Received Date : 26-Apr-2012

Revised Date : 19-Jun-2012

Accepted Date : 04-Jul-2012

Article type : Original Article

The new iminothiadiazole derivative VP1.14 ameliorates hippocampal damage after an excitotoxic injury

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/j.1471-4159.2012.07866.x

Abstract

Increased levels of glutamate causing excitotoxic damage accompany many neurological disorders. A well-characterized model of excitotoxic damage involves administration of kainic acid, which causes limbic seizure activity and subsequent neuronal death, particularly in the CA1 and CA3 areas of the hippocampus. Inhibition of the enzyme glycogen synthase kinase 3 (GSK-3) and cyclic AMP (cAMP) levels might play an important role in neuroprotection. Since intracellular cAMP levels depend, in part, on the activity of the phosphodiesterase enzymes (PDEs), these enzymes have recently emerged as potential therapeutic targets for the treatment of several diseases. In previous works we have shown a potent anti-inflammatory and neuroprotective effect of GSK-3 inhibition in a model of excitotoxicity, as well as a reduction of nigrostriatal dopaminergic neuronal cell death after phosphodiesterase 7 (PDE7) inhibition, which leads to an increase in cAMP levels. The present study was undertaken to determine whether simultaneous inhibition of GSK-3 and PDE7 by a novel 5-imino-1,2,4-thiadiazole compound, named VP1.14, could prevent the massive neuronal loss in the hippocampus evoked by intra-hippocampal injection of kainic acid. Here, we show that rats treated with VP1.14 showed a reduced inflammatory response after kainic acid injection, and exhibited a significant reduction in pyramidal cell loss in the CA1 and CA3 areas of the hippocampus. Studies with hippocampal HT22 cells *in vitro* also showed a clear neuroprotective effect of VP1.14 and an anti-inflammatory effect shown by a decrease in the nitrite liberation and in the expression of pro-inflammatory cytokines by primary cultures of astrocytes treated with lipopolysaccharide.

*Keywords:*Excitotoxicity; GSK-3; hippocampus; neuroinflammation; neuroprotection; PDE7

1. Introduction

Excitotoxicity is a well-characterized mechanism of neuronal death which has been implicated in different brain disorders, including neurodegenerative diseases (Coyle & Puttfarcken 1993, Diehl *et al.* 1994, Doble 1999, Meldrum 2000, Nakamura & Lipton 2010). An inflammatory response is often associated with kainic acid (KA)-induced hippocampal neurodegeneration. Activated glial cells increase the expression of genes implicated in the production of nitric oxide and proinflammatory cytokines, which in turn contribute to the expansion of brain injury and the increased loss of neurons (Oprica *et al.* 2003).

Administration of KA into the hippocampus is a well-established model of excitotoxicity. KA injection is known to induce a sequence of behavioral changes, which are followed by neurodegeneration in specific brain regions, such as the hippocampus, piriform cortex, thalamus, and amygdala. A well-characterized pattern of neurodegeneration takes place in the hippocampus where the CA1 and CA3 pyramidal neurons and the interneurons of the hilus of dentate gyrus are the most vulnerable (Coyle 1983, Sperk 1994, Lau & Tymianski 2010). Injection of KA into rodents also results in the activation of glial cells and inflammatory responses typically found in neurodegenerative diseases. Thus, this model has been widely used to analyze the cellular and molecular mechanisms that underlie central nervous system injury.

The suppression of neuroinflammation and induction of neuroprotection by GSK-3 inhibitors have been previously described (Luna-Medina *et al.* 2007a, Zhou *et al.* 2011). Moreover, GSK-3 inhibitors have been shown to possess preclinical efficacy in different animal models of neurodegenerative disorders, decreasing the microglial activation and reducing neuronal death (Martinez 2008). In fact, tideglusib, a thiadiazolidinidione GSK-3 inhibitor, is on clinical trial phase IIb for Alzheimer's disease (Martinez 2008, del Ser 2010).

Different studies have suggested that cyclic AMP (cAMP) can actively play an important role in neuroprotection and in the modulation of the neuroinflammatory response observed after a brain injury (Lonze & Ginty 2002, Volakakis *et al.* 2010). Thus, control of the levels of this nucleotide could generate the regulation of the pathological neuroinflammatory processes and, in consequence, the neuronal cell death that takes place in neurodegenerative disorders. Moreover, cAMP may activate the protein kinase A (PKA) signaling pathway leading to the inactivation of GSK-3 by its phosphorylation at Ser9 which, as commented above, also results in neuroprotection (Park *et al.* 2010, Hayashi & Sudo 2009).

The cellular levels of cAMP depend on their synthesis by adenyl cyclases and their hydrolysis by cyclic nucleotide 3', 5'-phosphodiesterases (PDEs) (Conti & Beavo 2007, Mehats *et al.* 2002). Among the eleven families of PDEs, specifically PDE7 hydrolyzes cAMP and is highly expressed in the brain, being relatively abundant in the olfactory bulb, hippocampus, striatum and cerebellum (Miro *et al.* 2001, Reyes-Irisarri *et al.* 2005, Sasaki *et al.* 2002). There is very little information regarding the physiological functions of PDE7. It has been suggested that this enzyme is involved in pro-inflammatory processes and that is necessary for the induction of T-cell proliferation.

Very recently, our group has shown a neuroprotective effect of PDE7 inhibition in *in vivo* models of stroke and Parkinson disease. Specifically, we have demonstrated that S14, a heterocyclic small molecule inhibitor of PDE7 belonging to the quinazoline type family, conferred significant neuronal protection against different insults both in the human dopaminergic cell line SH-SY5Y and in primary rat mesencephalic cultures. S14 treatment also reduced microglial activation, protected dopaminergic neurons and improved motor function in the lipopolysaccharide rat model of Parkinson disease (Morales-Garcia *et al.* 2011). Also, we have also shown that these quinazolines ameliorated brain damage and improved behavioral outcome in a cerebral artery occlusion stroke model (Redondo *et al.* 2012).

Given the limited understanding of PDE7 involvement in brain injury, the synergy recently shown between the increase of cAMP and the inhibition of GSK-3 (Lipina *et al.* 2012) and our previous speculation that both enzymes could play a crucial role in this process, in this work we sought to address the effects of VP1.14, a 5-imino-1,2,4-thiadiazole which inhibits both PDE7 and GSK-3 (Palomo *et al.* 2012), after an excitotoxic injury *in vivo* and *in vitro*. Our findings demonstrate that *in vivo* administration of VP1.14 results in a reduced inflammatory response after kainic acid injection, and in a dramatic reduction in pyramidal cell loss in the CA1 and CA3 subfields of the hippocampus. In addition, our *in vitro* studies show that VP1.14 inhibits inflammatory activation of primary cultures of astrocytes and rescues HT22 hippocampal cells from an excitotoxic insult.

