Running Title: PDE7 inhibition brings dopaminergic neurogenesis

Phosphodiesterase 7 inhibition induces dopaminergic neurogenesis in hemiparkinsonian rats.

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Abstract

Parkinson’s disease is characterized by a loss of dopaminergic neurons in a specific brain region, the ventral midbrain. Parkinson’s disease is diagnosed when about 50% of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) have degenerated and the others are already affected by the disease. Thus, it is conceivable that all therapeutic strategies, aimed at neuroprotection, start too late. Therefore there is an urgent medical need to discover new pharmacological targets and novel drugs with disease-modifying properties. In this regard, modulation of endogenous adult neurogenesis towards a dopaminergic phenotype may provide a new strategy to target Parkinson’s disease by partially ameliorating the dopaminergic cell loss that takes place in this disorder. We have previously shown that a PDE7 inhibitor called S14 exerts potent neuroprotective and anti-inflammatory effects in different rodent models of Parkinson’s disease indicating that this compound could represent a novel therapeutic agent to stop the dopaminergic cell loss that takes place during the progression of the disease. In this paper we show that, in addition to its neuroprotective effect, the PDE7 inhibitor S14 is also able to induce endogenous neuroregenerative processes towards a dopaminergic phenotype. We here describe a population of actively dividing cells, which give rise to new neurons in the SNpc of hemiparkinsonian rats after treatment with S14. In summary, our data identify S14 as a novel regulator of dopaminergic neuron generation.
Introduction

Parkinson’s disease (PD) is a chronic and progressive neurodegenerative disorder characterized by motor symptoms (muscular rigidity, resting tremor and bradykinesia or slowness of movement), and non-motor symptoms (visual hallucinations and dementia) [1]. The hallmark of PD is the gradual loss of dopamine-producing neurons, dopaminergic neurons, in a specific brain region, the ventral midbrain [2]. Although the selective loss of dopaminergic neuron within the substantia nigra pars compacta (SNpc) is the pathological characteristic of this disease, cell loss also takes place in other brain areas such as the locus coeruleus and dorsal nuclei of the vagus, among others [3]. The underlying cause of dopaminergic cell death and the mechanisms implicated remain elusive. Due to the slowly progressive way of this neurodegenerative process [4], PD is usually diagnosed when more than 50% of dopaminergic neurons of the SNpc have already degenerated and the others are affected by the disease. Since, so far, there are not treatments available that prevent the development of PD or modify its detrimental course (“disease-modifying agents”), and the ones used are only palliative and only leads to temporary improvement of the symptoms, there is an urgent medical need to discover new therapeutic strategies for PD which could slow, halt, or reverse the disease process.

Phosphodiesterases (PDEs) comprise a family of 21 members, which have been so far classified into 11 groups, according to their sequence homology, cellular distribution, and sensitivity to different PDE inhibitors [5, 6], being some of them expressed in the central nervous system [7]. PDE7 is a cAMP-specific PDE [5, 8] and it has been recently demonstrated that can be a target for the control of neuroinflammation [9]. PDE7 inhibition has recently emerged as a good therapeutic option for the treatment of
different neurodegenerative diseases. Several studies from our group have shown that different inhibitors of PDE7 are potent neuroprotective and anti-inflammatory agents in some animal models of neurodegenerative disorders, including PD [10-13]. Very recent data from our group show that PDE7 depletion in the SNpc, using specific shRNAs for PDE7, significantly protects dopaminergic neurons and improves motor function in LPS and 6-OHDA lesioned mice [14].

The ability of the adult central nervous system to produce new neurons is limited, rendering the brain particularly vulnerable to injury and disease. In mammals, the majority of neurons are born by the prenatal period, but it is well established that neurons continue to arise in two niches of the adult brain, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus [15, 16]. The generation of new neurons in these regions may contribute to endogenous repair mechanisms after brain damage and/or chronic disease [17]. It is known that dopamine regulates adult neurogenesis in the SVZ and hippocampus in rodents and human, and a decrease in new stem cell proliferation has been described in PD (review in:[18]). It has been shown a decreased proliferation of neural stem cells (NSCs) in the subventricular zone of human PD brains, and in two animal models of PD: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) [19-21], as a consequence of dopamine depletion caused by the loss of dopaminergic neurons in the SNpc. Consequently various therapeutic approaches, besides the classical use of dopaminergic agents, are now aimed at manipulating the resident stem/progenitor cells to produce neuroblasts, which could eventually differentiate into dopaminergic cells. The potential of this strategy, inducing endogenous neurogenesis by means of new therapeutic agents, holds great promise
since this increased neurogenesis could help to replace the dopaminergic cells lost in PD, which is already present by the time a patient is diagnosed.

