

Involvement of CRH Receptors in the Neuroprotective Action of R-Apomorphine in the Striatal 6-OHDA Rat Model

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ABSTRACT

The dopamine D1-D2 receptor agonist, R-apomorphine, has been shown to be neuroprotective in different models of Parkinson's disease. Different mechanisms of action for this effect have been proposed, but not verified in the striatal 6-hydroxydopamine rat model. In this study, the expression of a set of genes involved in 1) signaling, 2) growth and differentiation, 3) neuronal regeneration and survival, 4) apoptosis and 5) inflammation in the striatum was measured after a subchronic R-apomorphine treatment (10 mg/kg/day, subcutaneously, during 11 days) in the striatal 6-hydroxydopamine rat model. The expression of 84 genes was analysed by using the rat neurotrophins and receptors RT² Profiler™ PCR array. The neuroprotective effects of R-apomorphine in the striatal 6-hydroxydopamine model were confirmed by neurochemical and behavioural analysis. The expression data suggest the observed neuroprotection involved the alteration of the gene and the protein expression levels of the anti-inflammatory corticotropin releasing hormone receptor (CRHR) 1 and the pro-inflammatory CRHR2 receptor confirming its potential anti-inflammatory action.

Keywords: Apomorphine; Gene Expression; Inflammation; Neuroprotection; Parkinson's Disease; Striatal 6-Hydroxydopamine Rat Model

1. Introduction

Until today, the available treatments for Parkinson's disease do not stop or slow down the progressive nature of the disease and are based on dopamine (DA) replacement strategies, such as the use of DA agonists and/or the DA precursor, levodopa (L-DOPA) [1]. Due to the long pre-symptomatic phase of the disease, therapeutic interventions that result in the protection, restoration and/or rescuing of the dopaminergic neurons are of extreme importance to improve the quality of life of the patients [1,2].

Although R-apomorphine has been introduced as a drug a certain time ago, it is still available as treatment for patients with Parkinson's disease and with advanced Parkinson's disease and for the treatment of persistent and disabling motor fluctuations which do not respond to

L-DOPA [3-7]. It activates D1-like (D1, D5) and D2-like (D2, D3, D4) receptors, serotonin receptors (5HT1A, 5HT2A, 5HT2B and 5HT2C) and α -adrenergic receptors (α 1B, α 1D, α 2A, α 2B, α 2C) [8].

R-apomorphine has been shown to be neuroprotective both *in vitro* and *in vivo*, including the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [9-12], 6-hydroxydopamine (6-OHDA) [13-15] and methamphetamine (METH) [12,16] rodent models of Parkinson's disease and more recently, a mouse model of Alzheimer's disease [17]. The *in vivo* neuroprotective properties of R-apomorphine have been shown to be dose dependent, and mainly linked to the administration of high doses of R-apomorphine. These findings also suggest that the neuroprotective action may involve other pathways rather than activation of DA receptors. Among the proposed mechanisms of action are its radical scavenging activity [13,15,18,19] and iron chelating properties [20], inhibi-

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tion of mitochondrial iron-induced lipid peroxidation and protein oxidation [13,18,19], activation of nuclear transcription factor NF-E2-related factor 2 (Nrf2) [21], inhibition of monoamine oxidase (MAO)-A and MAO-B [22], its enhancement of glutathione peroxidase activity [23], anti-apoptotic [7,24], anti-inflammatory [9], mitogenic [25] and trophic effects [26-29]. We previously showed that a short-term treatment with R-apomorphine (10 mg/kg/day, s.c., during 11 days), started before or 24 hours after lesions, has neuroprotective actions in the striatal 6-OHDA rat model, as demonstrated by an improved motor behavior and a significant protection (20% - 35%) of the integrity of the nigrostriatal dopaminergic system at the level of the substantia nigra pars compacta and striatum [14]. As hydroxyl radical formation is considered as an important event in the neurotoxicity of 6-OHDA [30], several *in vivo* studies investigated the interference of R-apomorphine with the hydroxyl radical formation in the rat striatum [13-15]. However, these findings are not consistent. Furthermore, Battaglia *et al.* [11] have shown that R-apomorphine is still neuroprotective after 40 h following MPTP injections, and together with our previous findings that R-apomorphine is still neuroprotective 24 h after 6-OHDA administration [14], it can be suggested that the mechanism of action of R-apomorphine is not linked to the interference with the initial effects of the neurotoxins.

Although different mechanisms of action of R-apomorphine, as summarised above, have been proposed for its neuroprotective effect, these have not been extensively explored in the striatal 6-OHDA rat model. In this study, we screened the expression of a set of genes involved in 1) signaling, 2) growth and differentiation, 3) neuronal regeneration and survival, 4) apoptosis and 5) inflammation in the central nervous system after a sub-chronic R-apomorphine treatment (10 mg/kg/day, s.c., during 11 days) in the unilateral striatal 6-OHDA parkinsonian rat model.

Our data confirm the neuroprotective effects of R-apo

morphine in the unilateral striatal 6-OHDA parkinsonian rat model and suggest that they may involve the alteration of the striatal gene and the protein expression levels of the anti-inflammatory corticotropin releasing hormone receptor (CRHR) 1 and the pro-inflammatory CRHR2 receptor.

2. Experimental Procedure

All the chemical compounds where no supplier is mentioned, are supplied by Sigma-Aldrich, Brussels, Belgium.

2.1. Animals

In all experiments, male albino Wistar rats (Charles River, Sulzfeld, Germany) weighing 175 - 200 g were used. Animals were kept under standardised conditions (25°C, 12 h light-dark cycle) with free access to food and tap water. At the end of the experiments, rats were sacrificed with an overdose of pentobarbital (Nembutal®, Ceva Sante Animale, Brussels, Belgium). Animal experiments were carried out according to the national guidelines on animal experimentation and were approved by the Ethical Committee for Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. All efforts were made to minimise animal suffering and the minimal number of animals necessary to produce reliable scientific data was used.

2.2. Effects of R-Apomorphine on the Progress of Neurodegeneration in Rats Unilaterally Lesioned with 6-OHDA

2.2.1. Experimental Design

The experimental design is described in **Figure 1**. The rats were divided into 3 groups. Rats in group I were used as non-lesioned controls, receiving subcutaneous injections of saline. Rats in groups II were injected unilaterally with 3 µl of 6-OHDA solution (6.7 µg/µl in 0.1% ascorbic acid) in the left striatum and subcutaneously with saline. Rats in group III were lesioned with

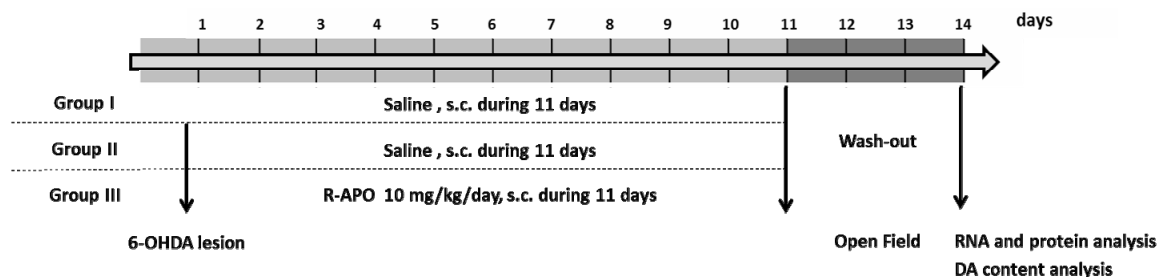


Figure 1. Experimental design. The rats were divided into 3 groups. Rats in group I were used as non-lesioned controls, rats in groups II were injected intrastrially with 6-OHDA and subcutaneously with saline, and rats in group III were lesioned with 6-OHDA similar to rats of group II, but also received daily subcutaneous injections of R-apomorphine (10 mg/kg/day). Further details are described in Materials and Methods. Abbreviations: R-APO: R-apomorphine, s.c. subcutaneously.

6-OHDA similar to rats of group II, but also received daily subcutaneous injections of R-apomorphine (10 mg/kg/day). The treatment with R-apomorphine started 15 min before the intrastriatal injection of 6-OHDA and continued for another 10 days. The dose of the R-apomorphine was based on previous findings [10,11,14]. Briefly, Grünblatt et al had already demonstrated that a low dose (0.5, 1, 2, and 2.5 mg/kg) of R-apomorphine does not protect against MPTP induced DA loss in mice, whereas the higher dose (10 mg/kg) restored the values to those of control mice [10]. Similar findings were reported in 2001 [22]. Furthermore, Fornai et al demonstrated a dose dependent neuroprotective effect of R-apomorphine in methamphetamine induced DA depletion. Based on these findings, we decided to use 10 mg/kg R-apomorphine in the 6-OHDA striatal rat model. Animals were visually checked at regular intervals and weighed daily before any manipulation until the end of the experiment (day 14). For all the groups there was a wash-out period of 3 days. Different sets of rats were used for the initial screening of the relative striatal gene expression levels on the one hand, and behavioural testing, assessment of DA and DOPAC content, and protein expression analysis on the other. mRNA of high quality was used during the gene expression studies, and therefore the purity and integrity of all mRNA samples were checked with advanced methods, such as UV spectrophotometry and a microfluidics based electrophoresis system. The specificity of the PCR reaction has been evaluated by analysing the PCR product (the amplicon) by conducting at the end of each RTqPCR experiment a melt curve analysis for each amplicon. The web based software of the company (SA Biosciences, MD) also automatically performed a quality control for each PCR run. The quality of the reference genes has been evaluated by the software program that was provided by the supplier (SA Biosciences, MD) of the PCR arrays. The selection of the potential candidate genes for the protein expression analysis was based on experimental and literature findings. To mitigate biological and technical variabilities during a gene expression study, the recommended number of biological replicates is minimum three, and for technical replicates minimum two [31]. We managed to have the minimum number of biological replicates for the gene expression study, but unfortunately technical replicates were not possible due to the limited sample volume. Despite a lower number of animals for the gene expression studies, our slightly higher number of animals for the protein expression favours our observations at the gene level.