Materials and methods

1.1. Animals

All procedures with animals were specifically approved by the 'Ethics Committee for Animal Experimentation' of the Instituto de Investigaciones Biomedicas (CSIC-UAM), license number SAF 2010/16365, and 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). All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult male Wistar rats (2-3-month old) and neonatal Wistar rats (2-day old), obtained from our breeding facilities (normative ES280790000188) were used throughout the study.

1.2. Cell culture and treatments

Rat primary astrocyte cultures were prepared from the cerebral cortex of 2-day old rats as described previously (Luna-Medina *et al*. 2005) with minor modifications. Briefly, after removal of the meninges the cerebral cortex was dissected, dissociated, and incubated with 0.25% trypsin/EDTA at 37ºC for 1 h. After centrifugation, the pellet was washed three times with HBSS (Gibco) and the cells were plated on poly-D-lysine (20 µg/ml) pre-treated 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 non-adherent microglial cells were removed. Then DMEM was added to the flasks, which were agitated at 260 rpm at 37°C overnight. 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 mouse monoclonal anti-

glial fibrillary acidic protein (GFAP; clone G-A-5; Sigma-Aldrich) to identify astrocytes.

On attaining semiconfluence, cells were treated with VP1.14 (3 μM; MW= 580 Da.; GSK-3 IC_{50}= 1.3±0.4 μM; PDE-7 IC_{50}=0.38 μM) (Palomo et al. 2012) (Fig 1) or BRL-50481 (30 μM, Tocris Bioscience) 2 h before exposure to glutamate (500 μM; Sigma-Aldrich) or lipopolysaccharide (LPS, 10 μg/ml; Sigma-Aldrich) for 24 h. The concentration of BRL-50481 was chosen based on it effectiveness in different previously published works (Morales-Garcia et al. 2011, Smith *et al.* 2004, Zhang *et al.* 2008). Dose-response analysis and viability of VP1.14 and BRL are shown in Supplementary Figures 1 and 2. After treatments, cells were processed for immunocytochemistry, nitrite release, cell viability assay, and western blot analysis.

The mouse hippocampal HT22 neuronal cell line (Invitrogen) was cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and incubated at 37ºC under 5% CO_2. To perform the experiments of cell viability HT22 cells were seeded onto 96-well microplates (5 000 cells/well) overnight. BRL-50481 (30 μM) or VP1.14 (3 μM) were added to the culture medium 2 h before exposure to glutamate (3 mM) and cells were incubated for 24 h before performing MTT assay.

1.3. Intrahippocampal injections

Adult male Wistar rats (2-3 month old) were used in this study. Rats ($n \geq 5$ per group) were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and medetomidine (0.4 mg/kg) and placed into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). KA (1 μg KA in 2.5 μl PBS, Sigma) alone or in

combination with VP1.14 (2.5 µg in 2.5 µl PBS) was delivered unilaterally into the left hippocampus at a speed of 1 µl/minute using the following coordinates from Bregma: posterior, -3.0 mm; lateral, -2.0 mm; depth, 3.5 mm; according to the atlas of Paxinos and Watson (Paxinos 1998). The amount of S14 injected was calculated taking into account the distribution volume of 1ml and a concentration of 4µM, which is effective in the *in vitro* experiments (Supplementary Figure 1). This concentration of VP1.14 did not significantly alter cell viability (Supplementary Figure 2). Control animals of the same age were injected with vehicle. Rats were then housed individually to recover.

1.4. Immunohistochemistry

Seventy two hours after stereotaxic injection, the animals were anesthetized and perfused transcardially with 4% paraformaldehyde solution. The brains were removed, post-fixed in the same solution at 4ºC overnight, cryoprotected in the paraformaldehyde solution containing 30% sucrose, frozen, and coronal sections (30 µm) were obtained in a cryostat. Free floating sections were stained with cresyl violet (Nissl staining) and Fluoro-Jade B or processed for immunohistochemistry using the diaminobenzidine method as previously described (Cortes-Canteli *et al.* 2008). The following antibodies were used: mouse monoclonal anti- CD11b (clone OX-42, Serotec; 1:200), mouse monoclonal anti-GFAP (Sigma; 1:400), and mouse monoclonal anti-NeuN (clone A60, Millipore; 1:200). The slides were examined with a Nikon eclipse 90i microscope. Neuronal integrity and the extent of gliosis were quantified as previously described (Morales-Garcia *et al.* 2009). The number of neurons Nissl-, NeuN-, or Fluoro Jade-B-positive cells in the CA1 and CA3 areas of the hippocampus, was assessed by counting five independent well-defined high-magnification (400X) fields per animal using computer-assisted image analySIS software and NIS-Elements BR 3.0 (Soft Imaging

System). The extent of microgliosis was quantified by counting the number of CD-11b-positive cells in five independent well defined high-magnification (400X) fields per animal, as described above. Astrogliosis was evaluated in sections immunostained with GFAP and using two different parameters: number of activated cells, based on the calculation of highly immunostained cell body profiles, and immunosignal intensity, based on the measurement of optical density. Individual cell bodies were manually traced, and their mean staining intensity was normalized against the background of the respective section, defined as tissue devoid of specific immunostaining. The procedure resulted in arbitrary values on a scale from 1 (background staining) to 256.

1.5. Immunocytochemistry

At the end of the treatment period, primary astrocyte cultures, grown on glass cover-slips, were washed with PBS, fixed for 30 min with 4% paraformaldehyde at 25°C and permeabilized with 0.1% Triton X-100 for 30 min at 37°C. After 1 h incubation with the corresponding primary antibody cells were washed with PBS and incubated with an Alexa-labeled secondary antibody (Invitrogen; 1:400) for 45 min at 37°C. Images were acquired using a LSM710 confocal microscope (Zeiss), with a 350 nm diode laser to excite DAPI (4, 6-diamidino-2-phenylindole) and a 488 laser to excite Alexa 488. Confocal microscope settings were adjusted to produce the optimum signal to noise ratio. To compare fluorescence signals from different preparations, settings were fixed for all samples within the same analysis. The following antibodies were used: goat anti-interleukin-6 (anti-IL-6, Santa Cruz Biotechnology; 1:200) and goat anti-cyclooxygenase type 2 (anti-COX-2, Santa Cruz Biotechnology; 1:200). A quantitative analysis of labeled cells was undertaken using the image analySIS software and

normalized to total nuclei. Areas to be counted were traced at high power (400X), and at least five different counting fields were selected at random per culture.

1.6. Nitrite measurements

To measure the levels of nitrite (NO_2^-) in astrocyte cultures after treatments, supernatants were collected and mixed with an equal volume of Griess reagent (Sigma-Aldrich).Samples were incubated for 15 min at room temperature and the absorbance was measured at 540 nm on a microplate reader.

1.7. Cell viability assay

For assessment of cell viability, a modified colorimetric MTT assay (Mosmann 1983) was used. This method is based on the ability of viable cells to reduce MTT (yellow solution) to formazan (dark blue crystals). After 24 h of treatment, HT22 cells were incubated with MTT solution (0.5 mg/ml PBS; Sigma-Aldrich) for 1 h at 37ºC, then aspirated and dimethyl sulfoxide was added and mixed thoroughly to dissolve dark blue crystals. The absorbance was read at 595 nm (wave long reference at 650 nm) with a microplate reader (Thermolab). The cell viability was expressed as a percentage relative to the control cells (cells not treated with glutamate).

