UNIVERSIDADE DA BEIRA INTERIOR

Ciências

Retinoic acid-loaded polymeric nanoparticles induce neuroprotection in a mouse model of Parkinson's disease

Marta Raquel Carrola Esteves

Dissertação para obtenção do Grau de Mestre em
Biotecnologia
(2º ciclo de estudos)

Orientador: Prof. Doutora Liliana Bernardino
Co-orientador: Prof. Doutora Graça Baltazar

Covilhã, junho de 2013

Agradecimentos

Concluída mais uma etapa não poderia deixar de manifestar o meu enorme agradecimento a todos aqueles que de certa forma me acompanharam e apoiaram e assim contribuíram para a concretização desta etapa.

Começo por agradecer à Prof. Doutora Liliana Bernardino, orientadora deste trabalho, pela partilha de conhecimento de científico, pelas críticas construtivas e por toda a motivação que se tornaram fundamentais na conclusão deste trabalho. Agradeço ainda a oportunidade, toda a ajuda e disponibilidade e por ter despertado em mim a curiosidade de querer saber mais.

À Prof. Doutora Graça Baltazar e à Prof. Doutora Carla Fonseca pela cooperação e partilha de conhecimentos.

À Sandra e à Tatiana por toda a ajuda que foi fundamental para a concretização deste trabalho, pela paciência e disponibilidade, partilha de conhecimentos e pelos bons momentos proporcionados.

A todos os meus colegas de laboratório que sempre se prontificaram a ajudar e esclarecer dúvidas.

A todos os meus amigos por toda a amizade, em especial à Zélia, Filipa e Ana por todo o apoio e pelos momentos de boa disposição.

Ao Bruno Mateus pelo companheirismo, apoio e motivação principalmente nos momentos difíceis.

Por último, e mais importante, um enorme obrigado aos meus pais por terem sido sempre o pilar da minha vida, pelo apoio incondicional, conforto e por sempre terem acreditado em mim. A vocês devo tudo o que sou hoje!

Resumo

O ácido retinóico (AR) desempenha uma função importante no desenvolvimento do sistema nervoso dos mamíferos e tem sido evidenciado como uma opção terapêutica para diversas doenças neurodegenerativas devido às suas propriedades neuroprotetoras, anti-inflamatórias e pro-neurogénicas. Contudo, o AR apresenta propriedades indesejáveis, tais como: a fraca solubilidade em água e o curto tempo de semi-vida. Por este motivo, as nanopartículas (NPs) apresentam-se como uma excelente alternativa de modo a contornar essas propriedades indesejáveis garantindo o transporte intracelular e a libertação controlada de AR. O objectivo deste trabalho foi avaliar os efeitos da administração intracerebral (estriado) de NPs carregadas com AR (NPs^+-AR) num modelo da doença de Parkinson (DP) em murganho utilizando a neurotoxina MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina), e a sua comparação com os efeitos da administração de AR solúvel. Curiosamente, em murganhos adultos observou-se que as NPs^+-AR reduziram significativamente a lesão provocada pelo MPTP, aumentando a percentagem de neurónios dopaminérgicos positivos para a tirosina hidroxilase (TH^+) na *substantia nigra* (*SN*) para níveis similares ao controlo, bem como, a intensidade e área ocupada pelas fibras TH^+ no estriado. Este efeito protetor mediado pelas NPs^+-AR foi mais robusto que o efeito proporcionado pelo AR solúvel. Estes efeitos foram acompanhados por um aumento da expressão na *SN* e estriado de RNAm de Nurr1 e Pitx3, ambos factores de transcrição envolvidos na especificação e sobrevivência neuronal dopaminérgica. O mesmo padrão de expressão de RNAm para Pitx3 foi detectado na *SN* de murganhos idosos. Em suma, as NPs^+-AR apresentam um efeito protetor robusto contra a lesão dopaminérgica quando comparadas com o AR solúvel, sugerindo que as NPs^+-AR podem ser uma boa estratégia para promover a reparação cerebral na DP.

Palavras-chave

Ácido retinóico, nanopartículas, neuroproteção, neurónios dopaminérgicos, MPTP, doença de Parkinson

Resumo alargado

O ácido retinóico (AR) é um produto resultante do metabolismo da vitamina A (retinol) que modula a transcrição genética envolvida na proliferação e diferenciação celular através da ativação de receptores nucleares do ácido retinóico (RAR) e a receptores retinóicos (RXR). Neste sentido, o AR desempenha uma função importante no desenvolvimento do sistema nervoso dos mamíferos e tem sido evidenciado como uma opção terapêutica para diversas doenças neurodegenerativas devido às suas propriedades antioxidantes, neuroprotetoras, anti-inflamatórias e pro-neurogénicas. Contudo, o AR apresenta propriedades indesejáveis, tais como: uma fraca solubilidade em água e um curto tempo de semi-vida devido à rápida metabolização pelas células. Por este motivo, as nanopartículas (NPs) apresentam-se como uma excelente alternativa de administração do fármaco garantindo o transporte intracelular e a libertação controlada de AR.

O objectivo deste trabalho foi avaliar os efeitos da administração intracerebral (estriado) de NPs carregadas com AR (NPs^+-AR) num modelo da doença de Parkinson (DP) em murganho e a sua comparação com os efeitos da administração de AR solúvel. Neste sentido, murganhos C57BL6 adultos foram injetados com 100 ng/ml NPs^+-AR, 100 ng/ml NPs desprovidas de AR ("blank") ou 10 μM AR solúvel no estriado lateral direito. Murganhos C57BL6 idosos também foram submetidos a injeção intrastriatal com 100 ng/ml NPs^+-AR. Ambos os grupos etários de murganhos foram submetidos a uma lesão aguda com MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina), administrado intraperitonealmente (i.p.) 3 dias após as injeções intrastriatais. Os murganhos foram sacrificados sete dias após a lesão aguda induzida por MPTP e as regiões da *substantia nigra* (*SN*) e do estriado foram recolhidas para posterior análise histoquímica ou de expressão de genes por PCR quantitativo.

A extensão da lesão foi avaliada por quantificação tanto do número neurónios dopaminérgicos positivos para tirosina hidroxilase (TH^+), na *SN,* como da imunoreactividade para fibras TH^+ no estriado. Como esperado, o MPTP provocou uma redução de cerca de 50% dos neurónios TH^+ na *SN* e uma redução dos terminais dopaminérgicos no estriado, quando comparado com os murganhos salinos (controlo). Curiosamente, as NPs^+-AR reduziram significativamente a lesão provocada pelo MPTP, aumentando a percentagem de neurónios TH^+ na *SN* para níveis similares ao controlo, bem como, a intensidade e área ocupada pelas fibras TH^+ no estriado. Este efeito protetor mediado pelas NPs^+-AR foi mais robusto que o efeito proporcionado pelo AR solúvel. Estes efeitos foram apoiados pela análise da expressão de RNAm de Nurr1 e Pitx3, factores de transcrição envolvidos na especificação e sobrevivência neuronal dopaminérgica, por PCR quantitativo. Na *SN* dos murganhos adultos expostos às NPs^+-AR e ao MPTP houve um aumento da expressão de RNAm de ambos os factores de transcrição, quando comparada com

células expostas apenas ao MPTP. Em murganhos idosos, foi obtido o mesmo padrão de expressão de RNAm de Pitx3. Verificou-se também que a lesão provocada pelo MPTP provocou um aumento na expressão dos marcadores dos astrócitos (GFAP) e da microglia (CD11b) assim como uma alteração de morfologia associada à reatividade glial. Curiosamente, as NPs^{+}-AR parecem diminuir a reatividade dos astrócitos e microglia em murganhos tratados com MPTP. Em suma, as NPs^{+}-AR apresentam um maior efeito protetor contra a lesão dopaminérgica induzida pelo MPTP quando comparadas com o AR solúvel. Este efeito protetor foi acompanhado por um aumento de expressão de factores de transcrição que são responsáveis pela sobrevivência e especificação neuronal. Estes resultados sugerem que as NPs^{+}-AR podem ser uma boa estratégia para promover a reparação cerebral na DP.

Abstract

Retinoic acid (RA) plays an important role in the developing mammalian nervous system and has been highlighted as a therapeutic option for some neurodegenerative diseases due to its neuroprotective, anti-inflammatory and pro-neurogenic properties. However, RA presents undesirable properties like poor water solubility and short half-life. Therefore, nanoparticles (NPs) are an excellent alternative to control the undesired side effects and to ensure intracellular transport and controlled release of RA. Thus, the aim of this work was to evaluate the effects of RA-loaded NPs (RA^+-NPs) in an *in vivo* mouse model of Parkinson's disease (PD) using a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxin, and to compare with effects of soluble RA. Interestingly, in adult mice, RA^+-NPs significantly reduced the MPTP lesion by increasing the percentage of tyrosine hydroxylase positive (TH^+) dopaminergic neurons in the *SN* to levels similar to control as well as increasing the intensity and area occupied by TH^+ fibers in the striatum. This protective effect mediated by RA^+-NPs was more robust than when compared with effect of soluble RA. These effects were accompanied by an increase in mRNA expression in *SN* and striatum of Nurr1 and Pitx3, both transcription factors involved in dopaminergic survival and specification. The same pattern of Pitx3 mRNA expression was found in the *SN* of old mice. In conclusion, RA^+-NPs show a robust protective effect against dopaminergic injury when compared to soluble RA, suggesting that RA^+-NPs could be a good strategy to boost brain repair in PD.