The aim of the present study was to investigate the effects of the PDE7 inhibitor S14, on adult neurogenesis in the 6-OHDA model of PD mimicking a severe dopaminergic striatal deficit. Here, we show that pharmacological manipulation of PDE7 in vivo induces a strong neurogenesis in the SNpc of 6-OHDA-lesioned animals towards a dopaminergic phenotype. In vitro, PDE7 inhibition increased the neuronal differentiation of neurospheres obtained either from embryonic ventral midbrain or adult SVZ. Then, by using PDE7 inhibitors, we could potentially contribute to up-regulate endogenous neurogenesis, and/or favor integration of new dopaminergic neurons to stimulate neurorepair in PD.
Materials and Methods.

Animals. Adult male Wistar rats (8–12 weeks old) were used in this study. All procedures with animals were specifically approved by the “Ethics Committee for Animal Experimentation” of the Instituto de Investigaciones Biomedicas (CSIC-UAM) 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). Adequate measures were taken to minimize pain or discomfort of animals.

Embryonic mesencephalic precursor isolation. Cultures were derived from the ventral mesencephalon (VM) of rat embryos at embryonic day 14 as previously described [10]. Briefly, ventral mesencephalon was isolated in ice-cold Hank's Balanced Salt Solution (HBSS) medium Ca\(^{2+}\) and Mg\(^{2+}\) free, washed several times and tissue digested 15 min at 37 °C in trypsin-EDTA + DNase (0.05%). VM was then gently minced and triturated with a micropipette and supernatant collected and centrifuged at 1200 xg/5 min. The pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM)/Hamks-F12 (1:1) containing 0.5mM glutamine, 200 U/mL penicillin and 200 µg/mL streptomycin (Gibco), 1% fungizone, 10 ng/mL epidermal growth factor (EGF, Peprotech, London, UK), 10 ng/mL fibroblast growth factor (FGF, Peprotech) and 1x B27 (Gibco). Cells were seeded onto 12-well plates (~40,000 cells per cm\(^2\)).

Adult SVZ precursor isolation. Primary precursor cultures were prepared from the subventricular zone (SVZ) of the lateral ventricle of adult Wistar rats as previously described [22]. Briefly, SVZ was microdissected, isolated and dissociated in DMEM (Invitrogen) containing glutamine, gentamicin and fungizone. After 15 min digestion at
37 °C in trypsin-EDTA, hialuronidase and DNAse, myelin was removed by using DPBS (Invitrogen). Precursor cells isolated were seeded into 12-well dishes at a density of ~40,000 cells per cm² in DMEM/F12 (1:1, Invitrogen) containing 10 ng/mL EGF, 10 ng/mL FGF and N2 medium (Gibco).

Neurospheres culture and treatments. After 3 days in culture, neural stem cells formed spherical cellular aggregates known as neurospheres (NS). At this moment we started to treat free-floating NS with BRL-50481 (30 µM, Tocris), S14 (10 µM) or vehicle during 7 days. The quinazoline S14 was synthesized following described procedures [23]. The effective dose of compounds was determined based on previous studies [10]. For measurements of growth and proliferation, NS were counted and their size was analyzed using the Nikon Digital Sight, SD-L1 software (Nikon, Japan). Number and diameter of neurospheres were then scored. In each experiment, six to eight wells per condition were tested and counted and the radius of 50 neurospheres determined. For cell differentiation studies, free-floating NS cultured during 7 days in the presence or not of the PDE7 inhibitors BRL-50481 or S14, were seeded onto poly-L-lysine (Sigma) precoated 6-well plates and/or coverslips. Seeded NS were further cultured for 3 days in the absence of exogenous growth factors in medium containing 1% fetal bovine serum and in the presence or absence of BRL-50481 or S14.

Immunoblot analysis. Proteins were isolated from cell cultures by standard methods. Briefly, cells were resuspended in ice-cold cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (Roche) and incubated for 15 to 30 min on ice. A total amount of 30 µg of protein was loaded on a 10% or 12% SDS-PAGE gel and transferred nitrocellulose membranes (Protran, Whatman). The membranes were
blocked in Tris-buffered saline with 0.05% Tween-20 and 5% skimmed milk, incubated with primary and secondary antibodies, and washed according to standard procedures. Primary antibodies were PDE7A (rabbit; Santa Cruz Biotech.), PDE7B (rabbit; Proteintech), p-CREB (rabbit; Cell Signaling), CREB (rabbit; Cell Signaling), Musashi1 (rabbit; Abcam), β-II-tubulin (Tuj1 clone, rabbit; Abcam), MAP-2 (mouse; Sigma), Nurr1 (rabbit; Sta. Cruz), tyrosine hydroxylase (rabbit; Millipore) and α-tubulin (mouse; Sigma). Secondary peroxidase-conjugated antibodies were: donkey anti-rabbit (Amersham Biosciences, GE Healthcare), rabbit anti-goat and rabbit anti-mouse antibodies (Jackson Immunoresearch). Values in figures are the average of the quantification of at least three independent experiments corresponding to three different samples.