2.2.2. Local Administration of 6-OHDA by Striatal Stereotaxic Microinjection

Rats were anaesthetized with a mixture of ketamine (50

mg/kg i.p.; Ketamine 1000 Ceva[®], Ceva Sante Animale, Brussels, Belgium) and diazepam (5 mg/kg i.p.; Valium[®], Roche Brussels, Belgium) and placed on a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, California, USA). The skull was exposed and a burr hole was drilled to introduce a syringe for a single injection of the 6-OHDA solution [containing 6.7 µg 6-OHDA per µl in 0.1% ascorbic acid, pH 5.0]. To minimize variability due to degradation of the toxin, the 6-OHDA solution was freshly prepared, kept on ice, and protected from exposure to light. The solution was injected in the left striatum at the following coordinates relative to the bregma L: -3.0, A: +1.0 and V: +5.0, according to the atlas of Paxinos and Watson [32]. A total volume of 3 µl 6-OHDA was injected at a flow rate of 1 µl/min. After injection, the syringe was left in place for 5 min and then slowly removed over a 1 - 2 min time period. The skin was sutured, the animals received ketoprofen (4 mg/kg i.p.; Ketofen[®], Merial, Brussels, Belgium) as analgesic and were allowed to recover before returning to the animal housing facilities [14].

2.3. Behavioral Analysis

Locomotor activity in Open Field

The spontaneous locomotor activity was monitored in a Plexiglas box (sides, 60 cm; height, 60 cm). At least 1 h prior to testing, rats were acclimated to the testing room. The recording started immediately after placing animals in the open field and continued for 60 min. To neutralise odor formation, the arena was disinfected and cleaned with 70% ethanol before each rat was tested. Experiments were performed between 09:00 AM and 06:00 PM. The rat was placed carefully in the center of the arena, and allowed to explore the field for 60 min. Each performance was automatically analysed using a video tracking system and Ethovision 3.0 tracking software (Noldus, the Netherlands). The following parameters were recorded: 1) distance moved, 2) movement time, 3) immobility, 4) velocity, 5) rearing and 6) relative meander. The assessment of spontaneous locomotor behavior was carried out during the wash-out period of the protocol, in order to avoid a possible modulatory effect of the DA agonist R-apomorphine on motor function, as previous studies have shown that following DA agonists significant alteration of spontaneous motor behavior occurs [33,34]. We preferred to use the open field test in this study to evaluate the animals in a drug-free state. Indeed, amphetamine induced rotation might influence the expression of genes, and our previous results already indicate that a subchronic R-apomorphine treatment of 6-OHDA striatally lesioned rats reduced the amphetamine induced ipsilateral rotations [14].

2.4. Sacrifice and Brain Prelevation

Seventy-two hours (wash-out period) after the last injection of R-apomorphine (day 14), the rats were killed with an overdose of pentobarbital (Nembutal[®], Ceva Sante Animale, Brussels, Belgium) and the brains were quickly removed without perfusion. From the rostral part (10 mm from front) of the brain the left and right striatum were dissected out on an ice cold chilled petri-dish, immediately snap frozen in dry-ice cooled isopentane and stored separately at -80°C until homogenisation and analysis of 1) striatal DA and DOPAC content, 2) striatal gene expression and 3) protein expression of CRHR1, CRHR2, and neuropeptide Y (NPY) receptors 1 and 2.

2.5. Neurochemical Determination of the Striatal DA and DOPAC Content

To establish the extent of DA and DOPAC depletion in the striatum after 6-OHDA lesioning, the liquid chromatography (LC) method previously described was used [14], with slight modifications.

The supernatant obtained during protein expression analysis (4.7) was diluted 5 times in antioxidant (0.05 M HCl, 0.5% $\text{Na}_2\text{S}_2\text{O}_5$ and 0.05% Na_2EDTA). 20 μl of samples were injected and analysed directly for DA and DOPAC content on a narrowbore (C18 column: 15 m, 150 mm \times 2.1 mm; Altima; Grace; Lokeren; Belgium) LC system. The mobile phase consisted of 0.1 M sodium acetate trihydrate, 20 mM citric acid monohydrate, 1 mM 1-octane sulfonic acid, 0.1 mM Na_2EDTA and 1 mM dibutylamine, adjusted to pH 3.7. Methanol 3% (v/v) was added as organic modifier. The flow rate was set at 0.2 ml/min. The electrochemical detection (Antec, The Netherlands) potential was +700 mV versus the reference electrode (Ag/AgCl). Sensitivity was set at 1 nA full scale. All samples were injected via a high precision auto-injector equipped with a cooling system (Kontron, San Diego, CA, USA). The integration of the chromatograms was done with the Data Apex Clarity software program (Antec). The tissue DA and DOPAC content were calculated and expressed as $\mu\text{g/g}$ wet weight of tissue.

2.6. Gene Expression Analysis

2.6.1. RNA Extraction

Total RNA was extracted from the striatal tissue using the RNeasy[®] Lipid Tissue Mini Kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands). The concentration and purity of RNA were determined by measuring the absorbance using the Nanodrop 1000 (Thermo Scientific). The quality of total RNA was assessed using the Agilent[®] Bioanalyzer RNA 6000 Nano Labchip[®] (Agilent Technologies, Palo Alto, CA).

For each RNA sample the presence of sharp bands/peaks present for both the 18S and 28S ribosomal RNAs were verified and only the samples with an RNA Integrity Number of 7 or higher were used.

One microgram of total RNA was subjected to first strand cDNA synthesis using the RT2 First Strand Kit (SA Biosciences, MD). For real-time PCR, a PCR components mix consisting of 2 \times RT2 SYBR Green Fluor qPCR Mastermix (1350 μl) (SA Biosciences), cDNA synthesis reaction (102 μl) and RNase-free water (1248 μl) was prepared. The final volume was 2700 μl , providing an excess volume to perform pipetting steps as precisely as possible to ensure that each well of the array receives the required volume. 25 μl of the mixture was added into each of the wells of the RT² Profiler PCR Arrays (PARN-031, SA Biosciences, MD).

2.6.2. Quantitative Real-Time PCR

Real-time quantifications were performed using the BioRad[®] iCycler[®] Real-time PCR system (Bio-Rad) using the recommended cycling conditions for the RT² Profiler PCR arrays (SA Biosciences, MD). Each PCR array contained 84 transcripts, a set of five housekeeping genes as internal controls and additional controls for efficiency of reverse transcription, PCR and the absence of contaminating genomic DNA.

Relative expression was determined with the $\Delta\Delta$ CT method using the PCR Array Data Analysis Web Portal (www.SABiosciences.com/pcrarraydataanalysis.php) and the web based software automatically performed quantification, including the quality control for each PCR run/array. For normalization of the expression levels of the genes of interest, the average CT value of the five housekeeping genes: Ribosomal protein, large, P1 (Rplp1); hypoxanthine phosphoribosyltransferase 1 (Hprt1); ribosomal protein L13A (Rpl13a); lactate dehydrogenase A (Ldha) and β -actin (Actb) was chosen. If the fold-change was greater than 1, the result is reported as a fold up-regulation. If the fold-change was less than 1, the negative inverse of the results is reported as a fold down-regulation.

2.7. Protein Expression Analysis

To measure CRHR1, CRHR2, NPY1R and NPY2R protein levels, the striatal tissue was homogenised in an ice-cold PBS-solution (0.02 M, pH 7.0 - 7.2) containing 2% protease inhibitor cocktail (Sigma, St-Louis, MO, USA) (25 mg tissue/ml) and subsequently sonicated for 1 min (Branson Sonifier 250). After centrifugation at 10,000 g at 4°C during 10 minutes (Sorvall RC5B refrigerated superspeed centrifuge, Dupont Instruments), the supernatants were collected and stored at -20°C until use. After decantation, the protein content of the supernatant

was determined by ELISA and the DA content by LC (4.5). Tissue levels of CRHR1, CRHR2, NPY1R and NPY2R were determined in 100 μ l aliquots by commercially available ELISA kits (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions. Absorbances were measured using a Bio-Rad 680 microplate (Bio-Rad Laboratories, Belgium) reader set at 450 nm. The protein content was determined using the Pierce® 660 nm Protein Assay Kit (Thermo Scientific, USA).

