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Dopamine receptor activation promotes adult neurogenesis in an acute Parkinson model

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Abstract

Cell proliferation of neural progenitors in the subventricular zone (SVZ) of Parkinson disease (PD) patients and animal models is decreased. It was previously demonstrated that the neurotransmitter dopamine modulates cell proliferation in the embryonic brain. The aim of the present study was to analyze whether oral treatment with the dopamine receptor agonist pramipexole (PPX) modulates adult neurogenesis in the SVZ/ olfactory bulb system in a dopaminergic lesion model. 6-Hydroxydopamine (6-OHDA) lesioned adult rats received either PPX (1,0 mg/kg) or PBS orally twice daily and bromodeoxyuridine (BrdU, a cell proliferation marker) for 10 days and were perfused immediately after treatment or 4 weeks after PPX withdrawal. Stereological analysis revealed a significant augmentation in SVZ proliferation by PPX. Consecutively, enhanced neuronal differentiation and more new neurons were present in the olfactory bulb 4 weeks after PPX withdrawal. In addition, dopaminergic neurogenesis was increased in the olfactory bulb after PPX treatment. Motor activity as assessed by using an open field paradigm was permanently increased even after long term PPX withdrawal. In addition, we demonstrate that D2 and D3 receptors are present on adult rat SVZ derived neural progenitors in vitro, and PPX specifically increased mRNA levels of epidermal growth factor receptor (EGF-R) and paired box gene 6 (Pax6).

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Oral PPX treatment selectively increases adult neurogenesis in the SVZ-olfactory bulb system by increasing proliferation and cell survival of newly generated neurons. Analyzing the neurogenic fate decisions mediated by D2/D3 signaling pathways may lead to new avenues to induce neural repair in the adult brain.

Introduction

Neural stem and progenitor cells are present in the hippocampal dentate gyrus and the subventricular zone (SVZ), where they continuously generate new neurons in the adult brain (Eriksson et al., 1998; Sanai et al., 2004). Cells born in the SVZ migrate through the rostral migratory stream towards the olfactory bulb, where they differentiate and functionally integrate as GABA-ergic and as dopaminergic interneurons (Carleton et al., 2003). Due to their capacity to detour to regions affected by disease processes (eg. striatal migration in ischemia and dopaminergic lesions, Arvidsson et al., 2002; Winner et al., 2008) these newborn cells are interesting targets for restorative therapies. Unfortunately, the neurogenic system is compromised in brains undergoing degeneration, as decreased proliferation in neurogenic regions was described in PD patient's hippocampus and SVZ (Hoglinger et al., 2004). This demonstrates that PD associated pathology not only has an impact on the degeneration of mature neurons but also influences the generation of neural progenitor populations in the adult brain. Therefore, stimulation of the endogenous stem- and progenitor cell population might be a promising means to restore some of the disease regions in PD. In that context, we and others recently demonstrated that intracerebral infusion of various growth factors induced striatal neuroblast migration toward the dopaminergic depleted striatum (Winner et al., 2008; Cooper and Isacson, 2004; Mohapel et al., 2005).

Dopamine agonists are first line symptomatic drugs for the treatment of PD patients and are deployed in early stages of the disease (Rascol et al., 2000). The therapeutic effects derive from binding of the dopamine agonists to the postsynaptic dopamine receptor subtypes in the striatum and the reduction of dopamine turnover in presynaptic dopaminergic neurons in the substantia nigra (SN) (Olanow et al., 1998).

During the development of the brain, dopamine receptors (mostly D3) are abundantly expressed in proliferative neuroepithelial zones (Diaz et al., 1997). In the adult brain, dopaminergic fibers contact the transit amplifying C-cells in the SVZ (Höglinger et al., 2004). Previous data on neurogenesis in 6-OHDA lesioned rats indicated a reduction in proliferation of neural progenitors (Hoglinger et al., 2004; Baker et al., 2004) as well as an increase in dopaminergic neurogenesis in the glomerular layer of the olfactory bulb (Winner et al., 2006). An additional effect of dopamine agonists may be related to their capacity to act on neural stem and progenitor cells and to promote neurogenesis. An increase in SVZ proliferation following dopaminergic stimulation was shown using 7-OH-DPAT (7-hydroxy-N,N-di-n-propyl-2-aminotetralin, a putative D3 receptor agonist) and levodopa in unlesioned and dopamine depleted rats (Höglinger et al., 2004, Van Kampen et al., 2004, O'Keeffee et al., 2009). This effect may be mediated by activation of D2-like receptors on transit amplifying C-cells most likely via the EGF receptor in conjunction with release of EGF in a

PKC-dependant manner (Coronas et al., 2004, O'Keeffe et al., 2009) or via ciliary neurotrophic factor dependant mechanisms (Yang et al., 2008). However, as unlesioned mice did not respond similarly to 7-OH-DPAT dopaminergic stimulation, there might be species differences (Baker et al., 2005).

Furthermore controversy exsists in the *in vitro* field, most probably due to different paradigms and cell conditions used in these studies: besides demonstrating a stimulatory effect on proliferation (Coronas et al., 2004, Hoglinger et al., 2004), it was shown that interruption of dopaminergic neurotransmission by D2-antagonists also increases proliferation of SVZ derived neuroblasts, and is speculated to be a SVZ B-cell induced effect (Kippin et al., 2005). Furthermore, using human midbrain precursor cells, 7-OH DPAT stimulation had no effect on the generation of dopaminergic neurons (Milosevic et al., 2007).

The aim of the present study is to investigate if the oral application of a clinically widely used dopamine agonist (PPX) has a modulatory effect on adult neurogenesis in the SVZ/olfactory bulb system in a PD model mimicking a severe dopaminergic striatal deficit. Moreover, we determined the functional consequences of PPX treatment by open field analysis. In addition, we aimed to analyze dopamine receptor (DR) expression of adult neural stem and progenitor (ANPs) cultures *in vitro*.

Material and Methods

Animals

Three-month old male Fisher F344 rats (Charles River Laboratories, Sulzfeld, Germany), weight 220 to 250 g, were used. For the *in vivo* experiment, rats (n=48) were randomly divided into six groups of n=8 each. Groups were defined by lesion (6-OHDA) and treatment with PPX or PBS as well as by the time point of perfusion immediately after or 4 weeks after the treatment period (Figures 1A, 2A, 3A). All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the local governmental commission for animal health. For the *in vitro* experiments, adult SVZ was isolated from rats (n=6) and ANP cultures were established and propagated as previously described (Wachs et al., 2006).

In vivo experiments

Proliferation in naive PPX treated animals—First, the impact of orally administered PPX (1,0 mg/kg) or PBS twice daily for 10 days in naive rats (n=8 per group) was tested to determine the potential of PPX to stimulate the proliferation of ANPs. Bromodeoxyuridine (BrdU) was administered over the same period (Figure 1A).