**Immunocytochemistry.** NS cultures were examined immunocytochemically as previously described [24]. Briefly, after 1 h incubation with the corresponding primary antibody, cells were washed with PBS and incubated with the appropriate AlexaFluor-labeled secondary antibody (AlexaFluor-488 or AlexaFluor-647; Molecular Probes; Leiden, The Netherlands) for 45 min at 37 °C. Later on, images were obtained using a LSM710 laser scanning spectral confocal microscope (Zeiss). Confocal microscope settings were adjusted to produce the optimum signal-to-noise ratio. Primary antibodies were directed against the following: β-III-tubulin (TuJ1 clone; rabbit; Abcam), MAP-2 (mouse; Sigma), Nurr1 (rabbit; Sta. Cruz) and tyrosine hydroxylase (rabbit; Millipore). Staining of nuclei was performed using 4′,6-Diamidino-2-phenylindole (DAPI).

**Neurogenic studies in vivo.** To determine the neurogenic effects of S14 in a model of dopaminergic cell loss, the animals were properly anesthetized and placed in a
stereotaxic apparatus (Kopf Instruments, CA). Rats (n=5/group) were injected into the right side of the striatum with 6-OHDA (9 µg in 2.5 µL PBS containing 0.02% ascorbic acid) based on the following coordinates: from Bregma, posterior −3.0 mm; lateral +1.0 mm; ventral +5.0 mm, according to the atlas of Paxinos and Watson [25]. Control animals of the same age were injected with PBS. Rats were then housed individually to recover and 15 days after lesioning S14 compound (10 mg/kg body weight) was intragastrically administered in a sodium carboxy methyl cellulose suspension, following the protocol shown in Figure 5A. This dose was chosen based on their effectiveness in different previously published works [10, 13]. Animals were sacrificed one month after lesioning. To label the entire population of proliferating cells, rats were intraperitoneally injected with 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg) at the indicated times before sacrifice (Figure 5A).

**Immunohistochemistry.** Animals previously anaesthetized were perfused transcardially with 4% paraformaldehyde, and brains were obtained, postfixed in the same solution at 4 °C overnight, cryoprotected, frozen, and finally 30 µm coronal sections were obtained in a cryostat. Free-floating sections were immunostained using immunofluorescence analysis or diaminobenzidine method as previously described [22]. Briefly, for BrdU detection, samples were first incubated with 2 M HCl for 30 min at 37° before blocking 1 h in PBS containing 5% normal serum, 0.1 M lysine, and 0.1% Triton X-100. Sections were then incubated with anti-BrdU mouse monoclonal (DAKO), anti-nestin rabbit polyclonal (Abcam), anti-tyrosine hydroxylase rabbit polyclonal (Millipore), anti-glial fibrillary acidic protein mouse monoclonal (GFAP; Sigma) and Texas Red Lycopersicon esculentum (tomato-lectin; Vector Labs) antibodies at 4°C overnight, washed three times and incubated with AlexaFluor-488 or AlexaFluor-647 secondary
antibodies for 1 h at room temperature. After rinses, sections were mounted with Vectashield. Images were obtained using a LSM710 laser scanning spectral confocal microscope (Zeiss). Confocal microscope settings were adjusted to produce the optimum signal-to-noise ratio. For doublecortin (DCX), cAMP response element-binding protein (CREB), and phosphorylated-CREB (p-CREB) detection floating sections were immersed in 3% H$_2$O$_2$ to inactivate endogenous peroxidase, blocked for 2 h at room temperature in 5% normal horse serum in PBS, containing 4% bovine serum albumin, 0.1 M lysine, and 0.1% Triton X-100. Afterwards, the sections were incubated overnight with an anti-DCX goat (Santa Cruz), anti-p-CREB polyclonal rabbit (Abcam) or anti-CREB (Cell Signaling) polyclonal rabbit antibody. After several rinses, sections were incubated for 1 h with the corresponding biotinylated secondary antibody and then processed following the avidin-biotin protocol (ABC, Vectastain kit, Vector Labs). The slides were examined with a Nikon eclipse 90i microscope, equipped with a DS-Fi1 digital camera. Five animals from each experimental group were analyzed.

Cell Counts. The total number of cells stained with a particular marker was determined as previously described [10] with some modifications. Immunoreactive cells in the SNpc, the SVZ or the midbrain aqueduct were counted in one-in-six series of 30-µm coronal sections (microtome setting) from the rostrocaudal extent of the SNpc, SVZ or aqueduct. The boundaries of each nervous system region were determined with reference to internal anatomic landmarks [25]. For each area of interest, DAB-stained images were analyzed under a light microscope (Nikon Eclipse 80i). Double labeling was examined with a confocal laser scanning microscope (Zeiss). Five well-defined high magnification ($\times$400) fields per animal were analyzed using a computer-assisted image analysis software (Soft Imaging System Corp). Positive cells, which intersected
the uppermost focal plane (exclusion plane) and the lateral exclusion boundaries of the counting frames, were not counted. Five animals/group were used.

