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Neuroprotection by neuropeptide Y in cell and animal models of Parkinson's disease

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Abstract

This study was aimed to investigate the potential neuroprotective effect of neuropeptide Y (NPY) on the survival of dopaminergic cells in both in vitro and in animal models of Parkinson's disease (PD). NPY protected human SH-SY5Y dopaminergic neuroblastoma cells from 6-hydroxydopamine-induced toxicity. In rat and mice models of PD, striatal injection of NPY preserved the nigrostriatal dopamine pathway from degeneration as evidenced by quantification of (1) tyrosine hydroxylase (TH)-positive cells in the substantia nigra pars compacta, levels of (2) striatal tyrosine hydroxylase and dopamine transporter, (3) dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) as well as (4) rotational behavior. NPY had no neuroprotective effects in mice treated with Y_2 receptor antagonist or in transgenic mice deficient for Y_2 receptor suggesting that NPY effects are mediated through this receptor. Stimulation of Y_2 receptor by NPY triggered the activation of both the ERK1/2 and Akt pathways but did not modify levels of brain derived neurotrophic factor (BDNF) or glial cell line-derived neurotrophic factor. These results open new perspectives in neuroprotective therapies using NPY and suggest potential beneficial effects in PD.

Keywords: Parkinson's disease; Neuropeptide Y; Neuroprotection

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder mainly characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leading to a loss of dopamine in the target structure striatum and development of motor symptoms, such as bradykinesia, rigidity, and tremor (Lang and Lozano, 1998). Current therapies in PD can provide temporary relief of motor symptoms but do not prevent disease progression. Research on new experimental treatment strategies for PD focused on either restoring dopamine level in the striatum by transplantation of fetal dopaminergic neurons (Gaillard and Jaber, 2011), or on preventing the degeneration of the dopaminergic neurons not yet affected by the pathology.

Neuropeptide Y (NPY), a 36-amino acid peptide, is the most abundant neuropeptide in the mammalian brain (Adrian et al., 1983; Allen et al., 1983) and is involved in many physiological and pathological conditions such as appetite regulation, memory, and seizure (Balasubramaniam, 1997; Vezzani et al., 1999; Wettstein et al., 1995). In vitro, NPY protects hippocampal organotypic cultures exposed to an excitotoxic insult (Silva et al., 2003a) and retinal cells against ecstasy-induced toxicity (Alvaro et al.,

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2008). Similarly, in vivo, NPY protects striatal neurons against methamphetamine-induced cell death (Thiriet et al., 2005). More recently, it has been shown that the C-terminal fragments of NPY protected neurons from the neurotoxic effects of the amyloid-β suggesting a potential interest of NPY in Alzheimer's disease (Rose et al., 2009). Changes in NPY expression have been observed in some pathological conditions such as PD. Indeed, the number of NPY messenger RNA (mRNA)-expressing interneurons was markedly increased in the nucleus accumbens and caudate nucleus of PD patients compared with healthy individuals (Cannizzaro et al., 2003). The NPY changes may reflect an endogenous, but ineffective, neuroprotective response of the brain against the ongoing neurodegenerative process. Similarly, in animal models of PD, it has been shown that the loss of nigrostriatal dopamine pathway led to a significant increase in the number of NPY-expressing cells in the striatum (Kerkerian et al., 1986, 1988). However, whether NPY can have neuroprotective effects on dopamine neurons and in animal models of PD has not yet been investigated.

In the present study, we hypothesized that an exogenous administration of NPY could be neuroprotective for nigral dopamine neurons when injected in the striatum of animals treated with 6-hydroxydopamine (6-OHDA). We show that NPY is neuroprotective in both in vitro and in vivo models of PD. Our behavioral, neuroanatomical, neurochemical, and imaging studies indicate that in vivo NPY treatment preserves nigrostriatal pathway against 6-OHDA-induced toxicity. The neuroprotective effect of NPY is preferentially mediated via the Y_2 receptor as evidenced using pharmacological agents and mice knockout for NPY receptors. In addition, NPY treatment induced the activation of both the ERK and the Akt pathways. Taken together, these results suggest a neuroprotective role of NPY on dopamine neurons via Y_2 receptor activation; they suggest a potential interest of NPY as a neuroprotective agent in PD.

2. Methods

2.1. Cell culture

Human SH-SY5Y dopaminergic neuroblastoma cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine at 37 °C in a 5% CO_2 atmosphere. Cell suspension was prepared in a 96-well plate at a density of 10^4 cells per well in 100 μL of medium. Cultures were grown for 48 hours before adding 6-OHDA (25, 50, or 100 μM, Sigma, Saint-Quentin Fallavier, France). NPY (23 μM, Bachem) was added 2 hours prior or simultaneously with 6-OHDA (50 μM). All molecules were used at approximately 20-μM concentration: Y_1 receptor agonist ([Leu31, Pro34] NPY, 24 μM, Bachem AG, Switzerland), Y_2 receptor agonist ($NPY_{3\text{-}36}$, 25 μM, Bachem), Y_1 antagonist (BIBP3226, 20 μM, Bachem) or Y_2 antagonist (BIIE0246, 25 μM, Tocris Biosciences, Bristol, UK) were added to the medium simultaneously with 6-OHDA and NPY.

2.2. Cell viability assay

Cell viability was determined using the MTS assay system (Promega, Les Bans, France) according to the manufacturer's protocol. Briefly, after 24-hour incubation with the different molecules, the medium was changed and 20 μL of MTS reagent was added to each well and let to incubate at 37 °C for 2 hours. The absorbance at 490 nm was measured using a Mithras LB 940 plate reader (Berthold, Thoiry, France). Control cells were cultured in the same way without toxin or peptide, and the values of absorbance were expressed as percentage of control. Wells without cells were used as blank and absorbance values were subtracted as background.

2.3. Animals

Most experiments were carried out on female C57BL/6 mice (4 months old) supplied by R. Janvier (Le Genest-Saint Isles, France). Y_1 receptor knockout mice (C57BL/6 background) were kindly provided by Dr T. Pedrazzini (Switzerland) (Pedrazzini et al., 1998) and Y_2 receptor knockout mice were kindly provided by Dr. H. Herzog (Australia) (Sainsbury et al., 2002). For better resolution in imaging studies with PE2I, 21 adult male Wistar rats weighing 250–300 g (Janvier, Le Genest-Saint Isles, France) were used. All animals were raised and housed under a 12-hours light/dark cycle with food and water ad libitum. All efforts were made to minimize the number of animals and their suffering. All animal treatments conformed to the guidelines of the French Agriculture and Forestry Ministry (decree 87849) and of European Communities Council Directive of 24 December 1986 (86/609/EEC).

2.4. Surgical procedure

Mice were anesthetized with intraperitoneal (i.p.) injection of Avertin (250 mg/kg) and placed in a stereotaxic frame. They were randomly assigned to 1 of the following groups: saline (NaCl 0.9%) (n = 40), 6-OHDA (8 μg/μL, Sigma) + saline (n = 40), NPY (23 μM, Bachem) (n = 40), 6-OHDA+NPY (n = 40), 6-OHDA+Y_1 receptor agonist ([Leu31, Pro34] NPY, 24 μM, Bachem) (n = 10), 6-OHDA+Y_2 receptor agonist ($NPY_{3\text{-}36}$, 25 μM, Bachem) (n = 10), 6-OHDA+Y_1 antagonist (BIBP3226, 22 μM, Bachem) (n = 8), or 6-OHDA+ Y_2 antagonist (BIIE0246, 25 μM, Tocris Biosciences) (n = 8). 6-OHDA (0.5 μL) was injected unilaterally in 3 different sites in the striatum at the following coordinates according to the stereotaxic atlas of Paxinos and Franklin (2001): site-1: anterior-posterior (A-P): 1 mm, lateral (L): 1.7 mm, profound (P): 2.2 mm; site-2: A-P: 0.26 mm, L: 1.8 mm, P: 2.5 mm; site 3: A-P: −0.2 mm, L: 2 mm, P: 2.8 mm. NPY, Y_1 receptor agonist/antagonist, Y_2 receptor agonist/antagonist, or saline solution

(0.5 μL) were injected immediately after 6-OHDA injection in sites 1 and 3.