Keywords

Retinoic-acid, nanoparticles, neuroprotection, dopaminergic neurons, MPTP, Parkinson's disease

Table of contents

List of figures ix

List of abbreviations x

CHAPTER 1 - INTRODUCTION 1

1.1 Neurodegenerative disorders 1
1.1.1 Parkinson's disease 1
1.1.2 Experimental animal models of PD 2

1.2 Transcription factors involved in dopaminergic survival 4

1.3 Retinoic acid 5
1.3.1 Retinoic acid receptors 6

1.4 Involvement of retinoic acid signaling in PD 8

1.5 Nanoparticles as a delivery system of retinoic acid 9

CHAPTER 2 - OBJECTIVES 11

CHAPTER 3 – MATERIALS AND METHODS 12

3.1 Animals 12

3.2 Intrastriatal injection 12

3.3 MPTP - induced lesion 13

3.4 Immunohistostainings 14
3.4.1 Tissue preparation 14
3.4.2 TH staining 14
3.4.3 Fluorescence immunohistostaining for glial cells 15

3.5 Cell countings and quantitative analysis 15

3.6 RNA isolation 16

3.7 Reverse transcription-polymerase chain reaction (RT-PCR) 16

3.8 Gene expression analysis by quantitative real-time PCR (qPCR) 17

3.9 Data analysis and statistics 17

CHAPTER 4 - RESULTS **19**

4.1 RA^+-NPs induce neuroprotection of the *SN* dopaminergic neurons against the MPTP-induced lesion **19**

4.2 RA^+-NPs induce neuroprotection of TH^+ striatal fibers against the MPTP-induced lesion **21**

4.3 Effects of RA^+-NPs in glial reactivity **23**

4.4 Effects of RA^+-NPs in Nurr1 and Pitx3 mRNA expression **25**
4.4.1 Nurr1 and Pitx3 mRNA expression in adult mice 25
4.4.2 Nurr1 and Pitx3 mRNA expression in old mice 27

CHAPTER 5 - DISCUSSION **29**

CHAPTER 6 - CONCLUSIONS **32**

6.1 Future Perspectives **32**

CHAPTER 7 - REFERENCES **33**

List of figures

Chapter 1

Figure 1.1 The pathology of Parkinson's disease. 2
Figure 1.2 Schematic representation of MPTP metabolism. 3
Figure 1.3 Mechanisms of MPTP neurotoxicity. 4
Figure 1.4 Retinoic acid synthesis and signaling. 6
Figure 1.5 Mode of action of retinoic acid. 7
Figure 1.6 Transmission electron microscopy image and release profile of RA^+-NPs. 10

Chapter 3

Figure 3.1 Schematic representation of experimental treatments and assays performed *in vivo*. 13
Figure 3.2 Experimental groups. 18

Chapter 4

Figure 4.1 RA^+-NPs pre-treatment increased survival of TH^+ cells in the MPTP-injured *SN*. 20
Figure 4.2 RA^+-NPs pre-treatment increases TH^+ fibers immunoreactivity in the MPTP-injured striatum. 22
Figure 4.3 Fluorescence immunohistostaining of astrocytes and microglia in striatum. 24
Figure 4.4 RA^+-NPs induce the expression of Nurr1 and Pitx3 mRNA expression in the *SN* of adult mice exposed to MPTP. 26
Figure 4.5 RA^+-NPs induce Nurr1 and Pitx3 mRNA expression in MPTP-treated old mice. 28

List of abbreviations

6-OHDA	6-hydroxidopamine
9cRA	9-cis retinoic acid
AD	Alzheimer's disease
ALDH	Aldehyde dehydrogenase
ATP	Adenosine triphosphate
atRA	All-trans retinoic acid
BBB	Blood-brain barrier
BMP-7	Bone morphogenic protein-7
BSA	Bovine serum albumin
CD11b	Alpha chain of $\alpha_M\beta_2$-integrin or cluster of differentiation molecule 11b
cDNA	Complementary DNA
CNS	Central nervous system
CRABP2	Cellular retinoic acid-binding protein 2
CRBP	Cellular retinol-binding protein
CYP26	Cytochrome P450 isoform 26
DA	Dopamine
DAB	3,3'-diaminobenzidine
DAT	Dopamine transporter
DMSO	Dimethyl sulfoxide
DS	Dextran sulfate
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
HPLC	High performance liquid chromatography
i.p.	intraperitoneal
LPS	Lipopolysaccharide
MAO-B	Monoamine oxidase-B
mDA	Midbrain dopaminergic
MPDP+	1-methyl-4-phenyl-2,3-dihydropyridinium
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
NaCl	Sodium chloride
NPs	Nanoparticles
Nurr1	Nuclear receptor related 1
OCT	Optimal cutting temperature gel
OCT-3	Organic cation transporter 3

PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline-Tween 20
PCR	Polymerase chain reaction
PD	Parkinson's disease
PEI	Polyethylenimine
PFA	Paraformaldehyde
Pitx3	Paired-like homeodomain 3
qPCR	Quantitative real-time polymerase chain reaction
RA	Retinoic acid
RA^{+}-NPs	Retinoic acid-loaded polymeric nanoparticles
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid-response element
RBP4	Retinol-binding protein
RDH10	Retinol dehydrogenase 10
RT	Room temperature
RT-PCR	Reverse transcription - polymerase chain reaction
RXR	Retinoid X receptor
SN	*Substantia nigra*
SNpc	*Substantia nigra pars compacta*
TBS	Tris buffer saline
TH	Tyrosine hydroxylase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VTA	Ventral tegmental area

Chapter 1

Introduction

1.1 Neurodegenerative disorders

Diseases that cause the degeneration of nerve cells in central nervous system (CNS) have a huge economic and social impact in aging populations all over the world. These debilitating and incurable conditions are characterized by the progressive loss of neuronal cell function and are often associated with atrophy of the affected nervous system structures. Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD), affecting more than 1% of the population over 60 years of age (von Campenhausen et al., 2005).

1.1.1 Parkinson's disease

PD occurs in an idiopathic manner in 95% of cases, whilst in the remaining 5-10% of cases a genetic mutation is present (Toulouse and Sullivan, 2008). Nevertheless the etiology of PD has not been completely understood yet. The causes of this disorder are likely to be multiple and to involve not only single factors alone, but instead several intrinsic and/or environmental factors acting together (Schapira, 2006).

In a healthy brain, the cell bodies of dopaminergic neurons reside within the *substantia nigra pars compacta* (*SNpc)*, region of the ventral midbrain, while the nerve terminals project to the dorsolateral striatum forming the nigrostriatal dopaminergic pathway which has an important role in the control of movements. However, PD is characterized mainly by a progressive and selective loss of the dopaminergic neurons that synthesize the neurotransmitter dopamine (DA) in the *SNpc*, resulting in the loss of dopaminergic nerve terminals, accompanied by DA deficiency in the striatum (Figure 1.1) (Jankovic, 2008).

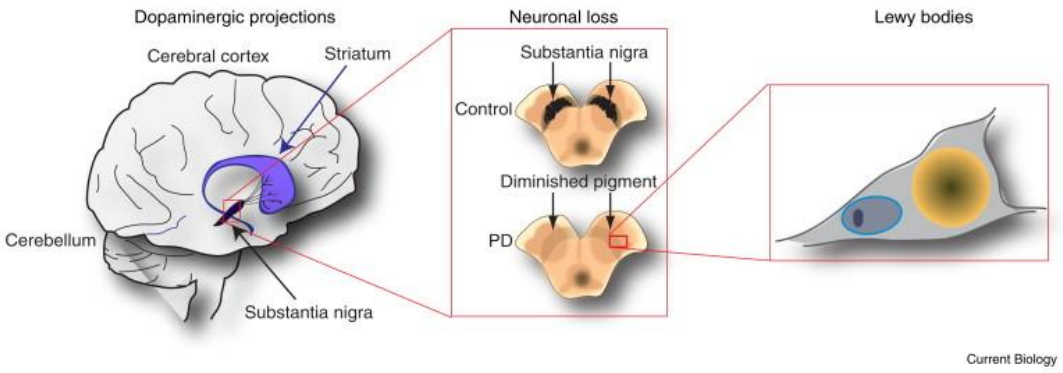


Figure 1.1 The pathology of Parkinson's disease. Simplified view of the main neuropathological events in PD at three levels from left to right. At the level of the brain, a major pathway is degeneration of the dopaminergic projections from the *substantia nigra* (in black) to the striatum (in purple), both of which are in the midbrain underneath the cerebral cortex. At the level of *substantia nigra*, the neurons that form the presynaptic portion of this pathway are normally melanized and are easily identified by this pigment in control brains (upper panel). In contrast, the loss of neurons in this region is so substantial that the whole area becomes depigmented in PD cases (lower panel). Of the few remaining cells, many show pathological changes, including the accumulation of proteins and lipids in Lewy bodies. Figure adapted from Cookson, 2012.

The resulting lack of the neurotransmitter DA leads to decreased signaling within the nigrostriatal pathway and produces abnormal motor behavior, including tremor, bradykinesia (slow movement), muscular rigidity and postural instability (Dauer and Przedborski, 2003). These motor manifestations can also be accompanied by non-motor symptoms such as sleep disturbances, neuropsychiatric symptoms and autonomic and cognitive dysfunction (Dauer and Przedborski, 2003, Reichmann et al., 2009). Although the loss of dopaminergic neurons within *SNpc* is the primary pathological feature of PD, widespread neuronal loss also occurs in the locus coeruleus, with a consequent loss of norepinephrine (Gesi et al., 2000). The histological hallmark of this disease is characterized by the accumulation of a protein called α-synuclein into neuronal eosinophilic insoluble cytoplasmic inclusions, known as Lewy bodies, in the residual neurons of the *substantia nigra* (*SN*) (Figure 1.1) (Dickson et al., 2009).

1.1.2 Experimental animal models of PD

In order to better elucidate the etiology, pathogenesis, mechanisms of cell death and to evaluate therapeutic strategies for PD, numerous animal models have been developed (Dauer and Przedborski, 2003). In this thesis, I will emphasize on common neurotoxic murine models in which toxic molecules are used to lesion the nigrostriatal dopaminergic system. Among the neurotoxic models, compounds that produce both reversible (reserpine) and irreversible (MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), 6-OHDA (6-hydroxidopamine), paraquat and rotenone) effects on dopaminergic neurons have been used widely.

MPTP administration (either acute or chronic) represents the most frequently neurotoxin-based model used to produce experimental models of PD in rodents and nonhuman primates

(Zigmond et al., 1989). In humans and nonhuman primates, depending on the regimen used, MPTP can produce an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD, including tremor, rigidity, bradykinesia, postural instability and freezing (Tetrud and Langston, 1989).

After systemic administration, MPTP which is highly lipophilic rapidly crosses the blood-brain barrier (BBB). Once in the brain, MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase-B (MAO-B) in glia and serotonergic neurons, the only cells that contain this enzyme (Vila et al., 2000). $MPDP^+$ is then converted to 1-methyl-4-phenylpyridinium (MPP^+), which is the active toxic compound. Thereafter, MPP^+ is released through the organic cation transporter 3 (OCT-3) into the extracellular space where it is taken up by the dopaminergic neurons via the dopamine transporter (DAT) (Figure 1.2) (Cui et al., 2009). Once accumulated in dopaminergic neurons, MPP^+ induces neurotoxicity primarily by inhibiting complex I of the mitochondrial electron transport chain resulting in ATP depletion and increases oxidative stress (Figure 1.3) (Nicklas et al., 1985, Mizuno et al., 1987). MPTP is usually systemically administered via intraperitoneal, subcutaneous, intravenous or intramuscular injection. For unknown reasons, rats are less sensitive to MPTP toxicity than mice (Giovanni et al., 1994).

In summary, although of its acute toxic property as seen with other neurotoxic PD models, MPTP will continue to play a major role in PD research based on its ability to produce PD-like effects in nonhuman primates and mice, its reproducible L-dopa-responsive lesion on the nigrostriatal system and its ease of administration.