*Rotation behavior analysis.* Animals were tested for apomorphine-induced contralateral rotations as previously described [10, 14]. Briefly, thirty days after unilateral 6-OHDA injection into the *Striatum*, apomorphine (Sigma, Spain) was subcutaneously administered at 0.5 mg/kg. After apomorphine injection, the rats were individually set into a hemispherical glass bowls (diameter 20 cm). Starting 10 min after apomorphine application, the numbers of full contralateral rotations were monitored for 40 min. Analysis of completed (360°) rotations was made offline and expressed as the number of contralateral net turns per minute. Three different experiments with at least 12 animals/experimental group were performed.

*Statistics Analysis.* Statistical comparisons for significance were performed by ANOVA using the SPSS statistical software package (version 20.0) for Windows (Chicago, IL) followed by Newman–Keuls' test. Differences were considered statistically significant at p < 0.05.
Results

Effect of PDE7 inhibition on the levels of PDE7 and P-CREB in neurosphere cultures.

We first performed Western blot analysis to determine the levels of PDE7 and CREB phosphorylation in vitro, using neural progenitors isolated from the embryonic ventral mesencephalon (VM). The progenitor cells divide forming small proliferating neurospheres (NS), visible after 3 days in culture. Since PDE7 comprises two genes, PDE7A and PDE7B, both isoforms were analyzed. As shown in Figure 1A, both isoforms were expressed in VM-derived NS, being the levels of PDE7B more prominent than that of PDE7A. No effect of S14 or BRL50481 (a commercially available PDE7 inhibitor used as standard reference) on PDE7 A and B expression was observed. We next examined the effects of S14 and BRL50481 on the phosphorylation levels of the cAMP response element binding protein (CREB), a well-known target of the cAMP signaling pathway, in order to confirm that effectively the S14 compound is acting through inhibition of PDE7 and subsequent induction of the cAMP pathway. To analyze this, NS cultures were treated or not with BRL50481 or S14 for 7 days and the phosphorylation of CREB was determined. The results presented in Figure 1B clearly show that treatment of NS with BRL50481 or S14 promoted a significant increase in the levels of P-CREB.

PDE7 inhibition induces proliferation and growth of embryonic ventral mesencephalic-derived neurospheres.

Next, we analyzed whether S14 affected neural stem cell proliferation of NS cultures. To assess the involvement of PDE7 inhibition in neurosphere formation, NS were cultured in floating conditions on non-adhesive dishes and cultured for 7 days in the
presence or absence of BRL50481 or S14 and the number and diameter of neurospheres was evaluated. The results depicted in Figure 2A show that PDE7 inhibition increased the rate of formation of NS and their size. Treatment with BRL50481 and S14 significantly increased the number of NS derived from embryonic VM (350±11 and 401±16), respectively, when compared with vehicle-treated cultures (180±13). The size of NS was also increased after BRL50481 (162.5±12.5 μm) or S14 (156.5±11 μm) treatments, as compared with non-treated cultures (101±9μm). Later, we analyzed the levels of musashi-1, a known marker of the NS undifferentiated state, in NS cultured under proliferative conditions in the presence or absence of PDE7 inhibitors for 7 days. The Western blot analysis shown in Figure 2B clearly shows a decrease in the amount of musashi-1 protein in those cultures treated with BRL50481 or S14, suggesting a diminution of the maintenance of NS stemness in those cultures treated with both compounds. Taking together, these results indicate that BRL50481 and S14 treatment promoted an increase in the number and size of neurospheres derived from embryonic VM, suggesting that PDE7 inhibition control the proliferation and growth of neural progenitors in this area of the brain.

**PDE7 inhibition up-regulates MAP-2 expression in neural stem cells derived from ventral mesencephalon.**

To investigate whether BRL50481 and S14 influenced neuronal differentiation after adhesion of NS, we analyzed by immunocytochemistry the expression of two neuronal markers, β-III-tubulin (a classical marker of immature neurons) and MAP-2 (a marker of more mature neurons). To this end, NS treated with BRL50481 or S14 during 7 days were adhered to a substrate and allowed to differentiate for 3 days, afterwards immunocytochemical analysis using specific antibodies were performed. Some cultures
were used for Western blot analysis and quantification of protein levels.

Immunocytochemical analysis showed no differences in β-III-tubulin expression
between controls and treated cultures (Fig. 3) in the outgrowth of the NS. On the
contrary, inhibition of PDE7 with BRL50481 and S14 resulted in an increase in the
number of MAP-2-positive cells, in comparison with basal levels. Western blot analysis
confirmed that PDE7 inhibition significantly promoted cell maturation of embryonic
neural stem cells towards a neuronal phenotype.