For imaging studies, 10 minutes before surgery, rats were injected i.p. with desipramine (25 mg/kg) and pargyline (50 mg/kg) (Sigma, Saint-Quentin Fallavier, France). Rats were anesthetized with isoflurane (4% then 2%, 500 mL/minute) and placed on a stereotaxic apparatus. Lesion was carried out by unilateral intrastriatal injection of 6-OHDA (1 μg/μL). Five microliters of 6-OHDA or saline was administered in 2 points of the right striatum at the following coordinates according to the stereotaxic atlas of Paxinos and Watson (1986): site-1: A-P: 0.5 mm, L: −2.5 mm, P: −5 mm; site-2: A-P: −0.5 mm, L: −4.2 mm, P: −5 mm. NPY (23 μM) or saline solution (0.5 μL) was injected immediately after 6-OHDA injection in these 2 sites. Injections were performed using a Hamilton microsyringe and solutions were allowed to diffuse before syringe removal. Three groups (saline, 6-OHDA and 6-OHDA + NPY) of 7 animals were lesioned.

2.5. Behavioral test

Four weeks after surgical procedure, mice were tested for drug-induced rotational behavior. According to the method previously described (Gaillard et al., 2009; Ungerstedt and Arbuthnott, 1970), mice received a subcutaneous injection of apomorphine (0.5 mg/kg), a dopamine D1/D2 receptor agonist. They were acclimatized in the bowls for 10 minutes prior to test. After apomorphine administration, full body turns were monitored over a period of 1 hour using an automated rotameter. Data were compiled using RotaCount 8 software (Columbus Instruments, OH, USA) and expressed as number of controlateral rotations per 60 minutes.

2.6. Immunohistochemistry

Four weeks after injections, mice were deeply anesthetized with Avertin (i.p., 500 mg/kg) and perfused transcardially with 0.9% saline followed by 4% ice-cold paraformaldehyde (0.1 M phosphate buffer [PB], pH 7.4). Brains were removed, postfixed 2 hours, and stored in phosphate buffered saline (PBS) containing 30% sucrose at 4 °C. Serial coronal 40 μm depth sections were cut on a sliding freezing microtome (RM2145, Leica, Nanterre, France), collected in a cryoprotective solution (20% glucose, 40% ethylene glycol, 0.025% sodium azide, 0.05 M PB, pH 7.4) and stored at −20 °C until immunohistochemical processing.

After 3 rinses in PBS 0.1 M, endogenous peroxidase activity was quenched for 5 minutes in 3% H_2O_2, 10% methanol in PBS. Free-floating sections were preincubated for 1.5 hours in bovine serum albumin 3%, Triton 0.3% in PBS to block nonspecific staining. Thereafter, sections were incubated overnight at room temperature with an anti-tyrosine hydroxylase (TH) primary antibody (1:5000, mouse, Incstar, MN, USA). This was followed by 3 rinses in tris-buffered saline (TBS) and then by incubation for 1.5 hours with a biotinylated anti-mouse secondary antibody (1:200, goat, Vector Laboratories, CA, USA). Immunoreactivity was detected using the Elite ABC Vectastain kit enhancing system (Vector Laboratories, CA, USA) and 3-3′-diaminobenzidine as a chromogen (Sigma). Sections were mounted on gelatin slides, dehydrated in toluene and coverslipped by using DPX montage medium (BDH Laboratories, Poole, England).

2.7. Stereological counting

Unbiased stereological estimation of the TH+ cells in the SNpc was performed using the optical fractionator method principle as previously described (West et al., 1991). Sampling was done using the Olympus, Cast-Grid system (Olympus, Ballerup, Denmark). The CAST-Grid software (version 1.09) was used to delineate the SNpc at 4× objective from −2.80 to −3.80 mm relative to bregma. Counting was performed at a 100× objective and a counting frame (5184 μm^2) was systematically moved through all counting areas until the entire delineated area was sampled. The estimate of the total number of neurons was calculated according to the optical fractionator formula (West et al., 1991). For every experimental condition, the number of TH+ cells in the contralateral untreated side was taken as control.

2.8. Autoradiographic analysis of the dopamine transporter

Twelve days postlesion, rats were sacrificed by decapitation and the brains were removed and frozen into isopentane cooled at −35 °C. The frozen tissue was cut into 20-μm thick sections with a cryostat (Reichert-Jung Cryocut CM3000; Leica, Rueil Malmaison, France), thaw mounted on SuperFrostPlus slides (CML, Nemours, France) and kept at −80 °C until use. Sections were taken from brain regions corresponding to the substantia nigra (SN) and striatum. Binding studies with [^{125}I]PE2I as dopamine transporter (DAT) ligand were performed as previously reported (Chalon et al., 1999). Sections were incubated for 90 minutes at 22 °C with 100 pM [^{125}I]PE2I in 100 μL of a pH 7.4 phosphate buffer (10.14 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4, 0.32 M sucrose). The nonspecific binding was defined on adjacent sections incubated in the presence of 1 μM cocaine. Three sections were used for each brain structure. Sections were then washed twice for 10 minutes in the buffer at 4 °C and rinsed in distilled water. After drying, sections were exposed to sensitive films (Biomax MR, Kodak, France) with standards (^{125}I-microscales; Amersham Biosciences AB, Uppsala, Sweden) in X-ray cassettes for 1 day for striatum and 2 days for SNpc. The films were analyzed using an image analyzer (Biocom, Les Ulis, France) after identifying anatomical regions of interest. The absorbance obtained was converted into apparent tissue ligand concentration with reference to standard and specific activity of the radioligand. The inten-

sity of [^{125}I]PE2I binding was then calculated in fmol/mg of equivalent tissue and results are expressed as the percentage of binding in the ipsilateral treated side compared with that in the contralateral intact side.

2.9. High performance liquid chromatography (HPLC) procedure

Four weeks after surgery, mice were beheaded and the striatum of both sides was rapidly dissected and frozen at −80 °C until HPLC analysis. Tissue samples were weighted and homogenized in a solution containing 0.1 M perchloric acid and 10^{-7} M dihydroxybenzylamine as an internal standard. After sonication, homogenates were centrifuged at 12,000 rpm for 10 minutes at 4 °C and supernatant was used to determine the concentration of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) using the HPLC apparatus (5200a Coulochem III, ESA, Chelmsford, MA, USA) (Bezard et al., 2000). Briefly, a column (Kromasil 100 C18 5 μm, Tecknokroma, Barcelona, Spain) was perfused with a mobile phase (100 mM sodium dihydrogenphosphate buffer, pH 3.0, 700 μM octane sulfonic acid, 200 μM ethylenediaminetetraacetic acid (EDTA), 25% methanol at a constant rate of 600 μL/minute) pumped by an ESA 582 solvent delivery module. Levels of dopamine and DOPAC were quantified by peak height comparison with standards that were run on the date of analysis. Concentrations were expressed as ng/mg wet weight of brain tissue and presented as a percentage of contralateral striatum.