2.8. Data Analysis

The web-based software of the company SA Biosciences has been used to analyse the gene expression data. All data are expressed as mean \pm S.E.M. Significant differences between all the experimental groups were determined using one-way analysis of variance (ANOVA) followed by the Bonferroni's post-hoc test. The significance of the change in gene expression between the groups was evaluated by unpaired Student t-test for each gene. All statistical analysis was performed with GraphPad Instat 3.0 (GraphPad Prism Software, Inc., San Diego, USA) at the 5% level of significance.

3. Results

3.1. Neuroprotective Effect of R-Apomorphine in the Striatal 6-OHDA Rat Model (Figures 2 and 3)

The neuroprotective effect of R-apomorphine was confirmed by determination of the striatal DA content and DA turnover (Figure 2) and in the open field test (Figure 3). Two weeks after injection of 6-OHDA, the DA content of the striata contralateral to the side of the lesion of rats receiving saline or R-apomorphine were not significantly different from those of the control animals (data not shown). However, the DA content of the le-

sioned striata of saline treated rats was significantly reduced after intrastriatal application of 6-OHDA by 60%, similar to our previous findings [14]. The DOPAC:DA ratio in the denervated striatum of the striatally lesioned rats was significantly increased. Treatment with R-apomorphine started 15 min before the intrastriatal injection of 6-OHDA significantly attenuated the striatal DA depletion and restored the DOPAC:DA ratio. The beneficial neurochemical effects of the treatment with R-apomorphine were similar to our previous findings [14].

Recording of the open field activity (Figure 3) revealed that R-apomorphine significantly attenuated the 6-OHDA induced reduction in total distance moved and increase in relative meander. There was a trend for R-apomorphine to improve the 6-OHDA lesion induced reduction in velocity ($p = 0.08$). R-apomorphine treatment had no effect on movement time, immobility time and rearing (data not shown).

3.2. Gene Expression Analysis

A list of the genes that have been screened with the corresponding fold changes is listed in Table 1.

3.2.1. Gene Expression Profile of the Striatal 6-Hydroxydopamine Rat Model

In the striatum, 20 genes out of the 84 genes on the array were changed in the striatally lesioned rats compared to non-lesioned rats. Significant differences were detected between the striatally lesioned rats and the non-lesioned rats for 14 out of the 20 genes ($p < 0.05$), with a further 6 genes nearing significance ($p < 0.1$). The majority of the genes (13) were upregulated in the striatal 6-OHDA rat model, whereas 7 were downregulated. Two weeks after the striatal administration of 6-OHDA, the expression of CRHR2, GFRA1, GFRA3, GMFG, CD40, CCKAR, TGFb1, CX3CR1, IL-10 α , IL-6 α , HSPB1, STAT1 and

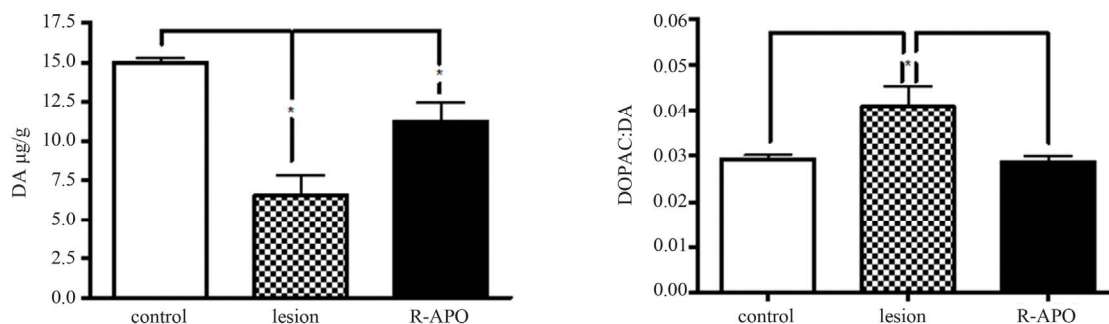


Figure 2. Dopamine levels and dopamine turnover. Dopamine (DA) content (left panel) and 3,4-dihydroxyphenylacetic acid (DOPAC) DOPAC:DA ratio (right panel) of the left striatum of control ($n = 6$), saline treated 6-OHDA striatal lesioned ($n = 6$) and R-apomorphine treated 6-OHDA striatally lesioned rats ($n = 6$). DA content was determined two weeks after striatal micro-injection of 6-hydroxydopamine (6-OHDA) and is expressed as μ g/g wet tissue (mean \pm S.E.M). *Significantly different (one-way ANOVA followed by a Bonferroni's Multiple Comparison Test: $p < 0.05$).

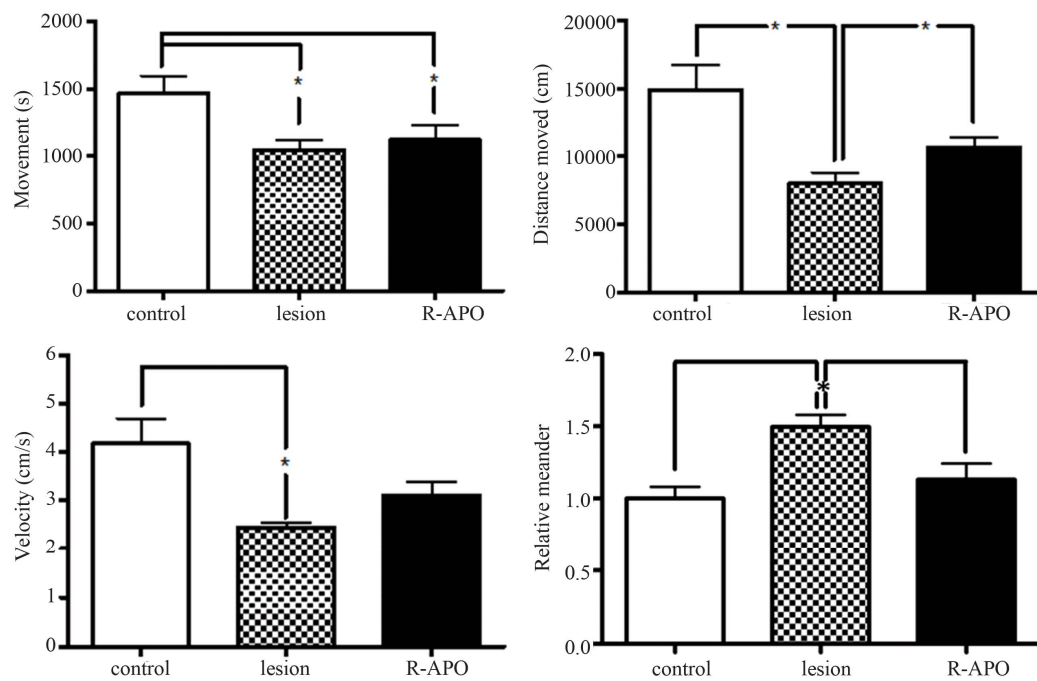


Figure 3. Open field activity. Locomotor activity of control ($n = 5$), saline treated 6-OHDA striatal lesioned ($n = 5$) and R-apomorphine treated 6-OHDA striatally lesioned rats ($n = 6$). The mean total distance moved (upper left panel), movement time (upper right panel), velocity (lower left panel) and relative meander (lower right panel) in the open field under un-habituated conditions are illustrated. The recording started immediately after placing animals in the open field and continued for 60 min. Each performance was automatically analyzed using a video tracking system and Ethovision 3.0 tracking software (Noldus, the Netherlands). Data are expressed as mean \pm S.E.M. *Significantly different (one-way ANOVA followed by a Bonferroni's Multiple Comparison Test: $p < 0.05$).

Table 1. Striatal gene expression results obtained by quantitative real-time PCR. Gene expression in the control ($n = 3$), saline treated 6-OHDA striatal lesioned ($n = 4$) and R-apomorphine treated 6-OHDA striatal lesioned rats ($n = 4$) was analyzed using the Neurotrophins and Receptors RT² Profiler PCR Arrays (SA Biosciences) as described in Material and Methods. Relative expression is determined with the $\Delta\Delta$ CT method. If the fold-change is greater than 1, the result is reported as a fold upregulation. If the fold-change is less than 1, the negative inverse of the results is reported as a fold downregulation. The p-values were calculated based on an unpaired Student's *t*-test for each gene. All the 84 genes and their functional classes are shown in the table.

| Description | Gene symbol | GenBank | Fold regulation 6-OHDA versus control | p-value | Fold regulation R-APO versus control | p-value |
|---|-------------|--------------|---------------------------------------|---------|--------------------------------------|---------|
| Neurotrophins and Receptors | | | | | | |
| Adenylate cyclase activating polypeptide 1 receptor 1 | Adcyap1r1 | NM_133511 | -1.11 | 0.6471 | 1.07 | 0.9809 |
| Artemin | Artn | NM_053397 | -1.11 | 0.6000 | -1.41 | 0.1508 |
| Brain-derived neurotrophic factor | Bdnf | NM_012513 | 2.22 | 0.3474 | -1.08 | 0.8819 |
| Ciliary neurotrophic factor | Cntf | NM_013166 | 1.10 | 0.5290 | 1.06 | 0.6760 |
| Ciliary neurotrophic factor receptor | Cntfr | NM_001003929 | -1.15 | 0.5044 | -1.15 | 0.5441 |
| Corticotropin releasing hormone | Crh | NM_031019 | -1.80 | 0.2127 | -2.15 | 0.1367 |
| Corticotropin releasing hormone binding protein | Crhbp | NM_139183 | -1.15 | 0.5033 | -1.16 | 0.4726 |
| Corticotropin releasing hormone receptor 1 | Crhr1 | NM_030999 | 1.04 | 0.7216 | 1.39 | 0.0632 |

