Silencing Phosphodiesterase 7B gene by lentiviral-shRNA interference attenuates neurodegeneration and motor deficits in hemiparkinsonian mice.

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Abstract

Different studies have suggested that the nucleotide cyclic adenosine 3', 5'-monophosphate (cAMP) can actively play an important role as neuroprotective and anti-inflammatory agent after a brain injury. The phosphodiesterase 7 (PDE7) enzyme is one of the enzymes responsible for controlling specifically the intracellular levels of cAMP in the immune and central nervous systems. Therefore this enzyme could play an important role in brain inflammation and neurodegeneration. In this regard, using different chemical inhibitors of PDE7 we have demonstrated their neuroprotective and anti-inflammatory activity in different models of neurodegenerative disorders, including Parkinson disease (PD). In the present study, we have used the toxin 6-hydroxydopamine (6-OHDA) and lipopolysaccharide (LPS) to model PD and explore the protective effects of PDE7B deficiency in dopaminergic neurons cell death. Lentivirus-mediated PDE7B deprivation conferred marked in vitro and in vivo neuroprotection against 6-OHDA and LPS toxicity in dopaminergic neurons, and preserved motor function involving the dopamine system in mouse. Our results substantiate previous data and provide a validation of PDE7B enzyme as a valuable new target for therapeutic development in the treatment of PD.

Keywords: Inflammation, new target, oxidative stress, Parkinson Disease, PDE7B, shRNA.

1. Introduction

The cellular levels of cAMP areregulated by its formation by adenylcyclases and its hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). Eleven families of PDEs, comprising multiple isoforms, hydrolyze cAMP and cGMP and have unique regulatory characteristics, cellular distribution, and sensitivity to inhibitors (Bender and Beavo, 2006). Specifically PDE7 hydrolyzes cAMP and is highly expressed in endothelial cells, immune system, and brain (Lee, et al., 2002,Miro, et al., 2000,Miro, et al., 2001,Sasaki, et al., 2004,Wang, et al., 2000). PDE7 is encoded by two genes, PDE7A and PDE7B which generate, by alternative splicing, several isoforms.Within the brain, PDE7 mRNA has been found in the cerebellum, olfactory bulb, dentate gyrus of the hippocampus and striatum, being PDE7B much more abundant than PDE7A (Johansson, et al., 2012,Lakics, et al., 2010).Our group has been the first to show that PDE7B protein is markedly induced in the *Substantia Nigra pars compacta* (SNpc) of adult rodents after a brain insult(Morales-Garcia, et al., 2011). There is relatively little information regarding the physiological role of PDE7B. It has been shown that PDE7B is involved in pro-inflammatory processes, in this regard it has been demonstrated that this enzyme can be a target for the control of neuroinflammation (Giembycz and Smith, 2006). Additionally PDE7B is necessary for the induction of T-cell proliferation (Nakata, et al., 2002).

cAMP signaling pathway is involved in different functions in the central nervous system, such as cellular growth, neuronal proliferation,and long-term memory formation (Lonze and Ginty, 2002). This nucleotide has also been shown to be a neuroprotective agent in different brain disorders, including neurodegenerative diseases such as Huntington, Alzheimer, and Parkinson disease (PD) (Bollen and Prickaerts,

2012,Morales-Garcia, et al., 2011,Volakakis, et al., 2010). Given the important role of cAMP in the brain, specific inhibitors ofPDEs are being analyzed as possible therapeutic targets for the treatment of different brain diseases (Garcia-Osta, et al., 2012,Menniti, et al., 2006,Sharma, et al., 2013). During the last years, we have shown that different inhibitors of PDE7 are potent neuroprotective and anti-inflammatory agents in several animal models of neurodegenerative disorders, including PD (Castano, et al., 2009,Morales-Garcia, et al., 2011,Redondo, et al., 2012,Susin, et al., 2012).

Parkinson disease is a devastating neurodegenerative disorder and the second most common neurodegenerative disease after Alzheimer disease. PD is characterized by the loss of dopamine producing neurons (dopaminergic neurons) in a specific brain region, the ventral midbrain (Schapira, 2009). Although the selective loss of dopaminergic neuron within the SNpc is the pathological hallmark of this disease, cell loss also takes place in other brain areas such as the locus coeruleus, dorsal nuclei of the vagus, among others (Damier, et al., 1999). Besides these sites, the whole nervous system is affected by the neurodegenerative process, according to the model of Braak et al (Braak, et al., 2003), in many cases in a slow, progressive, ascending way. The impact of the whole nervous system justifies the many non-motor symptoms that often affect the patients in a similar or even greater way than the motor symptoms (Chaudhuri, et al., 2006). Because of this slowly progressive neurodegenerative process, PD is usually diagnosed when about at least 50% of dopaminergic neurons of the SNpc have degenerated and the others are already affected by the disease. So far, we have no treatments that prevent the development of PD or modify the detrimental course of the disease ("disease-modifying agents"). Therapeutic options for PD are based on dopamine replacement, which reduces motor symptoms and alleviates associated depression and pain(Obeso, et al., 2010). However this pharmacological approach only leads to temporary, limited

improvement of patient's quality of life, because chronic treatment with L-DOPA has severe side effects. Therefore, the discovery of new and improved therapies, based in the identification of new targets and novel drugs, remains a challenging issue for both Parkinson research community and pharmaceutical industry.

As commented above, our group has been the first to show a potent neuroprotective and anti-inflammatory effect of different PDE7 chemical inhibitors in different animal models of PD (Morales-Garcia, et al., 2011,Susin, et al., 2012) suggesting that this enzyme could be a promising new therapeutic target for the treatment of this devastating disorder. In this work, we show that lentiviral-mediated delivery of PDE7B shRNA into the SNpc of adult mice protected 80% of dopaminergic neurons and decreased glial activation after an intranigral injection of lipopolysaccharide (LPS) or 6-hydroxydopamine (6-OHDA). Moreover, specific PDE7Bdepletion provides substantial improvement of behavioral deficits. Our data reveal that targeting specifically PDE7B can efficiently rescue dopaminergic neurons damage and provide further validation of the role of PDE7B enzyme as a promising new therapeutic target in PD.

2. Materials and Methods

2.1. Construction of shRNA and cloning into lentiviral vectors.

To knockdown PDE7 expression, two shRNA target sequences against PDE7B were obtained from Ambion (Life Techonologies). The selected interfering sequences used were the following: 1) shPDE7-1: 5'-GGCACATGCTCTCCAAAGT-3' (from 468 to 488) and 2) shPDE7-2: 5'-CTGGAAATCAGTCCTCTTT-3' (from 1,220 to 1,240) (GeneBank: AF AF190639.1) Annealed oligonucleotides were cloned into pGreenPuroTMshRNA lentiviral vector (Cloning and Expression Lentivector; SBI, System Biosciences), according to the manufacturer's protocol, in which shRNA was expressed under the control of the H1 promoter. The control pGreenPuro™ construct with the Luciferase shRNA Template provided by System Biosciences was used as control in all the experiments. To obtain lentiviral particles, HEK-293T cells were transiently transfected with the appropriate lentiviral expression vectors and the third generation packaging vectors pMD2-G, pMDLg/pRRE, and pRSV-Rev. The medium containing lentiviruses was recovered after 36 hours post transfection, filtered through a 0.22-µm filter and concentrated by ultracentrifugation using a Beckman SW28 ultracentrifuge rotor for 2 hours at 20,000 r.p.m at 4ºC. The concentrated virus stocks were titered by flow citometry (FACS) on HEK-293T cells.

2.2. Viral infection of SH-SY5Y cells.

SH-SY5Y human neuroblastoma cells were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM L-Glutamine, and 5 mg/ml penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2. One day before infection, SH-SY5Y cells were seeded in 10-cm diameter tissue culture plates and used at approximately 70-80% confluence. Cells were

infected with shPDE7-1 or SH-PDE7-2 lentiviral particles, cultured in normal media and subjected to puromycin selection using a concentration of 2 μg/mL. Positively selected cells were grown in medium containing 2 μg/mL puromycin.

2.3. Immunoblot analysis.

Proteins were isolated from puromycin selected SH-SY5Y cell cultures by standard methods. Total protein extraction and western blot analysis was performed as previously described (Morales-Garcia, et al., 2011). Membranes were incubated overnight with a rabbit polyclonal specific anti-PDE7B antibody (1:1000; ProteinTech) with gently shaking at 4°C. The proteins were detected with the chemiluminescence detection system according to the manufacturer's instructions using a secondary peroxidase-conjugated donkey anti-rabbit antibody (Amersham Biosciences). α-tubulin was used as a loading control. The band intensity was quantified with a densitometric scanner and values expressed as the average of the quantification of at least five independent experiments.

2.4. Cell viability assay and nitrite measurement.

Transfected SH-SY5Y cultures, after puromycin selection, were seeded at a density of $3x10^4$ in 96-well plates and maintained on standard conditions. On attaining semiconfluence, cells were treated or not with 6-OHDA (35 μM, Sigma) for 16 h. Cell viability was measured using the MTT assay (Roche Diagnostic, GmbH), based on the ability of viable cells to reduce yellow MTT to blue formazan. The extent of reduction of MTT was quantified by absorbance measurement at 595 nm according to the manufacturer's protocol. Nitrite production was measured by Griess reaction. Briefly, culture supernatant was mixed with an equal volume of Griess reagent (Sigma-

Aldrich). Samples were then incubated at room temperature for 15 minutes and absorbance read using a plate reader at 492/540 nm. Each data point represents the mean ± s.d. of six replications in three different experiments.