1.8. Western blot

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 (Cortes-Canteli *et al.* 2004). The primary antibodies used were the following: rabbit monoclonal anti-pGSK3 (Cell Signaling Technology) and mouse monoclonal anti-GSK-3 (BD

Bioscience) and the secondary peroxidase-conjugated mouse anti-rabbit and rabbit anti-mouse antibodies respectively were from Jackson Immunoresearch. Quantification analysis was performed using the Scion Image software. Values in the Figure are the mean of at least three independent experiments corresponding to three different samples.

1.9. Statistical Determinations

Statistical comparisons for significance among different groups of animals were performed by ANOVA followed by Newman-Keuls'test for multiple comparisons. Student *t* test was used to analyze statistical differences between cells. Differences were considered statistically significant at $p<0.05$. Data are given as the mean ± SEM of at least five different animals per group or three different experiments in vitro.

2. Results

2.1. Neuroprotective role of VP1.14 after excitotoxic brain injury

We studied the effect of VP1.14 in an established excitotoxic model *in vivo*. Adult rats received intrahippocampal injections of vehicle (PBS), KA or KA plus VP1.14 and animals were killed 72 h after injection. We first evaluated the extent of cell loss in the CA1 and CA3 regions of the hippocampus by Nissl staining (Fig 2A). We observed an almost total preservation of CA1 neurons in VP1.14-injected rats, compared with more than 80% loss in KA-injected animals (Fig. 2B). Regarding the CA3 region approximately 30% of the neurons survived in the VP1.14-injected animals. We further analyzed the extend of pyramidal neuronal cell death after excitotoxicity by staining with anti-NeuN antibody to detect surviving neurons, or with Fluoro-Jade B (Schmued *et al.* 1997) to detect degenerating neurons (Fig 3). The VP1.14-injected rats exhibited

significantly less damage in the CA1 and CA3 regions compared with the KA-injected rats, indeed there was no neuronal loss in CA1 and only a 30% loss in NeuN $^{+}$ cells in CA3 in contrast with a loss of 20 and 43% respectively in CA1 and CA3 of KA treated rats (Fig. 3A, C). In agreement with these results, VP1.14-treated animals presented a diminished Fluoro-Jade B fluorescence in these regions of the hippocampus 72 h after KA injection (Fig 3B, D), compared with the strong damage observed in KA-injected rats.

2.2. VP1.14 treatment inhibits glial activation

One of the events that take place in the hippocampus following excitotoxic injury is the sequential activation of microglia and astroglia. Microglial and astrocyte cells are considered to be key players in the induction of the neuronal damage following excitotoxic injury. Analysis of hippocampal sections revealed the expected accumulation of GFAP-positive astrocytes, 72 hours after KA injection, relative to vehicle-injected animals (Figure 4A). This increase in GFAP immunoreactivity was mainly detected in the CA1 and CA3 regions of the hippocampus. By contrast, VP1.14-treated rats displayed much less staining for GFAP, indicating a reduced astroglial response to KA-induced injury. Quantification of the data revealed a 2.4-fold decrease in the number of strongly-stained GFAP^{+} astrocytes in the CA1 and CA3 regions. We also detected a similar decrease on GFAP staining intensity (Fig. 4C, D).

In addition, the evaluation of CD11b staining for microglial infiltration revealed a considerably larger response in KA-injected rats than that found in those animals treated with VP1.14 (Fig. 4B). Quantitative studies (Fig. 4E) showed a 4-fold decrease in the number of CD11b-positive cells in the CA1 and CA3 of VP1.14-treated rats, compared with KA-injected rats

2.3. VP1.14 protects hippocampal neurons from cell death and inhibits inflammatory activation of astrocyte cultures

We next examined the effect of VP1.14 *in vitro* on cell death in the murine hippocampal cell line HT22. As shown in Figure 5A, glutamate treatment resulted in a loss of cell viability, as assessed by a decline in MTT, as compared with control untreated cells. Incubation with VP1.14 or BRL-50481, a well-known inhibitor of PDE7, caused a neuroprotective effect against glutamate-induced injury, as shown by an elevation in the cell viability of 20% and 27%, respectively (Fig 5A).

The potential anti-inflammatory activity of VP1.14 was tested by studying nitrite production and the expression levels of two pro-inflammatory agents, IL-6 and COX-2, in primary cultures of astrocytes treated with glutamate or lipopolysaccharide (LPS). To measure nitrite production, astrocytes were incubated with VP1.14 or BRL-50481 for 2 h, and then cells were cultured for another 24 h with LPS. As shown in Fig. 5B, LPS treatment resulted in a 3.2-fold increase in the concentration of nitrites in the culture medium, which was significantly prevented by treatment with VP1.14 and BRL-50481.

To further evaluate the role of PDE7 inhibition in the inflammatory response in neural tissues, we tested the effects of VP1.14 and BRL-50481 on the expression levels of IL-6 and COX-2 in astrocyte cultures treated with glutamate (Fig. 6A,B) or LPS (Fig. 6 C,D). To this end, we performed immunofluorescence analysis followed by confocal microscopy. In basal conditions, IL-6 and COX-2 levels were barely detectable in astrocyte cultures, and their content was significantly induced after glutamate or LPS treatment. However, the number of astrocytes expressing IL-6 was significantly reduced

(7.8- and 2.3- fold) in cultures treated with glutamate plus VP1.14 or BRL-50481, respectively. Similar results were observed in those cultures treated with LPS plus VP1.14 or BRL-50481. In parallel, we also observed a significant reduction in the number of cells expressing the pro-inflammatory enzyme COX-2 in those cultures treated with VP1.14 or BRL-50481, compared to glutamate-treated cultures. The effects of VP1.14 and BRL-50481 were not caused by a loss of cell viability, because the 24 h exposure of astrocyte cells to these compounds did not modify cell viability (Supplementary Figure 2).

2.4. VP1.14 phosphorylates glycogen synthase kinase-3 (GSK-3)

It has been previously shown a synergistic interaction between PDE4, another cAMP hydrolyzing enzyme, and GSK-3 (Lipina et al. 2012). We then analyzed whether VP1.14 could phosphorylate, and in consequence inactivate, GSK-3. As shown in Figure 7, VP1.14 treatment of astrocytes results in an enhancement of GSK-3 phosphorylation in serine9. Treatment of cells with H89 (a protein kinase A inhibitor (PKA)) partially reversed GSK-3 phosphorylation, suggesting that this phosphorylation was mediated, at least in part, by an activation of PKA.