Nigrostriatal lesions and PPX administration—In a second set of experiments, the potential of PPX to modulate adult neurogenesis was analyzed in 6-OHDA lesioned animals. Unilateral elimination of the dopamine (DA)-containing neurons in the SN pars compacta was achieved by stereotactic injection of two deposits of 2µl of 6-OHDA hydrobromide (Sigma, St. Louis, MO, USA) into the medial forebrain bundle (MFB) as described (Winner et al., 2006). Following 6-OHDA injection, dopaminergic neurons of the SN degenerate

within 12 hours (Faull and Laverty, 1969; Schober, 2004). Thus, dopaminergic neurons already degenerated before the first BrdU injection (50 mg/kg, Sigma) was applied eight days after the lesion, and BrdU was continued daily for 10 days. In addition, PPX (1,0 mg/kg) or PBS was administered orally twice daily for the same period. The early group (used to determine the proliferation rate) was perfused at the end of the treatment period (Figure 2A) and the late group (used to determine survival of new neurons) was perfused 4 weeks after PPX withdrawal (Figure 3A) (Winner et al., 2006). The olfactory bulbs were transected anterior to the septum and cut sagittally in 20 μm sections, the remaining hemispheres of the brains were cut coronal into 40 μm sections.

Immunohistochemistry and immunofluorescence—Immunohistochemistry and triple-labeling immunofluorescence for free floating sections was performed as recently described (Winner et al., 2006). The following primary antibodies were used: BrdU, rat monoclonal, 1:500 (Oxford Biotechnology, Oxford, UK), NeuN, mouse monoclonal 1:500, tyrosine hydroxylase (TH) rabbit monoclonal 1:1000 (both Chemicon, Temecula, CA, USA), doublecortin (DCX) goat polyclonal 1:500 and proliferating cell nuclear antigen (PCNA), mouse monoclonal, 1:1000 (both Santa Cruz, Santa Cruz, CA, USA).

Counting procedures—For quantification, a systematic, random counting procedure was applied using a semi-automatic stereology system as described recently (Stereoinvestigator, MicroBrightField, Colchester, VT, USA) (Winner et al., 2006), (Gundersen et al., 1988). An observer blinded to the treatment history performed the counting. PCNA-positive cells in the SVZ and BrdU-positive cells in the granule cell layer of the olfactory bulb were counted by using the optical fractionator (Winner et al., 2006). The glomerular layer and the hippocampal dentate gyrus were counted exhaustively. On average 100 BrdU-positive cells were analyzed for neuronal differentiation by using a confocal scanning laser microscope (Leica TCS-NT, Bensheim, Germany).

Open field behavioral assessment—In addition to the modulatory effect of dopamine agonists on basal-ganglia dependant motor function, previous studies showed that following dopamine agonists result in a significant alteration of spontaneous motor behavior (Millan et al., 2004). Increased motor activity has a stimulatory effect on hippocampal, but not olfactory bulb neurogenesis (van Praag et al., 1999, Brown et al., 2003). The goal of the open field study was to control the effect of D2/D3 agonists on this behavioral read out.

Open field assessments were performed 1 day before perfusion. At this time, the animals had been continuously treated with PPX for 10 days or were 4 weeks after PPX withdrawal. Open field activity was measured by counting the number of grid lines crossed by both hind feet for 5 min in a 100 × 100 cm^2 box marked with a grid of 20 cm^2 squares (Figures 1C, 2C, 3B).

ANP cell cultures—SVZ derived ANPs cultures from male Fischer 344 rats were established as described (Wachs et al., 2006). Cells were grown in DMEM/F12 media (Cellgro, Manasas, VA, USA) supplemented with B27 and Penstrept (GIBCO, Carlsbad, CA, USA) and containing 20 ng/mL FGF-2 (R and D Systems, Abingdon, U.K.), 20 ng/mL EGF (R and D Systems), and 20 ng/mL heparin (Sigma, Taufkirchen, Germany). Cells were

seeded in T-25 culture flasks and cultures were maintained at 37°C in an incubator with 5% CO_2. Single cells began to form spheres within 5 to 7 days of growing in suspension. Cells from passage number 3 to 6 were used for the experiments. For passaging of cells, 200 μL of Accutase® was used (Innovative Cell Technologies Inc., San Diego, CA, USA). For immunostainings ANPs were grown in neurosphere cultures and after first passage plated under adherence inducing conditions using polyornithine (250 μg/ml) and laminin (5μg/ml)-coated glass coverslips in 12-well plates at a cell density of 10^5 cells/well/ml and grown to adherence overnight. Cells were fixed with phosphate-buffered 4% paraformaldehyde (37° C, pH 7.4; 4% w/v paraformaldehyde, 100 nm NaH_2PO_4, 0.4 mm $CaCl_2$, 50mm sucrose) for 30 min and processed for immunohistochemistry. Samples were permeabilized in 1% Triton X-100 containing TBS (Sigma) and blocked for additional 30 min in 1.5% normal donkey serum containing TBS at room temperature. The specimens were incubated overnight at 4° C with the primary antibodies at the following dilutions: Dopamine D2-receptor, D3-receptor (1:100, both Santa Cruz, Santa Cruz, CA, USA) and EGF-R (1:100 A488 labeled EGF, Molecular probes, Invitrogen, Carlsbad, CA, USA). The secondary fluorochrome-conjugated antibodies were diluted 1:500 (donkey anti-goat; Dinova, Germany). Nuclear counterstaining was performed with 4′,6′-diamidino-2-phenylindole dihydrochloride hydrate at 0.25 μg/μl (DAPI; Sigma). After the last wash, the samples were briefly rinsed with PBS and mounted on slides using Prolong (Molecular Probes, The Netherlands).

RNA extraction—Total RNA was extracted from ANPs treated with 0, 1 or 10 mM PPX for 24 h. Three independent sets of RNA were isolated for each treatment condition using RNAeasy kit (QIAGEN, Germantown, MD, USA) as per manufacturer's instructions. All the samples were treated with DNAseI to eliminate genomic DNA contamination. RNA quantification was determined by spectrophotometer readings. The ratio of OD_{260}/OD_{280} was used to evaluate the purity of the nucleic acid samples and the quality of the extracted total RNA was determined using agarose gel electrophoresis.

Quantification of mRNA by Real Time-PCR (RT-PCR) analysis—For cDNA synthesis, 1 μg total RNA from SVZ derived ANPs was reverse transcribed using iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA). Specific primers were designed for each studied sequence using OligoPerfect software (Invitrogen, Carlsbad, CA, USA). The specificity of the primers to bind the desired sequence was determined by BLAST against the NCBI nucleotide database. Standard curves were generated for each gene of interest using serial dilutions of rat SVZ derived cDNAs. All primers used showed efficiencies >90% and < 118% and R^2 values > 0.97, parameters calculated by linear regression analysis of the Ct vs log[template] blots using Graph Pad Prism 3.0 software.