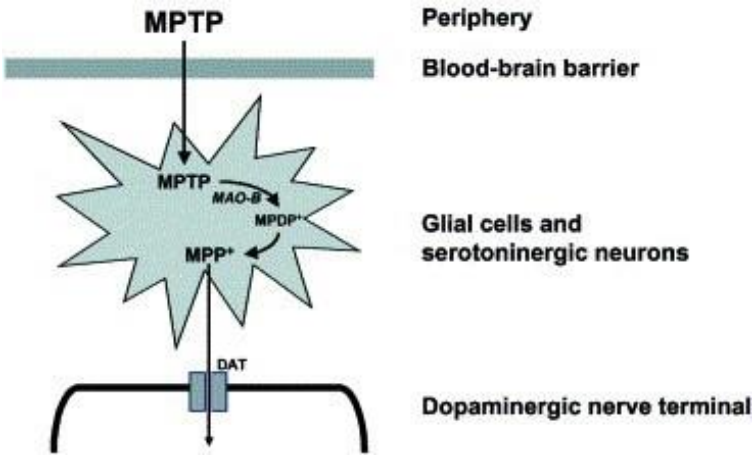


Figure 1.2 Schematic representation of MPTP metabolism. After its systemic administration, MPTP crosses the BBB. Once in the brain, MPTP is converted to $MPDP^+$ by MAO-B within non-dopaminergic cells, and then to MPP^+. Thereafter, MPP^+ is released in the extracellular space. From there, MPP^+ is taken up by the DAT and enters into dopaminergic neurons. Figure adapted from Przedborski and Vila, 2001.

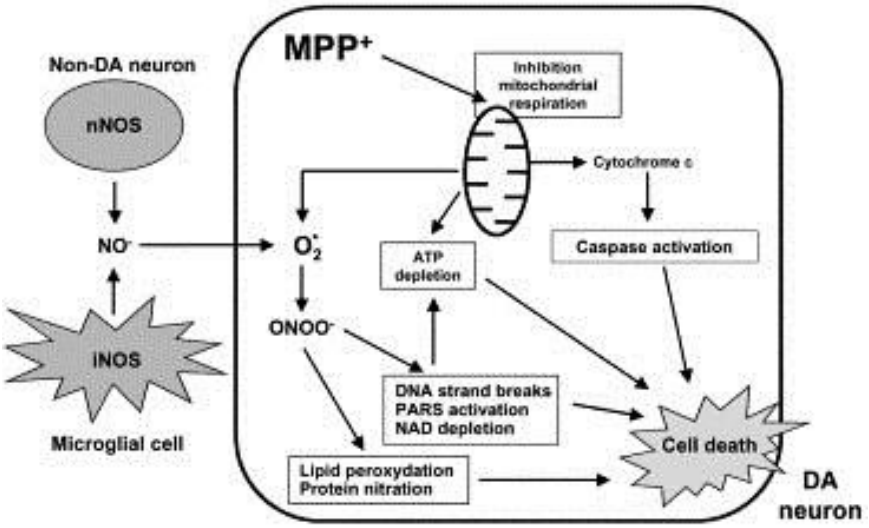


Figure 1.3 Mechanisms of MPTP neurotoxicity. Within dopaminergic neurons, MPP^+ inhibits enzymes in the mitochondrial electron transport chain, resulting in ATP deficit and increased 'leakage' of superoxide (O_2) from the respiratory chain. Superoxide remains in the cell in which it is produced. On the other hand, NO, which is produced by nNOS and iNOS outside dopaminergic neurons, is membrane-permeable and can diffuse into neighboring neurons. If the neighboring cell has elevated levels of superoxide, then there is an increased probability of superoxide reacting with NO to form peroxynitrite, which can damage lipids, proteins, and DNA. Damaged DNA stimulates PARS activity, which further depletes ATP stores. On the other hand, MPP^+ may induce the release of cytochrome c from the mitochondria to the cytosol where it initiates a cascade of caspase activation. Figure adapted from (Przedborski and Vila, 2001).

1.2 Transcription factors involved in dopaminergic survival

Some transcriptional factors have been described to be involved in the survival, functionality and maturation of dopaminergic neurons in the nigrostriatal pathway, and so, consequently altered in PD conditions. Among them, Nurr1 is a critical transcription factor for the development and functional maintenance of dopaminergic neurons and is also considered as a crucial regulator for the expression of several genes involved in PD pathology including DAT and tyrosine hydroxylase (TH), which is the rate-limiting enzyme in DA synthesis. Nurr1 has also been indicated to have a role in the neuroprotection of mature dopaminergic cells in several studies (Smits et al., 2003, Jankovic et al., 2005). This transcription factor is highly expressed in dopaminergic neurons and other cells including microglia, where it is involved in the modulation of the inflammatory response (Saijo et al., 2009). Nurr1 appears to interact with other transcription factor, Pitx3, which is specifically expressed in dopaminergic neurons. Pitx3 is highly expressed in *SN* and ventral tegmental area (VTA) of midbrain and is essential for the development and survival of these neurons (Smidt et al., 2004). Recently, Volpicelli *et al*. reported that Nurr1 can act as either a monomer or homodimer in controlling Pitx3 expression and, moreover, also demonstrated that Nurr1 RNA silencing reduced Pitx3 transcripts, leading to the hypothesis that Nurr1 may regulates Pitx3 expression by binding to its promoter (Volpicelli et al., 2012).

Nurr1 and Pitx3 have been shown to play a crucial role in the maturation, specification and survival of midbrain dopaminergic (mDA) neurons, and both of them are potential susceptibility genes for PD. The Nurr1-null mice failed to generate mDA neurons, leading to 98% decrease of DA in the striatum and reduced expression of Nurr1 increased the vulnerability of mDA neurons to MPTP-induced injury (Le et al., 1999b) . MPTP also decreases TH and Pitx3 gene expression leading to selective loss of dopaminergic neuronal population in the *SNpc* (Le et al., 1999a, Luk et al., 2013) and consequently to the loss of projections from de *SN* to the striatum (Nunes et al., 2003).

1.3 Retinoic acid

Until now, there is no cure for PD and current therapies can provide temporary relief of motor symptoms but do not prevent disease progression. Thus, new therapeutic agents and new approaches are desperately needed in PD. The origin of dopaminergic cell degeneration present in this disease is largely unknown, but is suggested to be caused by agents causing oxidative damage and energy depletion in the nigrostriatal pathway (Langston, 1998). Preventing the neurodegeneration, especially in nigrostriatal area, is important for controlling of the disease. Thus, besides the other therapeutic approaches, treatment with antioxidants gains gradually importance in pharmacotherapy of PD. In this context, a molecule of particular interest is retinoic acid (RA), specially the all-trans retinoic acid (atRA) isoform.

RA is a metabolic product of vitamin A ingested in the diet and plays an important role in the developing mammalian nervous system (Xu and Drew, 2006). RA has been highlighted as a therapeutic option for some neurodegenerative disorders due to its neuroprotective, anti-inflammatory and pro-neurogenic properties (Xu and Drew, 2006, Maia et al., 2011, Yin et al., 2012). RA exists in several stereoisomeric forms including predominantly atRA, 13-cis RA and less-stable isomer such as 9-cis-RA (9cRA).

Tissue distribution of RA is tightly regulated by a complex metabolic pathway, consisting of multiple steps of synthesizing and catabolic enzymes. When vitamin A is ingested in the diet as retinyl esters, these compounds are stored mainly in the liver; animals are unable to synthesize retinoids by other mechanisms. Depending on cell needs, retinyl esters are cleaved into retinol (the liposoluble form of vitamin A) and this molecule is secreted by the liver, transported into the blood and can cross the BBB to target tissues bound to plasma retinol-binding protein (RBP4) (Ruberte et al., 1993, Vogel et al., 2001). Retinol enters cells via a specific membrane receptor STRA6 (Kawaguchi et al., 2007) and in the cytoplasm, retinol is converted to retinaldehyde by retinol dehydrogenase 10 (RDH10) and consequently to atRA via the enzyme retinaldehyde dehydrogenase (RALDH1/ALDH1). RA can then be released from the cytoplasm and taken up by a receiving cell (paracrine signaling) or can act directly on its

cell nucleus (autocrine signaling). In its way to the nucleus, RA has to interact with the cellular retinoic acid-binding protein 2 (CRABP2) (Budhu and Noy, 2002) that facilitates uptake of RA and transport to the nucleus where RA binds to the RA receptors.

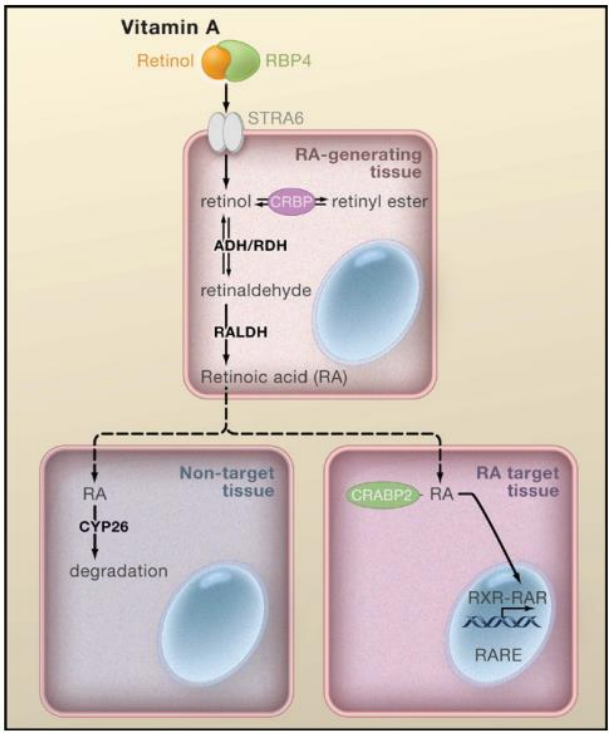


Figure 1.4 Retinoic acid synthesis and signaling. Depicted is the paracrine mechanism of retinoic acid (RA) signaling. Retinol is transported in plasma by retinol-binding protein (RBP4) secreted from the liver. Retinol enters cells via a specific receptor STRA6, and cellular retinol-binding protein (CRBP) facilitates conversion of retinol to retinyl esters for storage. In RA-generating tissues, retinol is oxidized to retinaldehyde by either alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH), and retinaldehyde is oxidized to RA by retinaldehyde dehydrogenase (RALDH). RA is then released and taken up by surrounding cells. Cells that express cytochrome P450 (CYP26) initiate the further oxidation of RA for degradation and excretion and are not target cells. Some RA target cells express cellular RA-binding protein 2 (CRABP2) that facilitates uptake of RA and transport to the nucleus where RA binds the RA receptor (RAR). The ternary complex of ligand-bound RAR with RXR and a retinoic acid response element (RARE) regulates transcription of RA target genes. Figure adapted from Duester, 2008.

1.3.1 Retinoic acid receptors

It is well known that RA signal is transduced by binding to specific nuclear receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR), which are members of the nuclear receptor superfamily. Both RAR and RXR include three subtypes designated as α, ß and γ, and expression of these receptor subtypes is observed in several brain regions in the adult CNS. RAR bind and are activated by atRA and its 9cRA isomer while RXR are only activated by the 9cRA (Allenby et al., 1993).