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|--|---------|--------------|-------|--------|-------|--------|
| Fibroblast growth factor receptor substrate 3 | Frs3 | NM_001017382 | 1.08 | 0.5453 | 1.22 | 0.1740 |
| Glial cell derived neurotrophic factor | Gdnf | NM_019139 | -1.05 | 0.7042 | -1.17 | 0.4756 |
| GDNF family receptor alpha 1 | Gfra1 | NM_012959 | 1.50 | 0.0999 | 1.40 | 0.1511 |
| GDNF family receptor alpha 2 | Gfra2 | NM_012750 | -1.00 | 0.9577 | -1.14 | 0.6205 |
| GDNF family receptor alpha 3 | Gfra3 | NM_053398 | 1.79 | 0.0435 | 1.26 | 0.0801 |
| Glia maturation factor, beta | Gmfb | NM_031032 | 1.10 | 0.4245 | 1.26 | 0.0867 |
| Glia maturation factor, gamma | Gmfg | NM_181091 | 1.70 | 0.0072 | 1.83 | 0.0032 |
| Hypocretin (orexin) receptor 1 | Hcrtr1 | NM_013064 | 1.19 | 0.4072 | -1.08 | 0.9414 |
| Hypocretin (orexin) receptor 2 | Hcrtr2 | NM_013074 | -1.05 | 0.8012 | -1.13 | 0.6321 |
| Metallothionein 3 | Mt3 | NM_053968 | -1.11 | 0.4487 | -1.27 | 0.2408 |
| Nerve growth factor (beta polypeptide) | Ngfb | XM_227525 | 1.20 | 0.2602 | 1.36 | 0.1124 |
| Nerve growth factor receptor (TNFR superfamily, member 16) | Ngfr | NM_012610 | -1.06 | 0.8455 | 1.12 | 0.6659 |
| Nerve growth factor receptor (TNFRSF16) associated protein 1 | Ngfrap1 | NM_053401 | -1.48 | 0.0437 | -1.72 | 0.0339 |
| Nuclear receptor subfamily 1, group I, member 2 | Nr1i2 | NM_052980 | -1.23 | 0.3919 | -1.11 | 0.7157 |
| Neuregulin 1 | Nrg1 | NM_031588 | -1.20 | 0.3026 | -1.26 | 0.2108 |
| Neuregulin 2 | Nrg2 | XM_344662 | -1.01 | 0.9586 | 1.20 | 0.3032 |
| Neurotrophin 3 | Ntf3 | NM_031073 | 1.19 | 0.6292 | 1.78 | 0.1067 |
| Neurotrophin 5 | Ntf5 | NM_013184 | -1.25 | 0.3904 | -1.96 | 0.4401 |
| Neurotrophic tyrosine kinase, receptor, type 1 | Ntrk1 | NM_021589 | -1.82 | 0.0828 | -1.42 | 0.3921 |
| Neurotrophic tyrosine kinase, receptor, type 2 | Ntrk2 | NM_012731 | -1.17 | 0.3840 | -1.44 | 0.1327 |
| Persephin | Pspn | NM_013014 | -1.03 | 0.7075 | 1.04 | 0.7371 |
| Prostaglandin E receptor 2 (subtype EP2) | Ptger2 | NM_031088 | -1.13 | 0.6021 | 1.08 | 0.9750 |
| Trk-fused gene | Tfg | NM_001012144 | 1.01 | 0.9790 | 1.03 | 0.8562 |
| CD40 molecule, TNF receptor superfamily member 5 | Cd40 | NM_134360 | 2.68 | 0.0003 | 3.27 | 0.0001 |
| Fas (TNF receptor superfamily, member 6) | Fas | NM_139194 | 1.29 | 0.3149 | 1.64 | 0.1195 |
| Urocortin | Ucn | NM_019150 | -1.17 | 0.5582 | -1.15 | 0.4268 |
| VGF nerve growth factor inducible | Vgf | NM_030997 | -1.36 | 0.3562 | -1.42 | 0.1827 |
| Zinc finger protein 110 | Zfp110 | NM_001024775 | -1.43 | 0.1192 | -1.68 | 0.0479 |
| Zinc finger protein 91 | Zfp91 | NM_001169120 | -1.30 | 0.0316 | -1.53 | 0.0526 |
| <u>Neuropeptides and Receptors</u> | | | | | | |
| <i>Bombesin Receptors</i> | | | | | | |
| Gastrin releasing peptide receptor | Grpr | NM_012706 | -1.79 | 0.0484 | -1.77 | 0.1867 |
| <i>Cholecystokinin Receptors</i> | | | | | | |
| Cholecystokinin A receptor | Cckar | NM_012688 | 2.00 | 0.0897 | 2.15 | 0.1202 |
| <i>Galanin Receptors</i> | | | | | | |
| Galanin receptor 1 | Galr1 | NM_012958 | -2.10 | 0.3045 | -1.83 | 0.1559 |

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|--|--------|--------------|-------|--------|-------|--------|
| Galanin receptor 2 | Galr2 | NM_019172 | 1.03 | 0.7421 | 1.01 | 0.7771 |
| <i>Tachykinin Receptors</i> | | | | | | |
| Tachykinin receptor 1 | Tacr1 | NM_012667 | -1.24 | 0.1941 | -1.06 | 0.8856 |
| <i>Other Neuropeptides and Receptors</i> | | | | | | |
| Neuropeptide FF receptor 2 | Npffr2 | NM_023980 | -1.11 | 0.9903 | -1.32 | 0.8297 |
| Hypocretin | HcRt | NM_013179 | -1.48 | 0.2752 | -1.23 | 0.3425 |
| Melanocortin 2 receptor | Mc2r | NM_001100491 | 1.65 | 0.3436 | 1.86 | 0.1214 |
| Neuropeptide Y | Npy | NM_012614 | -1.10 | 0.4672 | -1.06 | 0.7078 |
| Neuregulin 1 | Nrg1 | NM_031588 | -1.20 | 0.3026 | -1.26 | 0.2108 |
| <u>Neurogenesis</u> | | | | | | |
| <i>Central Nervous System Development</i> | | | | | | |
| Chemokine (C-X-C motif) receptor 4 | Cxcr4 | NM_022205 | 1.19 | 0.4159 | -1.11 | 0.4334 |
| Fibroblast growth factor receptor 1 | Fgfr1 | NM_024146 | 1.25 | 0.1320 | 1.26 | 0.1858 |
| Nerve growth factor receptor (TNFR superfamily, member 16) | Ngfr | NM_012610 | -1.06 | 0.8455 | 1.12 | 0.6659 |
| Neurotrophin 3 | Ntf3 | NM_031073 | 1.19 | 0.6292 | 1.78 | 0.1067 |
| <i>Peripheral Nervous System Development</i> | | | | | | |
| Artemin | Artn | NM_053397 | -1.11 | 0.6000 | -1.41 | 0.1508 |
| Glial cell derived neurotrophic factor | Gdnf | NM_019139 | -1.05 | 0.7042 | -1.17 | 0.4756 |
| GDNF family receptor alpha 3 | Gfra3 | NM_053398 | 1.79 | 0.0435 | 1.26 | 0.0801 |
| Nerve growth factor (beta polypeptide) | Ngfb | XM_227525 | 1.20 | 0.2602 | 1.36 | 0.1124 |
| Neuregulin 1 | Nrg1 | NM_031588 | -1.20 | 0.3026 | -1.26 | 0.2108 |
| Neurotrophin 3 | Ntf3 | NM_031073 | 1.19 | 0.6292 | 1.78 | 0.1067 |
| <i>Axon Guidance</i> | | | | | | |
| Artemin | Artn | NM_053397 | -1.11 | 0.6000 | -1.41 | 0.1508 |
| GDNF family receptor alpha 3 | Gfra3 | NM_053398 | 1.79 | 0.0435 | 1.26 | 0.0801 |
| Nerve growth factor receptor (TNFR superfamily, member 16) | Ngfr | NM_012610 | -1.06 | 0.8455 | 1.12 | 0.6659 |
| <i>Gliogenesis</i> | | | | | | |
| Fibroblast growth factor 2 | Fgf2 | NM_019305 | 1.24 | 0.3390 | 1.47 | 0.0259 |
| Neuregulin 1 | Nrg1 | NM_031588 | -1.20 | 0.3026 | -1.26 | 0.2108 |
| Neurotrophin 3 | Ntf3 | NM_031073 | 1.19 | 0.6292 | 1.78 | 0.1067 |
| <i>Dendrite Morphogenesis</i> | | | | | | |
| Brain-derived neurotrophic factor | Bdnf | NM_012513 | 2.22 | 0.3474 | -1.08 | 0.8819 |
| Metallothionein 3 | Mt3 | NM_053968 | -1.11 | 0.4487 | -1.27 | 0.2408 |
| <i>Other Neurogenesis Genes</i> | | | | | | |
| Bcl2-associated X protein | Bax | NM_017059 | -1.13 | 0.6256 | -1.09 | 0.5521 |
| FBJ osteosarcoma oncogene | Fos | NM_022197 | 1.74 | 0.1261 | 3.29 | 0.0102 |