RT-PCR experiments were performed using the iQ5 Detection System (BioRad, Hercules, CA, USA). Amplification was performed on a cDNA amount equivalent to 25 ng total RNA with 1 × iQ SYBRGreen Supermix (BioRad, Hercules, CA, USA) containing dNTPs, $MgCl_2$, Taq DNA polymerase, and forward and reverse primers. PCR reactions were performed on three independent sets of template. Experimental samples and no-template controls were all run in duplicate. The PCR cycling parameters were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s, 60°C for 1 min. Finally, a dissociation protocol

was also performed at the end of each run to verify the presence of a single product with the appropriate melting point temperature for each amplicon. To further ascertain the specificity and size of the PCR products, the amplicons were run alongside molecular weight markers on a 2% agarose gel in 1 × TAE. The amount of studied cDNA in each sample was calculated by the comparative threshold cycle (Ct) method and expressed as 2exp(Ct) using rat actin as an internal control.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t* test (Prism Graph Pad Software, San Diego, CA, USA). The data is expressed as mean values ± standard deviation (S.D.). The significance level was assumed at $p < 0.05$, unless otherwise indicated.

Results

Oral application of PPX in naive rats increases SVZ proliferation

To investigate the effect of orally applied PPX on adult neurogenesis in the SVZ/ olfactory bulb system, we first determined the consequences of PPX on cell proliferation in the SVZ of naïve rats (Figure 1 A). SVZ proliferation was assessed using two different approaches: i) BrdU was applied over a period of 10 days labeling all cells that were proliferating during that time period, and animals were perfused at the end of the labeling period. ii) PCNA-immunohistochemistry was used to label cells that were proliferating at the time of perfusion (Takasaki et al., 1981). Directly after PPX treatment, the number of PCNA-positive profiles is increased by about 40% in the SVZ of naive rats compared to PBS controls (Figure 1 B). This effect is present in both hemispheres. Moreover, an increased proliferation was confirmed by analysis of BrdU-positive profiles. Here a significant increase of about 37% was present compared to the PBS controls (Supplemental Table 1).

Oral application of PPX in 6-OHDA lesioned rats selectively increases SVZ proliferation and survival of newly generated neurons in the olfactory bulb

In a second set of experiments we examined the effects of PPX on neurogenesis in a 6-OHDA lesion model. The influence on cell proliferation was analyzed in the early group (10 days of PPX treatment followed by perfusion, Figure 2A). Effects on cell fate (survival and differentiation) were analyzed in the late group (10 days of PPX treatment, perfusion of animals 4 weeks after PPX withdrawal, Figure 3A). To ensure consistency and minimize variations due to the experimental procedures, we evaluated TH-staining of the SN and the striatum in all lesioned animals and detected a similar staining pattern for TH in all 6-OHDA injected animals.

At the end of the PPX treatment the SVZ proliferation was increased in the treated rats (Figure 2B). A 46% increase in PCNA-positive mitotic profiles was present in the SVZ on the lesioned side of the PPX administered rats (Figure 2D) compared to PBS controls. Complementary to PCNA-labeling, we also examined BrdU-positive profiles in the SVZ. Here, similar results of increased proliferation were found. A significant increase of BrdU-positive profiles was present both on the lesioned (31% increase, Figure 2D), as well as on the unlesioned side (46% increase, Supplemental Table 2).

Four weeks after PPX withdrawal, proliferation rates are not significantly different in the treated compared to the untreated groups (Supplemental Table 3). This indicates that the PPX-mediated increase of SVZ cells depends on the continuous presence. Interestingly, the effect of PPX on recruiting new cells was restricted to the SVZ. No changes in BrdU-positive profiles were present in the hippocampal dentate gyrus (Supplemental Table 2).

PPX increases olfactory bulb neurogenesis

SVZ derived newly generated cells integrate within the olfactory bulb in the granule cell layer as GABA-ergic and in the glomerular cell layer as dopaminergic neurons (Betarbet et al., 1996). In the present work, we analyzed the effects of PPX treatment on the fate of these two neuronal populations. Cell numbers of BrdU-positive profiles were determined and consecutively the neuronal identity of the newly generated cells was characterized. The total number of newly generated BrdU/ NeuN (new neurons) or BrdU/ TH positive cells (new dopaminergic neurons) was used to evaluate changes in neurogenesis.

In the olfactory granule cell layer, the site of integration for the vast majority (more than 90%) of newly generated SVZ-derived interneurons (Petreanu and Alvarez-Buylla, 2002, Winner et al., 2002), BrdU cell numbers in the 6-OHDA lesioned PPX treated group significantly increased compared to the 6-OHDA group (64% more BrdU-positive cells). In both groups, the large majority of the BrdU-positive profiles had differentiated into neurons at this time point, as determined by co-localization of BrdU and NeuN. There was a significant increase in neuronal differentiation in the PPX treated group, resulting in the increased number of BrdU/ NeuN coexpressing cells (about 50 000 more new neurons, indicating a 67% increase, Figure 3C–F, Supplemental Table 3). Taken together, these data indicate that more newly generated neurons were present in the olfactory bulb granule cell layer after PPX treatment, at a time point where physiologically most of the newly generated cells from the SVZ have reached the olfactory bulb.

Next, we evaluated dopaminergic differentiation of BrdU-positive cells in the glomerular layer. Here, no alteration in the differentiation pattern towards a dopaminergic phenotype was observed in the PPX treated group. However, due to more newly generated cells, the total number of BrdU/ TH positive cells was increased by 41% in the PPX treated group (Figure 3G–J). This indicates a net increase in olfactory bulb dopaminergic neurogenesis in the glomerular layer (Supplemental Table 3). As expected for the early group, the total number of new cells in the olfactory bulb granule cell layer and glomerular layer was not altered by PPX treatment since most of the BrdU-positive cells labeled have not reached the olfactory bulb at the 7th day of BrdU-treatment (Winner et al., 2002; Van Kampen et al., 2004).

Hippocampal neurogenesis is not altered in 6-OHDA lesioned animals following PPX treatment

In addition, no modulation of neurogenesis was observed in the hippocampal dentate gyrus in the PPX 6-OHDA group compared to the PBS 6-OHDA group (Supplemental Table 2). This result was not anticipated, since physiologically, in non-lesioned animals an increased motor activity results in increased hippocampal neurogenesis.