2.5. Animals.

In vivo experiments were carried out with adult (2 to 3 month old) male C57BL/6 mice obtained from our breeding facilities (normative ES280790000188). The animal protocols used in this study, as well as the overall mouse husbandry practices, were approved by the respective institutional animal care and use committees at Consejo Superior de Investigaciones Cientificas, in accordance with 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.

2.6. Injection of lentiviral particles in vivo.

The animals (at least 12 mice in each group) were anaesthetized and placed in a stereotaxic apparatus (Kopf Instruments, CA). LPS (10 µg in 2.5 µl PBS) or 6-OHDA (5µg in 2.5 µl PBS containing 0.02% ascorbic acid) in combination with shPDE7-2 or control non-targeting shRNA lentiviral particles (1 x 10^6 IU/animal) were injected into the right side of the SNpc (coordinates from Bregma: posterior − 3.2 mm; lateral + 2.0 mm; ventral: +4.7 mm), according to the atlas of Paxinos and Franklin (Paxinos and Franklin, 2001). Animals injected only with non-targeting shRNA were used as controls throughout all the *in vivo* experiments. Mice were then housed to recover and sacrificed at 3, 7 and 45 days after lesioning.

2.7. Histology and Immunohistochemistry.

Brains were processed as previously described (Morales-Garcia, et al., 2011). Briefly, after perfusion of the animals with 4% paraformaldehyde, brains were removed, postfixed in the same solution at 4 °C overnight, cryoprotected in 30% sucrose, frozen, and finally 30 μm coronal sections were obtained using a cryostat. Brain sections were stained with cresyl violet (Nissl stain) or used for immunohistochemistry. Immunofluorescence analysis in brain sections were performed as previously described (Cortes-Canteli, et al., 2008) and the images were acquired using a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA). To compare fluorescence signals from different preparations, confocal microscope settings were fixed for all samples within the same analysis and adjusted to produce the optimum signal-to-noise ratio. For single immunofluorescence analysis (Figures 2, 3, 5 and 6) the antibodies used were: rabbit polyclonal anti-tyrosine-hydroxylase (TH) (1/200; Chemicon/Millipore) followed by a secondary Alexa-Fluor546 goat anti-rabbit (Temecula, CA), goat polyclonal anti-PDE7B (1/100; Santa Cruz Biotech, USA) followed by a secondary Alexa-Fluor546 horse anti-goat and rabbit polyclonal anti-serine 133 P-CREB (1/200; Cell Signaling) followed by a secondary Alexa-Fluor488 goat anti-rabbit. Triple immunofluorescences (Figures 8 and 9) to detect astroglial and microglial cells (together with TH) were performed in the same sections and the antibodies used were: rabbit polyclonal anti-TH (1/200; Chemicon/Millipore) followed by a secondary Alexa-Fluor488 goat anti-rabbit, mouse anti-glial fibrillary acidic protein(GFAP) (1/300; Sigma, Germany) followed by a secondary Alexa-Fluor 647 goat anti mouse, and microglial cells were stained with Texas Red (emission at 546) Lycopersiconesculentum (tomato-lectin; 1/300; Vector Labs. USA). All secondary antibodies were used at 1/500. Neuronal integrity and specifically the extension of dopaminergic cell death was performed by counting Nissl-

stained and TH-positive cells, respectively, in the SNpc in five well-defined high magnification (×400) fields per animal, using a computer-assisted image analysis software (Soft Imaging System Corp). The extent of gliosis was quantified as previously described (Morales-Garcia, et al., 2009).

2.8. Stereological cell counting.

In addition, dopaminergic neuronal loss was also estimated by using unbiased stereology according to the optical fractionator method (West, et al., 1991). This method of cell counting is not affected by either the volume of the reference (SNpc) or the size of the counted elements. The number of TH+ cells was counted in a 1:5 series of sections across the rostro-caudal extent of the SNpc after TH staining using the DAB method. The stereology system consists of an Olympus BX51 microscope fitted with a XZY motorized computer stage (Prior ProScan, Rockland, MA), a digital microcator (Heidenhain, Schaumburg, IL), and the CAST-2 Stereological software (Olympus, Tokyo). Neurons were counted twice with the observer blind to the experimental group. To avoid double counting of neurons, TH-stained cells were counted only when their nuclei were optimally visualized. The sampling was performed according to unbiased stereological principles using a 100x100 μm step length and a counting frame. The total positive cell number (N) will be estimated using the equation: N= sumQ- x 1/ssf x 1/asf x 1/tsf, where sumQ- is the total number of neurons counted with the fractionator, ssf is the section sampling fraction, asf is the sampling fraction area, and tsf is the sampling fraction thickness. The reliability of the sampling scheme will be calculated to confirm that the coefficient of error is <0.1 (Tapia-Gonzalez, et al., 2011,Tapia-Gonzalez, et al., 2013). Results were expressed as the percentage of TH-immunoreactive cells relative to the uninjected side.

2.9. Behavioral testing.

Apomorphine-induced rotational behavioral test was performed 4 weeks following LPS or 6-OHDA lesioning of the SNpc. Mice were given a subcutaneous injection of apomorphine at a dose of 0.5 mg/kg (Sigma), placed in individual glass bowls with a diameter of 20 cm and rotation was monitored for 40 min. Analysis of completed (360°) rotations was made offline and expressed as the number of contralateral net turns per minute. Mice showing more than six turns per minute were considered as severely lesioned (Grealish, et al., 2010).Three different experiments with at least 12 animals/experimental group were performed. Results were expressed as the number of contralateral turns per minute.

2.10. Statistics Analysis.

The SPSS statistical software package (version 20.0) for Windows (Chicago, IL) was used for the ANOVA analyses. Statistical comparisons for significance among different groups of animals were performed by ANOVA followed by Newman−Keuls' test for multiple comparisons. Student's t-test was used to analyze statistical differences between cells. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Neuroprotective and anti−inflammatory effects of PDE7B gene silencing in SH-SY5Y cultures.

The human dopaminergic neuronal cell line SH-SY5Y has been described as an ideal system for modeling *in vitro* the characteristics of dopaminergic neuron since they have many features of *Substantia Nigra* neurons (Takahashi, et al., 1994). To study the role of PDE7B gene silencing on dopaminergic neurons death and inflammation, we infected SH-SY5Y cultures with lentiviral particles containing two different sequences targeting PDE7B (shPDE7-1 and shPDE7-2), as described in Material and Methods. We first validated by Western blot the effectiveness of both sequences used. Figure 1A shows that infection of SH-SY5Y cells with shPDE7-2 led to a drastic reduction of the targeted enzyme (68% decrease in PDE7B protein levels). This reduction was much lower in those cultures infected with shPDE7-1 (24% decrease). We next evaluated the effect of the two lentiviral constructs, shPDE7-1 and shPDE7-2, on the cell death induced by 6-OHDA. The treatment with this toxin led to a 41% loss of cell viability, as measured by the MTT assay (Figure 1B). Cells infected with lentiviral particles containing a non-targeting shRNA were used as controls. As can be seen in Figure 1B, infection of SH-SY5Y cells with shPDE7-1 did not have any effect on the loss of cell viability after 6-OHDA treatment, which is in accordance with the low levels of interference obtained with this sequence. However, when cells were infected with shPDE7-2 lentiviral particles a significant neuroprotective effect was observed. These results suggest that PDE7B gene silencing protects the human dopaminergic neuronal cell line SH-SY5Y from 6-OHDA-induced cell death. Cytotoxicity induced by 6-OHDA is frequently accompanied by a pronounced inflammatory activity. Then, we next evaluated the potential anti-inflammatory effect of silencing the PDE7B gene by

measuring the production of nitrites in 6-OHDA-treated SH-SY5Y cells. Again, as happened with cell viability, shPDE7-2 significantly reversed the increase in nitrite production elicited by 6-OHDA treatment, whereas infection with shPDE7-1 did not have any significant effect (Figure 1C). These results demonstrate that silencing PDE7B gen results in a blockade of the 6-OHDA-induced cell death and the subsequent inflammatory process.

3.2. Injection of shPDE7B in the SNpc of adult mice significantly reduces PDE7B induction by LPS and 6-OHDA and increases CREB phosphorylation.

In view of the results obtained *in vitro* suggesting a neuroprotective and anti-inflammatory effect of shPDE7-2 on dopaminergic cells, we next examined the role of PDE7B gene silencingin two clinically relevant models of Parkinson disease (PD). First, we analyzed the efficacy of shPDE7-2, since this construct was the most efficient in the *in vitro* studies, on PDE7 protein levels in the SNpc. To this end, adult mice were injected into the SNpc with lentiviral particles containing a non-targeting shRNA (control) or shPDE7-2 in combination with LPS or 6-OHDA and the expression of PDE7B was evaluated at days 3, 7 and 45 after injury. We found low levels of PDE7B in SNpc in control mice (Figure 2). However, mice lesioned with LPS or 6-OHDArevealed an increase in PDE7B protein levels, compared to the control group. This increase is clearly observed 3 days after injury and maintained at 7 and 45 days. These results are consistent with those previously described by our group in a similar model (Morales-Garcia, et al., 2011). When LPS (Figure 2A) or 6-OHDA (Figure 2B) were injected together with shPDE7-2, a dramatic decrease in PDE7B induction in the SNpc was observed. These results are in agreement with our previous observations showing that chemical inhibition of PDE7 by direct administration of the PDE7

inhibitor S14significantly decreases the induction of PDE7 after LPS treatment (Morales-Garcia, et al., 2011).