A classical mechanism of RAR action involves formation of heterodimer with RXR and binding to a DNA sequence called retinoic acid-response element (RARE) located in the promoter region of target genes (Bastien and Rochette-Egly, 2004). In the absence of ligand (RA), the RAR-RXR heterodimer is constitutively bound to DNA on RARE and associated with co-repressors complexes. These complexes induce transcriptional silencing through histone deacetylation associated with target sequences thus increasing chromatin condensation. The binding of RA to RAR induces conformational changes in the receptors bound to RARE causing the dissociation of co-repressors and the binding of co-activators. The co-activators subsequently mediate histone acetylation resulting in decondensation of the chromatin and activation of target gene expression (Figure 1.5) (Marletaz et al., 2006).

Although RAR agonists can autonomously active transcription through RAR-RXR heterodimers, RXR cannot respond to RXR-selective agonists in the absence of a RAR ligand (Chen et al., 1996). This phenomenon is generally referred to as RXR "subordination" or RXR "silencing". RAR and RXR are not active in their monomeric forms. While RAR are specifically involved in retinoid signaling, RXR also participate in many other signaling pathways by serving as heterodimerization partners not only for the RAR but also for other nuclear receptors, such as Nurr1 (Perlmann and Jansson, 1995, Chawla et al., 2001).

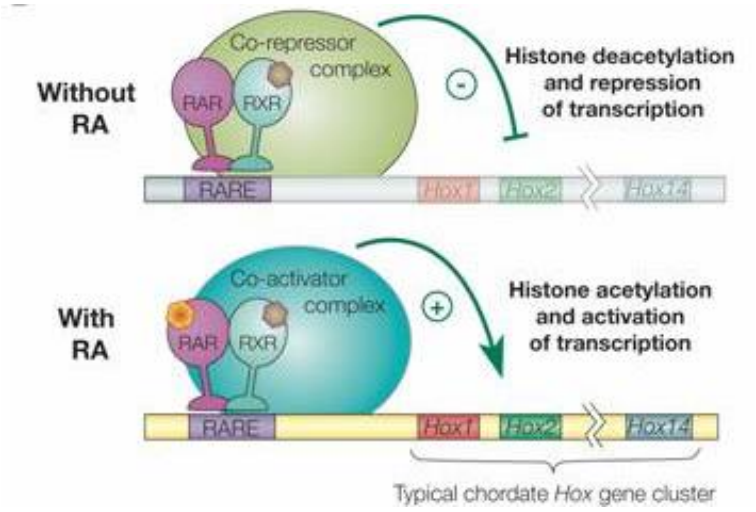


Figure 1.5 Mode of action of retinoic acid. The RAR-RXR heterodimer mediates the effects of RA. In the absence of ligand (RA), the RAR/RXR heterodimer is bound to DNA and co-repressors. This complex induces transcriptional repression through histone deacetylation. Binding of the ligand (RA) induces conformational changes and the binding of co-activators leading to histone acetylation and activation of transcription. Figure adapted from Marletaz et al., 2006.

1.4 Involvement of retinoic acid signaling in PD

RA is likely to be important for mDA neurons since receptors and RA-synthesizing enzymes are expressed at high levels both in the *SN* dopaminergic neurons and their target regions (McCaffery and Drager, 1994).

Several lines of evidences suggest an involvement of RA in the development, maintenance and protection of the nigrostriatal pathway. However the cellular and molecular mechanisms underlying these effects are not yet known. Regarding the expression of RA receptors, it is known that RARα and RARß are expressed in mDA neurons, suggesting that RA signaling may be involved in regulating gene expression in these neurons, whereas RARγ has not been detected in the nigrostriatal pathway. RXR (α, ß, γ) have been detected in the midbrain and striatum (Ruberte et al., 1993). Most importantly, it was shown that stimulation of RAR with a RAR agonist AM80 prevented dopaminergic cell loss induced by lipopolysaccharide (LPS) in the *SN* (Katsuki et al., 2009). In accordance, it was shown by Yin and collaborators that the intranasal delivery of RA reduces neurodegeneration of dopaminergic neurons induced by 6-OHDA (Yin et al., 2012).

Clinically, there is a marked reduction in the expression levels of the enzyme RALDH1 (ALDH1), which is necessary for the conversion of retinal to RA, in the *SN* dopaminergic neurons of post-mortem brain PD patients (Galter et al., 2003). The ALDH1 expression levels found in the peripheral blood have been recently reported as a candidate biomarker for PD diagnosis (Grunblatt et al., 2010). It is not possible to know if this decrease in ALDH1 gene expression precedes the onset of PD or is a consequence of the degenerative process. It may be possible that the reduced availability of RA in the midbrain, through reduced ALDH1 expression, increases the susceptibility of the mDA neurons to the degenerative processes, pushing the balance towards neuronal death instead of neuroprotection. Mutations in genes encoding for this enzyme were also proposed to represent a genetic risk factor for human PD either alone or in conjunction with environmental risk factors.

We should take in consideration that RA has also been pointed as a key neuroprotective therapeutic agent for other neurodegenerative diseases. It was shown that RA has neuroprotective effects against ischemic brain injury by the possible involvement of the trophic factor bone morphogenic protein-7 (BMP-7) (Shen et al., 2009). It also reduces mitochondrial oxidative damage, which is an important pathological factor of AD (Zhu et al., 2006).

1.5 Nanoparticles as a delivery system of retinoic acid

RA presents undesirable properties like poor water solubility, short half-life, and requires a fine-tuning of concentration window to achieve its results, posing difficulties in the delivery of therapeutic doses (Szuts and Harosi, 1991). Therefore, nanoparticles (NPs) are an excellent alternative to control the undesired side effects and to ensure intracellular transport and controlled release of RA. As has been said, retinoic acid receptors (RXR/RAR) are located on the nucleus and therefore RA needs to be delivered to the intracellular milieu. A successful approach was achieved by ~200 nm sized NPs, which were rapidly taken up by cells, delivering RA onto the cytoplasm (Maia et al., 2011). Several NPs formulations have been reported for the controlled release of this molecule (Castro et al., 2007, Narvekar et al., 2012) however, none of the formulations was designed to deliver RA within cells for dopaminergic neuroprotection.

Polymeric NPs were prepared through the electrostatic interaction of polyethylenimine (PEI) polycation and dextran sulfate (DS) polyanion (Maia et al., 2011). Since RA is a hydrophobic molecule, the presence of polycations, such as PEI, increase its water solubility due to electrostatic interactions, allowing the manipulation of significant quantities of this hydrophobic molecule. NPs can be internalized via endocytosis, macropinocytosis or phagocytosis, but these processes confine the compounds to closed vesicles (endosomes or phagosomes), where the pH is progressively lowered to 5.5-6.5 (Vasir and Labhasetwar, 2007, Breunig et al., 2008). Polycations, such as PEI, that absorb protons in response to the acidification of endosomes (i.e., cationic polymers with a pK around or slightly below physiological pH) can disrupt these vesicles via the "proton sponge" effect that promotes the osmotic swelling of the endosome resulting in the release of the NPs into the cytoplasm (Akinc et al., 2005). When polymeric NPs reach the cytosol, the bioactive molecule may be released by desorption, diffusion through the nanoparticle, or nanoparticle erosion and perform its function. It is was shown previously by our group that the internalization of the RA-loaded polymeric NPs (RA^+-NPs) has a minimal effect on cell viability and proliferation but enhanced neurogenesis at the subventricular zone stem cell niche, both *in vitro* and *in vivo* (Maia et al., 2011).

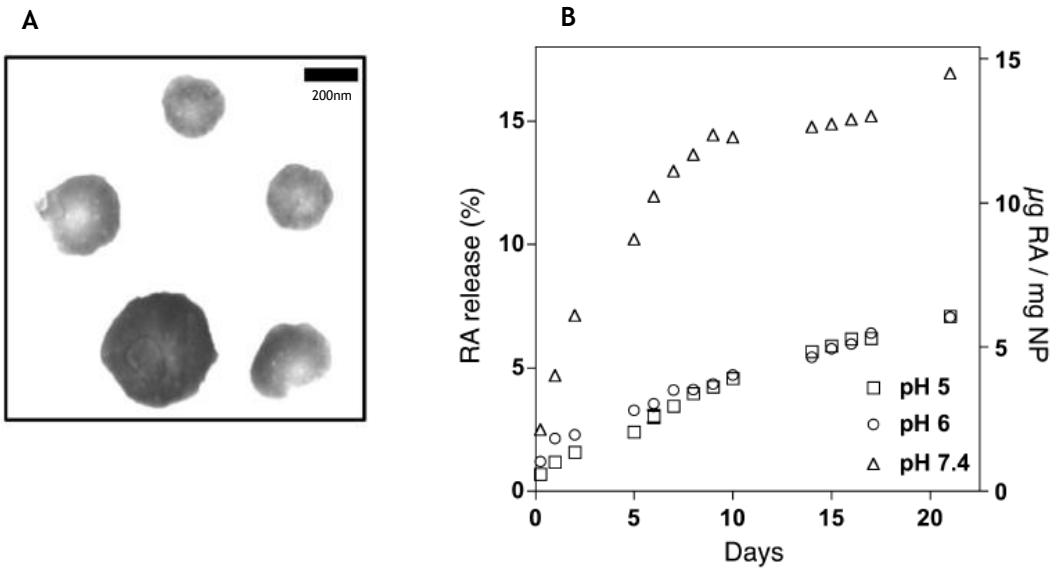


Figure 1.6 Transmission electron microscopy image (A) and release profile (B) of RA^{+}-NPs. Figure adapted from Maia et al., 2011.

Chapter 2

Objectives

RA offers great potential as a therapeutic molecule due to its numerous beneficial properties namely as antioxidant, anti-inflammatory, neuroprotective and proneurogenic. However, due to its hydrophobic nature, when is directly administrated into the body it is rapidly metabolized by cells thus reducing their bioavailability. In this sense, to maximize the therapeutic response, the administration of RA-containing NPs becomes an effective method for the delivery of RA.

Thus, the general aim of this work was to evaluate the neuroprotective effects of RA^+-NPs in a mouse model of PD and to compare with effects of soluble RA. For that purpose, a dopaminergic injury was induced in young adult or old C57BL6 mice by using the dopaminergic neurotoxin MPTP.

The specific objectives are as follows:

- To evaluate the extension of dopaminergic injury induced by MPTP in the nigrostriatal pathway and consequently the putative protective effects induced by RA^+-NPs or soluble RA;

- To determine whether the RA^+-NPs induce glial reactivity;

- To analyze the effects of RA^+-NPs in Pitx3 and Nurr1 mRNA expression.

Chapter 3

Materials and Methods

3.1 Animals

All animals were handled in accordance with protocols approved by the national ethical requirements for animal research, and in accordance with the Directive 2010/63/UE of the European Parliament and the Council on the protection of animals used for scientific purposes. All animals were kept in appropriate cages, under temperature controlled conditions (20±2°C) with a fixed 12h light/dark cycle (7:00 am/7:00 pm), with food and water freely available. All efforts were made to reduce the number of animals used and to minimize their suffering.

For this study were used 49 young adult (2-3 months-old) and 13 old (25-26 months-old) male C57BL6 mice.