3. Discussion

Here, we have shown that a novel thiadiazole derivative, VP1.14, which is a dual inhibitor of PDE7 and GSK-3, prevented both the glial activation and the neuronal cell death that occur in the hippocampus as a consequence of an excitotoxic insult. These results are further supported by *in vitro* studies directly demonstrating the anti-inflammatory and neuroprotective effects of this compound. These results establish PDE7 as a factor involved in the development of excitotoxic injury and suggest a role for PDE7 as a novel target for treatment of brain disorders such as epilepsy, ischemia, and neurodegenerative diseases, in which excitotoxicity is involved (Coyle & Puttfarcken 1993, Guo *et al.* 1999, Lynch & Dawson 1994, Hossmann 1994).

These data are in agreement with recent reports showing a neuroprotective role of PDE7 inhibition. Our laboratory has recently demonstrated an important neuroprotective role of PDE7 inhibition by quinazolines compounds in *in vitro* and *in vivo* models of stroke and Parkinson disease (Palomo et al. 2012, Morales-Garcia et al. 2011). Also, very recently Paterniti et al. have shown that inhibition of this enzyme by different compounds (quinazolines and 5-imino-1,2,4-thiadizoles) significantly reduced the degree of spinal cord inflammation and tissue injury in a model of spinal cord injury (Paterniti *et al.* 2011).

Glutamate is the driver of the excitotoxic process (Uryu *et al.* 2002). Although glutamate plays a central role in excitatory neurotransmission, alterations in glutamate homeostasis, with an excessive glutamate activity, can have significant repercussions on neurons through the generation of neurotoxic and excitotoxic cascades (Mody &

MacDonald 1995). (Dawson & Dawson 1996). Intrahippocampal injection of the excitotoxin kainic acid in rats is a common and well-characterized model of excitotoxicity. This treatment induces selective degeneration of hippocampal CA1 and CA3 pyramidal neurons and a significant fraction of dentate hilar neurons (Nadler *et al.* 1980, Shetty & Turner 1996). Our results show that dual GSK-3 and PDE7 inhibition clearly protects hippocampal neurons from KA-induced injury. In addition, VP1.14 protected the HT22 hippocampal neuronal cell line from glutamate injury.

One of the events associated to the neurodegeneration that takes place in several brain disorders is an inflammatory response, which is characterized by a reactive gliosis. Activation of glial cells leads to the production of toxic substances, including the enzyme COX-2 and cytokines such as IL1β, IL-6, and TNF-α, that further damage neurons, leading to a cycle of inflammatory damage that ultimately worsens the progression of the disease (Dawson & Dawson 1996). This view is further supported by epidemiologic data showing that long-term treatment with non-steroidal anti-inflammatory drugs may protect against Alzheimer disease and Parkinson disease (Townsend & Pratico 2005, Chen *et al.* 2003, Hald & Lotharius 2005). Unfortunately, current therapies directed to treatment of brain injury do not address this neuroinflammation process.

The results presented here, demonstrate that VP1.14 treatment significantly reduces the accumulation of reactive astrocites and microglia in the hippocampus and the production of COX-2 and IL-6 induced by glutamate and LPS treatment in primary astrocytes. In this regard, our group has shown that the thiadiazolidinone (TDZD) compounds, which are GSK-3 inhibitors and also activators of the nuclear receptor

PPARγ, also show anti-inflammatory and neuroprotective effects in KA induced injury (Luna-Medina *et al.* 2005, Luna-Medina *et al.* 2007b).

Our results showing a phosphorylation of GSK-3 in serine9 by VP1.14 suggest that the observed effects of this compound could also be mediated by an inhibition of GSK-3. In this regard it has been previously shown that an inhibitor of PDE4B and a GSK-3 blocker synergize to produce antipsychotic effects on the Disrupted-In-Squizophrenia-1-L100P genetic model (Lipina et al. 2012). In addition, our group has recently demonstrated that the novel family of 5-imino-1,2,4-thiadiazoles is able to inhibit GSK-3 in a substrate competitive manner (Palomo et al. 2012). Thus, our results indicate that VP1.14 could be acting through an inhibition of PDE7, which elevates cAMP levels, together with an inactivation of GSK-3 by a direct inhibition of this enzyme as well as by an inactivation through phosphorylation on Ser9 by the PKA enzyme. The fact that VP1.14 is targeting both PDE7 and GSK-3 enzymes may underlies the strong neuroprotective and anti-inflammatory activities of this compound.

In summary, our results demonstrate that a novel 5-imino-1,2,4-thiadiazole compound, through inhibition of PDE7 and GSK-3 enzymes, has a significant neuroprotective and anti-inflammatory effect in KA-injected rats, a well-characterized model of excitotoxicity. In addition, this inhibition diminished the induction of pro-inflammatory agents after glutamate or LPS treatment *in vitro*. Hence, PDE7 may be a potential new therapeutic target deserving further attention for the treatment of acute brain injury and neurodegenerative diseases, where excitotoxic and inflammatory processes are involved.

Acknowledgements

This work was supported by the Ministerio de Ciencia y Tecnología (SAF2010-16365 to A.P.-C., and SAF2009-13015-C02-01 and INC-0367 to A.M.) and by Instituto de Salud Carlos III (RD07/0060/015, RETICS program) to A.M. CIBERNED is funded by the Instituto de Salud Carlos III. We acknowledge Milagros Sonseca for technical assistance. The authors declare no conflict of interest.

References

Chen, H., Zhang, S. M., Hernan, M. A., Schwarzschild, M. A., Willett, W. C., Colditz, G. A., Speizer, F. E. and Ascherio, A. (2003) Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. *Arch Neurol,* **60,** 1059-1064.

Conti, M. and Beavo, J. (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem,* **76,** 481-511.

Cortes-Canteli, M., Wagner, M., Ansorge, W. and Perez-Castillo, A. (2004) Microarray analysis supports a role for ccaat/enhancer-binding protein-beta in brain injury. *J Biol Chem,* **279,** 14409-14417.

Cortes-Canteli, M., Luna-Medina, R., Sanz-Sancristobal, M., Alvarez-Barrientos, A., Santos, A. and Perez-Castillo, A. (2008) CCAAT/enhancer binding protein beta deficiency provides cerebral protection following excitotoxic injury. *J Cell Sci,* **121,** 1224-1234.

Coyle, J. T. (1983) Neurotoxic action of kainic acid. *Journal of neurochemistry,* **41,** 1-11.

Coyle, J. T. and Puttfarcken, P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science,* **262,** 689-695.

Dawson, V. L. and Dawson, T. M. (1996) Nitric oxide neurotoxicity. *J Chem Neuroanat,* **10,** 179-190.

del Ser, T. (2010) Phase IIa clinical trial on Alzheimer's disease with NP12, a GSK-3 inhibitor. *Alzheimer's Dementia,* **6**.

Diehl, A. M., Michaelson, P. and Yang, S. Q. (1994) Selective induction of CCAAT/enhancer binding protein isoforms occurs during rat liver development. *Gastroenterology,* **106,** 1625-1637.

Dirnagl, U., Iadecola, C. and Moskowitz, M. A. (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci,* **22,** 391-397.

Doble, A. (1999) The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacol Ther,* **81,** 163-221.