Since as expected, PDE7B interference will increase cAMP levels, we next analyzed the phosphorylation of the cAMP response element-binding protein (CREB), a well-known target of the cAMP signaling pathway. Our results clearly show (Figure 3) that intranigral infection with shPDE7-2lentivirus causes a major and sustained increase in the levels of phospho-CREB. This increase was observed at 3, 7 and 45 days after viral infection; no increase was observed in animals infected with the non-targeting control virus or animals lesioned with LPS or 6-OHDA (Figure 3).

3.3. Intranigral delivery of shPDE7-2 lentivirus protects dopaminergic neurons from LPS- or 6-OHDA-induced neuronal death.

To assess the potential neuroprotective role of PDE7B gene silencing in these animal models, we first carried out a histological analysis to evaluate the extent of neuronal cell loss in the SNpc of the different groups of animals (Figure 4). Quantification of the cresyl-violet stained cells showed that LPS (Figure 4A) and 6-OHDA (Figure 4B) injection resulted in a significant reduction in the number of neurons in the SNpc, in comparison with control animals. RNAi-mediated inhibition of PDE7B expression by shPDE7-2 led to substantial neuroprotection, and no significant differences were observed between control non-lesioned animals and LPS-lesioned animals infected with shPDE7-2 (Figure 4). To confirm that the neuronal death observed using Nissl staining corresponded to dopaminergic neurons, we performed immunohistochemistry analysis using an antibody against tyrosine hydroxylase (TH), to specifically stain dopaminergic neurons in the SNpc and dopaminergic fibers in the *Striatum* (Figure 5 and 6). As expected, after LPS or 6-OHDA injection, a substantial loss of dopaminergic cells in the

SNpc was observed, as compared with control animals at all times analyzed (3, 7 and 45 days). In contrast, in animals injected with shPDE7-2 lentiviral particles together with LPS or 6-OHDA, no significant loss in dopaminergic cell number was observed. In accordance with these data, a parallel changes in TH staining were observed in the striatum (Figures 5, 6). Indeed, a marked reduction in the intensity of tyrosine hydroxylase innervation in the striatum was only observed in mice injected with LPS or 6-OHDA and the control lentivirus. Additionally, we have performed unbiased stereological determination of the number of TH+ neurons in the SNpc. The results obtained are shown in Figure 7. As can be observed LPS- or 6-OHDA lesion reduced the number of dopaminergic neurons at all times analyzed and shPDE7-2 injection resulted in a remarkable neuronal protection. Overall, these results indicate that PDE7B gene silencing has significant neuroprotective effects of dopaminergic cells in the SNpc and also in its dopaminergic fibers projecting to the striatum, and validate our previous results showing a strong prevention of dopaminergic injury by chemical PDE7 inhibitors.

3.4. Intranigral delivery of shPDE7-2 lentivirus significantly reduces LPS- and 6-OHDA-induced glial activation.

The loss of dopaminergic neurons in the SNpc as a consequence of hyperactivation of the surrounding glial cells has been widely described (Castano, et al., 1998,Herrera, et al., 2000,Iravani, et al., 2005,Qin, et al., 2007). Since one of the events that takes place in the SNpc after LPS and 6-OHDA injury is the activation of the glial cells and the subsequent release of pro-inflammatory factors in this area of the brain, which is in part responsible for the ongoing dopaminergic cell degeneration (Hunter, et al., 2009), we

next analyzed the expression of glial fibrillary acidic protein (GFAP), as a marker of astroglialcells (Figure 8), and tomato-lectin (Figure 9) to detect microglial activation. Concerning astrogliosis, resting astrocytes were detected around the SNpc of control animals. After LPS (Figure 8A) or 6-OHDA (Figure 8B) injury, a dramatic increase in GFAP immunoreactivity was detected in the same area. This increase in astrogliosis was absent after silencing of PDE7B, and the pattern of GFAP immunostaining in these animals was indistinguishable from that of control animals. Quantification of the data when LPS was used revealed a 54- and 63-fold decrease in both the number of strongly GFAP-positive astrocytes and staining intensity, respectively, in the shPDE7-2-injected mice, compared with the control group (Figure 8A). Similar results were obtained using the 6-OHDA animal model.6-OHDA-lesioned animals injected with shPDE7-2 presented a number of GFAP$^+$ cells and staining intensity quite similar to that observed in control mice (Figure 8B).

Microglial cells (identified as tomato lectin-positive cells) (Figure 9) were almost undetectable in control non-targeting shRNA-injected animals. However, after LPS (Figure 9A) or 6-OHDA (Figure 9B) injection, a high tomato lectin signal was clearly detected in the SNpc. As observed with astroglial activation, this strong microgliosis was completely abrogated in those animals injected with shPDE7-2 lentiviral particles. Altogether, these data reinforce our hypothesis that targeting PDE7B can ameliorates PD progression, not only by directly preserving dopaminergic cells, but also diminishing the subsequent harmful inflammatory process.

Finally, we studied the effect of PDE7 gene silencing on rotational behavior in mice, as a measurement of motor alterations induced by LPS- and 6-OHDA-injection (Figure 10). To this end, 45 days after lesion mice were injected with apomorphine, which is known to induce contralateral rotational behavior in denervated animals. A significant

increase in the number of contralateral turns per minute following apomorphine administration was detected in those animals lesioned with LPS (Figure 10A) or 6-OHDA (Figure 10B), compared with control mice. Remarkably, RNAi-mediated inhibition of PDE7B expression by shPDE7-2 led to substantial attenuation of the asymmetric motor behavior in both animal models of Parkinson´s disease.

4. Discussion

PD is characterized by the progressive loss of dopaminergic neurons and currently there are not effective therapies to stop this process. Treating PD is still one of the main unmet medical needs and currently available therapies may stabilize the symptoms for some years but they are not able to significantly modify the course of the disease. Dopamine replacement with levodopa treatment helps to alleviate some of the motor problems but dopamine-producing neurons continue to die and the neurodegenerative disorder gets worse (Obeso, et al., 2010). Therefore identification of new therapeutic targets which can slow or halt the progression of the disease is of upmost importance. Chemical genetic screens allow for the discovery of small molecules that provide lead compounds for novel therapies; however identification of the mechanistically relevant targets and their validation remain a major experimental challenge. Accordingly with our previous results showing a neuroprotective effect of PDE7 inhibitors in PD (Morales-Garcia, et al., 2011) here we found that RNAi-mediated depletion of PDE7B results in a neuroprotection of dopaminergic cells and attenuates glial activation in two different models of PD. These results point to PDE7B as a new valuable target for novel treatments of this disease.

The experimental system we have employed involves lentivirus-mediated gene transfer of a shRNA specific for PDE7B *in vitro* and in two *in vivo* animal models of PD. We initially analyzed the potential neuroprotective and anti-inflammatory effects of PDE7B depletion in the human dopaminergic cell line SH-SY5Y. Human neuroblastoma SH-SY5Y cells exposed to 6-OHDA are used as *in vitro* model for PD, due to the fact that similar cellular processes occur in the degenerating dopaminergic neurons (Ouyang and Shen, 2006). We show that infection with shRNA lentiviral particles resulted in a significant reduction in 6-OHDA-induced neuronal cell death and in a decrease in nitrite

liberation after LPS treatment. A clear difference was observed between shPDE7-1 and shPDE7-2 in PDE7B reduction and cell protection. The reason why Seq 1 was not very effective may relay in the fact that the structural character of siRNA molecules might be an important determinant of siRNA efficiency. For reasons that are unclear, some siRNAs work better tan others. This efficiency is largely determined by a number of factors. In particular it depends upon the siRNA nucleotide composition. Thus, whatever the reason, we continued our in vivo studies using shRNA-2 since it was the most efficient in the in vitro studies. These results are consistent with our previous study indicating that S14, an inhibitor of PDE7, eliminated 6-OHDA-induced cell death and nitrite production (Morales-Garcia, et al., 2011).

To evaluate the *in vivo* relevance of the abovementioned cellular effects, the anti-inflammatory and neuroprotective effects of direct administration of PDE7 shRNA lentiviral particles into the SNpc of adult mice were assayed in two classical rodent models of PD: unilateral stereotaxic injection of LPS (inflammatory model) or 6-OHDA (oxidative stress model) injection into the SNpc of adult mice. The treatment with LPS leads to a loss of dopaminergic cells and activation of astrocytes and microglial cells with the subsequent release of neurotoxic factors (Dutta, et al., 2008,Kim, et al., 2000,McCoy, et al., 2006). 6-OHDA injection into the SNpc of rodents causes the destruction of the nigrostriatal pathway, mediated by an oxidative stress with a result of a loss of dopaminergic input to the striatum (Blandini, et al., 2008,Deumens, et al., 2002,Kirik, et al., 1998). Here, we show that injection of lentivirus shRNA targeting PDE7B into the SNpc of adult rats lesioned with 6-OHDA or LPS rescued dopaminergic neurons from cell death and considerably reduces the associated neuroinflammation. Importantly, depletion of PDE7B levels by lentiviral delivery of PDE7 shRNA significantly ameliorates the motor impairment subsequent to

dopaminergic cell loss. Previous results from our group demonstrate that chemical inhibition of PDE7 trigger neuroprotection and diminish neuroinflammation in different models of brain diseases, including PD (Castano, et al., 2009,Morales-Garcia, et al., 2011,Redondo, et al., 2012,Susin, et al., 2012). Other authors have shown a neuroprotective effect of PDE inhibitors in neurodegenerative disease causing motor disorders (Sharma, et al., 2013).The cAMP phosphodiesterase4 (PDE4) has been also a target of study for therapy of motor disorders. Specifically PDE4 inhibitors have been shown to be neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) murine model of PD (Yang, et al., 2008) and in 3-nitropropionic acid (3-NP) induced experimental Huntington's disease in rats (Thakur, et al., 2013). However, and in contrast with PDE7, targeting PDE4 has been unsuccessful in clinical trials due to their side effects such as nausea and emesis.