Lack of migration of newly generated neuroblasts to the nigro-striatal system

The rate of adult neurogenesis is intimately linked to the number of neuroblasts, since these cells have the potential to generate new neurons. We additionally searched for neuroblasts and young neurons in the striatum and SN. First, we searched for DCX-labeled cells in the striatum of the 6-OHDA lesioned groups immediately after PPX treatment or 4 weeks after PPX withdrawal. DCX-expressing cells were rarely found in the striatum in all studied animals (less than one cell per section). We extended our study and analyzed colocalization of BrdU and TH in the SN. The analysis of 50 BrdU-positive cells per animal showed no TH/ BrdU co-expressing cells in the substantia nigra of 6-OHDA lesioned rats.

Increased exploratory motor activity by PPX treatment irrespective of the lesion

Motor activity was assessed by counting grid lines crossed by the rat in a walled square box for 5 min. A significant increase in motor activity was observed in the PPX treated naive group. In addition, PPX significantly augmented exploratory motor behavior after the lesion (Figures 1C and 2C, Supplemental tables 1 and 2). Contrary to what was expected, an increased motor activity persisted in the PPX treated 6-OHDA lesioned animals even four weeks after PPX withdrawal (Figure 3C, Supplemental table 3). This might be an indication for a long lasting effect of PPX on exploratory motor activity.

Effects of PPX treatment on the expression of dopamine receptor genes and EGF-R/Pax6 in ANPs

In order to assess if the observed changes in SVZ neurogenesis after PPX treatment may be mediated by dopamine receptors, we investigated dopamine receptor expression at the mRNA and protein levels in SVZ derived ANPs. Under proliferating conditions, SVZ derived ANPs showed expression of D1R and D2-R, with lower abundance of D3-R transcripts. Immunohistochemistry showed abundant presence of D2-R and D3-R in ANPs cytoplasm and processes (Figure 5 C, D). Additionally, expression of EGF-R and Pax6 was also found at the mRNA and protein levels in SVZ derived ANPs (Figure 5A, B, and E).

To determine if PPX affects gene expression, SVZ-derived ANPs were treated with 1 or 10 μM PPX for 24 h before RNA extraction and qPCR. Figures 5A and B show that while 1 μM PPX treatment had almost no effect, treatment with 10 μM PPX significantly increased the expression of EGF-R and Pax6 genes. Taken together, these results indicate that D2 and D3 receptors are expressed in SVZ-derived ANPs and that PPX related effects in neurogenesis may be mediated by the upregulation of gene expression of EGF-R and Pax6 in ANPs.

Discussion

Oral application of the dopamine agonist PPX is able to stimulate SVZ proliferation in naive animals and more importantly in animals with a severe dopaminergic deficit. Our data provide evidence that the PPX mediated proliferation depends on the continuous presence of PPX as the increased number of mitotic cells in the SVZ is no longer present 4 weeks after withdrawal (see schematic drawing Figure 4).

PPX treatment not only increases cell proliferation but also increases the total number of new cells in the granule cell layer and glomerular layer adopting an increased neuronal differentiation. Importantly, the PPX induced increase in neurogenesis is restricted to the SVZ/ olfactory bulb system. No modulation in neurogenesis was found in the hippocampal dentate gyrus. Moreover, no DCX-positive neuroblasts are found in the striatum or SN. In addition, we show that dopamine receptors are present on SVZ derived ANPs and EGF-R expression is increased by PPX treatment.

Dopamine receptor mediated changes in adult neurogenesis

PPX is a widely used dopamine agonist for the treatment of PD, restless legs syndrom and depression. Activation of the D2 and/ or D3 receptors improves motor functions in PD. Indications for neuroprotection for D2/D3 agonists are based on the following findings: (1) in animal models PPX and further D3 receptor preferring agonists were found to be neuroprotective agents against MPTP-induced neurodegeneration in mice and primates as well as in 6-OHDA lesioned rats (Joyce et al., 2004; Vu et al., 2000; Ramirez et al., 2003; Iravani et al., 2006) and (2) more recently, human imaging studies described a reduced loss of dopaminergic terminals by long term PPX administration (Parkinson Study Group, JAMA, 2002).

Dopamine receptors are G protein-coupled receptors that are classified as D1-like (D1 and D5) and D2-like (D2, D3 and D4). D2 like receptors signal primarily through the $G\alpha_{i/o}$ class of heterotrimeric G proteins to elicit a number of cellular responses (for review see Neve et al., 2004). D2-like receptors activate extracellular signal-regulated kinases (ERKs) by transactivation of different receptors, in neurons mostly due to transactivation of the EGF-R (Wang et al., 2005). Interestingly, increased expression of EGF-R could also be demonstrated by PPX treatment in ANPs in our study.

In addition, our study for the first time addressed the crucial issue of oral bioavailability. Lower stimulatory effects were observed for alternate routes of 7-OH DPAT administration: intraventricular infusion doubled SVZ cell proliferation whereas intraperitonal injections led to a 25% increase (Van Kampen et al., 2004). However, 7-OH DPAT is not feasible for a clinical application.

Oral administered PPX was more effective to increase SVZ proliferation than intraperitonal application of 7-OH DPAT (Van Kampen et al., 2004). The effect of orally applied PPX resulted in an increase of proliferation of about 37% in naive rats. 7-OH DPAT has an 80-fold stronger receptor affinity to the D3 receptor (Levesque et al., 1992), whereas PPX has an 8-fold stronger receptor affinity to the D3 receptor (reviewed in Kvermo et al., 2006) and this difference may contribute to the different results in neurogenesis.

Effect of D3 stimulation on ANPs in a PD model

Besides studying the effect of PPX in naive animals, the focus of our study was to determine the PPX effect in animals with a severe dopaminergic deficit. Here, a PPX dependent rescue in explorative motor activity was noted. Therefore, one might argue that the PPX induced increase in SVZ proliferation might depend on the increased motor activity. Previous studies have shown, that physical activity is capable to increase hippocampal neurogenesis (van

Praag et al., 1999; van Praag et al., 2002), but neurogenesis in the SVZ/ olfactory bulb system remains unaffected (Brown et al., 2003). Interestingly, adult neurogenesis in the hippocampal dentate gyrus was not increased by PPX treatment in our 6-OHDA lesioned rats despite the increased motor activity. These results indicate major differences between hippocampal and SVZ progenitors in regard to dopaminergic stimulation in the 6-OHDA model. Decreased of expression of dopaminergic receptors in the dentate gyrus (Bouthenet et al., 1991) might explain this result. Independent of the lesion, the rodent brain retains the capacity to generate new SVZ derived cells, indicating a source of endogenous cell replacement not only in the intact, but also in the brain with a dopaminergic deficit. It is important to note, however, that this effect is not restricted to the lesion side but also present on the unlesioned side. PPX treatment in 6OHDA treated animals lead to levels of proliferation comparable to non-treated animals (31% vs. 37% increase for BrdU and 45% increase for PCNA). If one compares the absolute numbers of PCNA positive cells in PBS treated naïve rats with PBS treated 6OHDA rats there is no difference. Although a decreased proliferation after 6-OHDA lesioning has been reported, the present data cannot confirm this finding, possibly due to different animal strains and experimental designs.