3.2 Intrastriatal injection

Both adult and old C57BL6 mice were subjected to intrastriatal administrations of RA^+-NPs, blank (void formulation) NPs, soluble RA or 0.1 M sterile phosphate buffer saline (PBS[1]).

Animals were anesthetized by intraperitoneal (i.p.) injection of ketamine (90 mg/Kg) and xylazine (10 mg/Kg). Then, the animals were placed on the stereotaxic frame and were unilaterally injected in the right lateral striatum with 1 µl of 100 ng/ml RA^+-NPs (dissolved in PBS), 100 ng/ml blank NPs, 10 µM soluble atRA (dissolved in dimethyl sulfoxide (DMSO); final dilution of 1:10000) or 0.1 M sterile PBS through a 10 µl Hamilton syringe at a speed of 0.2 µl/min. The needle was retained in place for 3 min after injection. After the needle was removed and the incision sutured, mice were kept arm during recovery (27°C). RA^+-NPs and soluble atRA solutions were prepared freshly in the morning just before the injections and the atRA solution was protected from light and kept in ice until the beginning of the surgery. The coordinates for intrastriatal injection were: +0.6 mm posterior to the bregma (X,AP), -1.8 mm lateral to the midline (Y,ML) and - 2.8 mm below the dura surface (Z,DV) according to the mouse brain atlas of Paxinos and Franklin, 2001.

[1] PBS: NaCl 140 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM and Na_2HPO_4 8.1 mM, pH 7.4).

3.3 MPTP - induced lesion

Acute MPTP administration was made 3 days after intrastriatal injections. MPTP (Sigma Aldrich) was dissolved in sterile 0.9% NaCl and was injected in both adult and old mice in 4 i.p. injections at 2h intervals using a dose of 15 mg/Kg body weigh in adult mice (Kong et al., 2008) and 7 mg/Kg body weight in old mice (Peng and Andersen, 2011), to the total dose of 60 and 28 mg/Kg, respectively. Saline animals, subjected to the same procedure, received an equivalent volume of sterile 0.9% NaCl. Animals were sacrificed 7 days following the MPTP acute intoxication protocol (Figure 3.1).

At the end of experiment, the animals were divided into two groups. The first group was deeply anesthetized with an overdose of ketamine and killed by transcardial perfusion with 0.1 M PBS followed by perfusion with 4% paraformaldehyde (PFA). Brains were then removed surgically for immunohistostainings. The second group was sacrificed by spinal cord dislocation and the brains were removed and the regions of interest, *SN* and striatum, were quickly microdissected from both hemispheres and stored at -80°C for gene expression analysis.

MPTP was handled in accordance with "Protocol for the MPTP mouse model of Parkinson's disease" that provide a detailed protocol as well as a list of recommendations and guidelines to handle and produce MPTP mouse model of PD in a reliable and safe manner (Jackson-Lewis and Przedborski, 2007).

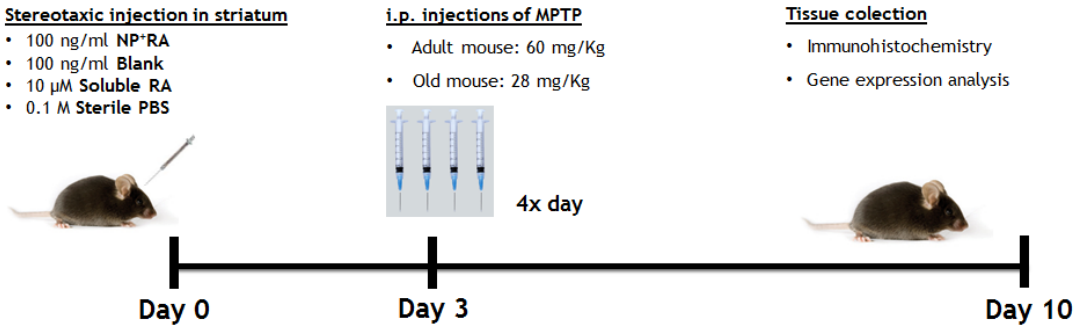


Figure 3.1 Schematic representation of the experimental treatments and assays performed *in vivo*.

3.4 Immunohistostainings

3.4.1 Tissue preparation

Brains were post-fixed by immersion in the 4% PFA solution for 24h at 4°C. After fixation, brains were then transferred to 30% sucrose solution (in 0.1 M PBS) for cryoprotection and were kept at 4°C until they sank.

Brains where then frozen, embedded in optimal cutting temperature (OCT) gel and were cut into coronal sections at a thickness of 35 µm from the frontal pole to the midbrain on a freezing cryostat-microtome (Leica CM 3050S, Leica Microsystems) at -20°C. The sections corresponding to the *SN* and striatum of each animal were collected sequentially in six compartments of 24-well plate (Orange Scientific), free-floating in PBS supplemented with 0.02% sodium azide at 4°C, until processing for immunohistostainings.

3.4.2 TH staining

This immunohistochemistry assay was used to detect dopaminergic neurons in *SN* and striatal fibers in striatum by TH staining.

Sections were incubated on a 10 mM citrate solution (pH 6.0) at 80°C for 30 min for antigen retrieval. After cooled to room temperature (RT) inside the solution, sections were placed in water for 5 min and were then washed for 10 min in PBS-Tween 20 (PBS-T). Then, the sections were permeabilized and blocked with PBS containing 10% fetal bovine serum (FBS) and 0.1% Triton X-100 for at least 1h at RT and then washed with PBS-T for 20 min. For the inhibition of endogenous peroxidase activity, sections (protected from visible light) were incubated with 3% hydrogen peroxide (H_2O_2) in water for 10 min at RT and then washed with PBS-T for 20 min. Sections were incubated with primary antibody mouse anti-TH (dilution 1:1000, Transduction Laboratories) diluted in PBS containing 5% FBS. Incubation with primary antibody was performed overnight at 4°C. After several rinses with PBS-T for 30 min, the sections were incubated with the secondary biotinylated goat anti-mouse antibody (dilution 1:200, Vector Laboratories) diluted in PBS containing 1% FBS for 1h at RT. Subsequently, the sections were washed with PBS-T for 30 min and were then incubated with avidin-biotin peroxidase complex reagent (Vectastain ABC KIT, Vector Laboratories Inc.) for at least 30 min at RT. The sections were first washed with PBS-T for 30 min and then with tris buffer saline solution (TBS[2]) for 10 min. The reaction product was visualized using 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich) in TBS containing 0.08% H_2O_2 until color develops (5-10 min) and the reaction was stopped by adding TBS. Sections were mounted onto slides (Thermo Scientific),

[2] TBS: 20 mM Tris and 137 mM NaCl solution, pH 7.6

dried, and dehydrated in graded ethanol (70%-->80%-->95%-->100%), cleared in xylene and coversliped using a permanent mounting medium, Entellan (Merck) for light microscopy. Digital images of the TH staining were acquired in the Zeiss Axiovert 200 imaging Microscope (Axiobserver Z1, Zeiss) at the 4x magnification.

3.4.3 Fluorescence immunohistostaining for glial cells

Double immunofluorescence staining against GFAP (glial fibrillary acidic protein) and CD11b (alpha chain of $\alpha_M\beta_2$-integrin or cluster of differentiation molecule 11b) was performed to reveal astrocytes and microglia cells, respectively, on coronal sections of the striatum. Sections were initially permeabilized with 0.1 M PBS containing 1% Triton X-100 for 45 min. For blocking of non-specific bindings sites, sections were incubated with PBS solution containing 10% FBS at RT for at least 30 min. Then, slices were incubated with primary Rat monoclonal anti-CD11b (dilution 1:600, Serotec) and Rabbit monoclonal anti-GFAP (dilution 1:200, Sigma) antibodies, both diluted in PBS containing 10% FBS for 24h at 4°C. After rinses with PBS containing 1% Triton X-100 for 45 min, sections were incubated with secondary Anti-Rat 594 (dilution 1:200, Invitrogen) and anti-Rabbit 488 (dilution 1:200, Invitrogen) antibodies diluted in PBS, for 1h30min at RT. After rinses with PBS for 45 min, sections were incubated with Hoechst-33342 (15 µg/ml; Invitrogen) in PBS at RT for 5 min. Sections were then washed with PBS for 10 min and mounted in Dako fluorescent mounting medium (DAKO). Digital images were acquired at the Zeiss inverted confocal microscope (AxiobserverZ1, Zeiss) under a 40x magnification.

3.5 Cell countings and quantitative analysis

Quantitative analysis of dopaminergic neurons in the *SN* was carried out by serial section analysis of the total number of TH-positive (TH^+) neurons throughout the rostro-caudal axis. The *SN* does not have exactly well-defined borders with adjacent brain structures in all mesencephalic sections, so the region corresponding to the *SNpc* was carefully delineated and the total number of TH^+-neurons in the full extent of structure was counted *per* section in each hemisphere. The total number of TH^+-neurons for each representative mesencephalic section (4 coronal sections *per* mouse from -2.80 to -3.80 mm relative to bregma) was calculated under the magnification of 10x at the Zeiss Axiovert 200 imaging microscope (Axiobserver Z1, Zeiss).

The quantitative analysis of the intensity and area occupied by TH^+-fibers staining was carried out in 4 coronal sections of the striatum, from 1.10 to 0.38 mm relative to bregma, of each mouse, selected throughout the rostro-caudal axis, under the magnification of 5x at the Zeiss Axiovert 200 imaging microscope (Axiobserver Z1, Zeiss). Quantitative analysis of striatal TH-fiber staining was performed using ImageJ program wherein striatal images converted to gray

scale were delineated and the intensity and area occupied by the TH staining were assessed for the entire region of the striatum. Background intensities of TH staining were subtracted from every measurement.

3.6 RNA isolation

Total RNA was extracted from the striatum and *SN* using illustra RNAspin Mini KIT (GE Healthcare) according to manufacturer's protocol.

Briefly, the samples were first lysed in Lysis Solution containing guanidine thiocyanate which ensured the inactivation of RNases. Samples were applied to spin mini filters to filtrate the lysate and the remaining filter was discarded. Afterwards, ethanol 70% was added to the filtrate to complex nucleic acids so that forms a stringy visible precipitate. Samples were then transferred to spin mini columns where total RNA bound to the membrane. Then, salts were removed from silica membrane by the addition of desalting buffer and this step makes the subsequent DNA digestion with DNase I much more effective. After incubation with DNase I, the column was washed and dried by the addition of a series of wash buffers promoting inactivation of DNase I and removing contaminants from de membrane-bound RNA, allowing the purification of high-quality mRNA enriched solution. At the end, mRNA samples were dissolved in 25 µl of RNase-free water and stored at -80°C until quantification. The total amount of mRNA was quantified spectrophotometrically by the Nanophotometer (Implen) at 260 nm, and the purity was determined by measuring the 260/280 nm ratio.

3.7 Reverse transcription-polymerase chain reaction (RT-PCR)

cDNA synthesis was performed using Transcriptor First Stand cDNA Synthesis KIT (Roche) according the manufacturer's instructions.