The results presented here further demonstrate the feasibility of PDE7B as a potential therapeutic target to accomplish dopaminergic cell protection in PD, and represent a functional validation of this target. Importantly, our findings support the proposition that the PDE7 enzyme is important in dopaminergic cell death and identify this enzyme as a therapeutic target for amelioration of dopaminergic injury and motor deficits associated to PD. Since inflammation and oxidative stress play a central role in the pathogenesis of different brain disorders and neuronal injury (Barone and Feuerstein, 1999,del Zoppo, et al., 2000,Dutta, et al., 2008,Hanrott, et al., 2006,Hunter, et al., 2009,Kim, et al., 2000,Rodriguez-Pallares, et al., 2007) our results suggest that inhibition of PDE7B could be of therapeutic value not only in PD but also in those brain diseases where inflammation and oxidative processes are involved. In this regard, it is interesting to mention that human genetic studies have point to an important role of PDE7Bin schizophrenia (Torri, et al., 2010).

This neuroprotective effect of PDE7B silencing is also in accordance with previous finding showing that cAMP signaling pathway ameliorates cell death in several neurodegenerative disorders. There is evidence showing a clear involvement of protein kinase A in neuroprotection (Lee, et al., 2005,Stetler, et al.) and that activation of CREB by this kinase is necessary for neuronal survival and axonal growth in different neuronal populations (Lonze and Ginty, 2002).

Collectively, our findings clearly support the proposition that the PDE7B enzyme is important in dopaminergic cell death and identify this enzyme as a therapeutic target for amelioration of dopaminergic injury and motor deficits associated to PD. Our data provide compelling reasons to accelerate chemical research approaches to identify new drugs which selectively target PDE7B and could prevent the PD progression, avoiding the side-effects of the conventional treatments and slow down the progression of the disease.

Disclosure Statement

The content of this article represents an original scientific work. There are no conflicts of interest to declare.

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Figure legends

Figure 1. **Gene silencing of PDE7 has a neuroprotective and anti-inflammatory effect in SH-SY5Y cells.** Cells were infected with lentiviral particles containing a non-targeting shRNA (control) or with two shRNAs targeting PDE7B (shPDE7-1 and shPDE7-2). (**A**) PDE7Blevels were measured by Western blot 7 days after infection. Representative Western blot and quantification analysis are shown. Each data point represents the mean ±s.d. of five different experiments. ***$p \leq 0.001$, versus control. (**B**) and (**C**) SH-SY5Y infected cultures were exposed to 6-hydroxydopamine (6-OHDA, 35 μM) for 24h and cell viability and nitrite concentration determined, as indicated in Materials and Methods. Each data point represents the mean ± s.d. of six replications in three different experiments. *$p \leq 0.05$; ***$p \leq 0.001$, versus non-treated control cultures; ###$p \leq 0.001$, versus 6-OHDA-treated control cultures.

Figure 2. Injection of shPDE7-2 in the SNpc of adult mice significantly reduces PDE7B induction in *in vivo* models of Parkinson's disease. Mice were injected into the SNpc with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with (**A**) LPS (10 μg) or (**B**) 6-OHDA (5μg). After 3, 7 and 45 days animals were sacrificed and the brains processed for immunohistochemistry, as indicated in Material and Methods. Representative images of both hemispheres (contra- and ipsilateral) showing PDE7B expression (red) is shown. Nuclei were stained with DAPI. Scale bar, 100 μm.

Figure 3. Injection of shPDE7-2 lentiviral particles in the SNpc of adult mice induces the phosphorylation of CREB in *in vivo* models of Parkinson's disease. Mice were injected into the SNpc with lentiviral particles containing non-targeting

shRNA (control) or shPDE7-2 in combination with **(A)** LPS (10 μg) or **(B)** 6-OHDA (5 μg). After 3, 7 and 45 days brains were isolated and p-CREB immunodetected as indicated in Material and Methods. Representative images of ipsilateral coronal sections showing p-CREB expression (green) are shown. Nuclei were stained with DAPI. Scale bar, 100 μm.

Figure 4. Gene silencing of PDE7B preserves neuronal cell death after LPS- or 6-OHDA-induced toxicity. Mice were injected into the SNpc with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with **(A)** LPS(10 μg) or **(B)** 6-OHDA (5μg). After 45 days brains were removed and coronal sections processed for Nissl staining to label neurons. Scale bars, 500 μm. Quantification of the numbers of Nissl-stained cells in the SNpc, as described in Materials and Methods, is shown. Values represent the mean ± s.d. from three different experiments and five animals/experiment/experimental groups. ***p≤ 0.001, versus vehicle-injected control animals; $^{###}$p≤ 0.001, versus LPS-, or 6-OHDA-injected animals.

Figure 5. Gene silencing of PDE7B protects dopaminergic neurons of the SNpc and its striatal terminals against LPS- induced toxicity. Mice were injected into the SNpc with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in in combination with LPS(10 μg). After 3, 7 and 45 days brains were removed and coronal sections processed for tyrosine hydroxylase (TH) immunoreactivity to label dopaminergic neurons in the SNpc or dopaminergic striatal fibers density (red). Scale bar, 100 μm. Quantification of the numbers of TH-immunoreactive (THir) neurons in the SNpc, as described in Materials and Methods is shown. Values represent the mean ±

s.d. from three different experiments and five animals/experiment/experimental groups.[***]p≤ 0.001, versus vehicle-injected control animals; [###]p≤ 0.001, versus LPS-, or 6OHDA-injected animals.

Figure 6. Gene silencing of PDE7B protects dopaminergic neurons of the SNpc and its striatal terminals against 6-OHDA- induced toxicity. Mice were injected into the SNpc with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with 6-OHDA (5μg). After 3, 7 and 45 days brains were removed and coronal sections processed for tyrosine hydroxylase (TH) immunoreactivity to label dopaminergic neurons in the SNpc or dopaminergic striatal fibers density (red). Scale bar, 100 μm. Quantification of the numbers of TH-immunoreactive (THir) neurons in the SNpc, as described in Materials and Methods, is shown. Values represent the mean ± s.d. from three different experiments and five animals/experiment/experimental groups,[***]p≤ 0.001, versus vehicle-injected control animals; [###]p≤ 0.001, versus LPS-, or 6-OHDA-injected animals.

Figure 7. Gene silencing of PDE7B protects dopaminergic neurons of the SNpc against LPS- or 6-OHDA-induced toxicity: stereological analysis. Mice were injected into the SNpc with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with **(A)** LPS (10 μg) or **(B)** 6-OHDA (5μg). After 3, 7 and 45 days brains were removed and coronal sections processed for tyrosine hydroxylase (TH) immunoreactivity to label dopaminergic neurons using DAB staining. A representative image at 7 days after injection is shown. Scale bar, 500 μm. Quantification of the numbers of TH-immunoreactive (THir) neurons in the SNpc was performed using stereological methods, as described in Materials and Methods. Values

represent the mean ± s.d. from three different experiments and five animals/experiment/experimental groups. [***]p≤ 0.001, versus vehicle-injected control animals; [###]p≤ 0.001, versus LPS-, or 6-OHDA-injected animals.

Figure 8. Gene silencing of PDE7B significantly reduces astroglial activation in the SNpc after LPS or 6-OHDA injections. Immunostaining showing the expression of the astrogial marker GFAP (magenta) and tyrosine hydroxylase (TH, green) in the SNpc. Mice were injected with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with **(A)** LPS (10 μg) or **(B)** 6-OHDA (5μg). After 45 days animals were sacrificed and the brains processed as indicated in Materials and Methods. Representative images showing the expression of GFAP (magenta) and tyrosine hydroxylase (TH, green) are shown. Quantification of the numbers of GFAP^{+} cells and their immunostaining intensity in the SNpc, as described in Materials and Methods, is shown. Values represent the mean ± s.d. from three different experiments and five animals/experiment/experimental group. [***]p≤ 0.001, versus vehicle-injected control animals; [###]p≤ 0.001, versus LPS-, 6-OHDA-injected animals. Nuclei were stained with DAPI. Scale bar, 200 μm.

Figure 9. Gene silencing of PDE7B significantly reduces microglial activation in the SNpc after LPS or 6-OHDA injections. Immunostaining showing the expression of the microglial marker tomato lectin (red) and tyrosine hydroxylase (TH, green) in the SNpc. Mice were injected with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with **(A)** LPS (10 μg) or **(B)** 6-OHDA (5μg). After 45 days animals were sacrificed and the brains processed as indicated in Materials and Methods. Representative images showing the expression of tomato lectin (red) and

tyrosine hydroxylase (TH, green) are shown. Quantification of the numbers of reactive microglial cells, as described in Materials and Methods, is shown. Values represent the mean ± s.d. from three different experiments and five animals/experiment/experimental group. ***p≤ 0.001, versus vehicle-injected control animals; $^{###}$p≤ 0.001, versus LPS-, 6-OHDA-injected animals. Nuclei were stained with DAPI. Scale bar, 200 μm.