Guo, Q., Fu, W., Sopher, B. L., Miller, M. W., Ware, C. B., Martin, G. M. and Mattson, M. P. (1999) Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. *Nature medicine,* **5,** 101-106.

Hald, A. and Lotharius, J. (2005) Oxidative stress and inflammation in Parkinson's disease: is there a causal link? *Exp Neurol,* **193,** 279-290.

Hayashi, H. and Sudo, T. (2009) Effects of the cAMP-elevating agents cilostamide, cilostazol and forskolin on the phosphorylation of Akt and GSK-3beta in platelets. *Thromb Haemost,* **102,** 327-335.

Hossmann, K. A. (1994) Glutamate-mediated injury in focal cerebral ischemia: the excitotoxin hypothesis revised. *Brain pathology (Zurich, Switzerland),* **4,** 23-36.

Lau, A. and Tymianski, M. (2010) Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch,* **460,** 525-542.

Lipina, T. V., Wang, M., Liu, F. and Roder, J. C. (2012) Synergistic interactions between PDE4B and GSK-3: DISC1 mutant mice. *Neuropharmacology,* **62,** 1252-1262.

Lonze, B. E. and Ginty, D. D. (2002) Function and regulation of CREB family transcription factors in the nervous system. *Neuron,* **35,** 605-623.

Luna-Medina, R., Cortes-Canteli, M., Alonso, M., Santos, A., Martinez, A. and Perez-Castillo, A. (2005) Regulation of Inflammatory Response in Neural Cells in Vitro by Thiadiazolidinones Derivatives through Peroxisome Proliferator-activated Receptor {gamma} Activation. *J Biol Chem,* **280,** 21453-21462.

Luna-Medina, R., Cortes-Canteli, M., Morales-Garcia, J. A., Martinez, A., Santos, A. and Perez-Castillo, A. (2007a) Effect of GSK-3β inhibition on neural stem cell proliferation and differentiation. In: *17th ESN (European Society for Neurochemistry) Meeting – 3rd Conference on Advances in Molecular Mechanisms of Neurological Disorders*, (J. o. Neurochemistry ed.), Vol. 101, pp. 56. blackwell publishing, 9600 garsington rd, oxford ox4 2dq, oxon, england, Salamanca
Spain.

Luna-Medina, R., Cortes-Canteli, M., Sanchez-Galiano, S., Morales-Garcia, J. A., Martinez, A., Santos, A. and Perez-Castillo, A. (2007b) NP031112, a thiadiazolidinone compound, prevents inflammation and neurodegeneration under excitotoxic conditions: potential therapeutic role in brain disorders. *The Journal of neuroscience,* **27,** 5766-5776.

Lynch, D. R. and Dawson, T. M. (1994) Secondary mechanisms in neuronal trauma. *Current opinion in neurology,* **7,** 510-516.

Martinez, A. (2008) Preclinical efficacy on GSK-3 inhibitors: Towards a future generation of powerful drugs. *Med Res Rev*.

Mehats, C., Andersen, C. B., Filopanti, M., Jin, S. L. and Conti, M. (2002) Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocrinol Metab,* **13,** 29-35.

Meldrum, B. S. (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr,* **130,** 1007S-1015S.

Miro, X., Perez-Torres, S., Palacios, J. M., Puigdomenech, P. and Mengod, G. (2001) Differential distribution of cAMP-specific phosphodiesterase 7A mRNA in rat brain and peripheral organs. *Synapse,* **40,** 201-214.

Mody, I. and MacDonald, J. F. (1995) NMDA receptor-dependent excitotoxicity: the role of intracellular Ca2+ release. *Trends Pharmacol Sci,* **16,** 356-359.

Morales-Garcia, J. A., Luna-Medina, R., Martinez, A., Santos, A. and Perez-Castillo, A. (2009) Anticonvulsant and neuroprotective effects of the novel calcium antagonist NP04634 on kainic acid-induced seizures in rats. *Journal of neuroscience research,* **87,** 3687-3696.

Morales-Garcia, J. A., Redondo, M., Alonso-Gil, S., Gil, C., Perez, C., Martinez, A., Santos, A. and Perez-Castillo, A. (2011) Phosphodiesterase 7 inhibition preserves dopaminergic neurons in cellular and rodent models of Parkinson disease. *PLoS One,* **6,** e17240.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods,* **65,** 55-63.

Nadler, J. V., Perry, B. W., Gentry, C. and Cotman, C. W. (1980) Loss and reacquisition of hippocampal synapses after selective destruction of CA3-CA4 afferents with kainic acid. *Brain Res,* **191,** 387-403.

Nakamura, T. and Lipton, S. A. (2010) Redox regulation of mitochondrial fission, protein misfolding, synaptic damage, and neuronal cell death: potential implications for Alzheimer's and Parkinson's diseases. *Apoptosis,* **15,** 1354-1363.

Nicotera, P., Leist, M. and Manzo, L. (1999) Neuronal cell death: a demise with different shapes. *Trends Pharmacol Sci,* **20,** 46-51.

Oprica, M., Eriksson, C. and Schultzberg, M. (2003) Inflammatory mechanisms associated with brain damage induced by kainic acid with special reference to the interleukin-1 system. *J Cell Mol Med,* **7,** 127-140.

Palomo, V., Perez, D. I., Perez, C. et al. (2012) 5-imino-1,2,4-thiadiazoles: first small molecules as substrate competitive inhibitors of glycogen synthase kinase 3. *Journal of Medicinal Chemistry*, **55**, 1645-1661

Park, C. H., Moon, Y., Shin, C. M. and Chung, J. H. (2010) Cyclic AMP suppresses matrix metalloproteinase-1 expression through inhibition of MAPK and GSK-3beta. *J Invest Dermatol,* **130,** 2049-2056.

Paterniti, I., Mazzon, E., Gil, C. et al. (2011) PDE 7 inhibitors: new potential drugs for the therapy of spinal cord injury. *PLoS One,* **6,** e15937.

Paxinos, G. a. W., C. (1998) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San-Diego.

Redondo, M., Zarruk, J. G., Ceballos, P. et al. (2012) Neuroprotective efficacy of quinazoline type phosphodiesterase 7 inhibitors in cellular cultures and experimental stroke model. *Eur J Med Chem,* **47,** 175-185.

Reyes-Irisarri, E., Perez-Torres, S. and Mengod, G. (2005) Neuronal expression of cAMP-specific phosphodiesterase 7B mRNA in the rat brain. *Neuroscience,* **132,** 1173-1185.

Sasaki, T., Kotera, J. and Omori, K. (2002) Novel alternative splice variants of rat phosphodiesterase 7B showing unique tissue-specific expression and phosphorylation. *Biochem J,* **361,** 211-220.

Schmued, L. C., Albertson, C. and Slikker, W., Jr. (1997) Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res,* **751,** 37-46.

Shetty, A. K. and Turner, D. A. (1996) Development of fetal hippocampal grafts in intact and lesioned hippocampus. *Prog Neurobiol,* **50,** 597-653.