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|--|---------|--------------|-------|--------|-------|--------|
| Galanin receptor 2 | Galr2 | NM_019172 | 1.03 | 0.7421 | 1.01 | 0.7771 |
| GDNF family receptor alpha 1 | Gfra1 | NM_012959 | 1.50 | 0.0999 | 1.40 | 0.1511 |
| GDNF family receptor alpha 2 | Gfra2 | NM_012750 | -1.00 | 0.9577 | -1.14 | 0.6205 |
| NEL-like 1 (chicken) | Nell1 | NM_031069 | -1.08 | 0.5581 | 1.01 | 0.9296 |
| Neurotrophin 5 | Ntf5 | NM_013184 | -1.25 | 0.3904 | -1.96 | 0.4401 |
| Neurotrophic tyrosine kinase, receptor, type 1 | Ntrk1 | NM_021589 | -1.82 | 0.0828 | -1.42 | 0.3921 |
| Neurotrophic tyrosine kinase, receptor, type 2 | Ntrk2 | NM_012731 | -1.17 | 0.3840 | -1.44 | 0.1327 |
| <u>Synaptic Transmission</u> | | | | | | |
| Cerebellin 1 precursor | Cbln1 | NM_001109127 | -1.38 | 0.9382 | -1.64 | 0.6224 |
| <u>Growth and Differentiation</u> | | | | | | |
| <i>Growth Factors and Receptors</i> | | | | | | |
| Artemin | Artn | NM_053397 | -1.11 | 0.6000 | -1.41 | 0.1508 |
| Brain-derived neurotrophic factor | Bdnf | NM_012513 | 2.22 | 0.3474 | -1.08 | 0.8819 |
| Fibroblast growth factor 2 | Fgf2 | NM_019305 | 1.24 | 0.3390 | 1.47 | 0.0259 |
| Fibroblast growth factor 9 | Fgf9 | NM_012952 | 1.24 | 0.4508 | 1.17 | 0.5779 |
| Fibroblast growth factor receptor 1 | Fgfr1 | NM_024146 | 1.25 | 0.1320 | 1.26 | 0.1858 |
| Glial cell derived neurotrophic factor | Gdnf | NM_019139 | -1.05 | 0.7042 | -1.17 | 0.4756 |
| Glia maturation factor, beta | Gmfb | NM_031032 | 1.10 | 0.4245 | 1.26 | 0.0867 |
| Glia maturation factor, gamma | Gmfg | NM_181091 | 1.70 | 0.0072 | 1.83 | 0.0032 |
| Interleukin 10 | Il10 | NM_012854 | 1.35 | 0.4768 | -1.02 | 0.7271 |
| Interleukin 1 beta | Il1b | NM_031512 | 2.50 | 0.1685 | 3.16 | 0.0035 |
| Interleukin 6 | Il6 | NM_012589 | -1.21 | 0.3849 | 1.46 | 0.6407 |
| Leukemia inhibitory factor | Lif | NM_022196 | 1.57 | 0.3027 | 2.17 | 0.0687 |
| Metallothionein 3 | Mt3 | NM_053968 | -1.11 | 0.4487 | -1.27 | 0.2408 |
| Nerve growth factor (beta polypeptide) | Ngfb | XM_227525 | 1.20 | 0.2602 | 1.36 | 0.1124 |
| Neuregulin 2 | Nrg2 | XM_344662 | -1.01 | 0.9586 | 1.20 | 0.3032 |
| Neurotrophin 3 | Ntf3 | NM_031073 | 1.19 | 0.6292 | 1.78 | 0.1067 |
| Neurotrophin 5 | Ntf5 | NM_013184 | -1.25 | 0.3904 | -1.96 | 0.4401 |
| Persephin | Pspn | NM_013014 | -1.03 | 0.7075 | 1.04 | 0.7371 |
| Transforming growth factor alpha | Tgfa | NM_012671 | 1.23 | 0.1619 | 1.22 | 0.1567 |
| Transforming growth factor, beta 1 | Tgfb1 | NM_021578 | 2.90 | 0.0029 | 3.36 | 0.0001 |
| Transforming growth factor beta 1 induced transcript 1 | Tgfb1i1 | XM_341934 | 1.21 | 0.2067 | 1.20 | 0.3595 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| VGF nerve growth factor inducible | Vgf | NM_030997 | -1.36 | 0.3562 | -1.42 | 0.1827 |
| <i>Cell cycle</i> | | | | | | |
| Fibroblast growth factor 2 | Fgf2 | NM_019305 | 1.24 | 0.3390 | 1.47 | 0.0259 |
| Fibroblast growth factor 9 | Fgf9 | NM_012952 | 1.24 | 0.4508 | 1.17 | 0.5779 |

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|--|--------|--------------|-------|--------|-------|--------|
| Interleukin 1 beta | Il1b | NM_031512 | 2.50 | 0.1685 | 3.16 | 0.0035 |
| Neurotrophic tyrosine kinase, receptor, type 1 | Ntrk1 | NM_021589 | -1.82 | 0.0828 | -1.42 | 0.3921 |
| Transforming growth factor alpha | Tgfa | NM_012671 | 1.23 | 0.1619 | 1.22 | 0.1567 |
| Transforming growth factor, beta 1 | Tgfb1 | NM_021578 | 2.90 | 0.0029 | 3.36 | 0.0001 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| <i>Cell Proliferation</i> | | | | | | |
| Bcl2-associated X protein | Bax | NM_017059 | -1.13 | 0.6256 | -1.09 | 0.5521 |
| Chemokine (C-X-C motif) receptor 4 | Cxcr4 | NM_022205 | 1.19 | 0.4159 | -1.11 | 0.4334 |
| Fibroblast growth factor 2 | Fgf2 | NM_019305 | 1.24 | 0.3390 | 1.47 | 0.0259 |
| Fibroblast growth factor 9 | Fgf9 | NM_012952 | 1.24 | 0.4508 | 1.17 | 0.5779 |
| Gastrin releasing peptide receptor | Grpr | NM_012706 | -1.79 | 0.0484 | -1.77 | 0.1867 |
| Interleukin 10 | Il10 | NM_012854 | 1.35 | 0.4768 | -1.02 | 0.7271 |
| Interleukin 1 beta | Il1b | NM_031512 | 2.50 | 0.1685 | 3.16 | 0.0035 |
| Myelocytomatosis oncogene | Myc | NM_012603 | 1.26 | 0.2614 | 1.67 | 0.0109 |
| Signal transducer and activator of transcription 4 | Stat4 | NM_001012226 | 1.64 | 0.2469 | 1.38 | 0.4436 |
| Transforming growth factor alpha | Tgfa | NM_012671 | 1.23 | 0.1619 | 1.22 | 0.1567 |
| Transforming growth factor, beta 1 | Tgfb1 | NM_021578 | 2.90 | 0.0029 | 3.36 | 0.0001 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| <i>Cell Differentiation</i> | | | | | | |
| Ciliary neurotrophic factor | Cntf | NM_013166 | 1.10 | 0.5290 | 1.06 | 0.6760 |
| Fibroblast growth factor 2 | Fgf2 | NM_019305 | 1.24 | 0.3390 | 1.47 | 0.0259 |
| Fibroblast growth factor 9 | Fgf9 | NM_012952 | 1.24 | 0.4508 | 1.17 | 0.5779 |
| Neurofibromin 1 | Nf1 | NM_012609 | -1.36 | 0.0706 | -1.34 | 0.0954 |
| Neuregulin 1 | Nrg1 | NM_031588 | -1.20 | 0.3026 | -1.26 | 0.2108 |
| Signal transducer and activator of transcription 3 | Stat3 | NM_012747 | 1.27 | 0.3093 | 1.33 | 0.2001 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| Zinc finger protein 91 | Zfp91 | NM_001169120 | -1.30 | 0.0316 | -1.53 | 0.0526 |
| <u>Cytokines and Receptors</u> | | | | | | |
| Chemokine (C-X3-C motif) receptor 1 | Cx3cr1 | NM_133534 | 1.67 | 0.0315 | 1.70 | 0.0635 |
| Chemokine (C-X-C motif) receptor 4 | Cxcr4 | NM_022205 | 1.19 | 0.4159 | -1.11 | 0.4334 |
| Interleukin 10 | Il10 | NM_012854 | 1.35 | 0.4768 | -1.02 | 0.7271 |
| Interleukin 10 receptor, alpha | Il10ra | NM_057193 | 1.73 | 0.0500 | 1.71 | 0.0292 |
| Interleukin 1 beta | Il1b | NM_031512 | 2.50 | 0.1685 | 3.16 | 0.0035 |
| Interleukin 1 receptor, type I | Il1r1 | NM_013123 | 1.22 | 0.2624 | 1.20 | 0.4294 |
| Interleukin 6 | Il6 | NM_012589 | -1.21 | 0.3849 | 1.46 | 0.6407 |
| Interleukin 6 receptor | Il6r | NM_017020 | 1.60 | 0.0157 | 1.40 | 0.1988 |
| Interleukin 6 signal transducer | Il6st | NM_001008725 | 1.08 | 0.3498 | 1.10 | 0.3305 |