Total mRNA extracted from adult (0.2 µg) and old mice (0.4 µg) tissue samples was mixed with 1 µl anchored-oligo (dT) 18 primers, 4 µl reverse transcriptase reaction buffer 5x, 0.5 µl RNase inhibitor, 2 µl deoxynucleotide mix (dNTPs), 0.5 µl reverse transcriptase and sterile water in a 20 µl final volume. The reaction was performed at 55°C for 30 min and stopped at 85°C for 5 min step by a thermal cycler (Biometra). The samples were then stored at -80°C until further use.

3.8 Gene expression analysis by quantitative real-time PCR (qPCR)

The qPCR assays for gene expression analysis of Nurr1 in the striatum and *SN* and Pitx3 in the *SN* were performed by adding 2 µl of sample cDNA, 10 µl SYBR Green Supermix (BioRad), 1/10 dilution of each primer (accordingly to primers datasheet) and RNAse free water to a 20 µl total volume. The reaction was initiated with activation of Taq polymerase by heating at 94°C during 3 min followed by 40 cycles of a 15 seconds denaturation step at 94°C and a 30 seconds annealing and elongation step at 60°C. Validated primer sets (GAPDH, Nurr1 and Pitx3) for use in qPCR were obtained from selected QuantiTect Primer Assays (Qiagen).

The fluorescence was measured after the extension step by the iQ5 Multicolor Real-time PCR detection system (BioRad). After the thermocycling reaction, a melting curve was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 seconds, up to 95°C, with continuous measurement of fluorescence, allowing detection of possible nonspecific products. The assay included a non-template control (sample was substituted by RNase- Dnase-free sterile water). All reactions run in duplicates.

The threshold cycle (C_t) was measured in the exponential phase and therefore was not affected by the possible limiting components in the reaction. Data analysis was performed with BioRad iQ5 software (BioRad). Fluorescent reading from qPCR was quantitatively analyzed by determining the difference of C_t (ΔC_t) between C_t of the target gene and GAPDH control housekeeping gene using the comparative C_t method as described by Pfaffl's formula (Pfaffl, 2001).

3.9 Data analysis and statistics

Statistical analysis of group differences was performed using GraphPad Prism 5.0 (GraphPad Software Inc.) by one-way analysis of variance (ANOVA) followed by Dunnett's test for comparison with control condition. Control and MPTP values correspond to the pool of all contralateral sides of saline- or MPTP-treated mice, respectively. All other conditions correspond to ipsilateral sides of saline- or MPTP-treated mice. Blank NPs were used as negative control (Figure 3.2). Data are expressed as percentages of values obtained relative to the control or MPTP and are presented as the means ± standard error of mean (SEM). Statistical significance was considered relevant for p value < 0.05.

Saline Group

Contralateral

- Saline i.p.

Ipsilateral

- Saline i.p. + RA^{+}NPs
- Saline i.p. + soluble RA
- Saline i.p. + blank Nps
- Saline i.p. + saline

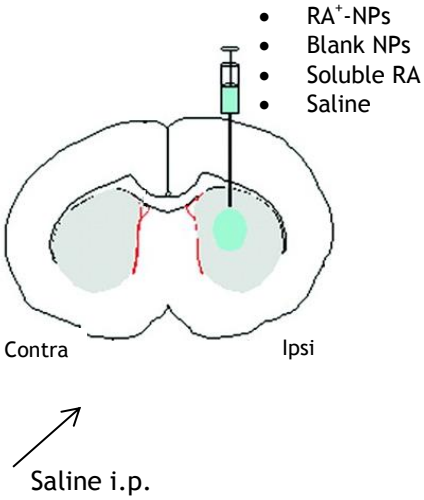
MPTP Group

Contralateral

- MPTP i.p.

Ipsilateral

- MPTP i.p. + RA^{+}NPs
- MPTP i.p. + soluble RA
- MPTP i.p. + blank Nps



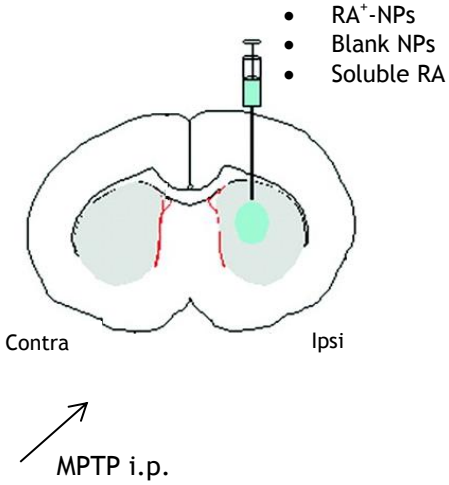


Figure 3.2 Experimental groups.

Chapter 4

Results

To investigate whether RA^+-NPs protects dopaminergic neurons *in vivo*, we used the MPTP mouse model of PD. For this purpose adult and old C57BL6 male mice were injected with MPTP (60 and 28 mg/Kg, respectively) or saline (equal volume of 0.9% NaCl) in 4 i.p. injections at 2h intervals 3 days after intrastriatal injections with 100 ng/ml RA^+-NPs, 100 ng/ml blank NPs, 10 μM soluble RA or 0.1 M sterile PBS in the right lateral striatum (ipsilateral side). The *SN* and striatum regions were collected 7 days after the neurotoxin injection and processed for immunohistostainings or qPCR analysis.

4.1 RA^+-NPs induce neuroprotection of the *SN* dopaminergic neurons against the MPTP-induced lesion

To analyze the extension of dopaminergic lesion produced by MPTP and consequently the neuroprotective effects driven by RA^+-NPs against this neurotoxin, we performed TH immunohistostainings in midbrain coronal brain sections of adult mice (Figure 4.1C). The percentages of TH^+ cells were analyzed in saline- and MPTP-treated mice groups as described in the figure 3.2.

In the *SN* of saline mice (i.p. 0.9% NaCl) we could not found any difference in the percentage of TH^+ cells when comparing ipsilateral side - i.e. intrastriatal injection with RA^+- or blank-NPs - with the contralateral side (98±14.4% and 87.6±8.3% of control, respectively) (Figure 4.1A). These results suggest that both RA^+- and blank-NPs *per se* did not interfere with the dopaminergic neuronal survival. By contrast, exposure to MPTP triggered about 50% reduction of TH^+ cells in *SN* as compared with saline mice (control group) (51.3±3.7% of control) (Figure 4.1B, C-left panel). Intrastriatal injection with RA^+-NPs before MPTP i.p. administration significantly reduced the MPTP-induced lesion by increasing the percentage of TH^+ cells in ipsilateral *SN* to levels similar to control (94.5±6.6% of control) (Figure 4.1B, C-right panel). This neuroprotective effect was more robust than when compared with soluble RA (63.2±4.7% of control) (Figure 4.1B). Moreover, intrastriatal injections of blank NPs, used as a negative control, were not able to protect TH^+ neurons against the MPTP-induced lesion. These results suggest that the prophylactic administration of RA^+-NPs was very effective in protecting *SN* dopaminergic neurons against the MPTP-induced lesion.

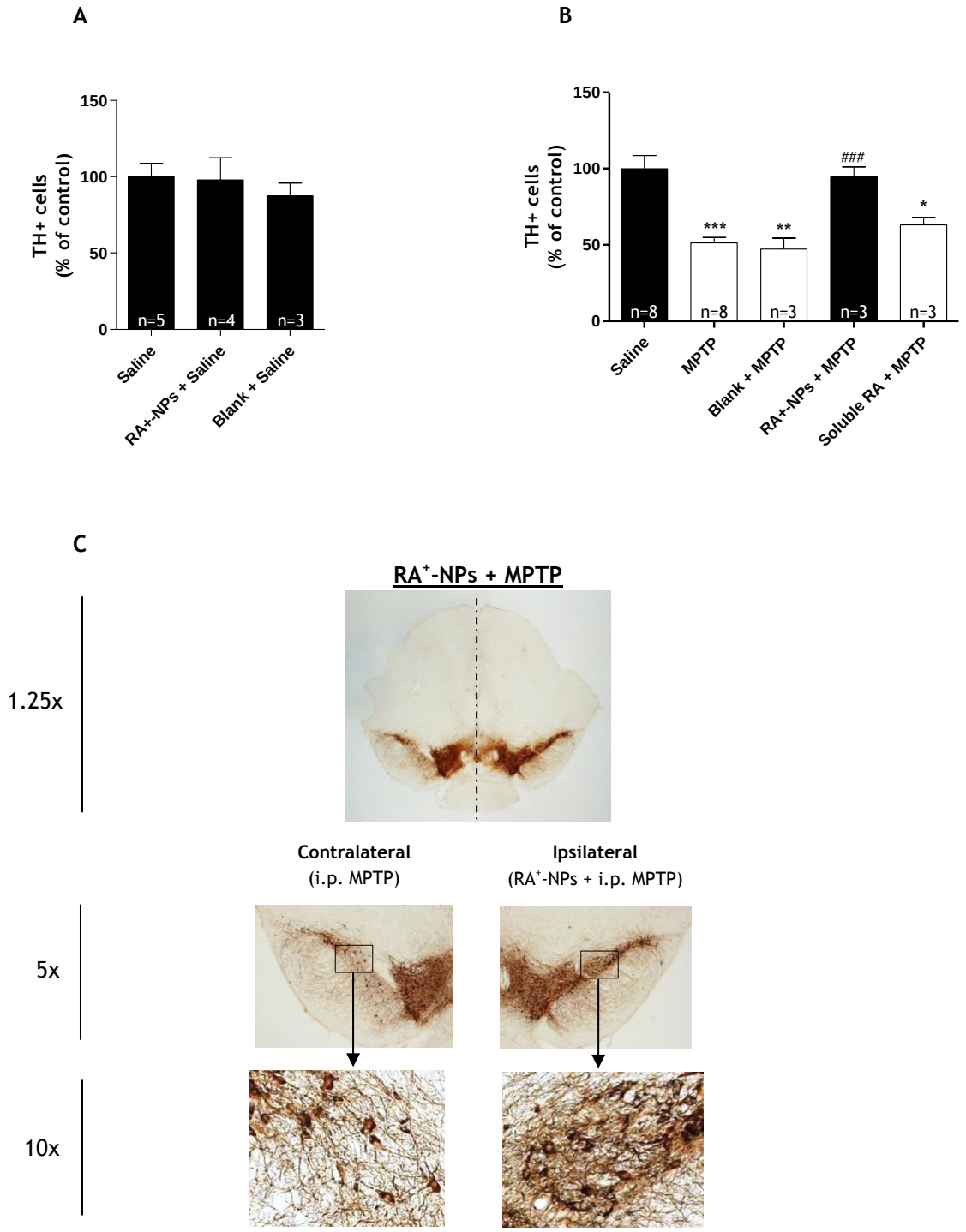


Figure 4.1 RA$^+$-NPs pre-treatment increased survival of TH$^+$ cells in the MPTP-injured *SN*. Adult mice received RA$^+$-NPs, blank NPs or soluble RA by stereotaxic injections in the right striatum. MPTP or saline (0.9% sterile NaCl) were administered intraperitoneally (i.p.) three days after stereotaxic injections. Seven days after MPTP or saline i.p. injections, coronal brain slices containing the *SN* region were collected for TH staining. Therefore, the contralateral *SN* contains MPTP-only exposed cells, whereas, the ipsilateral *SN* receives the influence of both MPTP and NPs. (A) Quantitative analysis of TH$^+$ cells in the *SN* of control mice (saline, i.p.) injected or not with RA$^+$-NPs (RA$^+$-NPs + saline) or blank NPs (blank + saline). The saline condition corresponds to the contralateral *SN* whereas both RA$^+$-NPs and blank conditions correspond to the ipsilateral *SN*. (B) Quantitative analysis of TH$^+$ cells in the contralateral *SN* of saline or MPTP-treated mice and in the ipsilateral *SN* of RA$^+$-NPs-, blank- and soluble RA-treated MPTP mice (C) Representative photomicrographs of midbrain sections immunostained for TH of a mouse injected with RA$^+$-NPs in the right striatum followed by the i.p. MPTP-induced lesion. Data are expressed as the percentage of control and represent the mean±SEM (n=3—8 mice). Statistical analysis was performed using one way ANOVA followed by Dunnett's test. ***P<0.0001, **P<0.001 and *P<0.05 compared to control and $^{###}$P<0.0001 compared to MPTP.