2.10. Western blotting

Mice were sacrificed at different time points: 24 hours, 2 days, 1 week, and 3 weeks after surgical procedure. Brains were rapidly removed, ipsi- and contralateral striatum and ventral mesencephalon were dissected and frozen on dry ice. Tissues were homogenized in a 50 mM 3-(N-morpholino) propanesulfonic acid (MPOS) buffer containing 1% nonidet P-40 (NP-40), 100 mM sodium pyrophosphate, 250 mM NaCl, 3 mM ethylene glycol tetraacetic acid (EGTA), 0.2 mM sodium orthovanadate, 1 mM NaF, protease inhibitors and phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 13,000*g* for 10 minutes at 4 °C. Supernatants were collected and stored at −80 °C until use. Protein concentration was determined using the detergent compatible (DC) protein assay kit (Bradford method). Thirty micrograms of protein were boiled at 100 °C for 5 minutes in loading buffer, separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred on a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). After blocking for 3 hours in Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin, membranes were incubated overnight at 4 °C with 1 of the following primary antibodies: rabbit anti-glial cell line-derived neurotrophic factor (GDNF) (1:500, Santa Cruz Biotechnology), rabbit anti-brain derived neurotropic factor (BDNF) (1:500, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-ERK1/2 (1:1000, Cell Signaling, MA, USA), rabbit anti-phospho-ERK1/2 (1:1000, Cell Signaling), mouse anti-TH (1:5000, Incstar, MN, USA), rabbit anti-Akt (1:1000, Cell Signaling), or rabbit phospho-Akt (1:1000, Cell Signaling) in combination with a mouse anti-actin (1:20,000, Sigma) or a mouse anti-β3-tubulin (1:3000, Sigma). After washing for 30 minutes in Tween 20 with gentle agitation, membranes were incubated for 1 hour at room temperature with a goat anti-mouse or goat anti-rabbit Alexa 568 nm secondary antibody (1:1000, Invitrogen, Cergy Pontoise, France). Signal was detect using Typhoon Trio system (GE Healthcare, France). Band intensities were quantified by densitometric analyses using ImageJ software (NIH, Bethesda, MD, USA).

2.11. Statistical analysis

Figures show group means ± standard error of the mean (SEM). Data were analyzed using 1-way analysis of variance (ANOVA). For all analyses, upon confirmation of significant main effects, differences among individual means were analyzed using the Tukey-Kramer post hoc test. For all analyses, significance was accepted at $\alpha = 0.05$.

3. Results

3.1. Neuroprotective effect of NPY on SH-SY5Y dopaminergic cell line

We first investigated the potential neuroprotective effect on NPY against 6-OHDA-induced toxicity in vitro. The cell survival study was carried out on SH-SY5Y cells that are the most extensively used model of dopaminergic neurons to study neuroprotective properties of NPY in vitro (Shimohama et al., 2003). Cell viability was analyzed using an MTS assay. ANOVA analysis revealed a main effect of treatments on cells survival [$F(10,24) = 233.98$; $p < 0.0001$]. Increasing concentrations of 6-OHDA were added to the medium in order to determine the dose-response toxicity as well as the adequate concentration of the toxin for the neuroprotection study. Cell viability was analyzed 24 hours after intoxication (Fig. 1). 6-OHDA-induced cell death increased in a dose-dependent manner (79 ± 5% at 25 μM, 36 ± 3% at 50 μM, and 17 ± 3% at 100 μM of 6-OHDA; ps < 0.001). Neither saline nor NPY (23 μM) alone affected cell viability (96 ± 3% for saline, 107 ± 5% for NPY; ps > 0.5). Incubation with NPY 2 hours prior to the addition of 6-OHDA (50 μM) significantly prevented cell death (70 ± 14%) ($p < 0.001$ vs. 6-OHDA). Similarly, simultaneous treatment of the cells with the toxin and the peptide significantly protected the cells from 6-OHDA-induced toxicity (73 ± 17%) ($p < 0.001$ vs. 6-OHDA 50 μM). We then investigated which NPY receptor subtype might be involved in this neuroprotection. SH-SY5Y cells express the Y_1 and the Y_2 receptors (Chen and Cheung, 2004), 2 receptors also expressed by dopamine neurons along the nigrostriatal pathway (Shaw et al., 2003). Treatment with a specific Y_2 receptor agonist (NPY3-36, 25 μM) protected SH-SY5Y cells

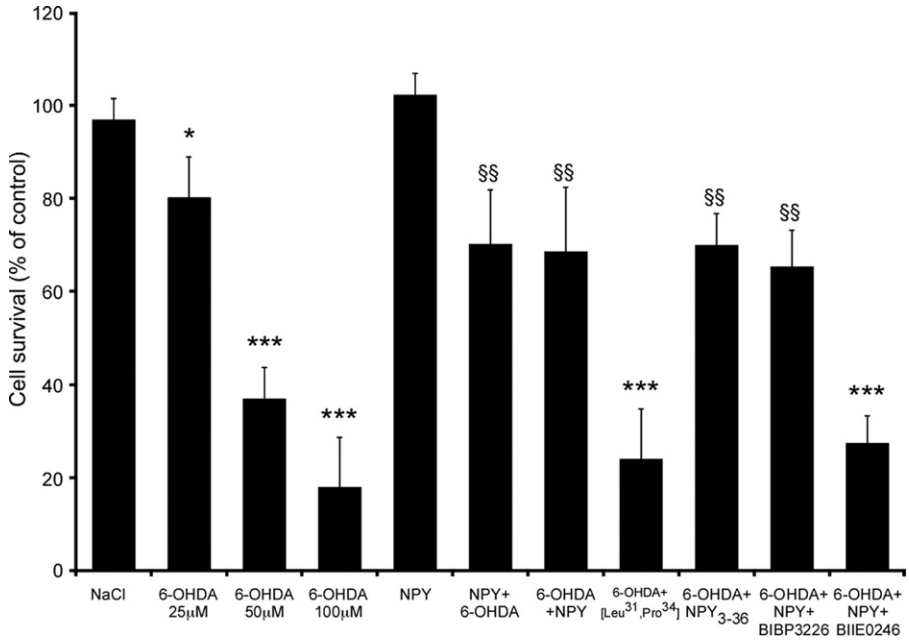

Fig. 1. Neuropeptide Y (NPY) protects SH-SY5Y cells from 6-hydroxydopamine (6-OHDA) toxicity. Human neuroblastoma cells were incubated with NPY, Y_1 receptor agonist ([Leu31, Pro34] NPY) or antagonist (BIBP3226), Y_2 receptor agonist (NPY_{3-36}) or antagonist (BIIE0246) and/or 6-OHDA. Toxicity of 6-OHDA was evaluated using increasing concentration of the toxin (25, 50, and 100 μM). Cultures were treated with NPY or its specific receptor agonists (20 μM approximately) simultaneously (6-OHDA+NPY) or prior to intoxication (NPY+6-OHDA). Cell viability was evaluated 24 hours following treatments using an MTS (Promega) assay. Each condition was performed in triplicate, data were calculated from 6 different experiments and expressed as mean ± standard error of the mean (SEM). Values are presented as percentage of control group. * $p < 0.05$; *** $p < 0.001$ compared with saline group; §§ $p < 0.01$ compared with 6-OHDA 50 μM group.

from 6-OHDA-induced toxicity (68 ± 7%) ($p < 0.001$ vs. 6-OHDA 50 μM) with the same efficacy observed with NPY treatment ($p = 0.88$ vs. 6-OHDA+NPY). In contrast, Y_1 receptor specific agonist ([Leu31, Pro34] NPY, 24 μM) failed to prevent cell death (32 ± 11%) ($p = 0.72$ compared with 6-OHDA 50 μM). The use of specific Y_1 or Y_2 receptor antagonist further confirmed the role of Y_2 receptor in the neuroprotective effect of NPY. Indeed, while addition of Y_1 receptor antagonist (BIBP3226; 22 μM) failed to abolish the NPY-induced neuroprotection ($p = 0.81$ vs. 6-OHDA+NPY), Y_2 receptor antagonist (BIIE0246; 22 μM) blocked NPY-induced neuroprotection leading to a significant cell loss (27 ± 4%) ($p < 0.001$ vs. 6-OHDA+NPY; $p = 0.084$ vs. 6-OHDA 50 μM). Thus, NPY protected SH-SY5Y cells against 6-OHDA-induced toxicity through the activation of the Y_2 receptor.