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|--|---------|--------------|-------|--------|-------|--------|
| Leukemia inhibitory factor | Lif | NM_022196 | 1.57 | 0.3027 | 2.17 | 0.0687 |
| Leukemia inhibitory factor receptor alpha | Lifr | NM_031048 | -1.43 | 0.1643 | -1.47 | 0.1204 |
| Neuregulin 1 | Nrg1 | NM_031588 | -1.20 | 0.3026 | -1.26 | 0.2108 |
| Signal transducer and activator of transcription 4 | Stat4 | NM_001012226 | 1.64 | 0.2469 | 1.38 | 0.4436 |
| <u>Apoptosis</u> | | | | | | |
| <i>Anti-apoptosis</i> | | | | | | |
| B-cell CLL/lymphoma 2 | Bcl2 | NM_016993 | -1.16 | 0.4948 | -1.34 | 0.2575 |
| Brain-derived neurotrophic factor | Bdnf | NM_012513 | 2.22 | 0.3474 | -1.08 | 0.8819 |
| Interleukin 10 | Il10 | NM_012854 | 1.35 | 0.4768 | -1.02 | 0.7271 |
| <i>Caspase Activation</i> | | | | | | |
| Bcl2-associated X protein | Bax | NM_017059 | -1.13 | 0.6256 | -1.09 | 0.5521 |
| Myelocytomatosis oncogene | Myc | NM_012603 | 1.26 | 0.2614 | 1.67 | 0.0109 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| <i>Induction of Apoptosis</i> | | | | | | |
| Bcl2-associated X protein | Bax | NM_017059 | -1.13 | 0.6256 | -1.09 | 0.5521 |
| Myelocytomatosis oncogene | Myc | NM_012603 | 1.26 | 0.2614 | 1.67 | 0.0109 |
| Nerve growth factor receptor (TNFR superfamily, member 16) | Ngfr | NM_012610 | -1.06 | 0.8455 | 1.12 | 0.6659 |
| Nerve growth factor receptor (TNFRSF16) associated protein 1 | Ngfrap1 | NM_053401 | -1.48 | 0.0437 | -1.72 | 0.0339 |
| Fas (TNF receptor superfamily, member 6) | Fas | NM_139194 | 1.29 | 0.3149 | 1.64 | 0.1195 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| <i>Other Apoptosis Genes</i> | | | | | | |
| Heat shock protein 1 | Hspb1 | NM_031970 | 3.86 | 0.0231 | 4.21 | 0.0075 |
| Interleukin 6 | Il6 | NM_012589 | -1.21 | 0.3849 | 1.46 | 0.6407 |
| CD40 molecule, TNF receptor superfamily member 5 | Cd40 | NM_134360 | 2.68 | 0.0003 | 3.27 | 0.0001 |
| <u>Immune response</u> | | | | | | |
| <i>Acute-phase response</i> | | | | | | |
| Interleukin 6 | Il6 | NM_012589 | -1.21 | 0.3849 | 1.46 | 0.6407 |
| Signal transducer and activator of transcription 3 | Stat3 | NM_012747 | 1.27 | 0.3093 | 1.33 | 0.2001 |
| <i>Inflammatory response</i> | | | | | | |
| Interleukin 10 | Il10 | NM_012854 | 1.35 | 0.4768 | -1.02 | 0.7271 |
| Interleukin 1 beta | Il1b | NM_031512 | 2.50 | 0.1685 | 3.16 | 0.0035 |
| Transforming growth factor, beta 1 | Tgfb1 | NM_021578 | 2.90 | 0.0029 | 3.36 | 0.0001 |
| <i>Lymphocyte activation</i> | | | | | | |
| Interleukin 10 | Il10 | NM_012854 | 1.35 | 0.4768 | -1.02 | 0.7271 |
| CD40 molecule, TNF receptor superfamily member 5 | Cd40 | NM_134360 | 2.68 | 0.0003 | 3.27 | 0.0001 |
| <i>Other immune response genes</i> | | | | | | |

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| | | | | | | |
|--|-----------|--------------|-------|--------|-------|--------|
| Leukemia inhibitory factor | Lif | NM_022196 | 1.57 | 0.3027 | 2.17 | 0.0687 |
| Fas (TNF receptor superfamily, member 6) | Fas | NM_139194 | 1.29 | 0.3149 | 1.64 | 0.1195 |
| Transcription factors and regulators | | | | | | |
| <i>Positive regulation of transcription</i> | | | | | | |
| Fusion (involved in t(12;16) in malignant liposarcoma) (human) | Fus | NM_001012137 | -1.18 | 0.2780 | -1.14 | 0.3574 |
| Neurotrophin 3 | Ntf3 | NM_031073 | 1.19 | 0.6292 | 1.78 | 0.1067 |
| Transforming growth factor beta 1 induced transcript 1 | Tgfb1l1 | XM_341934 | 1.21 | 0.2067 | 1.20 | 0.3595 |
| <i>Transcription coactivator activity</i> | | | | | | |
| Melanoma antigen, family D, 1 | Maged1 | NM_053409 | -1.34 | 0.0279 | -1.36 | 0.0508 |
| Transforming growth factor beta 1 induced transcript 1 | Tgfb1l1 | XM_341934 | 1.21 | 0.2067 | 1.20 | 0.3595 |
| <i>Other transcription factors and regulators</i> | | | | | | |
| FBJ osteosarcoma oncogene | Fos | NM_022197 | 1.74 | 0.1261 | 3.29 | 0.0102 |
| Myelocytomatosis oncogene | Myc | NM_012603 | 1.26 | 0.2614 | 1.67 | 0.0109 |
| Nuclear receptor subfamily 1, group I, member 2 | Nr1i2 | NM_052980 | -1.23 | 0.3919 | -1.11 | 0.7157 |
| Similar to myocyte enhancer factor 2C | LOC685671 | XR_006259 | 1.08 | 0.5206 | 1.20 | 0.4123 |
| Signal transducer and activator of transcription 1 | Stat1 | NM_032612 | 1.82 | 0.0014 | 1.61 | 0.0042 |
| Signal transducer and activator of transcription 2 | Stat2 | NM_001011905 | 1.23 | 0.0877 | 1.22 | 0.2499 |
| Signal transducer and activator of transcription 3 | Stat3 | NM_012747 | 1.27 | 0.3093 | 1.33 | 0.2001 |
| Signal transducer and activator of transcription 4 | Stat4 | NM_001012226 | 1.64 | 0.2469 | 1.38 | 0.4436 |
| Tumor protein p53 | Tp53 | NM_030989 | -1.10 | 0.5051 | 1.10 | 0.6728 |
| Zinc finger protein 110 | Zfp110 | NM_001024775 | -1.43 | 0.1192 | -1.68 | 0.0479 |

STAT2 was upregulated, whereas the expression of NGFRAP1, NTRK1, ZFP91, GRPR, NPY2R, NF1, MAGED1 was downregulated.

3.2.2. Gene Expression Profile in the R-Apomorphine Treated Striatal 6-Hydroxydopamine Lesioned Rats

R-apomorphine treatment of the striatally lesioned 6-OHDA rats changed the striatal expression of 20 genes out of the 84 genes on the array compared to the non-lesioned rats. Significant differences were detected between the R-apomorphine treated striatally lesioned rats and the non-lesioned rats for 13 genes out of 20 genes ($p < 0.05$), with a further 7 genes nearing significance ($p < 0.1$). The majority of the genes (15) were upregulated in the R-apomorphine treated striatally lesioned rats, whereas 5 were downregulated. An upregulation was found for the genes CRHR1, GFRA3, GMFB, GMFG, CD40, FGF2, FOS, IL-1b, LIF, TGFb1, MYC, CX3CR1, IL10 α , HSPB1 and STAT1, whereas the expression of NGFRAP1, ZFP110, ZFP91, NF1 and MAGED1 was

downregulated.

The changes in expression level of some genes were similar in both the striatally lesioned and the R-apomorphine treated 6-OHDA striatally lesioned rats versus the non-lesioned rats, such as the upregulation of GFRA3, CD40, TGFb1, CX3CR1, IL-10 α , HSPB1 and STAT1, and the downregulation of NGFRAP1, ZFP91, NF1 and MAGED1.

The upregulation of GFRA1, CCKAR, IL-6 α and STAT2 in the striatum of striatally lesioned rats was either prevented or not different in the R-apomorphine treated striatally lesioned rats versus the non-lesioned rats.

Furthermore, a downregulation was observed for NTRK1, GRPR and NPY2R in the striatally lesioned rats, while the expression levels of these genes in the R-apomorphine treated striatally lesioned rats were not different from the non-lesioned rats. ZFP110 was significantly downregulated in the treated striatally lesioned rats versus the controls, whereas its expression in the striatally lesioned rats was not different from the non-lesioned rats.

Based on the global overview of the gene expression alterations, we chose to focus on the neuropeptide receptors CRHR1, CRHR2, NPY1R and NPY2R. These 4 receptors are all G protein coupled receptors (GPCRs) and have been implicated as potential drug targets in the treatment of Parkinson's disease, and are more extensively described in the discussion section. Regarding these neuropeptide receptors the following findings were observed. The expression level of CRHR1 was not significantly different between non-lesioned and striatally lesioned rats. However, in the R-apomorphine treated striatally lesioned rats an upregulation of the expression of CRHR1 ($p = 0.06$) was observed when compared to non-lesioned rats. Although this upregulation was nearing significance, there was no difference when compared to striatally lesioned rats (Figure 4).