Figure 10. Effect of PDE7B silencing on rotational behavior in hemiparkinsonian mice. Mice were injected in the SNpc with lentiviral particles containing non-targeting shRNA (control) or shPDE7-2 in combination with vehicle, LPS (10μg) (**A**) or 6-OHDA (5μg) (**B**). Three weeks after injection apomorphine-induced rotations were analyzed, as indicated in Materials and Methods. Values represent the mean ± s.d. from three different experiments. At least 12 animals/experimental group were evaluated.***p≤ 0.001, versus vehicle-injected control animals; $^{###}$p≤ 0.001, versus LPS- or 6-OHDA-injected animals.

All the authors want to declare that:

1) The manuscript is original and is not under consideration elsewhere. None of the manuscript content has been previously published and has not been submitted elsewhere while under consideration at Neurobiology of Aging.

2) All authors have read and approved all versions of the manuscript, its content, and its submission to *Neurobiology of aging*.

3) There are no conflicts of interest to declare.

4) The animal protocols used in this study, as well as the overall mouse husbandry practices, were approved by the respective institutional animal care and use committees at Consejo Superior de Investigaciones Cientificas, in accordance with national (normative 1201/2005) and international recommendations (normative 86/609 from the European Communities Council).

FIGURE 1

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A
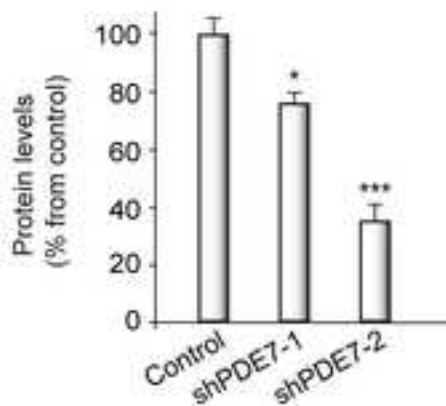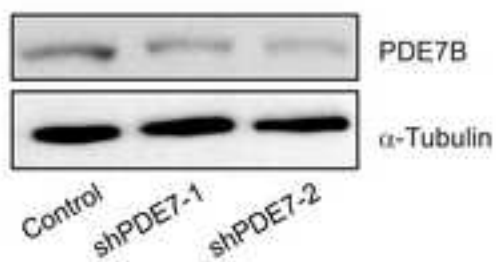
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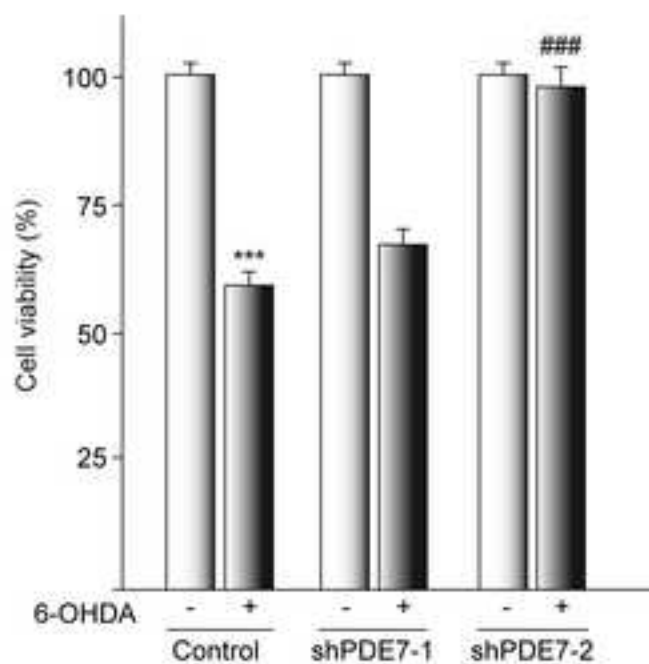
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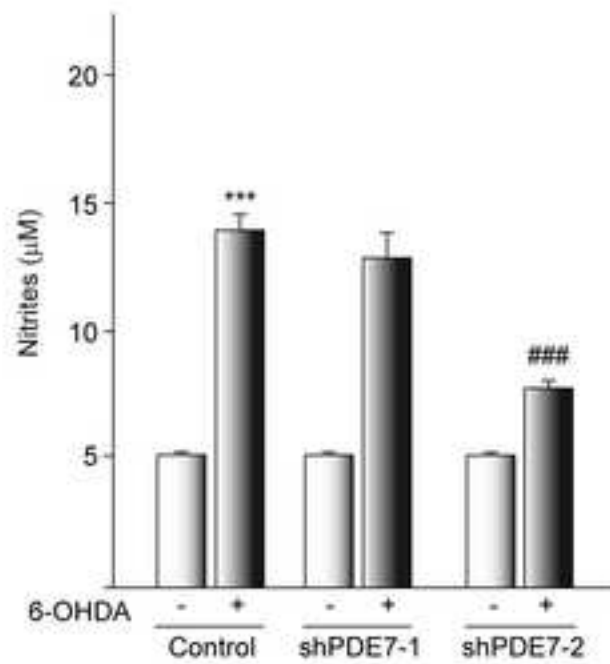
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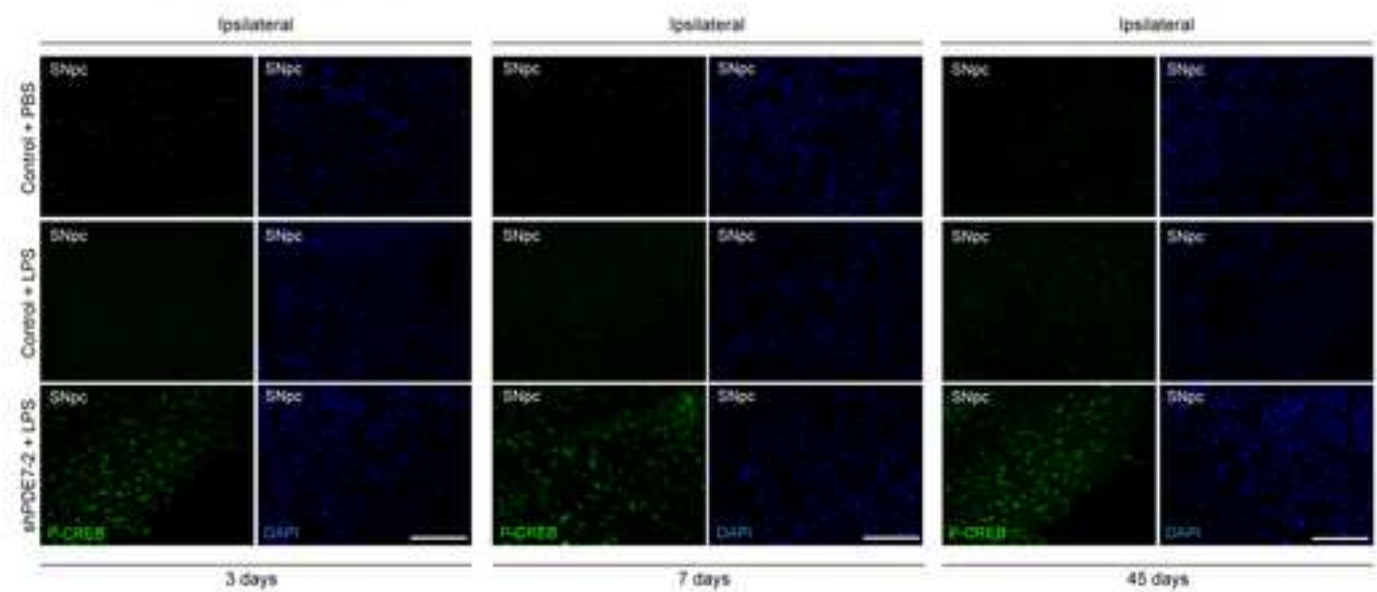
Morales-Garcia et al.

