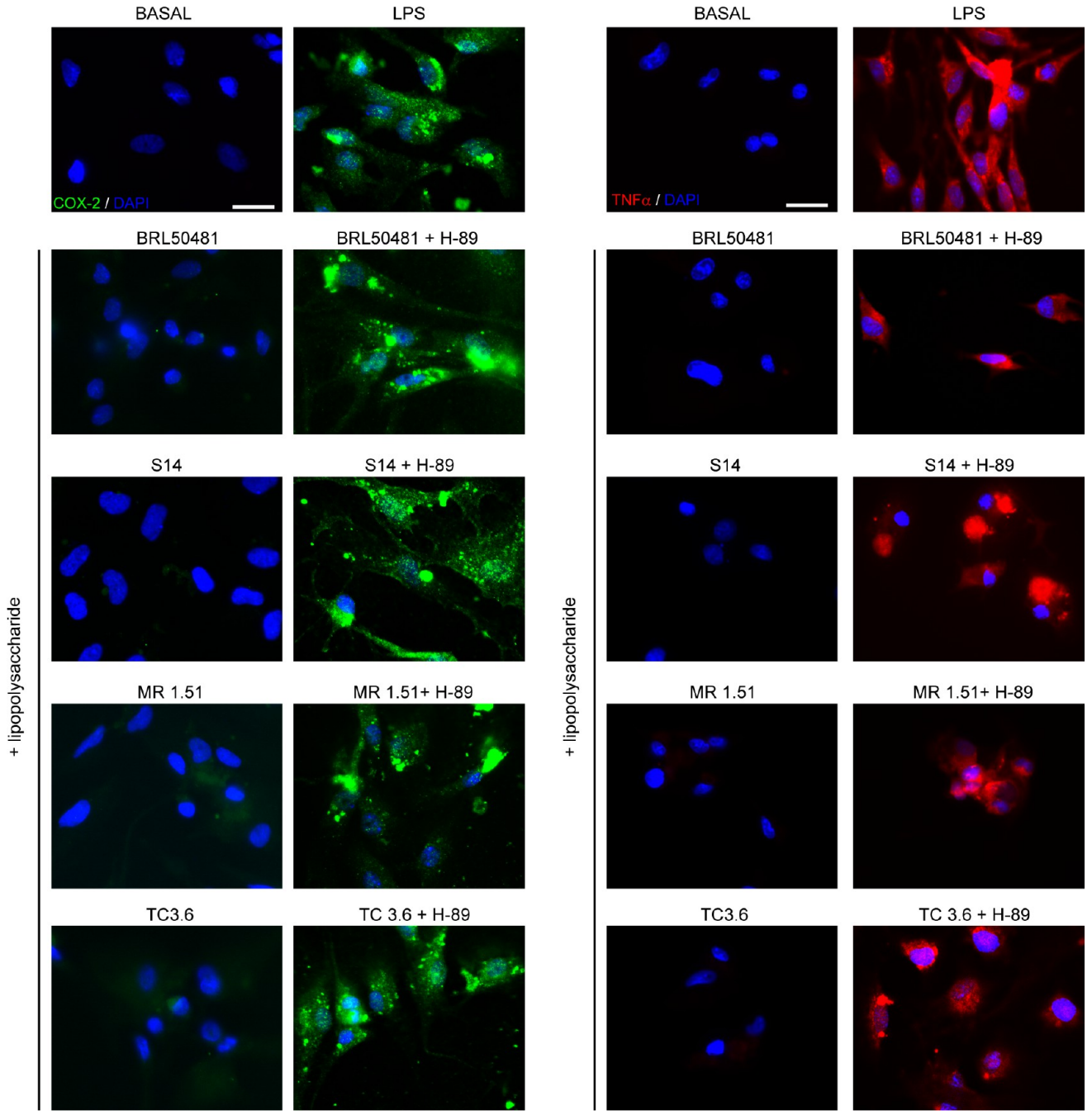

Figure 2. Anti-inflammatory effect of PDE7 inhibitors. Pro-inflammatory factors production was evaluated by immunofluorescence on astrocytes primary cultures that were treated for 24 h with lipopolysaccharide (10 μg/mL) in the presence of the different PDE7 inhibitors at 10 μM. Some of the cultures were preincubated with the PKA inhibitor, H-89. Representative images show the expression of COX-2 (green) and TNF-α (red). Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm.