3.2. NPY attenuates rotational behavior in 6-OHDA unilaterally lesioned mice

We then investigated the effect of a striatal injection of NPY in a mouse model of PD. To assess behavioral consequences of the different treatments, mice underwent an apomorphine-induced rotation test. Results are expressed as number of contralateral rotations during 60 minutes (Fig. 2). ANOVA analysis revealed a main effect of treatment on the rotational behavior [$F(9,65) = 76.81$; $p < 0.0001$]. As previously reported (Bensadoun et al., 2000), we observed that mice that received striatal injections of 6-OHDA (12 μg) performed a high number of rotations (207 ± 34) compared with saline injected mice (2 ± 1) ($p < 0.001$). This behavior was significantly decreased by the striatal injection of NPY (23 μM) in lesioned mice (81 ± 26; $p < 0.001$ vs. 6-OHDA group). Similarly, a reduction in the number of rotations was observed after the injection of the Y_2 agonist (25 μM) in lesioned mice (66 ± 25; $p < 0.001$ vs. 6-OHDA group; $p = 0.09$ vs. 6-OHDA+NPY group). This effect was abolished when mice were treated with both NPY and a Y_2 specific antagonist (BIIE0246; 22 μM) resulting in the persistence of a high number of rotations ($p < 0.001$ vs. saline group), while administration of NPY and a Y_1 antagonist did not show any motor improvement ($p = 0.22$ vs. 6-OHDA group). Striatal injection of a Y_1 agonist (25 μM) was also not efficient in reducing the number of apomorphine-induced rotations (161 ± 40) ($p = 0.24$ vs. 6-OHDA group).

In parallel to the pharmacological approach, we investigated the effect of similar treatments on transgenic mice knockout for the Y_1 or the Y_2 receptor of NPY (Fig. 2). We found that mice deleted for the Y_1 receptor gene exhibited a low number of apomorphine-induced rotations after injection of NPY and 6-OHDA ($p < 0.001$ vs. 6-OHDA group) further confirming that the presence of Y_2 receptor is mandatory for the effect of NPY in this model. Similar injections in Y_2 knockout mice resulted in a high number of rotations performed by these animals (250 ± 39) ($p = 0.89$ vs. 6-OHDA).

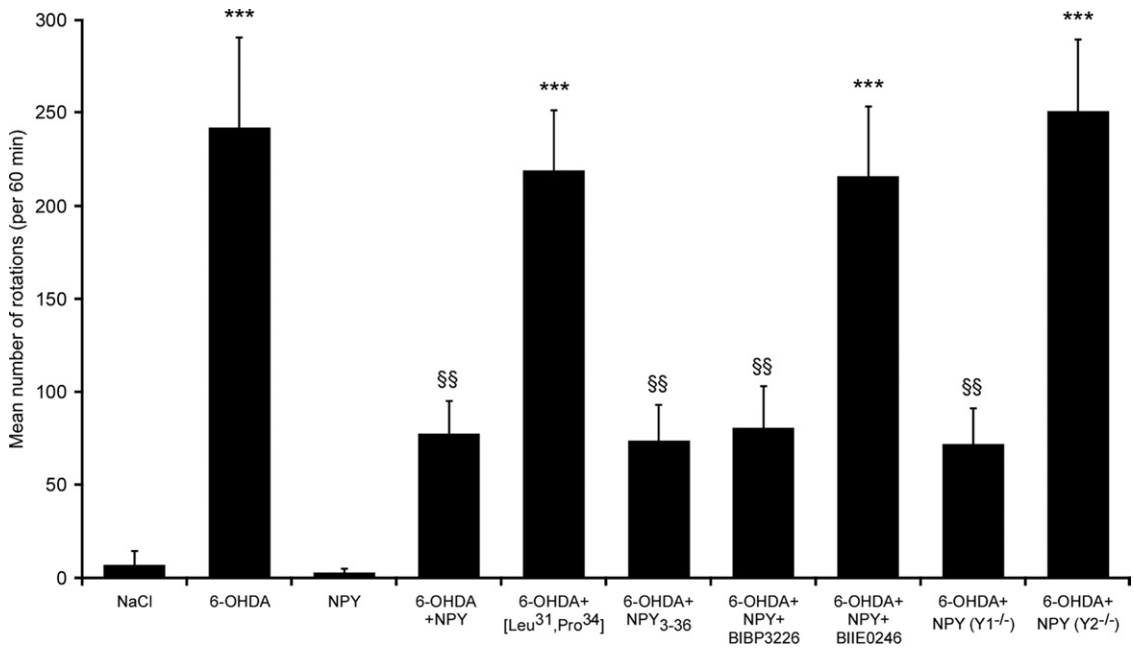

Fig. 2. Behavioral consequence of neuropeptide Y (NPY) treatment in 6-hydroxydopamine (6-OHDA) mice. Quantification of the number of apomorphine-induced rotation in wild type animals treated with saline, NPY, 6-OHDA, 6-OHDA+NPY (23 μM), 6-OHDA+ Y_1 receptor agonist ([Leu31, Pro34] NPY, 24 μM), 6-OHDA+Y_2 receptor agonist (NPY_{3-36}, 25 μM), 6-OHDA+ NPY+Y_1 antagonist (BIBP3226, 22 μM), or 6-OHDA+NPY+Y_2 antagonist (BIIE0246, 22 μM), and mice knockout for the Y_1 or the Y_2 receptor treated with 6-OHDA+NPY. Results as represented as the number of rotations in 60 minutes. Data are expressed as mean ± standard error of the mean (SEM). *** $p < 0.001$ compared with saline group; §§ $p < 0.01$ compared with 6-OHDA group.

3.3. NPY reduces the degeneration of nigral dopamine neurons following 6-OHDA lesion

After the behavioral tests, mice were sacrificed, brains were cut and sections were processed for TH immunodetection. The survival of the nigral dopamine neurons was examined by the stereological counting of TH+ cells in the SNpc. Comparisons were made on absolute number of TH+ cells and results were expressed as percentage of remaining dopamine neurons compared with the uninjected contralateral side.

ANOVA analysis revealed a main effect of treatment on the number of TH+ cells in the SNpc [$F(9,65) = 200.95$; $p < 0.0001$]. As previously demonstrated (Alvarez-Fischer et al., 2008), we found that striatal injection of 6-OHDA induced a retrograde degeneration of the nigrostriatal pathway leading to a significant loss of TH+ neurons into the SNpc (39 ± 3%) compared with saline group (99 ± 3%) ($p < 0.001$) (Fig. 3A, B, and K). Injection of NPY in the striatum immediately after 6-OHDA protected a large number of dopaminergic cells from 6-OHDA-induced toxicity (76 ± 6%) ($p < 0.01$ vs. 6-OHDA) (Fig. 3D and K). As demonstrated in our in vitro study, the neuroprotective effect observed after NPY treatment appears to be mediated through the activation of the Y_2 receptor because the striatal injection of the Y_2 agonist (25 μM) in 6-OHDA-lesioned mice resulted in only a partial degeneration of dopaminergic cells (71 ± 4%) ($p = 0.47$ vs. 6-OHDA+NPY group; $p < 0.01$ vs. 6-OHDA group) (Fig. 3F and K). In contrast, injection of the Y_1 agonist (24 μM) failed to prevent cell death in lesioned mice thereby leading to a massive loss of dopamine neurons in the SNpc similar to that observed in lesioned mice (38 ± 3%) ($p = 0.86$ vs. 6-OHDA) (Fig. 3E and K). The involvement of Y_2 receptor in the NPY-induced neuroprotective effect was further confirmed by the injection of Y_2 receptor antagonist (BIIE0246; 22 μM) in 6-OHDA+NPY treated mice that abolished the protective effect of NPY resulting in a significant degeneration of dopamine neurons (40 ± 5%) ($p < 0.001$ vs. saline; $p = 0.74$ vs. 6-OHDA) (Fig. 3H and K). In contrast, striatal injection of the Y_1 antagonist (BIBP3226, 22 μM) did not alter the positive effect of NPY on dopamine neurons survival as demonstrated by the significant number of surviving neurons (74 ± 4%) ($p = 0.68$ vs. 6-OHDA+NPY; $p < 0.01$ vs. 6-OHDA) (Fig. 3G and K).