Smith, S. J., Cieslinski, L. B., Newton, R., Donnelly, L. E., Fenwick, P. S., Nicholson, A. G., Barnes, P. J., Barnette, M. S. and Giembycz, M. A. (2004) Discovery of BRL 50481 [3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: in vitro studies in human monocytes, lung macrophages, and CD8+ T-lymphocytes. *Mol Pharmacol,* **66,** 1679-1689.

Sperk, G. (1994) Kainic acid seizures in the rat. *Prog Neurobiol,* **42,** 1-32.

Townsend, K. P. and Pratico, D. (2005) Novel therapeutic opportunities for Alzheimer's disease: focus on nonsteroidal anti-inflammatory drugs. *Faseb J,* **19,** 1592-1601.

Uryu, S., Harada, J., Hisamoto, M. and Oda, T. (2002) Troglitazone inhibits both post-glutamate neurotoxicity and low-potassium-induced apoptosis in cerebellar granule neurons. *Brain Res,* **924,** 229-236.

Volakakis, N., Kadkhodaei, B., Joodmardi, E., Wallis, K., Panman, L., Silvaggi, J., Spiegelman, B. M. and Perlmann, T. (2010) NR4A orphan nuclear receptors as mediators of CREB-dependent neuroprotection. *Proc Natl Acad Sci U S A,* **107,** 12317-12322.

Zhang, L., Murray, F., Zahno, A. et al. (2008) Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A,* **105,** 19532-19537.

Zhou, X., Zhou, J., Li, X., Guo, C., Fang, T. and Chen, Z. (2011) GSK-3beta inhibitors suppressed neuroinflammation in rat cortex by activating autophagy in ischemic brain injury. *Biochemical and biophysical research communications,* **411,** 271-275.

Figure legends

Figure 1. Chemical Structure of the 5-imino-1,2,4-thiadiazole VP1.14.

Figure 2. Neuroprotective effects of VP1.14 on KA-induced neuronal loss in the hippocampus. Rats were injected with vehicle, KA, or KA plus VP1.14 and the different groups were sacrificed 72 h post-injection. **(A)** Representative coronal sections stained with Nissl. Scale bars, 100 μm. **(B)** Quantification of the number of neurons stained by Nissl in the CA1 and CA3 regions of hippocampus. Values represent the mean ± SEM from at least five different animals and two different sections per animal. *** $p \leq 0.001$ *vs* KA injected rats.

Figure 3. VP1.14 protects hippocampal neurons from excitotoxic brain damage. Rats were injected with vehicle, KA, or KA plus VP1.14 and the different groups were sacrificed 72 h post-injection. Coronal sections were stained with anti-NeuN antibody **(A)** and Fluoro-Jade B **(B)**. Representative images are shown. Scale bars, 100 μm. Quantification of neurons stained with anti-NeuN specific antibody **(C)** and Fluoro-Jade B **(D)** in the CA1 and CA3 regions of hippocampus. Values represent the mean ± SEM from at least five different animals and two different sections per animal. * $p \leq 0.05$, ** $p \leq 0.01$ *vs* KA injected rats.

Figure 4. VP1.14 decreases glial activation induced by KA in the CA1 and CA3 regions of the hippocampus. Rats were injected with vehicle, KA, or KA plus VP1.14 and the different groups were sacrificed 72 h post-injection. Coronal sections were stained with mouse monoclonal antibodies directed against either GFAP **(A)** or CD11b **(B)** to detect astrocytes or microglial cells, respectively. Representative images are

shown. Scale bars, 100 μm. **(C, D)** Quantification of the number of reactive astrocytes and their immunostaining intensity. **(E)** Quantification of the number of reactive microglial cells. Values represent the mean ± SEM from at least five different animals and two different sections per animal. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ *vs* KA injected rats.

Figure 5. VP1.14 protects HT22 neuronal cultures and reduces nitrite production in astrocyte cultures after an excitotoxic or pro-inflammatory injury. **(A)** HT22 cells were treated for 24h with glutamate (3 mM) in presence or absence of VP1.14 (3 μM) or BRL-50481 (30 μM). Cell viability was measured by the MTT assay. Values represent the mean ± SEM of six replications in at least two different experiments. * $p \leq 0.05$ *vs* glutamate treated cells. **(B)** Nitrite production was measured by the Griess reaction in the supernatant of astrocytes that were treated for 24h with LPS (10 μg/ml) in the presence or absence of VP1.14 (3 μM) or BRL-50481 (30 μM). Values represent the mean ± SEM from six replications in at least two different experiments. * $p \leq 0.05$ *vs* LPS treated cells.

Figure 6. VP1.14 inhibits the expression of pro-inflammatory mediators in astrocyte cultures after LPS treatment. Rat primary astrocytes cultures were treated for 24 h with glutamate (500 μM) **(A)** or LPS (10 μg/ml) **(C)** in presence or absence of VP1.14 (3 μM) and BRL-50481 (30 μM). The expression of IL-6 and COX-2 were evaluated by immunofluorescence analysis and confocal microscopy using specific antibodies, as described in Materials and Methods. Representative confocal images are shown. Scale bar, 10 μm. Nuclei were counterstained with DAPI (blue). **(B, D)** Quantification of

labeled cells. Values represent the mean ± SEM of at least two different experiments * p ≤ 0.05, ** p ≤ 0.01 *vs* glutamate or LPS treated cells.

Figure 7. VP1.14 phosphorylates GSK-3 enzyme. **(A)** Representative Western blot showing an increase in p-GSK-3 in primary cultures of astrocytes treated with VP1.14 or VP1.14 plus H89 for 30 minutes. **(B)** Quantification analysis of three different blots. **p≤ 0.01 *vs* control cells, #p ≤ 0.05 *vs* VP1.14 treated cells.