cell-permeable small molecules with the ability to inhibit the targets under study: (1) PDE7 inhibitors named BRL-50481,[22] S14,[23] TC3.6,[23] and MR1.51;[10] (2) the GSK-3 inhibitors alsterpaullone,[24] AR-A014418,[25] TDZD-8,[26] VP0.7,[27] and VP2.51;[28] and (3) heterocyclic derivatives that inhibit both targets with similar potencies, VP1.14, VP1.15, VP1.21, and VP3.15.[29] Structures of all compounds are shown in Table 1 together with IC_{50} values of corresponding targets. The binding mode to the corresponding enzyme is also detailed in Table 1 where known. Compound selection considered diversity in chemical structure and in mechanism of enzyme inhibition. Thus, we used different heterocyclic compounds such as quinolines, arylfuranes, thiadiazoles, thiazoles, quinazolines, and so forth, that act as cAMP or ATP competitive inhibitors and/or allosteric modulators of both enzymes.

PDE7 and GSK-3 Inhibition Determination. To investigate cross inhibition between the selected PDE7 and GSK-3 inhibitors we determined the in vitro activity of these compounds for the other target under study. Thus, BRL-50481, S14, TC3.6, and MR1.51 were evaluated as potential GSK-3 inhibitors using a recently described luminescent technique.[30] These well-known PDE7 inhibitors did not show GSK-3 inhibition at the concentration tested (10 μM) (table 1). In the same way, the GSK-3 inhibitors alsterpaullone, AR-A014418,

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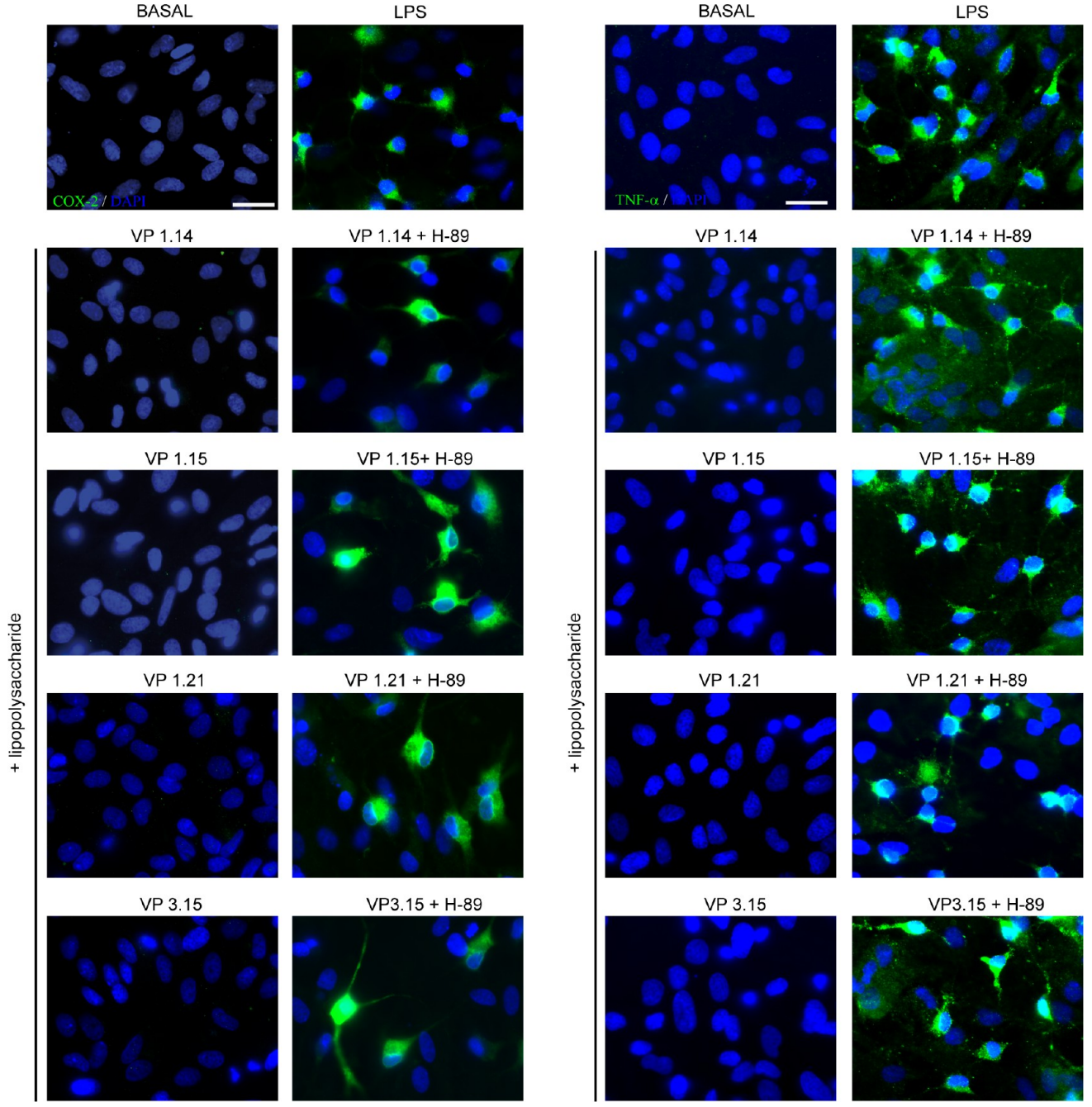