Complementary to the pharmacological studies using specific agonist and antagonists of the NPY receptors, we carried out a similar analysis on transgenic mice lacking the Y_1 or the Y_2 receptor. We found that nigral dopamine cells were also protected against 6-OHDA toxicity in $Y_1^{-/-}$ mice that received striatal injection of 6-OHDA and NPY (73 ± 4%) ($p < 0.01$ vs. 6-OHDA) (Fig. 3I and K). Conversely, similar treatment in mice knockout for the Y_2 receptor resulted in the absence of neuroprotective effect by NPY as illustrated by the massive loss of TH+ cells in the SNpc (31 ± 7%) ($p = 0.42$ vs. 6-OHDA; $p < 0.01$ vs. 6-OHDA+NPY) (Fig. 3J and K). Altogether, our in vivo results are in line with our in vitro findings demonstrating

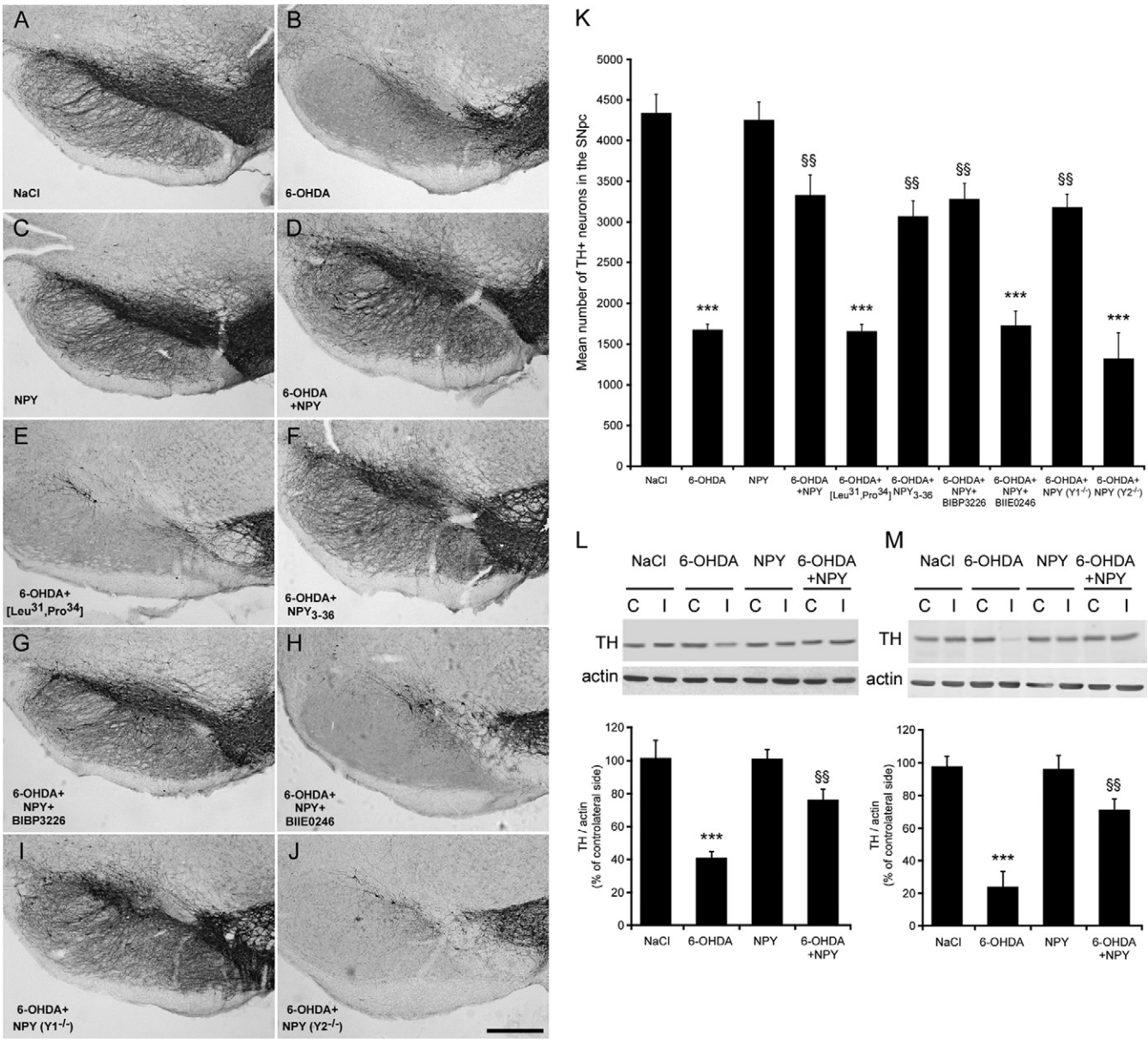

Fig. 3. Neuropeptide Y (NPY) partially rescues the nigrostriatal pathway from 6-hydroxydopamine (6-OHDA) toxicity. Immunohistochemistry of tyrosine hydroxylase (TH) in the substantia nigra pars compacta (SNpc) in (A) saline, (B) 6-OHDA, (C) NPY, (D) 6-OHDA+NPY (23 μM), (E) 6-OHDA+ Y_1 receptor agonist ([Leu31, Pro34] NPY, 24 μM), (F) 6-OHDA+Y_2 receptor agonist ($NPY_{3\text{-}36}$, 25 μM), (G) 6-OHDA+ NPY+ Y_1 antagonist (BIBP3226, 22 μM), (H) 6-OHDA+NPY+Y_2 antagonist (BIIE0246, 22 μM) treated wild type mice, and in (I) 6-OHDA+NPY treated $Y_1^{-/-}$ and (J) $Y_2^{-/-}$ mice. Scale bar: 400 μm (A–H). (K) Stereologic quantification of the number of TH-positive neurons in the SNpc in the different experimental groups. TH expression in the (L) SNpc and the (M) striatum was analyzed by Western blot in saline, 6-OHDA, NPY, and 6-OHDA+NPY injected mice. Data are expressed as mean ± standard error of the mean (SEM). C, controlateral side; I, ipsilateral injected side. *** $p < 0.001$ compared with saline group; §§ $p < 0.01$ compared with 6-OHDA group.

that NPY is efficient in preventing 6-OHDA-induced death through the selective activation of the Y_2 receptor.

3.4. NPY partially prevents degeneration of the nigrostriatal pathway induced by 6-OHDA

Results obtained with the stereological counting were further confirmed by an analysis of the level of the TH protein in both the SNpc and the striatum by Western blot (Fig. 3L) and the terminals (Fig. 3M). Data were presented as a percentage of TH expression in the treated side with respect to the contralateral intact side. ANOVA analysis revealed a significant effect of treatment on the level of TH in the SNpc [$F(3,16) = 33.76$; $p < 0.0001$] and the striatum [$F(3,16) = 50.23$; $p < 0.0001$]. Striatal injection of 6-OHDA induced a significant decrease of TH protein levels both in the SNpc (−58 ± 9%) (Fig. 3L) and in the

striatum (−72 ± 11%) (Fig. 3M) compared with saline injection (0.7 ± 12% for SNpc and 1.8 ± 12% for the striatum) (*p*s < 0.001). This difference was reduced by the injection of NPY in 6-OHDA-lesioned mice as demonstrated by the significant increase of TH expression in the SNpc (−24 ± 4.75%) ($p < 0.01$ vs. 6-OHDA) (Fig. 3L) and in the striatum (−32 ± 12%) ($p < 0.001$ vs. 6-OHDA) (Fig. 3M). Thus, 6-OHDA-lesioned mice treated with NPY had 2.4 times more TH proteins in the striatum than lesioned mice suggesting the protection of significant proportion of dopaminergic terminals. Striatal injection of NPY alone did not affect TH expression in the SNpc (*p*s > 0.7).

In the 6-OHDA rat model, we confirmed the degeneration of the nigrostriatal pathway measured by the stereological counting of TH+ cells in the SNpc and the striatum of animals.