**PDE7 inhibition up-regulates Nurr1 and tyrosine hydroxylase (TH) expression in
neural stem cells derived from ventral mesencephalon and SVZ.**

Taking into account the potential neurogenic effect of PDE7 inhibition, we next
investigated whether treatment with the PDE7 inhibitors BRL50481 and S14 could
specifically promote the differentiation of NS towards dopaminergic neurons. For this
purpose, we analyzed the expression levels of Nurr1 (a marker of dopaminergic
precursors) and TH (the rate-limiting enzyme in dopamine synthesis and marker of
dopaminergic neurons) in NS cultures derived from both embryonic midbrain and adult
SVZ. NS treated or not with BRL50481 or S14 during 7 days were adhered to poly-L-
lysine-coated plates and allowed to differentiate for 3 days, afterwards
immunocytochemical and Western blot analysis using specific antibodies were
performed. The results presented in Figure 4A show that, the number of Nurr1- and TH-
positive stained cells in embryonic VM cultures is greatly increased in those cultures
treated either with BRL50481 or S14 compounds, with respect to controls. Furthermore,
this increase was also observed in NS cultures derived from a well-established adult
neurogenic niche, the SVZ (Figure 4B). These results clearly indicate that PDE7
inhibition promotes the differentiation of neural stem cells in vitro towards a
dopaminergic phenotype.

**S14 induces proliferation and differentiation of adult progenitor cells in vivo in the**
**SNpc.**

Given the in vitro results showing a neurogenic effect of PDE7 inhibition and
demonstrating an increase in the number of de novo dopaminergic neurons, we next
assessed the efficacy of the PDE7 inhibitor S14 in vivo. It has been suggested that
neurogenesis is impaired in PD, therefore any treatment able to enhance endogenous
neurogenesis may have relevant disease-modifying effects in this disorder.

To analyze in vivo neurogenesis, adult rats were lesioned with 6-OHDA and 15 days
later received a daily oral dose of S14, which is known to cross the blood brain barrier
[13, 26] during another 15 days, following the experimental approach shown in Figure
5A. Rats were also-intraperitoneally injected with BrdU. To test whether neurons are
generated in the SNpc after S14 treatment, coronal sections containing the SNpc were
doubled-immunostained using anti-TH and anti-BrdU specific antibodies. Vehicle-
treated animals presented BrdU+ cells throughout the entire SNpc (Fig. 5B). These
results are in agreement with those previously described by others [27] indicating that
proliferating cells are present in this region. In accordance with these results, we found
BrdU-labeled cells predominantly in doublets, what suggests that cells had divided
locally. Rats lesioned with 6-OHDA also presented some scattered BrdU-labeled cells.
Interestingly, when 6-OHDA-lesioned rats were treated with S14, besides BrdU+ cells
we also could observe cells double-stained for BrdU and TH, suggesting that this
compound elicited an increase in the generation of new dopaminergic cells in the
injured SNpc of adult rats. Interestingly, in accordance with the increase in TH+ cells
elicited by S14 in the SNpc, a parallel increase in TH staining was observed in the striatum of treated animals (Figure 5C). Concerning neuroinflammation, an event that takes place after a brain injury, we observed a significant reduction in glial activation (astrocytes and microglial cells) in those animals lesioned with 6-OHDA and treated with S14 (Supplementary Figure 1). Taking into account these results, we next studied whether the new generation of dopaminergic cells in the SNpc together with the reinnervation observed in the striatum and the anti-inflammatory effect of S14 was associated with an improvement of the motor alterations induced by 6-OHDA-injection. To this end, 30 days after lesion rats were tested for apomorphine-induced contralateral rotations. Apomorphine subcutaneous administration induces contralateral rotational behavior in denervated animals. Our results clearly show a significant increase in the number of contralateral turns per minute following apomorphine administration in 6-OHDA-lesioned animals (Fig. 5D), compared with control animals. S14 administration led to substantial attenuation of the asymmetric motor behavior in lesioned animals.

The newborn cells found in the SNpc could originate from precursors already present in this area or from precursor cells existing in other areas of the brain. Therefore, we first examined the presence of possible neural progenitor cells (NPC) in the SNpc by analyzing the occurrence of nestin and doublecortin (DCX)-stained cells. Nestin is commonly used as a reliable biological marker of NPCs in vitro and in vivo [28-31]. DCX is a microtubule-associated protein, which is a valuable endogenous marker for dividing neuroblasts and immature neurons [32, 33]. The results shown in Figure 5E indicate the presence of nestin and DCX-labeled cells in the SNpc of vehicle- and 6-OHDA-injected animals. The presence of nestin-positive cells suggest that, as indicated by other authors, the SNpc of adult animals can also contain neural precursor cells. This
Figure also show that rats lesioned with 6-OHDA and treated with S14 presented an increase in the number of nestin-positive cells. Also the number of cells expressing DCX was increased in the S14-treated group of animals.

It is known that PDE7 inhibition increases cAMP levels, consequently we next analyzed the phosphorylation state of CREB, a well-known target of the cAMP signaling pathway. The results shown in Figure 6 clearly indicate that S14 oral administration increases the levels of phospho-CREB in the SNpc in this animal model of PD.

Immunodetection of total amount of CREB protein was used as a control of the phosphorylation state induction.