PPX promotes neuronal differentiation and increases new neurons in the olfactory bulb

Earlier studies were able to show that a modulation of olfactory neurogenesis is present in animal models of PD. Interestingly, an increase in dopaminergic neurogenesis in the glomerular layer of the olfactory bulb has been observed both in a 6-OHDA as well as in the MPTP model (Winner et al., 2006; Yamada et al., 2004). In contrast to previous studies (Van Kampen and Eckman, 2006) no new neurons where observed in the striatum and substantia nigra in our study. Four weeks after the end of PPX treatment, higher rates of neurogenesis in the olfactory bulb were detected. It is likely, that the increased number of newly generated cells depends on the continuous presence of PPX although we cannot exclude the possibility that the responsiveness to PPX may be of transient nature.

PPX has the capacity to foster neuronal differentation in the granule cell layer. In the glomerular layer we observe an increased differentiation towards a dopaminergic phenotype. This leads to an increase in dopaminergic neurogenesis. It is important to note, that even after a dopaminergic lesion of the brain, the ability to modulate adult neurogenesis by dopamine D2/D3 agonist enhancing small molecules persists. Under these conditions, PPX is capable to increase survival and differentiation of newly generated neurons in the olfactory bulb. Although on a functional level the olfactory bulb might not be the target region for functional recovery of motor symptoms, the survival effect of newly generated neurons is outstanding and might be a key element to investigate the regulation of adult dopaminergic neurogenesis in PD.

Moreover, increasing interest is converging towards the olfactory bulb in PD as hyposmia is one of the early symptoms of the disease. Olfactory deficits are currently proposed as an important premotor biomarker to define patients at risk to develop PD later in life. So far, no changes in olfactory function were described following dopaminergic medication (Roth et al., 1998). In PD brains, the SVZ may respond to neurodegenerative changes in adjacent

brain regions by decreasing progenitor cell proliferation (Hoglinger et al., 2004) and thus modulating neurogenesis.

Oral PPX treatment selectively affects neuroblasts in the SVZ-olfactory bulb system

This is the first report to study the effect of oral PPX administration on adult neurogenesis. Our study indicates that the application of small molecules with a good oral bioavailability is able to independently stimulate SVZ proliferation as well as differentiation and increases the number of newly generated neurons in the olfactory bulb. In the SN the newly generated cells do not express neuronal markers, these newly generated cells most likely adopt a glial fate (Lie et al., 2002).

Dopaminergic fibers preferentially contact EGF-R positive rapid amplifying C-cells in the SVZ (Hoglinger et al., 2004). We show that D2-R and D3-R are expressed on SVZ derived ANPs. Interestingly, PPX treatment increased the expression of EGF-R and Pax6 mRNA. These results suggest a PPX related upregulation of EGF-R and Pax6 in ANPs. In PD patients' brains as well as in 6-OHDA lesioned rats a decrease in EGF protein content was detected in the prefrontal cortex and striatum. Moreover, the expression of the EGF receptors ErbB1 and ErbB2 was down-regulated (Iwakura et al., 2005). Given the fact that EGF levels are reduced in PD patients, our results showing EGF-R increased expression induced by PPX point towards the potential therapeutic efficacy of EGF-based induction of the generation of endogenous ANPs. The similarities of the endogenous stem cell regions in mammalians therefore highlight the advantages of using this model for mechanisms of replacement in human PD. Investigating D2/D3 mediated signaling pathways may lead to new avenues to generate new neurons in the adult brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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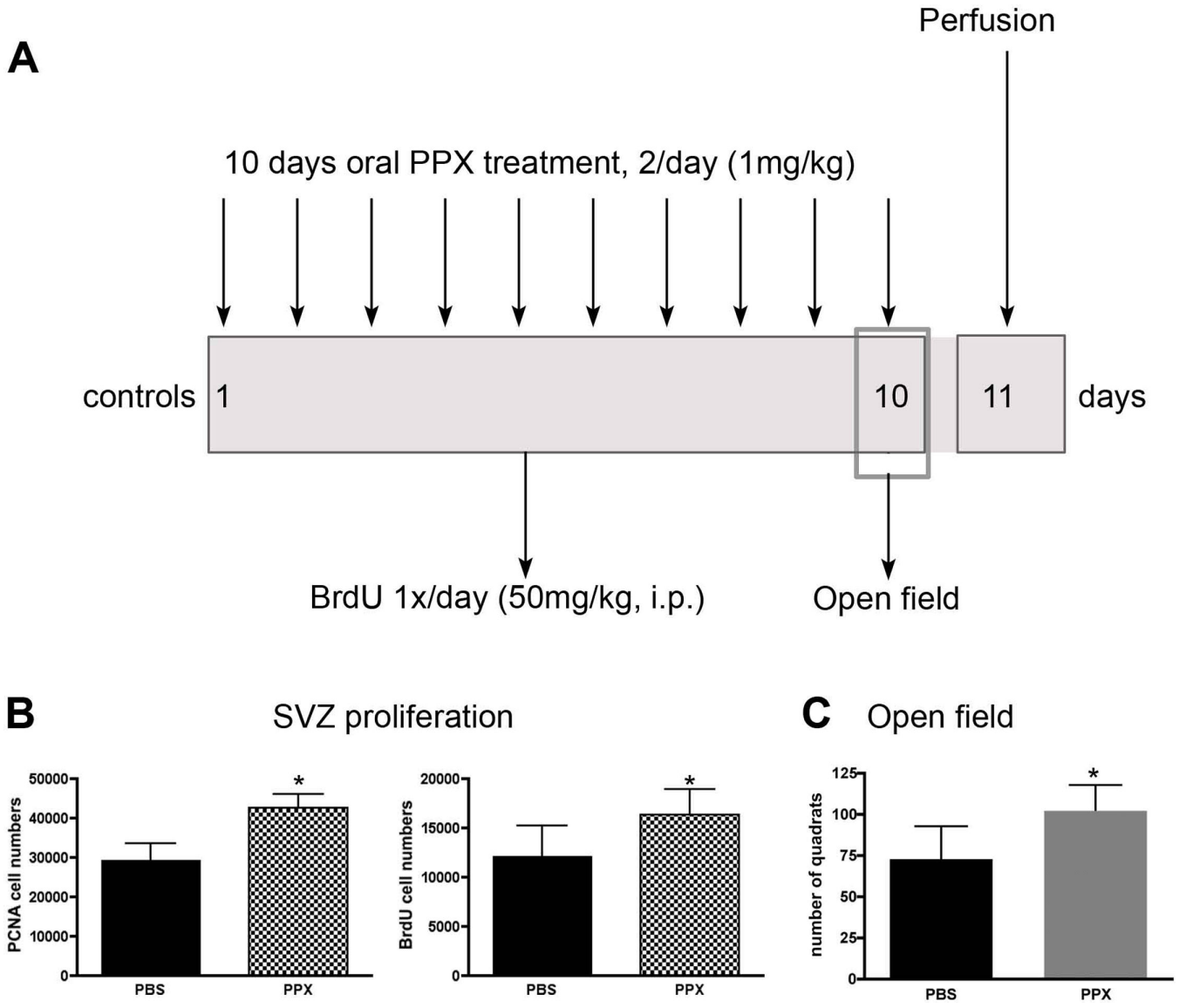