A Inflammatory animal model (LPS-injection)
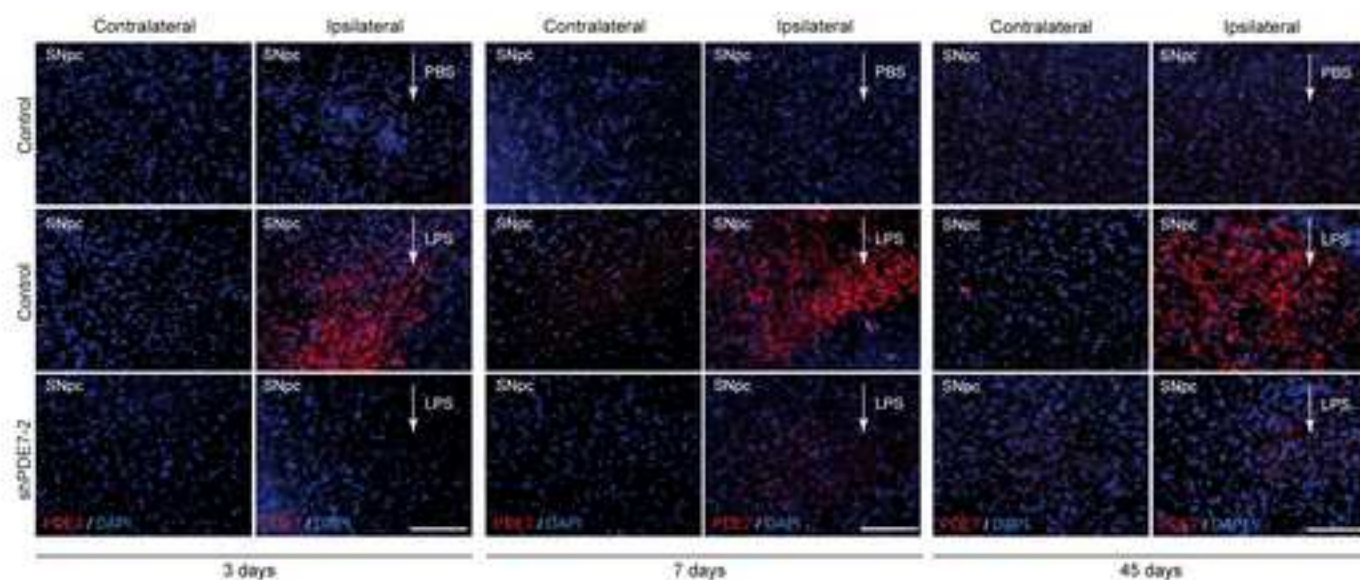
B Oxidative stress animal model (6-OHDA-injection)
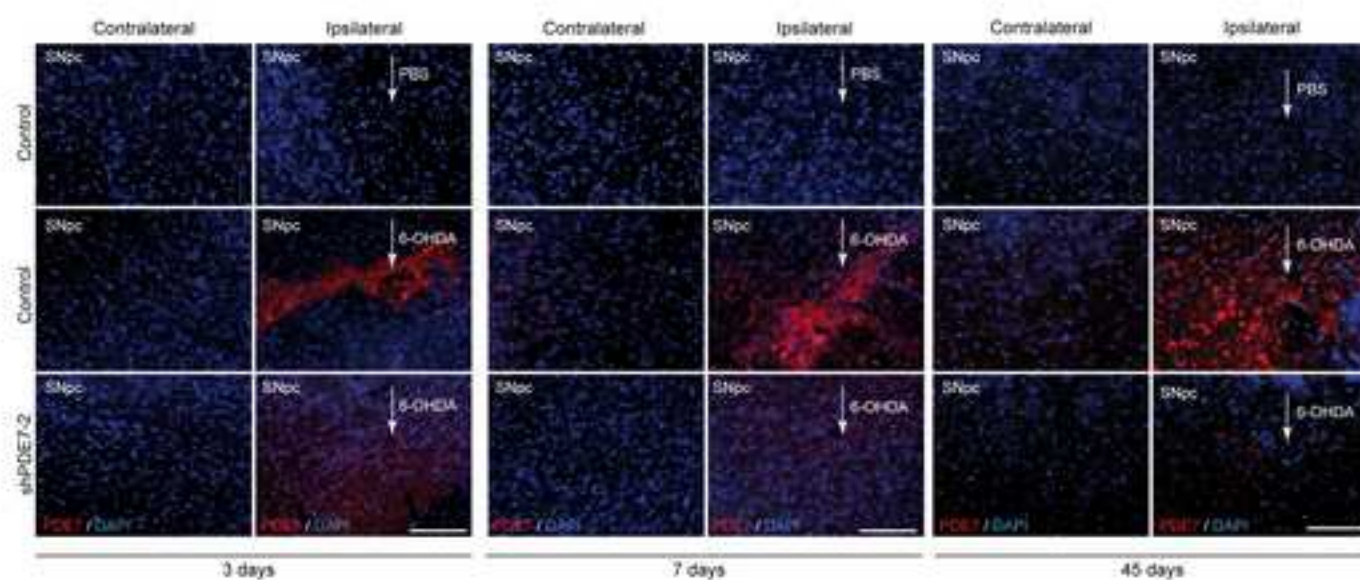