Figure 3. Anti-inflammatory effect of PDE7/GSK-3 dual inhibitors. Pro-inflammatory factors production was evaluated by immunofluorescence on astrocytes primary cultures that were treated for 24 h with lipopolysaccharide (10 μg/mL) in the presence of the different PDE7/GSK-3 dual inhibitors at 10 μM. Some of the cultures were preincubated with the PKA inhibitor, H-89. Representative images show the expression of COX-2 and TNF-α. Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm.

TDZD-8, VP0.7, and VP2.51 were evaluated using human recombinant PDE7A and did not show PDE7A inhibition at the concentration assayed (10 μM) (Table 1). The concentration of the different compounds was chosen based on its effectiveness in different previously published works.[8,22,31,46,47]

PDE7 and GSK-3 Inhibition Prevents Nitrite Production in Primary Astrocyte Cultures. We evaluated potential anti-inflammatory activity of the three different categories of small heterocyclic derivatives (PDE7 inhibitors without inhibitory potency for GSK-3, GSK-3 inhibitors without inhibition for PDE7 and dual GSK-3/PDE7 inhibitors), by evaluating the production of nitrites, an indirect quantification of NO generation, using cultured astroglial cells. To analyze the role of cAMP through activation of protein kinase A (PKA) in the effects of these compounds, we pretreated cell cultures with the PKA inhibitor H-89 before LPS exposure. In this way, we tested our working hypothesis about the inhibition of GSK-3 via an increase in intracellular cAMP levels and subsequent activation of PKA signaling pathway.

As shown in Figure 1, all compounds belonging to the three different families significantly inhibited the enhanced expression of nitrites in response to LPS. The effects of these compounds were not caused by a loss of cell viability because 24 h exposure