**S14 increases the number of nestin^+ cells in the mesencephalic aqueduct and induces proliferation and differentiation of adult progenitor cells in vivo in the SVZ.**

As commented above, the newborn cells found in the SNpc could also originate from precursors present in other brain areas such as the mesencephalic aqueduct and that could have migrated to the SNpc, as it has been suggested before [27, 34]. Also, there are several reports showing an impaired neurogenesis in the SVZ in PD patients and in several animal models of this disease. Consequently, we finally analyzed the presence of possible neural progenitor cells (NPC) in both regions (Figure 7). Figure 7A shows that S14 treatment considerably increased the number of Nestin-positive cells in the mesencephalic aqueduct of rats lesioned with 6-OHDA and treated later with S14, in comparison with control vehicle-treated animals. In the SVZ, S14 treatment considerably increased the number of Nestin/BrdU double-stained cells (Figure 7B). These results suggest that S14 stimulates the proliferation of new progenitors in the mesencephalic aqueduct and in the SVZ of adult rats. We also analyzed the presence of
DCX-stained cells in this neurogenic niche. As shown in Figure 7C, the increase in proliferating cells in the SVZ after S14 treatment correlates with a notably increase in the number of DCX$^+$ cells in this area. An increase in the migrating chain of cells was also observed.

**Discussion**

Similar to other neurodegenerative diseases, PD is diagnosed when more than 50% of dopaminergic neurons of the SNpc have already degenerated. There is currently no cure and no effective disease-modifying therapy; dopamine replacement treatment is only palliative leading to temporarily limited improvement of clinical symptoms, and the chronic treatment with dopaminergic drugs, such as L-DOPA, has severe side-effects as dyskinesia [35]. Consequently, new approaches to treat PD are being developed. Grafts of dopamine neurons derived from induced (iPS) or embryonic stem cells (hES) have been done to test the clinical potential of differentiated stem cells in PD. However, their value is largely questioned by data from transplanted patients, which indicate that the grafted neurons have compromised function and eventually acquire disease [36-40]. Another approach to achieve clinical benefit is an indirect way by activating precursor cells that are already present in the brain. In the present study we have investigated the potential effect of PDE7 inhibition on the promotion of dopaminergic cells in the 6-OHDA animal model of PD. Our results demonstrate a unique role for the PDE7 inhibitor S14 as a regulator of dopamine precursor cell proliferation and differentiation in the SNpc of 6-OHDA-lesioned adult rats, which could have potential implications for future innovative therapies in PD.
Previous work from our group has shown that the PDE7 inhibitor S14 significantly protects dopaminergic neurodegeneration and improves motor function in LPS-lesioned animals [10]. We here show that S14 regulates the expansion and differentiation of the stem cell population derived from VM. This is evident in vitro by an enhanced number and size of neurospheres and an induction of MAP2-positive cells. More interestingly we also found a significant increase in the number of Nurr1- and TH-positive cells, indicating that the S14 compound can elicit differentiation of VM stem cells towards a dopaminergic phenotype. We also observed an increase in Nurr1- and TH-labeled cells in the NS cultures derived from the SVZ of adult rats treated with the compound.

Our work shows that PDE7 inhibition by S14 has a dual function in neural stem cells: induction of proliferation and differentiation, and suggests that this compound is not only mitogen for neural stem cells, but is also an inducer of neuronal differentiation. It seems interesting that inhibition of PDE7 can promote both proliferation and differentiation. However, precedents for this phenomenon are seen in other cases, such as leukotriene B4 [41], mild hypoxia [42], BMPs [43], EGF/FGF2 [44], NGF/BDNF/bFGF [45], and the transcription factors Lmx1a and Lmx1b [46]. In this respect, we suggest that inhibition of PDE7 can represent a new strategy for restoring neurogenesis.

The mechanism of action of this compound seems to be an inhibition of PDE7, the subsequent activation of cAMP/PKA signaling pathway, and the activation of the transcription factor cAMP response element-binding protein (CREB) by phosphorylation. These results are in accordance with different studies showing that P-
CREB plays a considerable role in adult neurogenesis [47], particularly in the hippocampus [48, 49]. Our results add new and important data suggesting that activation of CREB following PDE7 inhibition results in a generation of new neurons with a dopaminergic phenotype.

Several studies have suggested that neurogenesis in the SVZ is impaired in PD, which might be due to the lack of dopamine in the subventricular zone [19, 20, 50]. An impairment in neurogenesis may have negative consequences for the development of new therapeutic approaches, since neural stem cells are a potential source for endogenous repair of the lost dopaminergic neurons. Our *in vivo* studies demonstrate an enhancement of neural stem cells proliferation and a larger population of new TH-positive cells in the *SNpc* of rats lesioned with 6-OHDA and treated with S14. Our findings that endogenous neurogenesis can be induced by PDE7 inhibition following a 6-OHDA lesion, raises the question of whether this can be utilized therapeutically in neurodegenerative diseases, and specifically PD. Other studies in rodents have suggested that promoting cell proliferation in the subventricular zone can have a positive effect in models of PD, probably mediated by a neurotrophic effect on the nigro-striatal system [51, 52]. Our results also show an increase, induced by the treatment with S14, in the number of double-labeled nestin/BrdU cells in the SVZ of lesioned rats, indicating an increase in the generation of new progenitor cells in this neurogenic niche. It has been shown that in adult humans new neurons integrate in the striatum, which is adjacent to the SVZ niche [53]. It is known that in the SVZ of humans there is a generation of neuronal precursors, however, unlike rodents, these new neurons are not added in the olfactory bulb of adult humans [54, 55]. This fact, poses the question of whether neuroblasts may migrate to another location close to the
ventricle, e.g. the striatum. Our data suggest that the newly generated neurons found in the SNpc of adult rats after S14 treatment may originate from the SVZ, as described in humans. In line with this notion there are data showing that, although the vast majority of neurons generated in the SVZ in rodents integrate in the olfactory bulb, also a number of striatal neurons are generated from the SVZ in both rodents and monkeys following a stroke [56-58].