Figure 1. PPX treatment for 10 days for naïve rats

(A) Paradigm for oral PPX treatment in naive rats. (B) PPX significantly increased proliferation in the SVZ of adult naive animals as indicated by proliferating cell nuclear antigen (PCNA) and BrdU cell numbers (* $p<0.001$). (C) The open field analysis revealed an increased motor activity after PPX treatment in naive animals (*$p<0.005$).

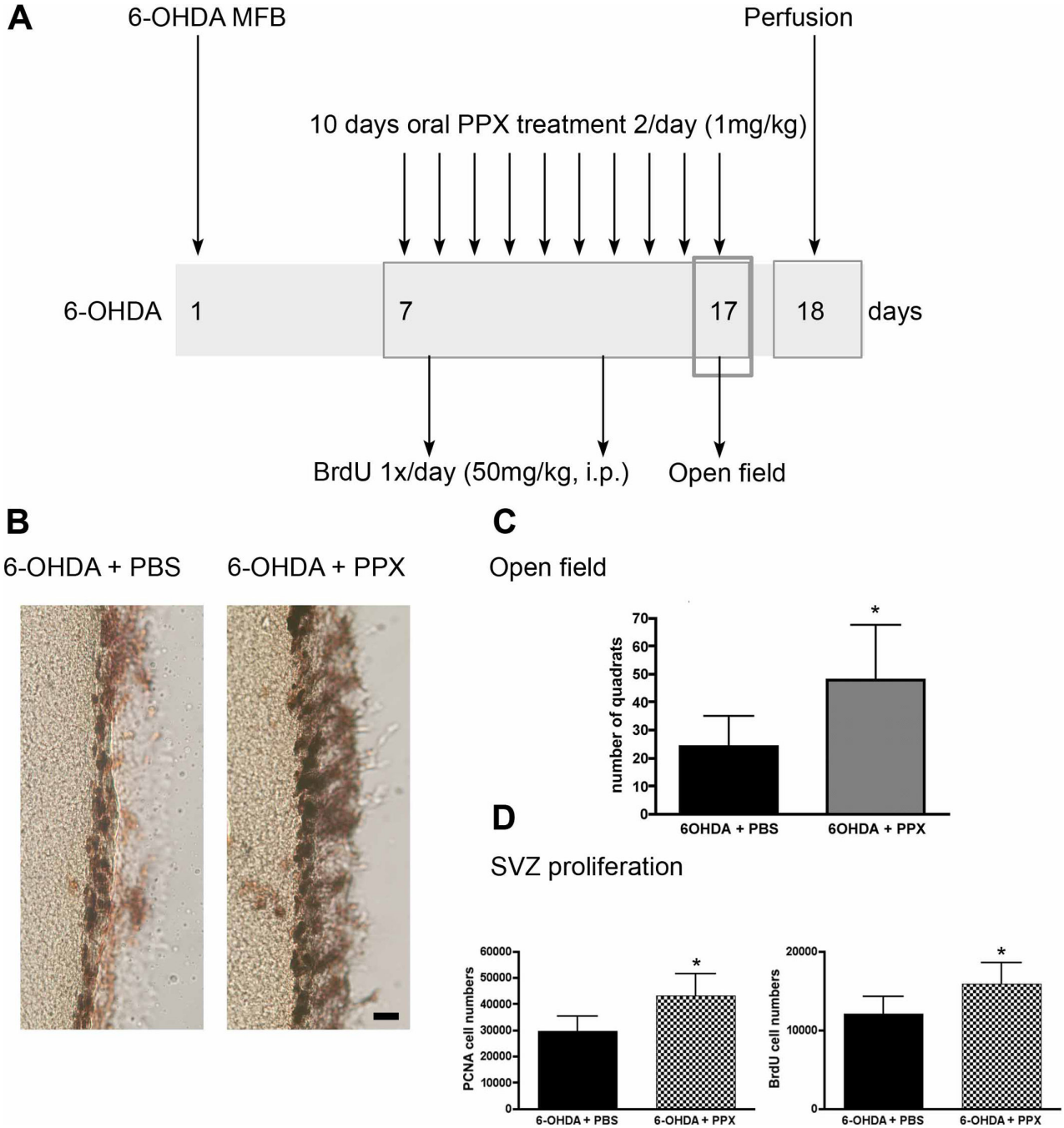

Figure 2. PPX treatment for 10 days in animals with a unilateral dopaminergic deficit

(A) Paradigm for oral PPX treatment in 6-OHDA MFB lesioned rats. The unilateral 6-OHDA lesion was applied to the right MFB 1 week before PPX treatment. (B) Representative sections of the SVZ region in PPX treated animals versus PBS controls: adjacent to the dopamine depleted striatum, PCNA-positive cells in the SVZ were quantified stereologically. Note the increase in cell density of PCNA-positive profiles in the PPX treated group (6-OHDA + PPX) compared to the control (6-OHDA + PBS). Scalebar represents 50μm. (C) The open field analysis revealed an increased motor activity after PPX

treatment in 6-OHDA treated animals (*p<0.005). (D) PPX treatment results in an increased proliferation in animals with a unilateral dopaminergic deficit as depicted by increased PCNA (* p<0.005) and BrdU cell numbers (* p<0.01).