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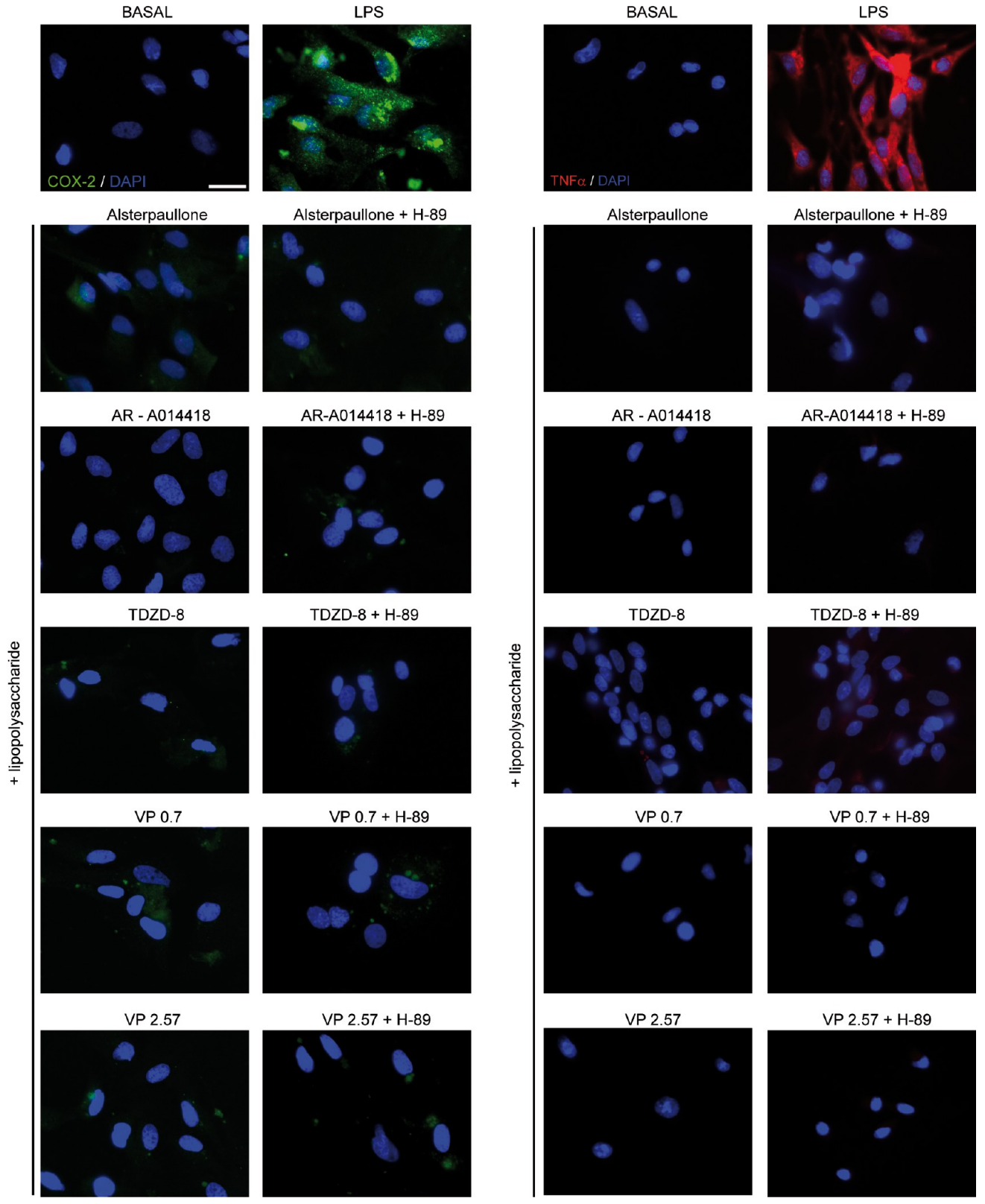

Figure 4. Anti-inflammatory effect of GSK-3 inhibitors. Pro-inflammatory factors production was evaluated by immunofluorescence on astrocytes primary cultures that were treated for 24 h with lipopolysaccharide (10 μg/mL) in the presence of GSK-3 inhibitors at 10 μM. Some of the cultures were preincubated with the PKA inhibitor, H-89. Representative images show the expression of COX-2 (green) and TNF-α (red). Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm.

of cells to these compounds did not alter cell survival (Supporting Information Figure 1). As seen in Figure 1A, the anti-inflammatory effect of PDE7 inhibitors was completely abrogated in cultures pretreated with H-89, suggesting that these compounds modulate the production of nitrites in astrocytes[31] at least in part through the cAMP/PKA signaling