Fig. 1

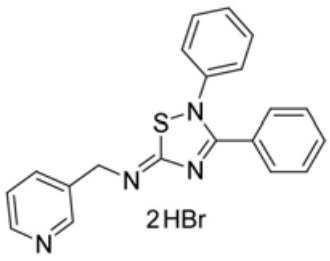


Fig. 2

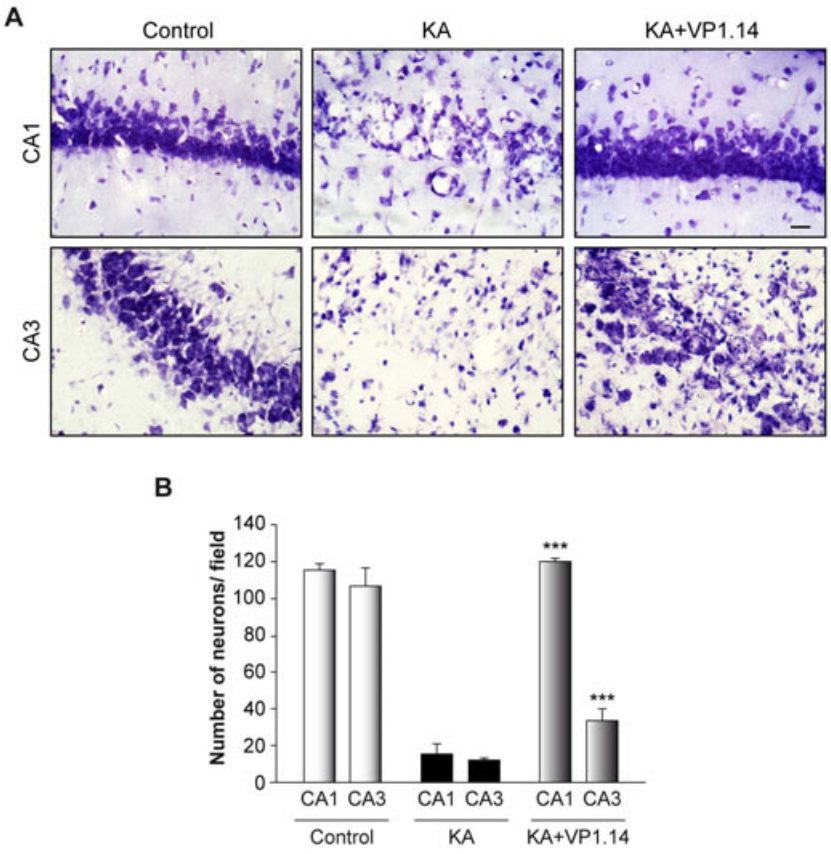
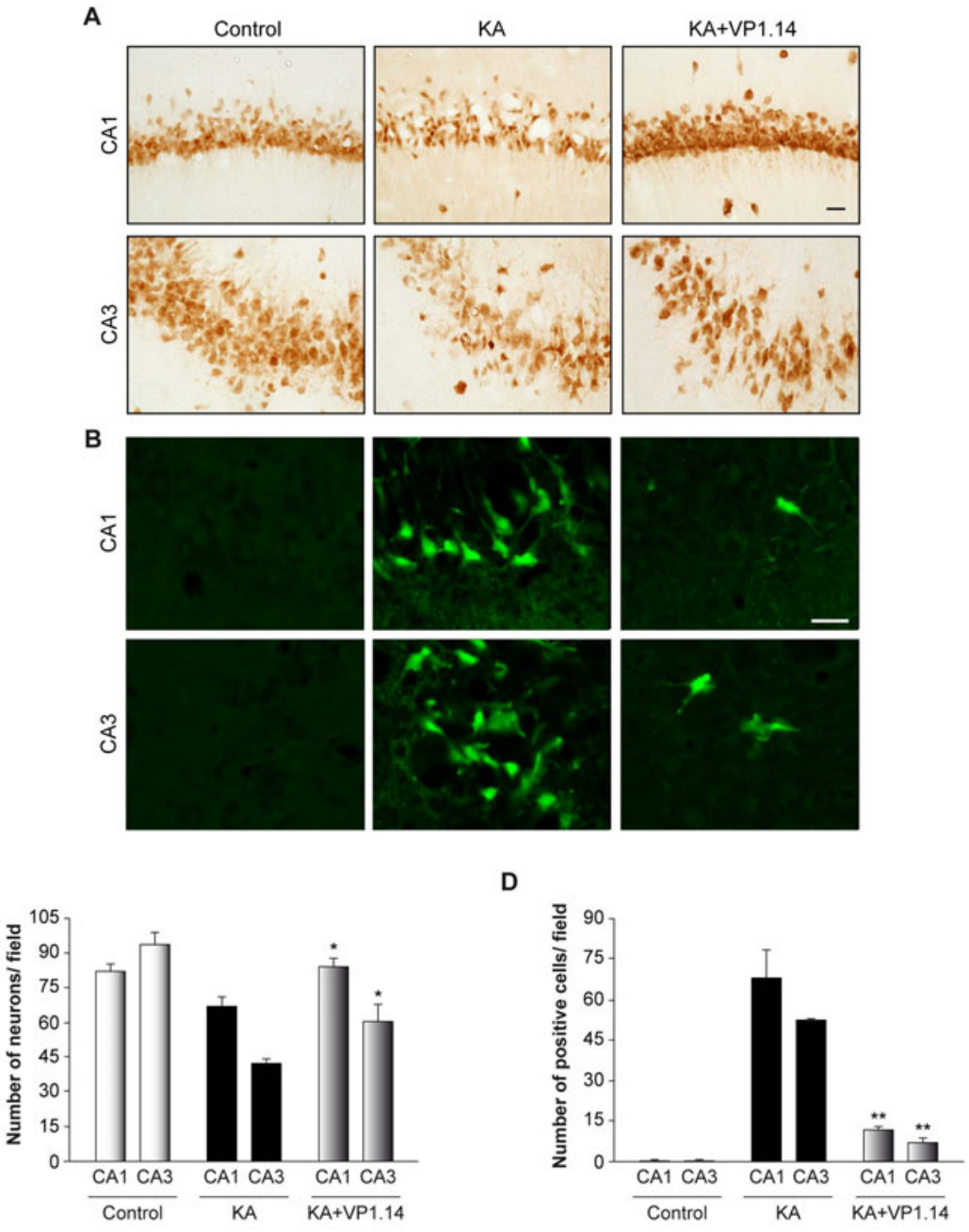