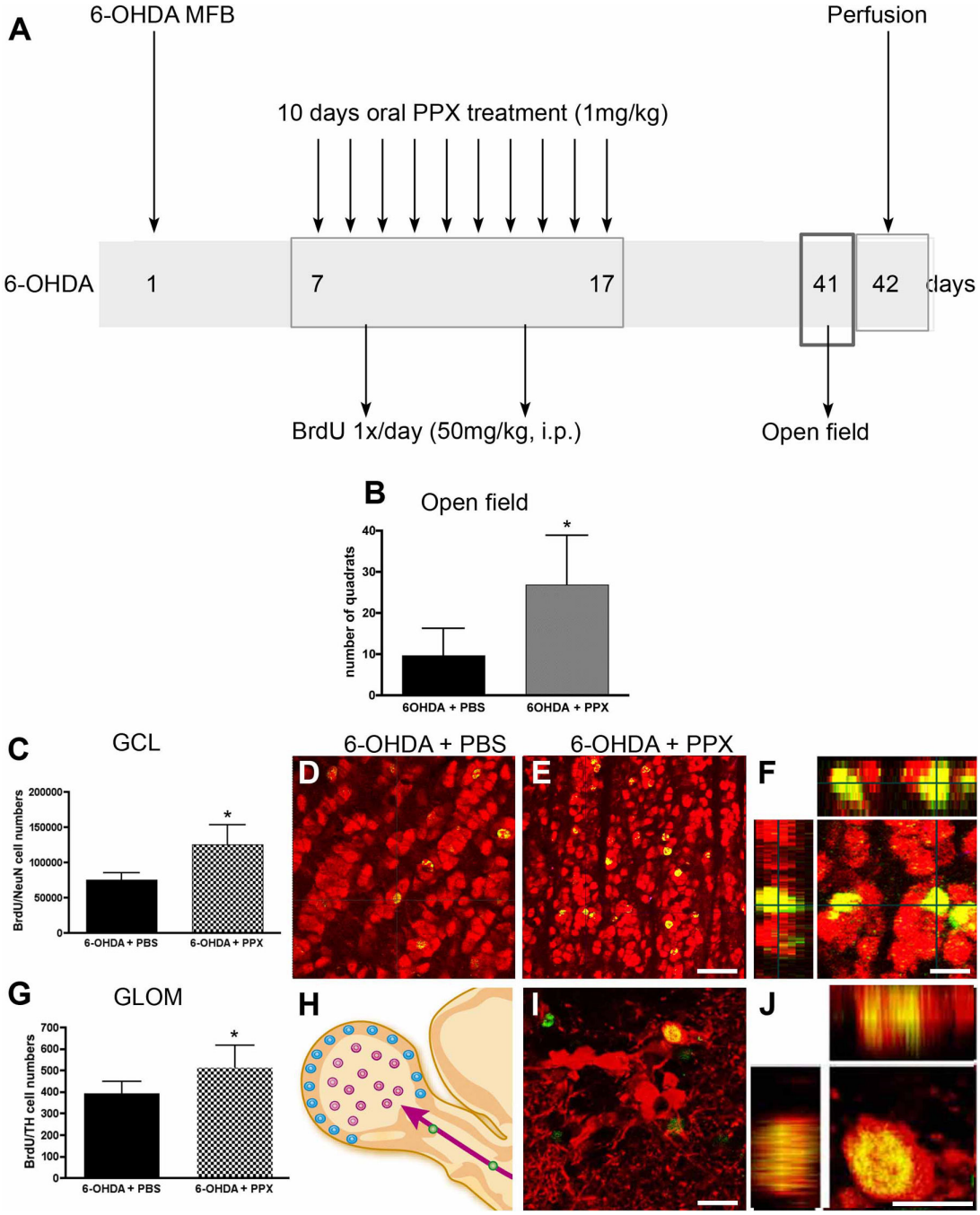

Figure 3. PPX treatment in animals with a unilateral dopaminergic deficit: analysis four weeks after PPX withdrawal

(A) Paradigm for oral PPX treatment in 6-OHDA MFB lesioned rats. The unilateral 6-OHDA lesion was applied to the right MFB 1 week before PPX treatment. Analysis was performed 4 weeks after withdrawal of PPX. (B) The open field analysis revealed an increase in motor activity four weeks after cessation of the PPX treatment in 6-OHDA treated animals (*p<0.05). This indicates a long-lasting behavioral effect of PPX on explorative motor activity. (C) PPX significantly increases new neurons (BrdU/ NeuN double labeled cells) in the granule cell layer (GCL, * p<0.005). (G) In the glomerular layer

(GLOM) PPX leads to an increased dopaminergic neurogenesis (* $p<0.05$). Note the increased number of BrdU/ NeuN positive cells (newborn neurons) in the GCL of PPX treated rats compared to PBS (D, E, scalebar 30μm in D, E). F depicts xyz reconstruction of confocal image depicting colabeling of a cell double labeled for BrdU (green) and NeuN (red, scalebar 15μm in F). H represents a schematic drawing of the olfactory bulb with the GCL (purple cells) and the GLOM (blue cells). GLOM double labeled cell (red: TH, green BrdU) identifies a newly generated dopaminergic neuron (I, overlay, J xyz reconstruction, scalebars 15μm in I, J).