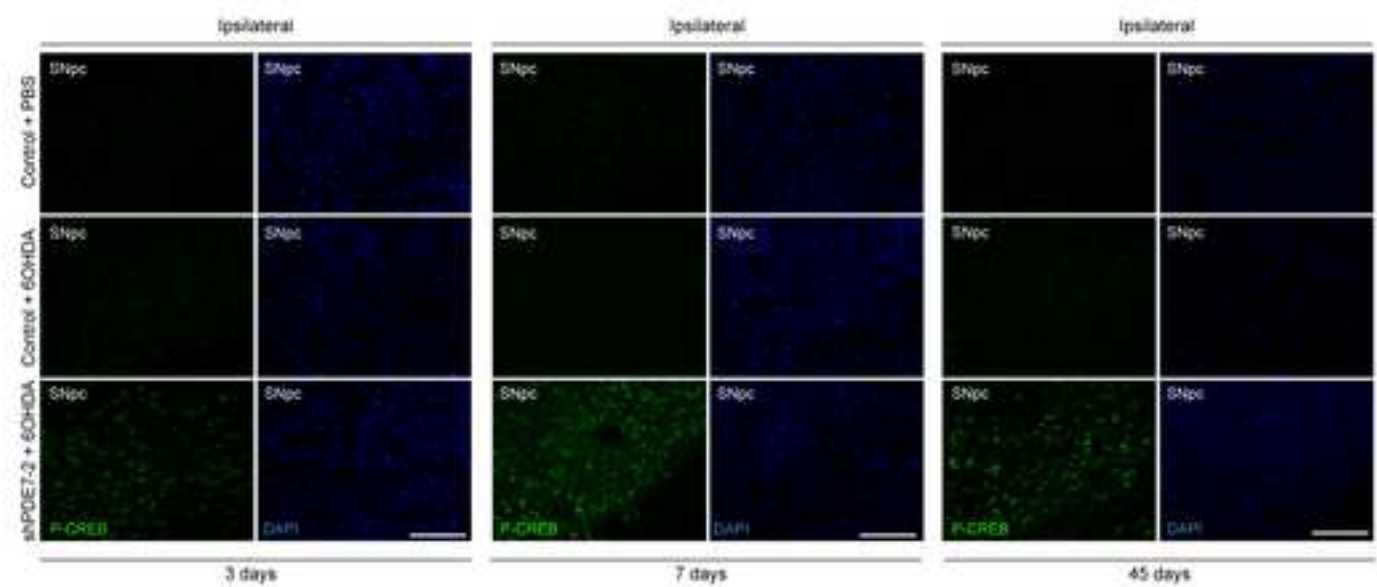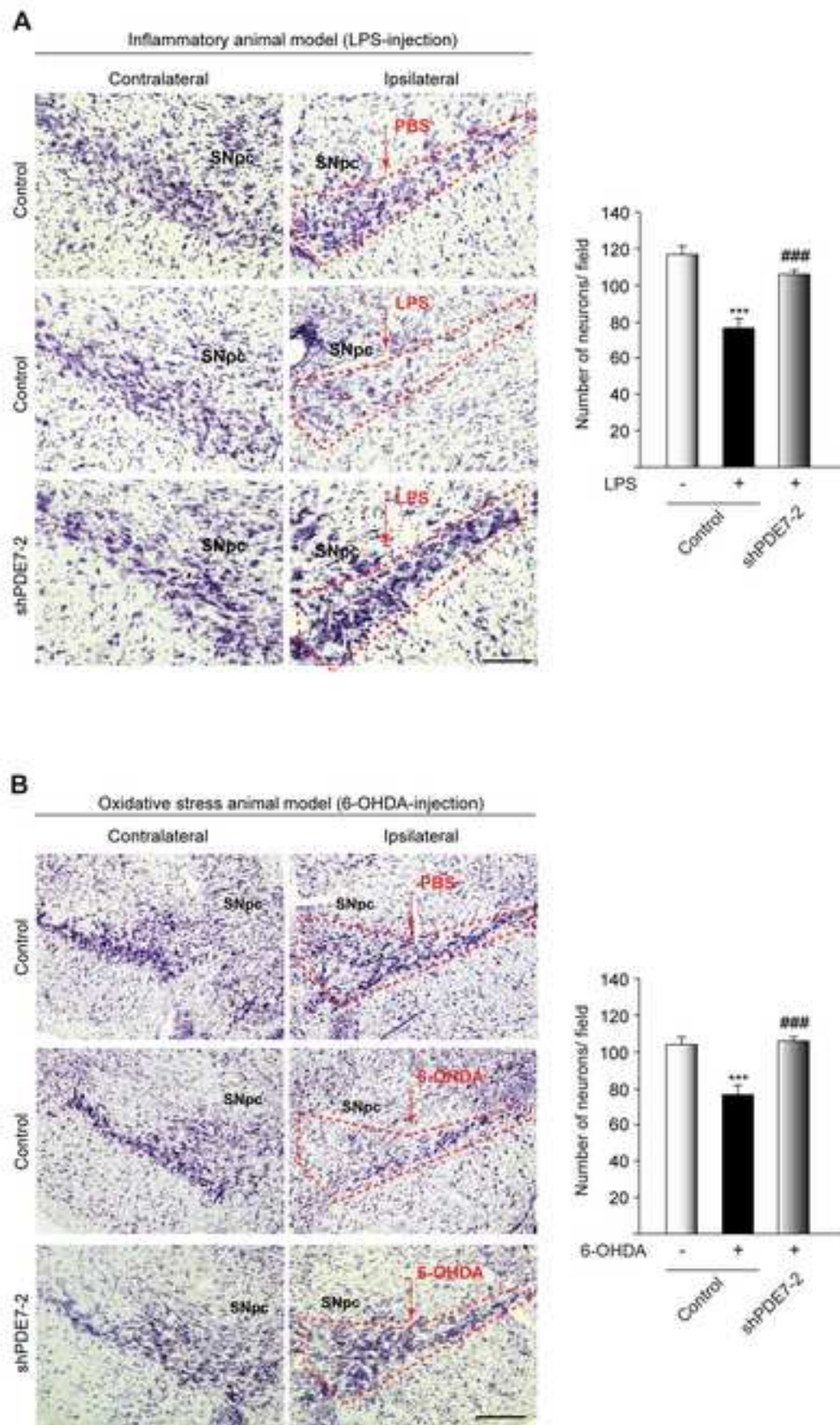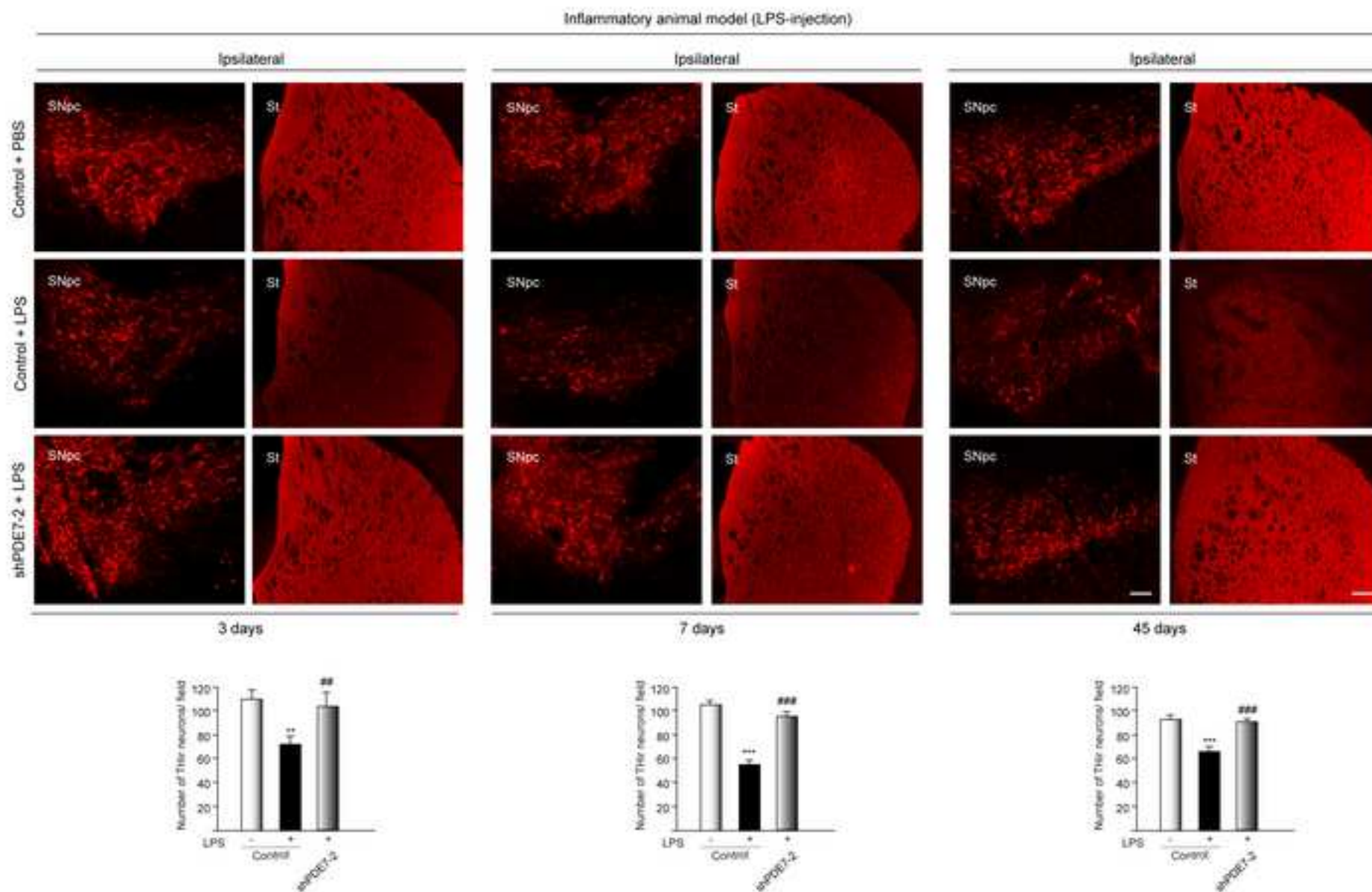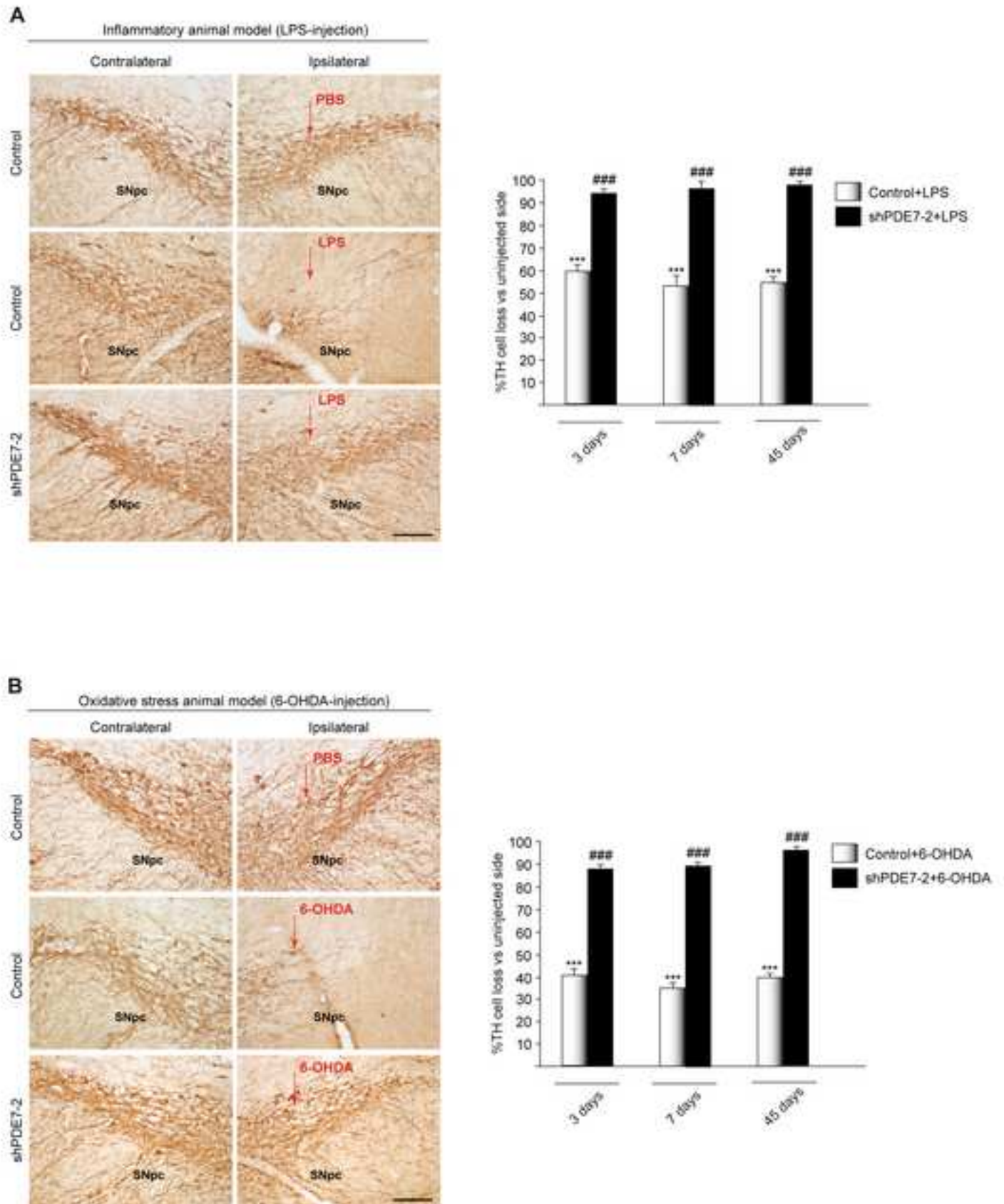FIGURE 3   Morales-Garcia et al.