We observed that striatal injection of 6-OHDA induced a significant loss of TH+ neurons into the SNpc (55 ± 3%) and of TH levels in the striatum (60% ± 9%) compared with saline group (90 ± 6% and 108 ± 6% respectively) ($p < 0.001$). Injection of NPY in the striatum immediately after that of 6-OHDA protected a large number of TH+ cells from 6-OHDA-induced toxicity in SNpc (81 ± 4%; $p < 0.001$ vs. 6-OHDA) as well as TH+ projections to the striatum (89 ± 4%; $p < 0.05$ vs. 6-OHDA) (data not shown).

3.5. NPY preserves dopamine transporter density in the substantia nigra and the striatum

The density of [^{125}I]PE2I binding sites was assimilated to DAT levels (Chalon et al., 1999) in the rat SNpc (Fig. 4A–C) and the striatum (Fig. 4E–G). Data are presented as a percentage of DAT expression in the treated side with respect to the contralateral intact side (Fig. 4D and H). In the SNpc of 6-OHDA-treated rat, the DAT density was significantly lower (60 ± 8%) compared with saline group (95 ± 3%) ($p < 0.005$, Fig. 4A, B, and D). In 6-OHDA rats treated with NPY, the DAT density was significantly higher (82 ± 4%) than in 6-OHDA group alone ($p < 0.05$ vs. 6-OHDA; Fig. 4B, C, and D). In the striatum, a significant decrease of DAT signal was observed in the ipsilateral side versus the controlateral side of 6-OHDA animals (57 ± 5%) compared with saline group (97 ± 2%, $p < 0.005$, Fig. 4E, F, and H). The injection of NPY induced a partial restoration of DAT levels in the ipsilateral striatal side (79 ± 3% in 6-OHDA+NPY rats, $p < 0.05$ vs. saline and $p < 0.05$ vs. 6-OHDA; Fig. 4F, G, and H).

3.6. NPY reduces dopamine loss following 6-OHDA lesion

We investigated the impact of the striatal injection of NPY in lesioned mice by analyzing the striatal levels of dopamine and its metabolite DOPAC by HPLC (Fig. 5). Dopamine content was measured in both treated and intact sides and expressed as ng/mg of tissue. ANOVA analysis

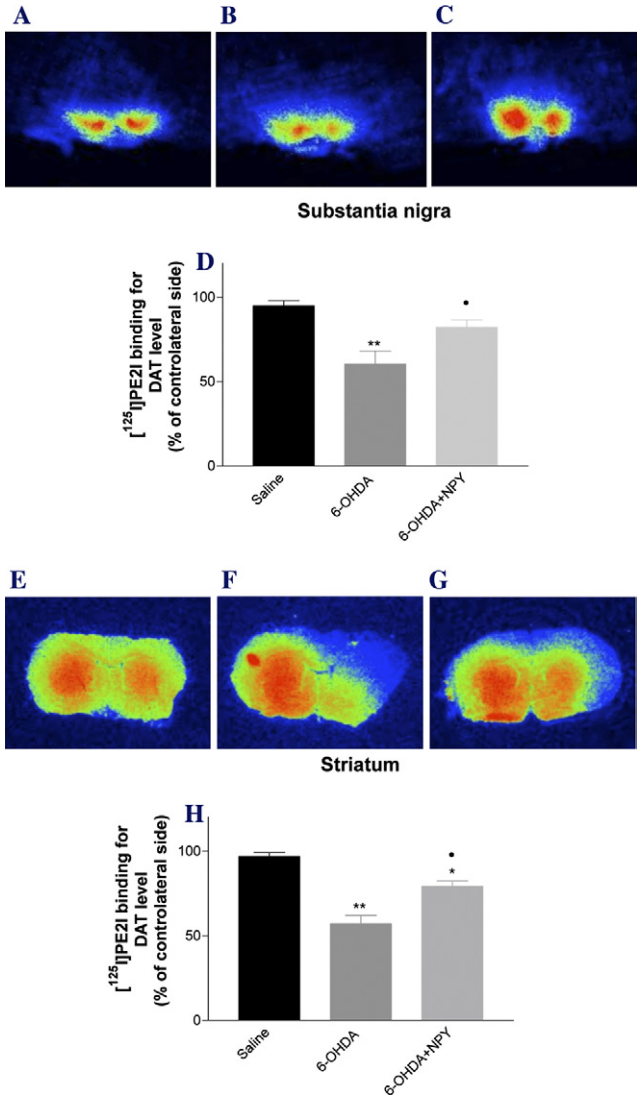

Fig. 4. Autoradiographic analysis of dopamine transporter (DAT) with [125I]-PE2I binding in substantia nigra and striatum. Representative autoradiographic images showing [125I]PE2I binding to the DAT in substantia nigra of (A) saline, (B) 6-hydroxydopamine (6-OHDA), (C) 6-OHDA+neuropeptide Y (NPY), and in striatum of (E) saline, (F) 6-OHDA, (G) 6-OHDA+NPY treated animals. The binding was quantified in the (D) substantia nigra and in the (H) striatum and results are expressed as the percentage of binding in the ipsilateral lesioned side compared with that in the contralateral intact side (mean ± standard error of the mean [SEM]). $n = 7$ for each group. * $p < 0.05$, ** $p < 0.005$, versus control saline animals; ● $p < 0.05$ versus 6-OHDA rats (1-way analysis of variance [ANOVA] followed by Tukey post hoc test).

showed that treatments induced a significant effect on the levels of dopamine [$F(3,11) = 11.98$; $p < 0.01$] and DOPAC [$F(3,16) = 10.54$; $p < 0.01$]. Four weeks after surgery, we observed that striatal injection of 6-OHDA induced a significant decrease in the striatal levels of dopamine (3.7 ± 0.8 ng/mg) and DOPAC (0.49 ± 0.11 mg/mg) compared with intact side (8.2 ± 0.7 ng/mg for dopamine; 0.89 ± 0.09 ng/mg for DOPAC) (*p*s < 0.01) (Fig. 5). Striatal injection of NPY in striatum of 6-OHDA-lesioned

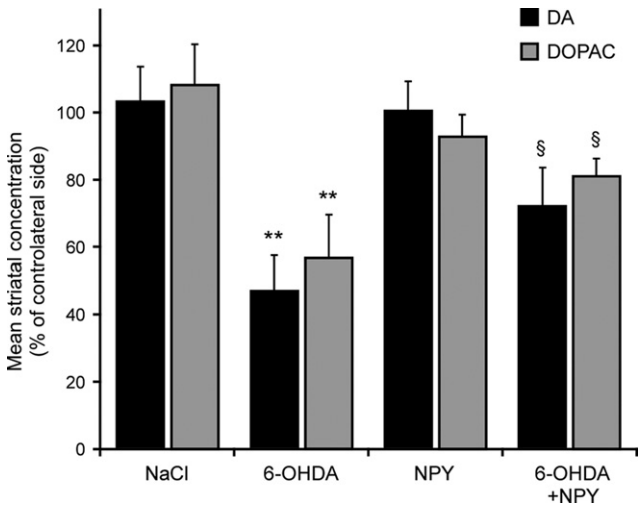

Fig. 5. Effect of neuropeptide Y (NPY) treatment on the striatal dopamine content. Analysis of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations by high performance liquid chromatography (HPLC) in treated and control sides in saline, 6-hydroxydopamine (6-OHDA), NPY, and 6-OHDA+NPY injected mice. Concentrations are measured in ng/mg of tissue and presented as a percentage of contralateral side. Data are expressed as mean ± standard error of the mean (SEM). *** $p < 0.001$; §§ $p < 0.01$ compared with 6-OHDA group.

mice resulted in a moderate decrease of dopamine (6.2 ± 0.4 ng/mg) and DOPAC concentrations (0.73 ± 0.08 ng/mg) compared with contralateral side (8.5 ± 0.5 ng/mg for dopamine; 0.91 ± 0.07 ng/mg for DOPAC) (*p*s < 0.05) (Fig. 5). Hence, NPY treatment significantly increased dopamine and DOPAC contents compared with the lesioned mice (*p*s < 0.05 vs. 6-OHDA group). NPY alone had no effect on the striatal content of dopamine (8.2 ± 0.9 ng/mg) or DOPAC (0.83 ± 0.15 ng/mg) compared with the contralateral side (8.3 ± 0.8 ng/mg for dopamine; 0.88 ± 0.11 ng/mg for DOPAC) (*p*s > 0.7) (Fig. 5).