In the same way, a significant upregulation was found for CRHR2 in the striatally lesioned rats versus the non-lesioned and the treated striatally lesioned rats, whereas no significant difference was found between the R-apomorphine treated striatally lesioned rats and the non-lesioned rats, suggesting that the treatment normalized or prevented the upregulation of CRHR2 (Figure 4).

Regarding the NPY1R, there was a downregulation in the striatally lesioned rats versus the non-lesioned rats, but this was not statistically significant. However, a significant downregulation was observed in the striatally lesioned rats compared to the R-apomorphine treated striatally lesioned rats. There was no significant difference between non-lesioned and R-apomorphine treated striatally lesioned rats, suggesting that R-apomorphine normalized or prevented changes in the expression level of NPY1R (Figure 5).

NPY2R was significantly downregulated in the striatally lesioned rats when compared to the non-lesioned rats. This downregulation was either prevented or normalized by R-apomorphine, as no significant differences were observed between the R-apomorphine treated striatally lesioned and non-lesioned rats (Figure 5).

3.3. Protein Expression of CRHR1, CRHR2, NPY1R and NPY2R

CRHR1 and CRHR2 protein levels, quantified with ELISA, confirmed the results of the gene expression analysis. The CRHR1 protein content in the denervated

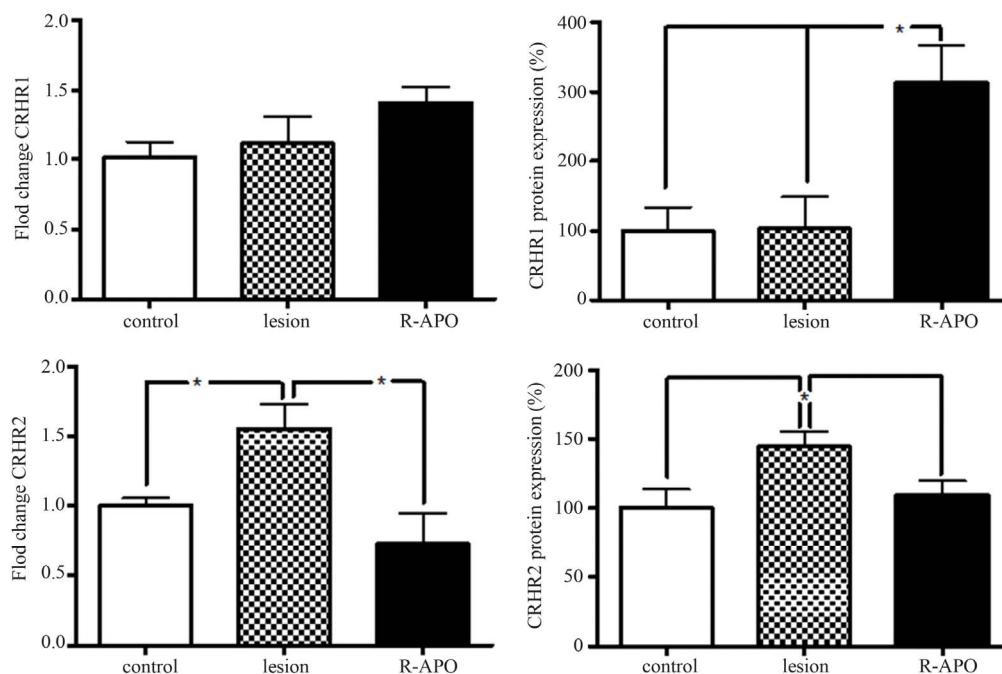


Figure 4. CRHR1 and CRHR2 mRNA and protein expression. CRHR1 (upper left panel) and CRHR2 (lower left panel) mRNA expression in the left striatum of control ($n = 3$), saline treated 6-OHDA striatal lesioned ($n = 4$) and R-apomorphine treated 6-OHDA striatally lesioned rats ($n = 4$) was determined using quantitative real-time PCR as described in the Methods. A fold-change greater than 1 is reported as up regulation, while lower than 1 is reported as down regulation. The protein expression of CRHR1 (upper right panel) and CRHR2 (lower right panel) in the left striatum in all the groups [(control ($n = 6$), saline treated 6-OHDA striatal lesioned ($n = 6$) and R-apomorphine treated 6-OHDA striatally lesioned rats ($n = 6$)] was determined using ELISA as described in the Methods. For each protein, the average control was set to 100%. Data are expressed as mean \pm S.E.M. *Significantly different (one-way ANOVA followed by a Bonferroni's Multiple Comparison Test: $p < 0.05$).

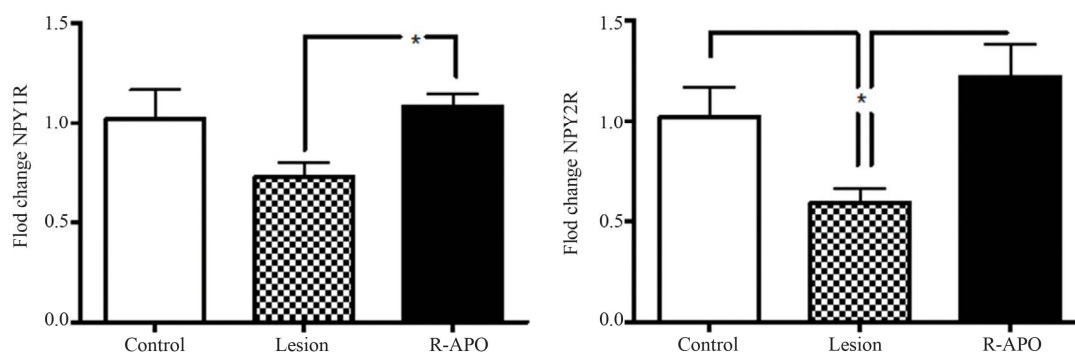


Figure 5. NPY1R and NPY2R mRNA expression. NPY1R (left panel) and NPY2R (right panel) mRNA expression in the left striatum of control ($n = 3$), saline treated 6-OHDA striatal lesioned ($n = 4$) and R-apomorphine treated 6-OHDA striatal lesioned rats ($n = 4$) was determined using quantitative real-time PCR as described in the Methods. A fold-change greater than 1 is reported as up regulation, while lower than 1 is reported as down regulation. Data are expressed as mean \pm S.E.M. *Significantly different (one-way ANOVA followed by a Bonferroni's Multiple Comparison Test: $p < 0.05$).

striata of the R-apomorphine treated striatal lesioned rats was significantly higher when compared to the left striata of the non-lesioned and the denervated striata of the striatal lesioned rats, while the CRHR1 protein content in the striata of non-lesioned rats and the denervated striata of striatal lesioned rats was not significantly different (**Figure 4**). CRHR2 protein levels of the denervated striata of the striatal lesioned rats were significantly higher when compared to the left striata of non-lesioned rats and the denervated striata of the R-apomorphine treated striatal lesioned rats, with no statistical difference between the R-apomorphine group and the control group (**Figure 4**).

No significant changes in NPY1R and NPY2R protein levels were detected in the left striata between the three groups (data not shown), suggesting that these receptors might not be involved in the biological action of R-apomorphine. However, this method does not monitor post-transcriptional and post-translational modifications, and it provides only a partial picture of the biological events [35].

4. Discussion

Our data show that R-apomorphine treatment (10 mg/kg/day, s.c., during 11 days) of rats striatal lesioned with 6-OHDA is associated with a partial restoration of the DA levels and the DOPAC:DA ratios in the ipsilateral striatum. Furthermore, in the open field test an improvement in distance moved and attenuation of the relative meander was observed. The decreased locomotor activity, such as in the lesioned rats, has been attributed to the loss of DA neurotransmission [36]. The reason that not all the investigated behavioural parameters assessed during the open field test normalised or improved, might be due to the fact that R-apomorphine does not fully restore the neuronal degeneration caused by 6-OHDA, which is in

line with the observed partial neuroprotection. These data confirm our previous findings where the same treatment with R-apomorphine significantly reduced the amphetamine-induced rotations, attenuated the DA levels and the DOPAC:DA ratios, and subsequently suggest that the functionality and the integrity of the nigrostriatal dopaminergic system have at least partially been preserved by a treatment with R-apomorphine [14].

The gene expression analysis demonstrated that different genes are altered in the lesioned striatum of the saline treated striatal lesioned rats and the R-apomorphine treated striatal lesioned rats. Genes that were altered in the saline treated striatal lesioned rats, are related to the regulation of neurotrophins, neuropeptides, immune response, neurogenesis, growth and differentiation, cell cycle, cell proliferation, apoptosis and transcription factors and regulators, suggesting that different major events occur which might be involved in the molecular pathways of the pathogenesis of Parkinson's disease and confirming the complexity of the disorder. Treatment with R-apomorphine of the striatal lesioned rats did not reverse all the gene alterations that were observed in the saline treated striatal lesioned rats.