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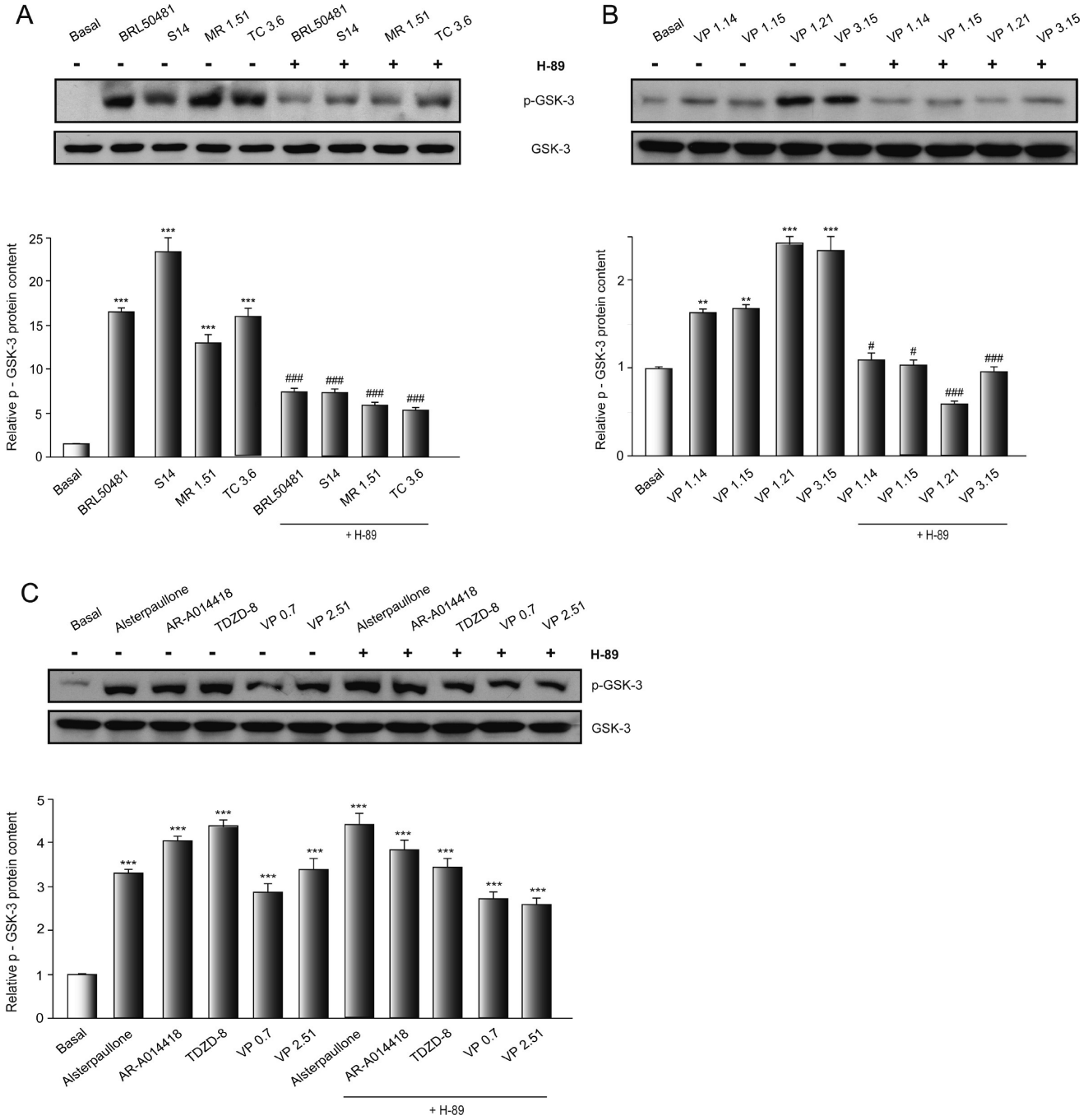

Figure 5. Western blot analysis of the expression of p-CSK3. Representative Western blot and quantification analysis showing expression levels of phosphorilated GSK-3 in primary cultures of astrocytes. Cells were cultured during 5 min with the different compounds (10 μM) in the presence or not of the inhibitor H-89. (A) PDE7 inhibitors. (B) PDE7/GSK-3 inhibitors. (C) GSK-3 inhibitors. The use of an antiserum that does not discriminate between GSK-3 and phospo-GSK3 indicates that the total levels of GSK-3 are not affected by the treatments. Each data point represents the mean ± SD of four replications in three different experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ versus nontreated (basal) cells; $^{\#}p \leq 0.005$, $^{\#\#\#}p \leq 0.001$ versus the values obtained in the absence of H-89-treated cultures.

pathway [ANOVA, $F(8,108) = 205.56$, $p < 0.0001$]. Cell cultures treated under the same conditions with the dual GSK-3/PDE7 inhibitors (Figure 1B) showed a mixed response. There was a decrease in the anti-inflammatory response due to the inhibition of the cAMP pathway, but the increase in nitrite production was not completely reversed in all cultures [ANOVA, $F(8,108) = 562.81$, $p < 0.0001$]. Interestingly, the decrease in nitrate production is not reversed in cell cultures treated with the GSK-3 specific-inhibitors and pretreated with the PKA inhibitor H-89 (Figure 1C), pointing to a cAMP independent mechanism of anti-inflammatory activity that is due to a direct inhibition of GSK-3 [ANOVA, $F(10,132) = 290.44$, $p < 0.0001$].