3.7. ERK1/2 and Akt pathways are involved in NPY-induced neuroprotection

The mitogen-activated protein kinase pathway (MAPK) was shown to be rapidly activated and thereby to be, at least in part, responsible for the neuroprotective action of the glial cell line-derived neurotrophic factor (GDNF) (Cavanaugh et al., 2006; Lin et al., 2008; Lindgren et al., 2008). In order to analyze the possible activation of the MAPK pathway in our study models, animals were sacrificed at different time points from 24 hours to 3 weeks after surgery (Fig. 6A and B). Twenty-four hours after striatal injection of NPY, we observed an increased phosphorylation (4-fold) of ERK1/2 compared with saline-injected animals ($p < 0.001$). Similar variations were observed following 6-OHDA injection (4.6-fold) ($p < 0.001$ vs. saline group) or combined injection of 6-OHDA+NPY (6.5-fold) ($p < 0.001$ vs. saline group) (Fig. 6A). In addition, we found similar increases in the phosphorylation of ERK1/2 in the SNpc 48 hours after striatal injection of 6-OHDA ($p < 0.05$), NPY ($p < 0.05$) or combined injection of the toxin and the peptide ($p < 0.01$) compared with saline-injected animals (Fig. 6B).

We then investigated the activation of Akt pathway that is known to be a key component of the neuroprotective intracellular cascade (Ries et al., 2006) (Fig. 6C). We found that, in parallel to the activation of the ERK1/2 pathway, NPY triggered an increased expression of the phosphorylated form of Akt as detected 2 days after injection of NPY in the striatum ($p < 0.05$ vs. saline group) (Fig. 6C). Activation of the Akt pathway was also observed after striatal injection of 6-OHDA ($p < 0.05$ vs. saline group) although of a lesser magnitude than after NPY injection (Fig. 6C). In addition, we observed a strong expression (2-fold) of phospho-Akt pathway in lesioned mice treated with NPY ($p < 0.01$ vs. saline group) (Fig. 6C). This suggests that the neuroprotection induced by NPY may be, at least in part, mediated by activation of the phospho-Akt pathway.

3.8. The neuroprotective effect NPY is GDNF- and BDNF-independent

GDNF and BDNF have been reported to be neuroprotective in animal models of PD (Kirik et al., 2004; Sun et al., 2005). NPY was shown to act as a neuroprotective factor in other models of toxicity than PD by altering the expression of BDNF (Xapelli et al., 2008). Thus, we investigated whether intrastriatal injection of NPY induces any variation in the expression of these 2 factors that may contribute for the neuroprotective effect of NPY. No changes in the expression of either GDNF (Fig. 6D) or BDNF (Fig. 6E) were observed 1 week after injection, or at any other time point investigated (2 days and 3 weeks, data not shown) in our different experimental conditions (*p*s > 0.1 vs. saline).

Therefore, the neuroprotective effect of NPY is likely to be due to the direct consequence of activation of the Y_2 receptors and the downstream transduction pathways involving ERK1/2 and Akt rather than to an indirect up-regulation of these factors.

4. Discussion

To the best of our knowledge, this is the first report showing that NPY is neuroprotective in both in vitro and in vivo models of PD. Indeed, our behavioral, neuroanatomical, neurochemical, imaging, and cellular studies indicate that NPY exerts a neuroprotective effect against 6-OHDA-induced toxicity both on nigral dopamine cell bodies and striatal terminals. This neuroprotection is preferentially mediated via the Y_2 receptor and likely implicates the activation of both the MAPK and the Akt pathways.

Striatal injection of 6-OHDA leads to a progressive and retrograde degeneration resulting, 4 weeks later, in the loss of about 60% of dopaminergic neurons in the SNpc which is comparable to what was observed previously with similar

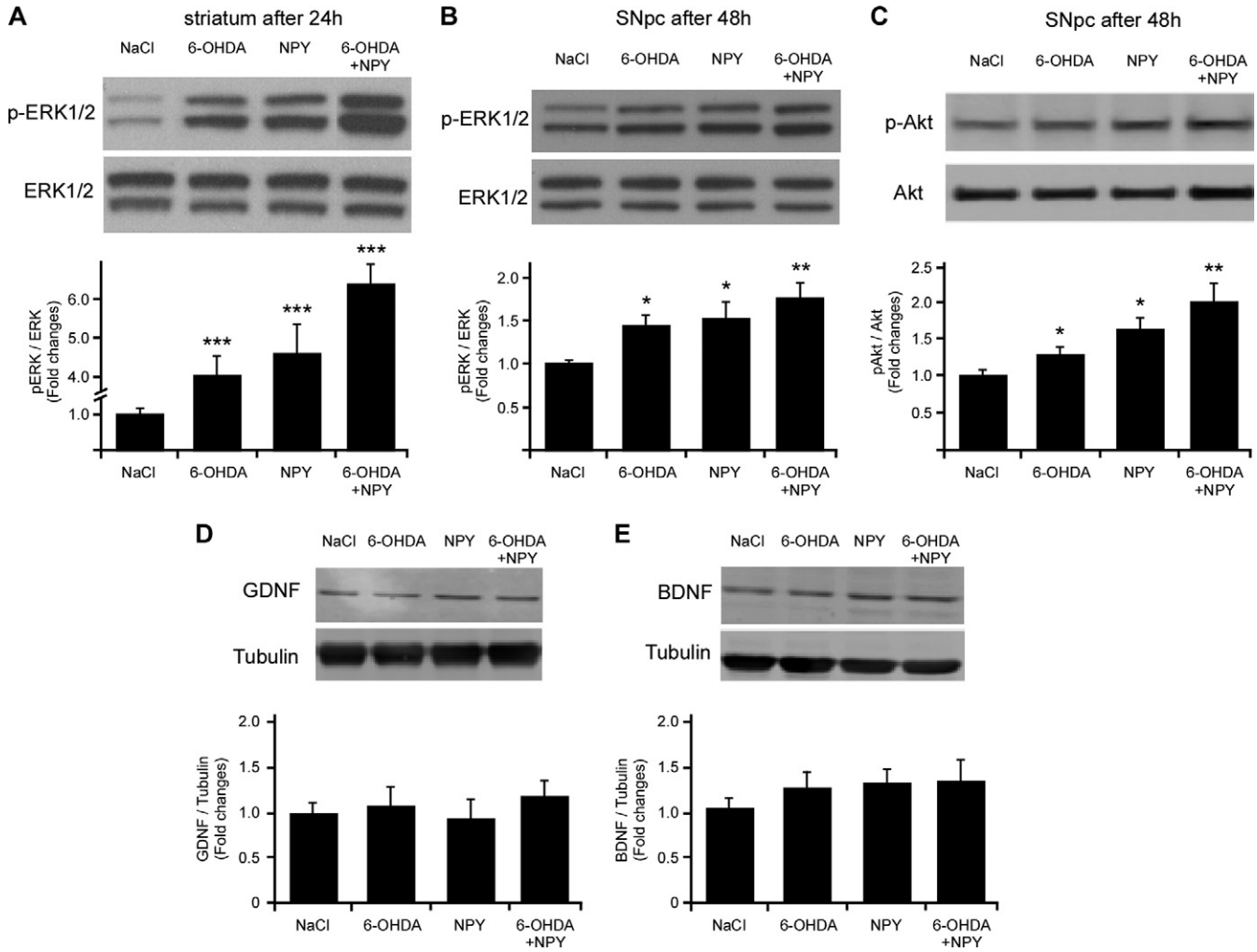

Fig. 6. Neuropeptide Y (NPY) induces the activation of mitogen-activated protein kinase pathway (MAPK) and Akt pathways. (A and B) Expression of ERK1/2 and phospho-ERK1/2 activation was analyzed in the (A) striatum after 24 hours and in the (B) substantia nigra pars compacta (SNpc) after 2 days. (C) Expression of Akt and phospho-Akt was determined in the SNpc 2 days after striatal injection of saline, NPY, 6-hydroxydopamine (6-OHDA), and 6-OHDA+NPY. (D) Glial cell line-derived neurotrophic factor (GDNF) and (E) brain derived neurotrophic factor (BDNF) expression was analyzed in the striatum 1 week after injection of saline, NPY, 6-OHDA, and 6-OHDA+NPY. Data are expressed as mean ± standard error of the mean (SEM). *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$ compared with saline group.