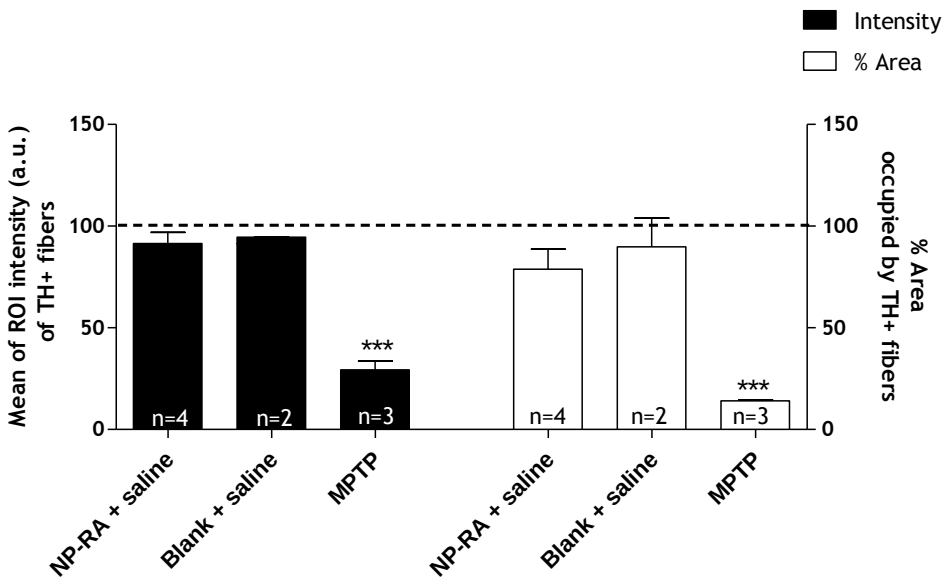
4.2 RA^+-NPs induce neuroprotection of TH^+ striatal fibers against the MPTP-induced lesion

We then performed TH immunohistostainings in striatal coronal sections of adult mice in order to analyze the extension of lesion produced by MPTP in dopaminergic striatal terminals and consequently the neuroprotective effects driven by RA^+-NPs against this neurotoxin (Figure 4.2C). The intensity and area occupied by TH^+ immunoreactive fibers were analyzed in saline and MPTP-treated mice groups (as described in the figure 3.2).

No statistical difference was found in the intensity (black bars) and area (white bars) occupied by TH^+ fibers in the contralateral striatum of saline animals (line set to 100%; Figure 4.2A) as compared with the ipsilateral side of the same animals injected with RA^+-NPs or blank NPs (intensity: 91.4±5.6% and 94.6±0.1% of control; area: 78.9±9.8% and 89.8±14.2% of control, respectively). As expected, MPTP caused a significant decrease in the intensity (29.3±4.3% of control) and in the percentage of area occupied by TH^+ fibers (14±0.6% of control) as compared with saline animals (set to 100%) (Figure 4.2A, C-right panel). Exposure to RA^+-NPs before MPTP-induced lesion significantly increased the intensity and area occupied by TH^+ fibers in the ipsilateral striatum (135.2±7.5% and 218.1±44.4% of MPTP, respectively) (Figure 4.2B, C-left panel) as compared with MPTP-only exposed contralateral striatum (set to 100%). As expected, the intensity and percentage of area occupied by TH^+ fibers found in the ipsilateral side of the striatum exposed to both blank NPs and MPTP was not different from the contralateral side of the same animals (MPTP-only exposed cells) (98.1±15.2% and 135.1±49.7% of MPTP, respectively) (Figure 4.2B). These data suggest that RA^+-NPs *per se* did not modulate TH^+ fibers staining in the striatum but they increased its immunoreactivity in the presence of MPTP-induced dopaminergic injury.

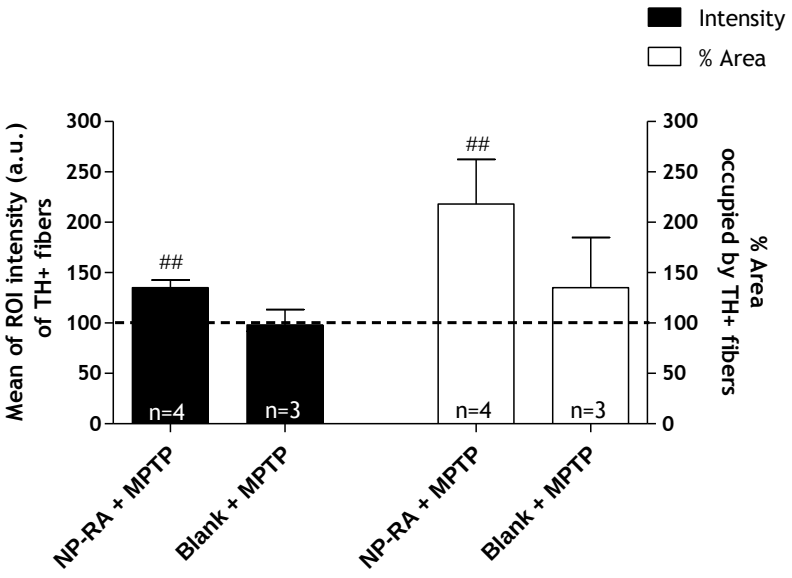
Relative to saline (set to 100%)

A



Relative to MPTP (set to 100%)

B



C

RA$^+$-NPs + MPTP

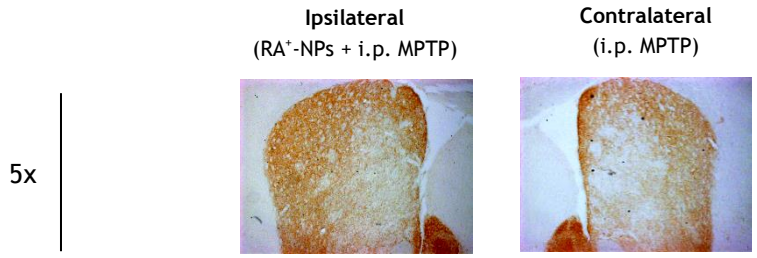


Figure 4.2 RA$^+$-NPs pre-treatment increases TH$^+$ fibers immunoreactivity in the MPTP-injured striatum. Adult mice received RA$^+$-NPs or blank NPs by stereotaxic injections in the right striatum. MPTP or saline were administered intraperitoneally three days after stereotaxic injections. The striatum region was collected for TH immunostaining seven days after MPTP or saline injections. (A) Quantitative analysis of the intensity and area occupied by TH$^+$ fibers in control (contralateral side; set to 100%) and in RA$^+$-NPs- or blank-treated saline mice (ipsilateral sides). (B) Quantitative analysis of the intensity and area occupied by TH$^+$ fibers in contralateral striatum of MPTP-treated mice (set to 100%) and in RA$^+$-NPs- and blank-treated MPTP mice (ipsilateral sides). (C) Photomicrographs of striatum sections immunostained for TH of a mouse injected with RA$^+$-NPs in the right striatum followed by the i.p. MPTP-induced lesion. Data are expressed as the percentage of control (A) or MPTP (B) (both contralateral sides) and represent the mean±SEM (n=2—4 mice). Statistical analysis was performed using one way ANOVA followed by Dunnett's test. ***P<0.0001 compared to control and $^{\#\#}$P<0.001 compared to MPTP.

4.3 Effects of RA$^+$-NPs in glial reactivity

To evaluate whether RA$^+$NPs induce glial reactivity *in vivo*, we then performed a fluorescence immunostaining against GFAP (astrocyte marker) and CD11b (microglia marker) in striatal coronal sections. Analysis were performed only in striatal sections where the injection site was detected and images were captured about 100-150 µm away from the injected site.

Immunohistochemistries suggest that the MPTP-induced lesion robustly increased the reactivity of astrocytes and microglia in the contralateral striatum (Figure 4.3). Interestingly, it seems that the RA$^+$-NPs decreased the reactivity of astrocytes and microglia by decreasing the GFAP and CD11b expression/staining in the ipsilateral side of RA$^+$-NPs-treated MPTP mice (Figure 4.3). However, in the ipsilateral side of RA$^+$-NPs- and saline-treated saline mice groups, some reactive astrocytes and microglia were also observe. This occurs probably due to the tissue lesion caused by the intrastriatal injection. These results suggest that RA$^+$-NPs decrease glial reactivity in the striatum in the presence of MPTP-induced dopaminergic injury.