PDE7 and GSK-3 Inhibition Abrogates LPS-Induced Glial Activation. To study further the inhibitory effects of the different classes compounds, we examined whether these compounds affected LPS-induced intracellular accumulation of TNF-α and COX-2, two well-known pro-inflammatory

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agents, in primary glial cultures. We studied this by immunofluorescence analysis followed by confocal microscopy. As shown in Figures 2−4, the levels of TNF-α and COX-2 were clearly increased after LPS treatment of astrocytes, and treatment of the cultures with compounds belonging to each of the three classes completely abrogated this effect, confirming the anti-inflammatory activity of PDE7 and GSK-3 inhibitors. Under basal conditions, TNF-α and COX-2 levels were barely detectable in astrocyte cultures. As mentioned above, pretreatment with the PKA inhibitor, H-89, reversed anti-inflammatory effects mediated totally or partially by cAMP (Figures 2, 3), whereas the decrease of proinflammatory agents TNF-α and COX-2 produced by GSK-3 inhibitors was not affected by PKA inhibitor pretreatment (Figure 4). These results suggest again that PDE7 inhibitors act by activation of PKA and subsequent inhibition of GSK-3, probably due to phosphorylation at Ser9.

Effects of PDE7, GSK-3, and Dual GSK-3/PDE7 Inhibitors on GSK-3 Phosphorylation in Astroglial Cultures. In view of the results above, we analyzed the ability of the test compounds belonging to the three different categories (PDE7, GSK-3, and dual GSK-3/PDE7 inhibitors) to phosphorylate the GSK-3 enzyme in cell cultures. The most common intracellular target of cAMP is PKA,[32] although other effects of this nucleotide are not mediated by this kinase.[33,34] Several kinases, including PKA, are able to phosphorylate GSK-3 at Ser9 leading to its inactivation.[35,36] Consequently, an increase in cAMP levels can activate PKA, and this in turn can result in a phosphorylation and inactivation of GSK-3. We therefore determined whether treatment of astrocyte cultures with inhibitors of PDE7 could lead to phosphorylation of GSK-3. To this end, astroglial cultures were treated for 5 min with each test compound and the cells were harvested for Western blot analysis. To know whether possible GSK-3 phosphorylation induced by the compounds in Table 1 was indeed mediated by PKA activation, some of the cultures were pretreated for 1 h with the PKA inhibitor H-89. As shown in Figure 5A, compounds targeted PDE7 phosphorylated GSK-3 at Ser9. When these cultures were grown in the presence of the PKA inhibitor, H-89, this phosphorylation was significantly reduced [ANOVA, $F(8,18) = 140\,329.47$, $p < 0.0001$]. As expected, dual GSK-3/PDE7 inhibitors also induced GSK-3 phosphorylation, which was partially reversed by incubation with H-89 (Figure 5B) [ANOVA, $F(8,18) = 16\,877.35$, $p < 0.0001$]. These results suggest that PDE7 inhibitors can act in two ways: by direct inhibition of the enzyme producing an increase in intracellular cAMP levels and also by indirect inhibition of GSK-3 through activation of the cAMP/PKA signaling pathway.

Finally, we observed that the inhibitors of GSK-3 used in this study also phosphorylated the Ser9 residue of this enzyme although their mechanism of action did not involve PKA activation since phosphorylation was not reversed by H-89 treatment (Figure 5C) [ANOVA, $F(10,22) = 62\,990.40$, $p < 0.0001$]. These results can be explained by considering other off-target effects of these compounds. For example, CDK inhibition in the case of alsterpaullone[37] or PPAR-γ activation by TDZD-8[38] can act to enhance the indirect inhibition of GSK-3 in cell cultures. Additionally, the observed GSK-3 phosphorylation at Ser9 after treatment with GSK-3 inhibitors can also occur via upstream pathways leading to activation of protein kinase B or protein kinase C, which are also known to phosphorylate GSK-3 at this residue.[39−42] Specifically, Kirshenboim et al.[41] have shown that treatment of HEK293 and PC12 cells with lithium indirectly results in phosphorylation of GSK-3 at Ser9 via activation of PKC-α.

CONCLUSIONS

When taken together, the results reported here provide new evidence that demonstrates that PDE7 inhibitors can also be considered as potent indirect inhibitors of GSK-3 through the cAMP/PKA signaling pathway and through phosphorylation of GSK-3 at Ser9 and subsequent inactivation of this enzyme. As such, PDE7 inhibitors represent a class of drugs with a dual mechanism of action and with therapeutic potential for regulating neuroinflammation processes in different brain injury paradigms. We have shown the in vivo efficacy of several chemically diverse PDE7 inhibitors in different murine models of neurodegenerative diseases such as Alzheimer's disease,[13] Parkinson's disease,[7] stroke,[10] and multiple sclerosis[43] and also the pharmacologic potential of dual GSK-3/PDE7 in a mice model of schizophrenia.[44] The PKA-dependent stimulation of GSK-3 phosphorylation by these cAMP-enhancing agents should be considered in their therapeutic translation to the clinic. Our results provide new insights into the mechanism of action of PDE7 inhibitors, and corroborate their potential as new therapeutic agents for the treatment of neurodegenerative disorders.