A Inflammatory animal model (LPS-injection)

FIGURE 4

Morales-Garcia et al.

A — Inflammatory animal model (LPS-injection)

B — Oxidative stress animal model (6-OHDA-injection)

FIGURE 5

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Inflammatory animal model (LPS-injection)

FIGURE 6

Morales-Garcia et al.

FIGURE 7

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FIGURE 8   Morales-García et al.
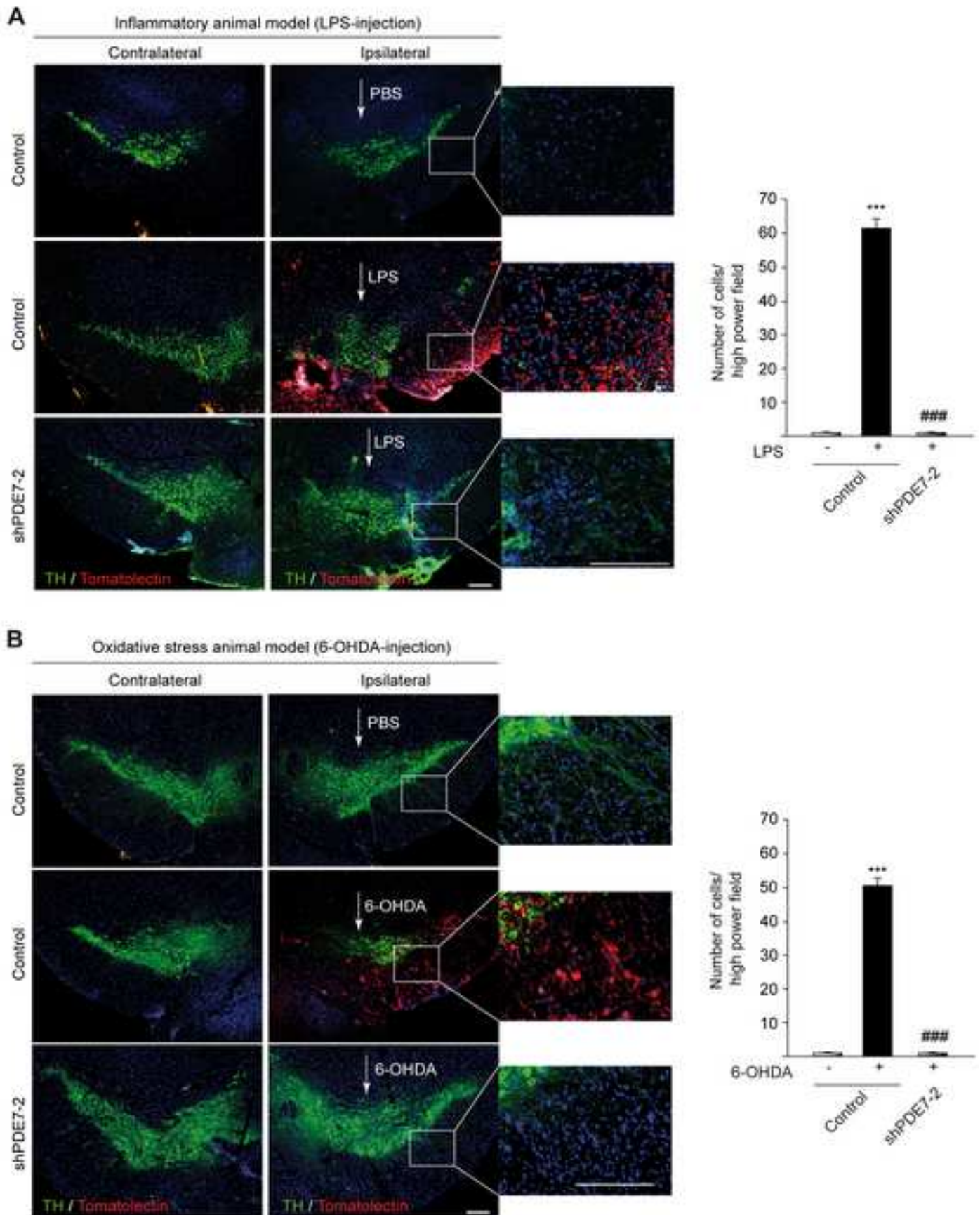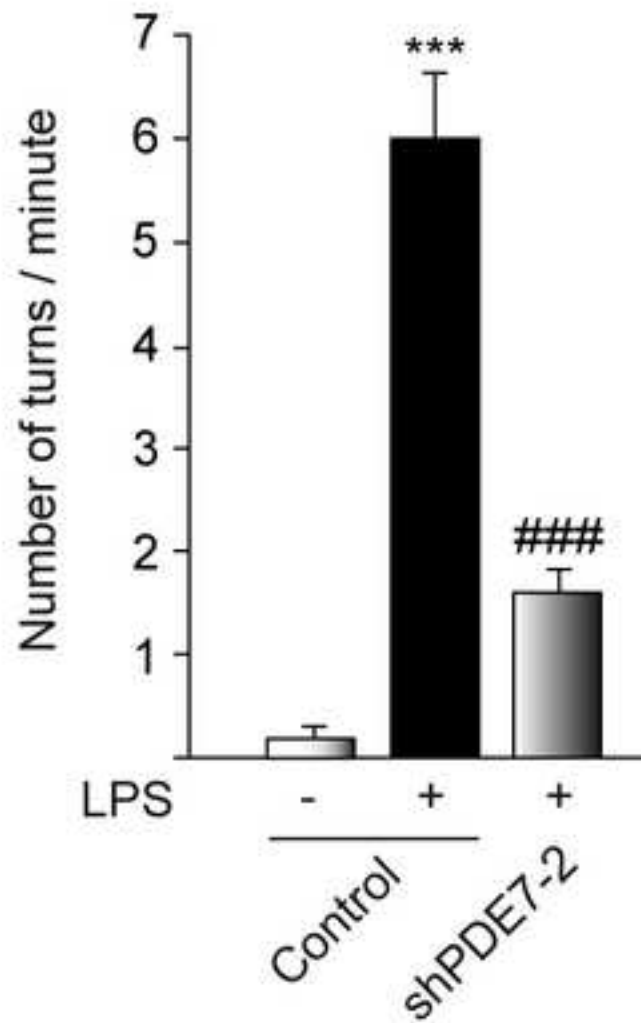
FIGURE 9
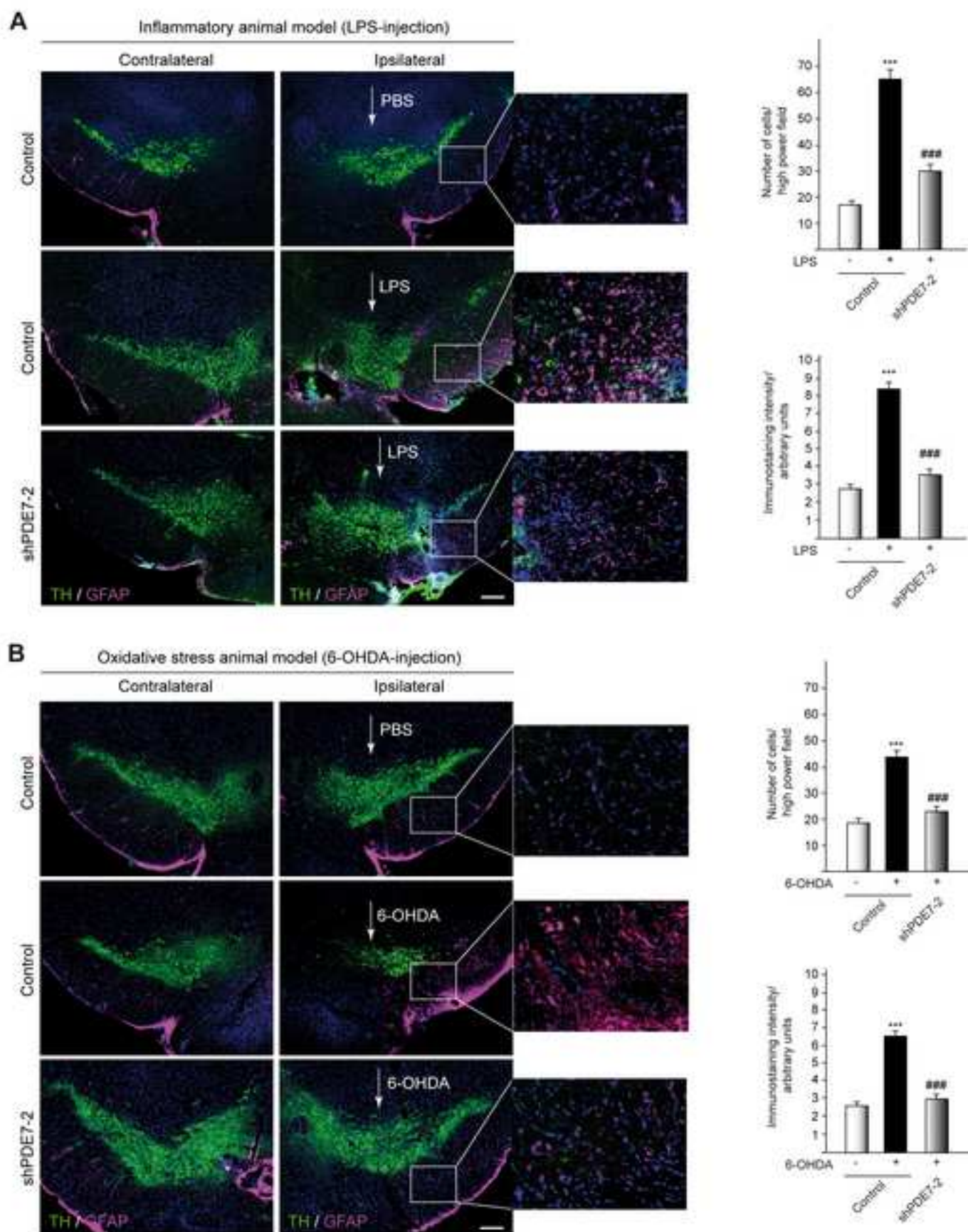
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FIGURE 10

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A

B