A role for inflammation in the striatal 6-OHDA rat model can be suggested from our data as several inflammatory-related genes were upregulated, such as CX3CR1, IL6R, IL10RA, TGFB1, and CD40. The concept of neuroinflammation as a primary response to 6-OHDA in both the striatum and the substantia nigra has been confirmed by Na *et al.*, 2010 [37]. Furthermore, the DA-ergic neuronal loss within the striatal 6-OHDA rat model is preceded by microglial activation [38,39], suggesting a temporal relationship between neurodegeneration and neuroinflammation [39]. Similarly, *in vivo* and postmortem observations from Parkinson's disease patients show that indeed activated microglia are present and that the

levels of pro-inflammatory mediators, such as TNF- α , IL-6, IL-1 β , are increased [40]. All these findings substantiate the hypothesis of a fundamental role of inflammation in neurodegeneration.

Similarly, the brain alteration in the MPTP mouse model of Parkinson's disease has been investigated by others using a cDNA expression array including 1200 genes fragments and identified 51 genes in the MPTP mouse model related to similar major events and alterations in genes, such as a general increase in IL-1 β , IL-6 and IL-7, as well as in IL-1R, IL-2R, IL-3R and IL-4R, which confirms the concept of inflammation in neurodegeneration [9]. Pretreatment with R-apomorphine (10 mg/kg/day, s.c.) for 5 days before MPTP (24mg/kg/day) injections, reversed most of the gene alterations, suggesting a possible anti-inflammatory action. Furthermore, an increase in anti-inflammatory cytokine IL-10 mRNA might reflect an attempt to protect the neurons from further degeneration [9]. Although, the pharmacogenetic profile of the drug R-apomorphine was already investigated in the above mentioned study, major differences with our experimental set-up are the rodent model, the administration mode of R-apomorphine and some of the selected genes for the screening. For instance, R-apomorphine was administrated before the MPTP injections for 5 consecutive days by Grunblatt *et al.* (2001), while in our study the treatment started 15 min before the lesion induction and continued for 10 days [9].

Based on the global overview of the gene expression alterations (**Table 1**), we chose to focus on the GPCR neuropeptide receptors CRHR1, CRHR2, NPY1R and NPY2R. GPCRs represent a class of proteins with significant clinical importance, as approximately 30% of all modern therapeutic treatments target these receptors [41]. Furthermore, literature reports, as discussed below, suggest that these four receptors might have relevance as potential drug targets in the treatment of Parkinson's disease.

It has been demonstrated that activation of CRHR1 is anti-inflammatory [42] and that urocortin, a CRH-like peptide, restores key deficits, such as motor behavior, striatal DA levels and dopaminergic cell death in a LPS rat model of Parkinson's disease via CRHR1 [43-45] and protects against excitotoxic cell death via the same receptor [46]. Furthermore, the neuroprotective and neurorestorative effects of urocortin in the 6-OHDA rat model are mediated via CRHR1 [43-45]. The anti-inflammatory properties of urocortin in the periphery were previously reported by Gonzalez-Rey [47]. Huang *et al.* have also shown that urocortin modulates dopaminergic neuronal survival via inhibition of glycogen synthase kinase-3 β and histone deacetylase [48]. In addition, Kim *et al.* confirmed the downstream properties of urocortin

on MPP⁺ treated neuroblastoma cells and its mediation via the activation of CRHR1 [49]. Further findings demonstrate that the activation of CRHR2 is pro-inflammatory in the periphery as it mediates the inflammatory responses via release of pro-inflammatory mediators at the colonocyte level [50,51]. Our data show that R-apomorphine treatment is associated with a downregulation of the pro-inflammatory CRHR2, and an upregulation of the anti-inflammatory CRHR1, both at mRNA and at protein levels.

Regarding the NPY receptors, Decressac *et al.* demonstrated that neuroproliferation *in vivo* is mediated by NPY1R [52]. Furthermore, NPY has an anti-inflammatory effect that is mediated by NPY1R *in vivo* [53]. More recently, Decressac *et al.* have shown that NPY is neuroprotective in *in vivo* models of Parkinson's disease via the NPY2 receptor via activation of both MAPK and Akt pathways [54]. Similarly, Thiriet *et al.* have shown that the intracerebral administration of NPY in mice blocked methamphetamine induced apoptosis and that this effect was mainly mediated by the stimulation of the NPY2 receptor and to a lesser extent by NPY1 receptors [55]. Moreover, it has been shown that the NPY receptors are involved in the attenuation of DA release *in vitro* [36,56] and *in vivo* [36,57,58] and that the NPY2 receptor is involved in DA synthesis in the rat striatum [36] and the inhibition of glutamate release [59]. Regarding these NPY receptors, our data demonstrates that R-apomorphine treatment is associated with an upregulation of the NPY1R and NPY2R at mRNA level, but not at the protein level. However, it is important to note that changes in mRNA levels are not always reflected by changes in protein expression since proteins can be synthesized in one brain area and afterwards transported to another [60]. Furthermore, post-transcriptional and post-translational modifications may occur [61].

R-apomorphine has been already tested *in vitro* and various different observations have been registered [21, 26,27,62]. It has been shown previously that R-apomorphine stimulates the synthesis and release of multiple trophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), in both mesencephalic and striatal neurons, thereby effectively preventing dopaminergic neuron loss *in vitro* [27,62]. In another study, it was shown that fibroblast growth factor-2 expression is robustly induced in cultured astrocytes in response to R-apomorphine [26]. It is worth noting that the study was performed on cultured astrocytes derived from newborn animals, while the astrocytes from aged brains of Parkinson's disease may react differently *in vitro*. The aforementioned findings were not confirmed by our gene expression studies. Furthermore, if astrocytes *in vivo* will respond to R-apo-

morphine treatment in the same way as reported in the aforementioned study needs to be investigated, as *in vitro* and *in vivo* findings do not always correlate.

The absence of sham lesioned rats within our experimental design may be considered by others as a study limitation. However, various publications indicate that sham lesioning of rodents has no effect on the main pathological hallmarks of Parkinson's disease, such as DA loss [30,37,43,63,64]. Furthermore, Na *et al.* [37] assessed the number of gene expression changes in vehicle and 6-OHDA lesioned striatum and substantia nigra at various time points by using cDNA microarray. They found that three days after vehicle administration there are profound changes in relative striatal gene expression levels (<50 genes). However, this gene expression pattern began to wane as a function of time, and fourteen days after vehicle administration, the number of altered genes was almost equal to zero. A similar pattern was observed for 6-OHDA striatally lesioned rats. However, within this group the number of altered genes was much higher after 3 days (>150 genes) and after 14 days (>50 genes) compared to the vehicle lesioned rats. These findings suggest that sham lesioning has an effect on mRNA expression, but that it disappears very quickly as a function of time [37]. In addition, needle insertion and vehicle injection does not alter functional protein levels of BDNF and CNTF [63,65]. However, some astrocytic reaction in the neostriatum of solvent injected rats close to the needle track has been observed, while in the neurotoxin lesioned animals this reaction was much more widespread [66-68]. Our own findings also show that neurodegeneration caused by a unilateral injection of 6-OHDA in the medial forebrain bundle or the striatum, has no effect on the striatal protein expression of GDNF at different time points after lesion in the intact and the denervated striata of the lesioned animals and that of control animals [60]. The aforementioned findings suggest that the insertion of the needle for lesioning does not cause significant alterations in terms of striatal neurotransmitters and behaviour. However, some acute, minor and temporary effects have been observed at the gene and protein level, but this is limited to the needle tract. In our study, we assessed the gene and protein expression fourteen days after surgery. Even though, there would be an effect of the needle insertion, the combination with the neurotoxin is much more invasive and destructive.

Similarly, we did not include intact rats which have been treated with R-apomorphine, as we wanted to investigate the effects of R-apomorphine in the striatum of diseased, parkinsonian animals. Previously, we have shown that intact rats treated with R-apomorphine do not have any significant differences in striatal DA and DOPAC content, and DA turnover when compared to

intact rats that didn't receive any treatment [14]. Furthermore, the number of tyrosine hydroxylase immunoreactive neurons in the SNpc were not different between intact rats and intact rats treated with R-apomorphine.

5. Conclusion

Our data confirm the neuroprotective effects of R-apomorphine in the unilateral striatal 6-OHDA parkinsonian rat model and suggest that they involve the alteration of the striatal gene and the protein expression levels of the anti-inflammatory CRHR1 receptor and the pro-inflammatory CRHR2 receptor. Furthermore, treatment with R-apomorphine led to an upregulation of the NPY1R and NPY2R at the mRNA level. These results provide a better insight for understanding our previously observed neuroprotective effects of R-apomorphine in the unilateral striatal 6-OHDA parkinsonian rat model and confirm its potential anti-inflammatory action. However, additional investigations, e.g. functional studies, are required in order to confirm these findings. Despite the value of the 6-OHDA rat model, as illustrated in this work, one should be aware that the mechanism of 6-OHDA probably only reflects a small fraction of the events occurring in human Parkinson's disease. 6-OHDA induces rather acute effects, which differ significantly from the slowly progressive pathology of human Parkinson's disease. Moreover, Lewy body pathology is not present in the surviving neurons, and no other brain areas involved in Parkinson's disease are affected, such as the olfactory bulb and the locus coeruleus [69-71]. Therefore, further work would be valuable in understanding the potential therapeutic value of targeting the CRH and NPY receptors in Parkinson's disease.

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