METHODS

Pharmacological Probes. PDE7, GSK-3, and dual GSK-3/PDE7 inhibitors were acquired from commercial suppliers or were synthesized in the neurodrug laboratories from the Medicinal Chemistry Institute following previously described procedures. BRL,[22] alsterpaullone,[24] and AR-A014418[25] were obtained from Calbiochem. The quinazolines S14 and TC3.6,[23] the PDE7 inhibitors MR1.51,[10] TDZD-8,[26] VP0.7,[27] and VP2.51,[28] and the 5-imino-1,2,4-thiadiazoles VP1.14, VP1.15, VP1.21, and VP3.15[29] were prepared to previously according reported synthetic methodologies.

Radiometric PDE7 Inhibition Assay. The methodology used for measuring human recombinant PDE7A1 activity was based on a scintillation proximity assay (SPA) from Perkin-Elmer (TRKQ7090). The activity of the phosphodiesterase was measured by coincubating the enzyme with [^{3}H]cAMP and the hydrolysis of the nucleotide was quantified by radioactivity measurements after binding of [^{3}H]AMP to scintillation binding beads. Thus, 0.02 units of PDE7A1 (Calbiochem # 524751) were incubated in a 96-well flexiplate with 5 nCi of [^{3}H]cAMP and inhibitors in 100 μL of assay buffer (contained in the kit) for 20 min at 30 °C. After incubation, 50 μL of a solution of SPA-beads (approximately 1 mg per well) was added to each well and plates were shaken for 1 h at room temperature. Finally, the beads were allowed to settle for 30 min, and radioactivity was detected in a Microbeta Trilux reader.

Inhibition of GSK-3. Human recombinant GSK-3β was purchased from Millipore (Millipore Iberica S.A.U.) The prephosphorylated polypeptide substrate was purchased from Millipore (Millipore Iberica S.A.U.). The kinase-Glo Luminescent Kinase Assay was obtained from Promega (Promega Biotech Ibérica, SL). ATP and all other reagents were from Sigma-Aldrich (St. Louis, MO). Assay buffer contained 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, and 15 mM magnesium acetate.

The method of Baki et al.[45] was followed to analyze the inhibition of GSK-3β. Kinase-Glo assays were performed in assay buffer using black 96-well plates. In a typical assay, 10 μL (10 μM) of each test compound (dissolved in dimethyl sulfoxide [DMSO] at 1 mM concentration and diluted in advance in assay buffer to the desired concentration) and 10 μL (20 ng) of enzyme were added to each well followed by 20 μL of assay buffer containing 25 μM substrate and 1 μM ATP. The final DMSO concentration in the reaction mixture did not exceed 1%. After a 30 min incubation at 30 °C, the enzymatic reaction was stopped with 40 μL of Kinase-Glo reagent. Glow-type

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luminescence was recorded after 10 min using a FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany) multimode reader. Activity was proportional to the difference between the total and consumed ATP. Inhibitory activities were calculated on the basis of maximal activities measured in the absence of inhibitor.

Animals. Neonatal Wistar rats (2 days old), obtained from our breeding facilities (normative ES280790000188), were used throughout the study. Animals were cared for according to the Guide for the Care and Use of Laboratory Animals specifically approved by the "Ethics Committee for Animal Experimentation" of the Instituto de Investigaciones Biomedicas (CSIC-UAM), license number SAF 2010/16365, and experiments were carried out in accordance with the protocols issued, which followed National (normative 1201/2005) and International recommendations (normative 86/609 from the European Communities Council). Adequate measures were taken to minimize pain or discomfort of animals.