Although some of the newly generated neurons found in the SNpc can be generated in the SVZ, other origins cannot be excluded. In this regard, the presence of progenitor cells in non-neurogenic regions, such as the cortex, septum, spinal cord, ventricular extension and the SN has also been demonstrated, but at a less appreciable level compared with the established neurogenic regions [59]. We here report that cells expressing the uncommitted neural precursor marker nestin are present not only in the SVZ but also in the SNpc and in the midbrain aqueduct. In a similar way we have found DCX⁺ cells, marker associated to dividing neuroblasts, in the SNpc after treatment with S14. We furthermore report a significant increase in the SNpc neurons in rats lesioned with 6-OHDA after treatment with S14. Similar results were previously reported by others, describing the presence of dopaminergic neurons with BrdU-positive nuclei in the SNpc, suggesting that these cells could have migrated from the midbrain aqueduct or arise from precursors already existing in the SNpc [34, 60, 61]. However other authors have not been able to reproduce these results and the occurrence of neurogenesis in the SNpc still remains controversial [18, 27]. These discrepancies among the different studies could be due to the different methodologies used. Our findings suggest that the new generated dopaminergic cells, in response to the S14 treatment, can originate from
precursor cells migrating either from the SVZ or the midbrain aqueduct towards the 
SNpc and/or from precursor cells already present in the SNpc.

**Conclusion**

Together, these observations suggest that PDE7 inhibition may represent a means of 
replacing neurons lost in the SNpc of PD patients and consequently could confer 
therapeutic benefit in this disease. In this regard, the PDE7 inhibitor S14 holds great 
promise as a therapeutic new strategy for PD since this compound, besides inducing 
replacement of dopaminergic neurons, is also able to induce a significant 
neuroprotection of the remaining cells.
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Disclosure of Potential Conflicts of Interest

All authors declare no competing financial interests.
References


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Figure legends

**Figure 1. PDE7 expression in embryonic ventral mesencephalic (VM) neurospheres.** VM-derived neurospheres were cultured as indicated in Materials and Methods and treated with vehicle, BRL50481 (BRL; 30 µM) or S14 (10 µM) during 7 days. **A)** Representative Western blot showing the levels of PDE7A and PDE7B. **B)** Western blot showing the levels of CREB phosphorylation. Quantification analysis is also shown. Results are mean values ± SD from three independent experiments. ***p ≤ 0.001 versus vehicle-treated (basal) cultures.

**Figure 2. Effects of PDE7 inhibition on embryonic ventral mesencephalic (VM) neurosphere formation.** VM-derived neurospheres were cultured, treated with vehicle, BRL50481 (BRL; 30 µM) or S14 (10 µM) during 7 days and the number and size determined as indicated in Materials and Methods. **A)** Representative phase-contrast micrographs and quantification analysis showing the number and size of neurospheres. Scale bar, 100 µm. **B)** Representative Western blot and quantification analysis showing expression levels of the precursor cell marker Musashi-1. Results are mean values ± SD from three independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***p ≤ 0.001 versus vehicle-treated (basal) cultures.

**Figure 3. Inhibition of PDE7 promotes neurogenesis of embryonic ventral mesencephalic neurospheres.** Neurospheres were cultured in the presence of vehicle, BRL50481 (BRL; 30 µM) or S14 (10 µM) for 7 days and later on adhered for 3 days to allow differentiation in the presence of inhibitors. Immunocytochemistry and Western blot were then performed on differentiated neurospheres as indicated in Materials and Methods.
Methods. Immunofluorescence images show the expression of the neuronal markers β-III-Tubulin (TuJ clone, early neurogenesis) in green and MAP-2 (mature neurons) in red. DAPI was used for nuclear staining. Scale bar, 20 µm. Western blot and quantification analysis are also shown. ***p ≤ 0.001 versus vehicle-treated (basal) cultures.