doses (Alvarez-Fischer et al., 2008). Similarly, the dose response effect obtained in vitro after 6-OHDA intoxication was comparable to that reported in previous Studies (Guo et al., 2005). With NPY treatment, 6-OHDA led to a more moderate degeneration of SH-SY5Y cells and of nigral dopamine neurons (−27% and −24% respectively). Thus, NPY can rescue a significant proportion (approximately 35%) of cells intoxicated with 6-OHDA both in vitro and in vivo.

The Y_2 receptor appears to be the main NPY receptor mediating the neuroprotective effect of NPY, as neuroprotection mediated by NPY treatment was abolished in mice treated with Y2 receptor antagonist or in mice deficient for Y_2 receptor. In contrast, neither administration of the Y_1 antagonist nor the genetic deletion of Y_1 receptor affects the neuroprotective effect of NPY. The involvement of Y_2 receptor in the NPY-induced neuroprotective effect was further confirmed by behavioral tests. Similarly to NPY, injection of the Y_2 agonist induced a reduction in the number of apomorphine-induced rotations. This effect was abolished in mice deficient for Y2 receptor or in mice treated with Y_2 antagonist. However, mice deficient for Y_1 receptor or treated with Y_1 antagonist did not show any motor improvement after injection of NPY and 6-OHDA further confirming that the presence of Y_2 receptor is crucial for the effect of NPY in this model.

Western blot analysis confirmed the beneficial effect of NPY on TH+-cell bodies and also suggested a protective action of the peptide on striatal dopaminergic terminals. This was also confirmed in a quantitative autoradiographic study using the high specific DAT marker PE2I. We showed that, similarly to our in vitro and in vivo experiments in

mice, NPY could rescue a significant proportion (approximately 35%) of DAT density both in the SNpc and striatum of 6-OHDA-lesioned rats. Neurochemical analysis further corroborates this hypothesis as striatal 6-OHDA injection led to a major decrease in the content of dopamine and DOPAC within the striatum while NPY treatment significantly increased dopamine and DOPAC contents compared with lesioned mice. The asymmetry in the dopaminergic transmission between both sides is illustrated at the behavioral level by a significant number of rotations in lesioned mice after apomorphine administration; this rotation was reduced in mice that also received NPY. Thus, neuroprotection of SNpc neurons by NPY is followed by an attenuated 6-OHDA-induced depletion of striatal TH and DAT as well as increased striatal dopamine and DOPAC levels and restoration of normal rotational behavior.

We then investigated the molecular pathways that might be involved in the neuroprotective effect of NPY. First, we studied the activation of the ERK1/2 as it has been shown to be responsible in part for the neuroprotective effect of various compounds such as glial cell line-derived neurotrophic factor or leptin (Cavanaugh et al., 2006; Lin et al., 2008; Lindgren et al., 2008; Weng et al., 2007). We observed an increased phosphorylation of ERK1/2, 24 hours after NPY injection in the striatum and 2 days postinjection in the SNpc. We also investigated whether NPY injection could also activate what is believed to be the most important neuroprotective cascade, namely the Akt pathway (Ries et al., 2006; Timmons et al., 2009). Striatal NPY injection resulted in an increased expression of the phosphorylated form of Akt after 2 days. Taken together, our results indicate that the neuroprotective effect of NPY is mediated, at least in part, by the activation of both the MAPK and the PI3K/Akt pathways which may result in an increased survival of the nigral dopaminergic neurons. To our knowledge, this is the first demonstration of activation of these pathways by NPY in this model. However, other parallel molecular pathways involving calcium dependent second messenger cannot be excluded. For instance, NPY was shown to exert its neuroprotective action on hippocampal neurons intoxicated with kainate by acting on calcium influx (Silva et al., 2003b, 2005a). Furthermore, depletion of striatal dopaminergic innervation by dopamine receptor blockade or 6-OHDA-induced lesion, increases striatal excitatory amino acid activity such as that of glutamate (Meshul et al., 1999). The excitotoxic effect of glutamate contributes to motor deficits, such as dyskinesia in PD patients. The neuroprotective action of NPY on hippocampal neurons exposed to kainate was shown to be due to an inhibitory effect of the peptide on glutamate release therefore limiting the excitotoxic effect of the neurotransmitter (Silva et al., 2005a, Silva et al.,2005b). Thus, giving that Y_2 receptor is strongly expressed in the striatum (Stanić et al., 2006), and by dopaminergic neurons (Shaw et al., 2003), NPY may be exerting its neuroprotective effect not only by acting directly on dopaminergic cells and terminals via this Y_2 receptor but also potentially by acting on glutamatergic cortical afferents to reduce excitotocity.

The results presented here are in line with previous studies showing that NPY can be neuroprotective in other models of neurotoxicity. In these models, NPY preserved neurons from death through the activation of Y_2 receptor subtype (Silva et al., 2003a; Smiałowska et al., 2009; Thiriet et al., 2005). NPY expression was increased in the dopamine-denervated striatum, which was proposed to be an endogenous reaction indicating that NPY may be contributing to brain neuroprotective mechanisms (Cannizzaro et al., 2003; Kerkerian et al., 1986; Obuchowicz et al., 2003). In the same line, we recently demonstrated that administration of NPY in a transgenic mouse model of Huntington's disease improves survival, motor performances and pathology, suggesting that NPY plays a prosurvival effect in other systems (Decressac et al., 2010).

In a degenerative microenvironment, glial cells release several neurotrophins such as GDNF and BDNF contributing in large part to the neuroprotective processes in various models of PD (Sun et al., 2005; Yurek and Fletcher-Turner, 2001). Therefore, we investigated variations in GDNF and BDNF expression in the striatum 2 days, 1 week, and 3 weeks after treatments. We did not observe any alteration in the expression of neither GDNF nor BDNF, suggesting that the NPY-induced neuroprotective effect is mainly due to a direct action of the peptide itself via the activation of Y_2 receptor that triggers intracellular neuroprotective cascades in dopamine neurons.

Among all the molecules tested so far, and taking into consideration the criteria required by the Committee to Identify Neuroprotective Agents in Parkinsons (CINAPS), NPY appears to be a promising candidate for a neuroprotective approach in PD. Indeed, we provide here the first evidence that NPY protects dopaminergic neurons and terminals both in vitro and in animal models of PD. In addition, like GDNF, NPY is an endogenous molecule that is expressed in the nigrostriatal pathway. However, unlike GDNF, NPY has the capacity to cross the blood-brain barrier (Kastin and Akerstrom, 1999) which may facilitate its use in therapeutic approaches without being impeded by the same methodological problems that still face the administration of identified neuroprotective factors such as glial cell line-derived neurotrophic factor. In order to ensure specific effects however, interventions using targeted overexpression of NPY or the Y2 agonist can be used. This can be achieved for instance through the use of adeno-associated viral vector (AAV)-NPY vectors as previously reported in various models of epilepsy (Noe' et al., 2007).

Disclosure statement

The authors report no actual or potential conflicts of interest.

This study was approved by the Institutional Review Board of the Samsung Medical Center. All animal treatments conformed to the guidelines of the French Agriculture and Forestry Ministry (decree 87849) and of European Communities Council Directive of 24 December 1986 (86/609/EEC).

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