Cell Culture and Treatments. Rat primary glial cultures were prepared from the cerebral cortex of 2 day old rats as described previously[38] with some minor modifications. Briefly, after removal of the meninges, the cerebral cortex was dissected, dissociated, and incubated with 0.25% trypsin/EDTA at 37 °C for 1 h. After centrifugation, the pellet was washed three times with HBSS (Gibco) and the cells were plated in poly-D-lysine (20 μg/mL) pretreated flasks (75 cm^2). After 7−10 days, the flasks were agitated in an orbital shaker for 4 h at 230 rpm at 37 °C and nonadherent microglial cells were isolated and plated. Then DMEM was added to the flasks, which were agitated in a horizontal shaker at 260 rpm at 37 °C. After overnight agitation, the supernatant (oligodendrocytes and some remaining microglial cells) was removed, and astrocytes (adherent cells) were collected. The purity of the cultures was >95%, as determined by immunofluorescence analysis using an antiglial fibrillary acidic protein (GFAP; clone G-A-5; Sigma-Aldrich) antibody to identify astrocytes, an anti-CD11b (Serotec) antibody to identify microglial cells, and an anti-O4 (Millipore) antibody as a oligodendrocyte marker.

On attaining semiconfluence, cells were treated with the test compounds 2 h before exposure to lipopolysaccharide (LPS, 10 μg/mL; Sigma-Aldrich) for 24 h. Vehicle-treated cultures were used as a control. The concentration of the different compounds was chosen based on their effectiveness in different previously published works.[8,22,31,46,47] Previously to the treatment with the test compounds, some plates were also preincubated with the PKA inhibitor H-89 (20 μM, BIOMOL Research Laboratories). After treatments, cells were processed for Western blot analysis, immunocytochemistry, or nitrite release.

Nitrite Measurements. To measure the accumulation of nitrites in media, supernatants of glial cultures after treatments were collected and mixed with an equal volume of Griess reagent (Sigma-Aldrich). Samples were then incubated at room temperature for 15 min and absorbance was read at 540 nm on a microplate reader.

Immunocytochemistry. Cells were processed for immunocytochemistry at the end of the treatment period as previously described.[48] Briefly, primary glial cultures grown on glass coverslips were washed with PBS, fixed for 30 min with 4% paraformaldehyde at 25 °C and permeabilized for 30 min with 0.1% Triton X-100 at 37 °C. After 1 h incubation with the corresponding primary antibody, cells were washed with PBS and incubated with Alexa-488 or Alexa-647 secondary antibodies (Invitrogen; 1:400) for 45 min at 37 °C. Images were acquired using a LSM710 confocal microscope (Zeiss). Confocal microscope settings were adjusted to optimize signal-to-noise ratios. To compare fluorescence signals from different preparations, settings were fixed for all samples within the same analysis. The following primary antibodies were used goat anti-cyclooxygenase type 2 (anti-COX-2, Santa Cruz Biotechnology; 1:200) and goat anti-tumor necrosis factor alpha (anti-TNFα; Santa Cruz Biotechnology; 1:200).

Immunoblot Analysis. Proteins were isolated from cell cultures by standard methods. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran, Whatman) and blots were probed with the indicated primary antibodies, as previously described.[31] The antibodies used were the following: rabbit monoclonal anti-pGSK3 (Cell Signaling Technology), mouse monoclonal anti-GSK3 (BD Bioscience), and secondary peroxidase-conjugated antibodies (Jackson Immunoresearch). Quantification was performed using Scion Image software. Values in Figure 5 are the means of at least three independent experiments corresponding to three different samples.

Statistical Determinations. The SPSS statistical software package (version 20.0) for Windows (Chicago, IL) was used for the ANOVA analyses. The data were analyzed using a one-way ANOVA with drug treatment as the between-subject factor followed by Tukey's post hoc test and Bonferroni's correction for multiple testing.

■ ASSOCIATED CONTENT

Supporting Information

Graphic showing that the different compounds used do not alter astrocyte cell viability. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥V.P. and M.R. contributed equally to this work. A.P.-C. and A.M conceived and designed the experiments, analyzed the data, and wrote the manuscript. J.A.M.-G. helped design experiments and wrote the manuscript, analyzed the data, and performed the experiments. V.P. and M.R. synthesized and optimized the compounds. C.G. synthesized and optimized the compounds and analyzed the data. S.A.-G. conducted experiments.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

cAMP, cyclic adenosine monophosphate; CDK, cyclin-dependent kinase; COX-2, cyclooxygenase 2; GSK-3, glycogen synthase kinase-3; LPS, lipopolysaccharide; PDE7, phosphodiesterase7; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumoral necrosis factor α

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