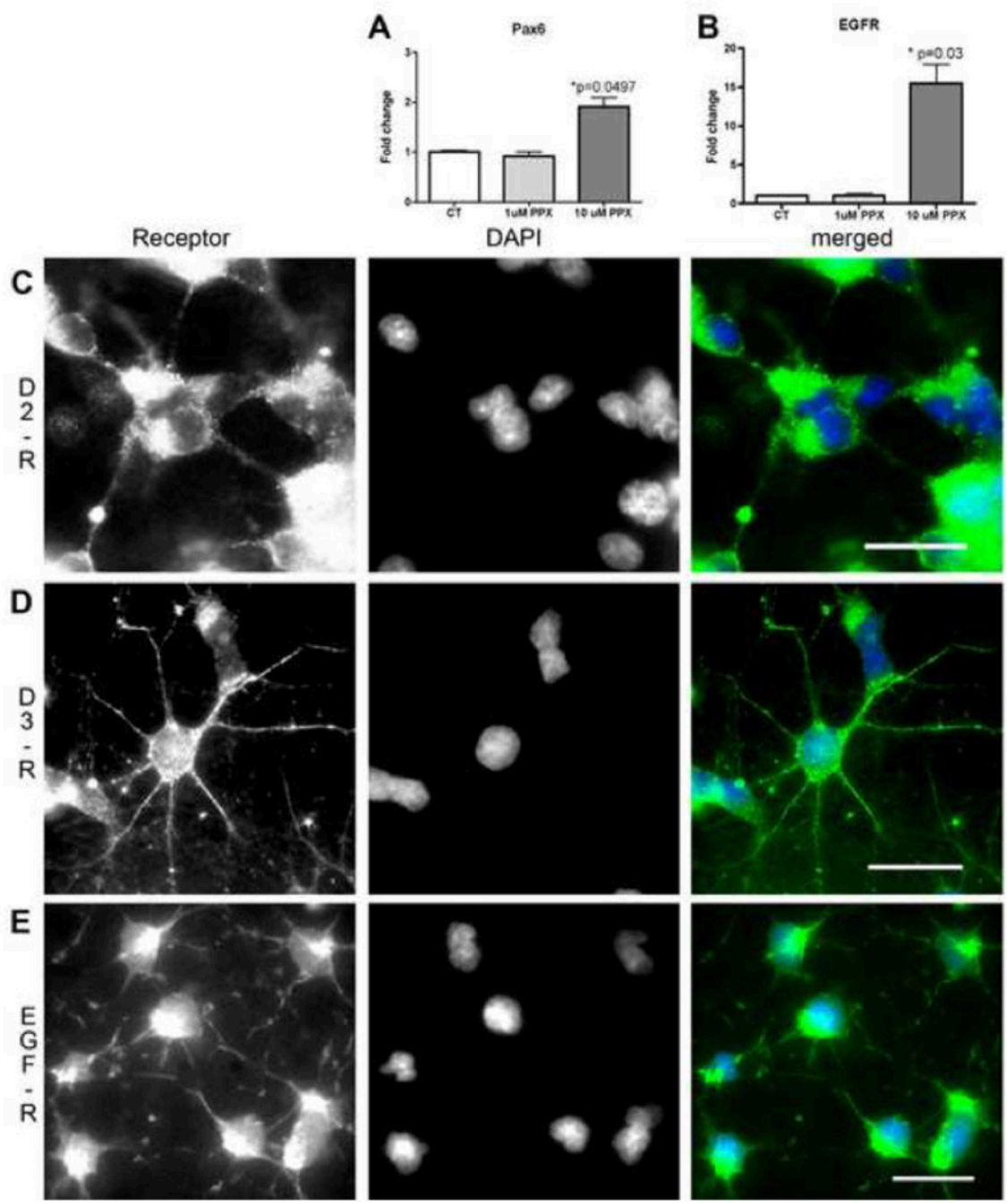

Figure 4. Schematic representation of changes in neurogenesis after PPX treatment

PPX induces an increase in SVZ proliferation (green cells, C compared to B). There is an increase in newly generated cells migrating to the olfactory bulb via the rostral migratory stream (colored arrow in D, E). This is reflected by an increase in new neurons in the granule cell layer (purple cells, E compared to D) and new dopaminergic neurons in the glomerular layer of the olfactory bulb (blue cells, E compared to D).

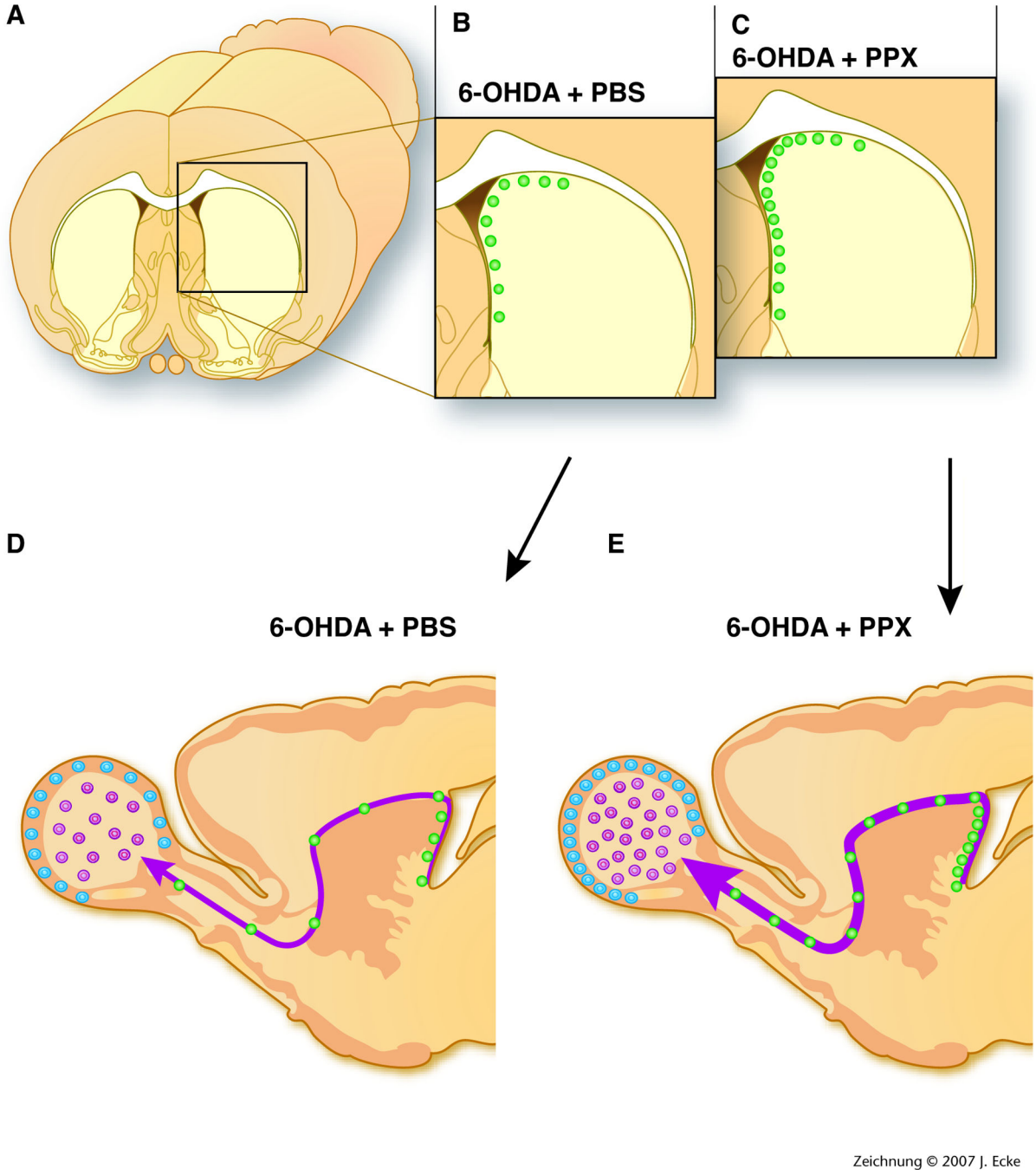

Figure 5. Presence of dopamine receptors and EGF-R/ Pax6 in ANPs

Relative quantification of mRNAs by RT-PCR showing increased EGF-R (A) and Pax6 (B) gene expression after 24 hours PPX exposure. Significant differences in gene expression were determined by Student's *t* test (paired, two tailed). Asterisks denote significant differences in expression between control and treated cells: *, $p<0.05$; **, $p<0.001$. Immunodetection of the D2-R (C), D3-R (D), and EGF-R (E, left panels). Nuclei were

counterstained using DAPI (middle panels), merged in right panels (blue: DAPI, green: receptors, scalebars 20μm).