Figure 4. Inhibition of PDE7 promotes dopaminergic neurogenesis on embryonic ventral mesencephalic (VM) and adult subventricular zone (SVZ) neurospheres. Neurospheres were cultured in the presence of vehicle, BRL50481 (BRL; 30 µM) or S14 (10 µM) for 7 days and later on adhered for 3 days to allow differentiation in the presence of inhibitors. Immunocytochemistry and Western blot were then performed on differentiated neurospheres from VM (A) or SVZ (B). Immunofluorescence images show the expression of Nurr1 in green and tyrosine-hydroxylase (TH) in red. DAPI was used for nuclear staining. Scale bar, 20 µm. Representative Western blots and quantification analysis are shown. **P ≤ 0.01; ***p ≤ 0.001 versus vehicle-treated (basal) cultures.

Figure 5. Effect of the inhibition of PDE7 on neurogenesis in the Substantia nigra pars compacta (SNpc) in an animal model of Parkinson disease. A) Experimental approach. 6-OHDA (9µg) or vehicle was injected unilaterally into the striatum of adult rats. Two weeks after lesion, animals received a daily intragastrical dosis of S14 (10mg/kg) until brain isolation. Before sacrifice, animals were intraperitoneally injected with BrdU (50mg/kg) at the indicated times. B) Immunofluorescence images and quantification showing the double expression of BrdU^+ (green) and TH^+ (tyrosine-hydroxylase, red) in cells in the SNpc. Scale bar, 25µm. C) Ipsilateral coronal sections
processed for tyrosine hydroxylase (TH) immunoreactivity labeling dopaminergic striatal (St) fibers density (red). Scale bar, 100 µm. DAPI was used for nuclear staining.

D) On day 30 after injury, apomorphine-induced rotation test were performed. Values represent the mean ± SD from 3 different experiments. At least 12 animals/experimental group were evaluated. ***p ≤ 0.001 versus 6-OHDA-injected animals. E) Neural stem cells markers expression in the SNpc. Representatives images showing nestin and doublecortin (DCX)-expressing cells immunohistochemistry. Scale bar, 25µm. Quantification of the number of nestin\(^+\) and DCX\(^+\) cells in the SNpc is shown. Values in all quantifications represent the mean ± SD from 3 different experiments and 5 animals/experiment/experimental group. **p ≤ 0.01 versus 6-OHDA-injected animals.

**Figure 6. S14 oral administration induces the phosphorylation of CREB in an animal model of Parkinson's disease.** Adult rats were treated following the experimental approach described in Figure 5A. Serial coronal sections were made and consecutive sections containing the Substantia nigra pars compacta (SNpc) were immunostained using anti-p-CREB or anti-CREB antibodies. Representative images of ipsilateral sections showing p-CREB (upper panel) or CREB (lower panel) are shown. Scale bar, 100 µm.

**Figure 7. Effect of the inhibition of PDE7 on neurogenesis in the adult midbrain aqueduct (Aq) and in the subventricular zone (SVZ) in an animal model of Parkinson disease.** The experimental approach used was that described in Figure 5A. A) Brain coronal sections showing nestin-expressing cells in the Aq. Scale bar = 100 µm. B) Coronal sections of the SVZ showing the immunofluorescence expression of BrdU\(^+\) (green) and nestin\(^+\) (red) cells. C) Doublecortin (DCX)-expressing cells in the
SVZ. Insets show higher magnifications of representatives selected areas. Scale bar, 75 µm. Quantification of the number of nestin$^+$ cells in the Aq (A); BrdU$^+$/nestin$^+$ cells (B), and DCX$^+$ cells (C) in the SVZ is shown. Values represent the mean ± SD from 3 different experiments and 5 animals/experiment/experimental group. *p ≤ 0.05, **p ≤ 0.01 ***p ≤ 0.001 versus 6-OHDA-injected control animals.
Figure 1
Perez-Castillo, A

A

- PDE7A
- PDE7B
- α Tubulin

B

- p-CREB
- CREB

Bar graph showing the p-CREB protein content (relative to basal) for Basal, BRL, and S14 conditions.

135x312mm (300 x 300 DPI)
Figure 3

Top

Perez-Castillo, A

150x130mm (300 x 300 DPI)
Figure 5

A

B

C

D

E

159x259mm (300 x 300 DPI)
Figure 6

Top

Perez-Castillo, A

VEHICLE  6-OHDA  6-OHDA + S14

p-CREB  p-CREB  p-CREB

CREB  CREB  CREB

126x82mm (300 x 300 DPI)
Top

Perez-Castillo, A
Supplemental Figure 1

VEHICLE
GFAP / Tomato / DAPI
SNpc

6-OHDA
GFAP / Tomato / DAPI
SNpc

6-OHDA + S14
GFAP / Tomato / DAPI
SNpc

104x131mm (300 x 300 DPI)
Supplemental Figure 1. Anti-inflammatory effect of PDE7 inhibition against 6-OHDA-induced toxicity in adult rats. Animals were injected into the SNpc with 6-OHDA (9 µg) or vehicle and 15 days later orally treated with S14 (10mg/kg), as described in Figure 5A. Representative images of the Substantia nigra pars compacta (SNpc) showing the expression of the astroglial marker GFAP (green) and the microglial marker tomato lectin (Tomato, red). DAPI was used for nuclear staining. Scale bar, 100 µm.