Fig. 3



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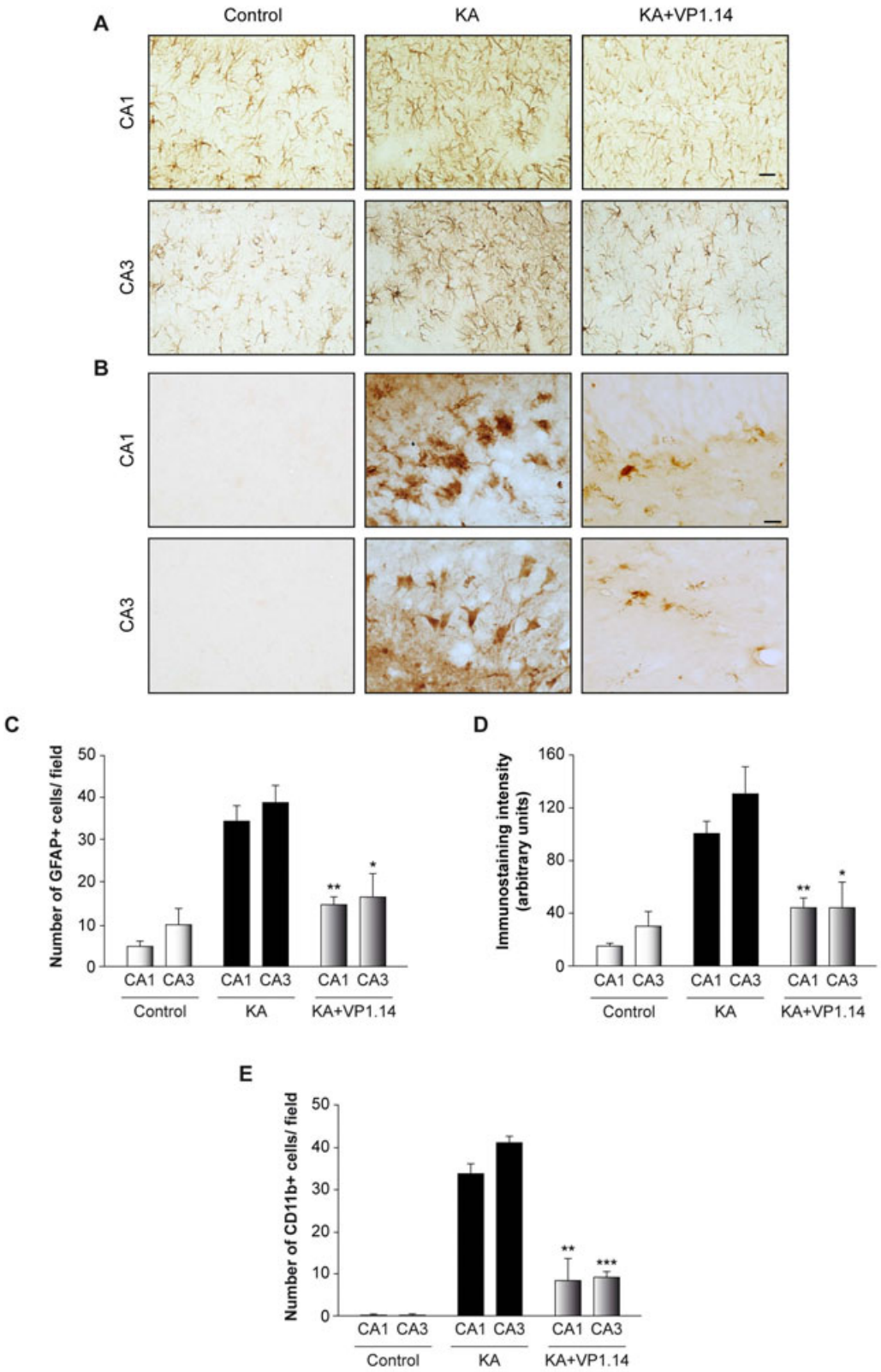

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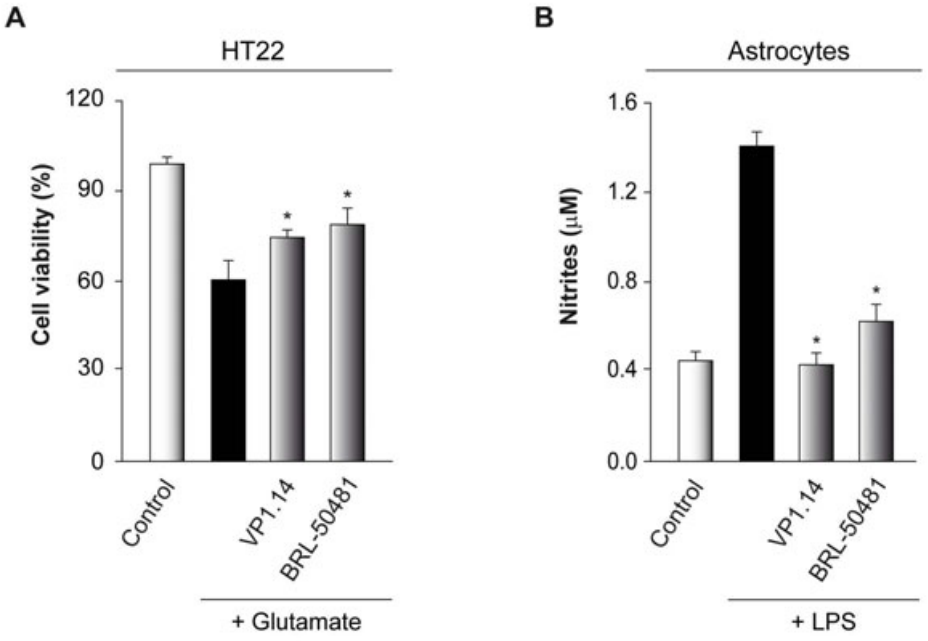
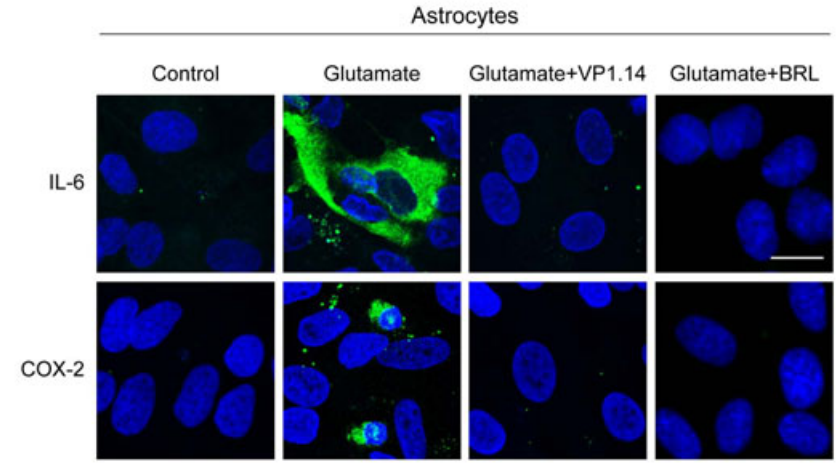


Fig. 6

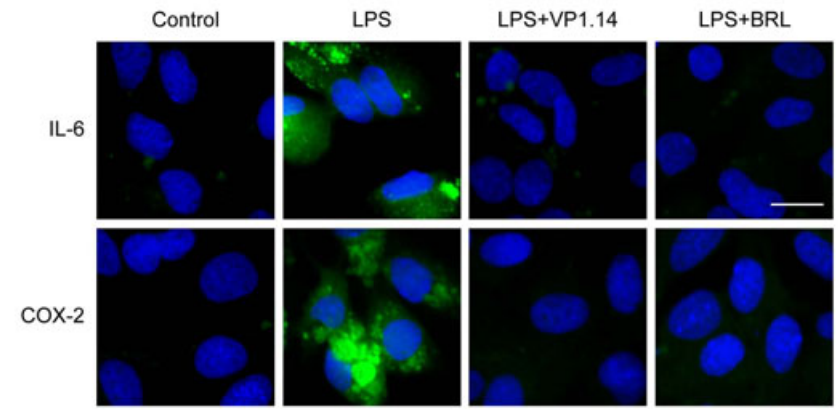
A



B Percentage of labeled cells

	Control	Glutamate	Glutamate+VP1.14	Glutamate+BRL
IL -6	9 ± 1**	47 ± 3	6 ± 1**	20 ± 0.5*
COX-2	13 ± 1**	53 ± 2	6 ± 3**	23 ± 7*

C



D Percentage of labeled cells

	Control	LPS	LPS+VP1.14	LPS+BRL
IL -6	11 ± 2**	74 ± 8	28 ± 13*	13 ± 3**
COX-2	4 ± 1**	55 ± 5	2 ± 1**	11 ± 1*

Fig. 7