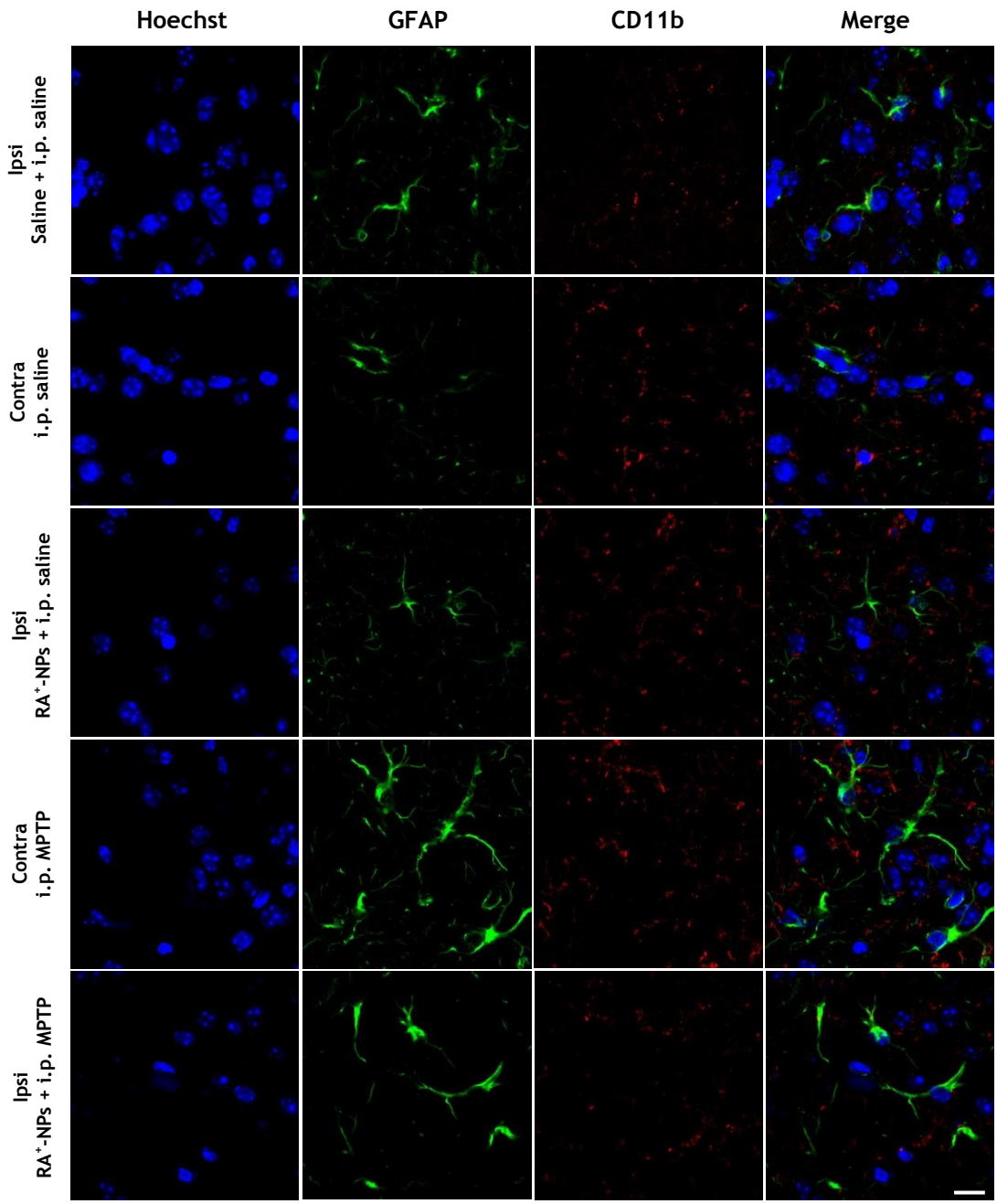


Figure 4.3 Fluorescence immunostaining of astrocytes and microglia in the striatum. Representative images of fluorescence immunostaining against GFAP (green) and CD11b (red) in the striatum of adult mice that received RA^+-NPs or saline by stereotaxic injections in the right striatum. MPTP or saline were administered intraperitoneally three days after stereotaxic injections. Hoechst 33342 staining (blue) was performed to detect cell nuclei. Scale bar: 10 µm.

4.4 Effects of RA^+-NPs in Nurr1 and Pitx3 mRNA expression

To investigate the role of RA^+-NPs in the expression of transcription factors that are involved in dopaminergic survival and specification, *SN* and striatum regions were then collected from adult and old mice. For this purpose, Nurr1 and Pitx3 mRNA expression was examined through qPCR in saline- and MPTP-treated mice groups (Figure 3.2). Nurr1 mRNA expression was evaluated in *SN* and striatum whereas Pitx3 mRNA expression was evaluated only in *SN*.

4.4.1 Nurr1 and Pitx3 mRNA expression in adult mice

As shown in figure 4.4 (A, B), the intrastriatal injection with RA^+-NPs in saline-treated adult mice (ipsilateral side) did not changed the mRNA expression levels of Nurr1 in the striatum and *SN* and of Pitx3 in the *SN* (108.2±4.7%; 107±3.7% and 112.2±21.5% of control, respectively) as compared with the contralateral hemisphere (saline i.p.; set to 100%). As expected, when mice were exposed to MPTP there was a significant decrease in Nurr1 transcript levels in both striatum (68.4±8.7% of control) and *SN* (72.9±7.2% of control) and also in the Pitx3 mRNA expression levels found in the *SN* (38.2±5.7% of control).

In the *SN* of mice exposed to both RA^+-NPs and MPTP there was an increase of Nurr1 and Pitx3 mRNA levels as compared to MPTP, however no statistically significant differences were detected (201.1±62.9% and 153.7±35.7% of MPTP) (Figure 4.4 C, D). In same conditions, no changes were detected in Nurr1 mRNA levels in the striatum exposed to both RA^+-NPs plus MPTP (ipsilateral) when compared with MPTP-only exposed cells (contralateral; set to 100%) (Figure 4.4 C). In accordance with previous data, blank NPs did not change neither Nurr1 (striatum: 72±14.4% of MPTP; *SN*: 85.4±11.9% of MPTP) nor Pitx3 mRNA expression (106.6±11% of MPTP) as compared with MPTP-only exposed cells (Figure 4.4 C, D).

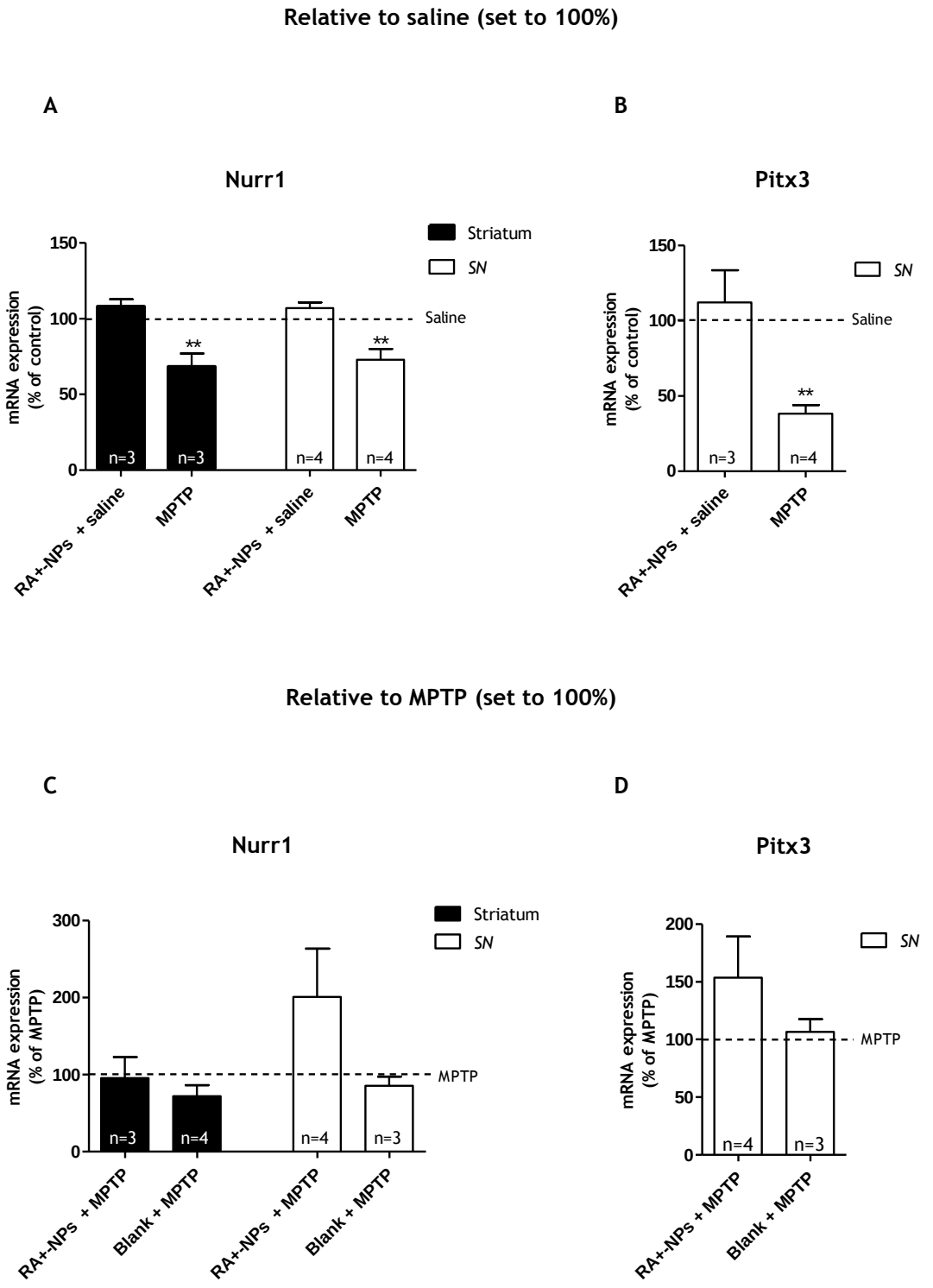


Figure 4.4 RA$^+$-NPs induce the expression of Nurr1 and Pitx3 mRNA in the *SN* of adult mice exposed to MPTP. Adult mice received RA$^+$-NPs or blank NPs by stereotaxic injections. MPTP or saline (0.9% NaCl) were administered intraperitoneally three days after stereotaxic injections in the right striatum (ipsilateral side). Seven days after MPTP or saline injections, the *SN* and striatum regions were collected for measurement of Nurr1 and Pitx3 mRNA expression by qPCR. The expression levels were normalized to the GAPDH housekeeping gene. (A, B) Bar graphs indicate the percentage of Nurr1 and Pitx3 mRNA expression, respectively, expressed as the percentage of control (saline, contralateral). (C, D) Bar graphs indicate the percentage of Nurr1 and Pitx3 mRNA expression, respectively, expressed as the percentage of MPTP. Data represent the mean±SEM (n=3—4 mice). Statistical analysis was performed using one way ANOVA followed by Dunnett's test. **P<0.001 compared to control.

4.4.2 Nurr1 and Pitx3 mRNA expression in old mice

The same analysis regarding the expression of both Nurr1 and Pitx3 mRNAs was also performed in old mice (25-26 months). The aim of these experiments was to better mimic what happens in PD, since the most predominant form of this disease (idiopathic) occurs mainly in older people and not in young adults.

First we showed that the striatal administration of RA^+-NPs in saline mice, did not significantly alter both *SN* and striatal mRNA expression of Nurr1 (84.1±3.3% and 77.9±14.1% of control, respectively) and *SN* mRNA expression of Pitx3 (92.3±41.7% of control) (Figure 4.5 A, B) as compared with saline animals (control; set to 100%). As expected, a robust decrease in Nurr1 mRNA expression in the striatum and *SN* (21±2% and 54.1±7.5% of control, respectively) as well as a decrease in Pitx3 mRNA levels in *SN* (42.6±3.7% of control) was found in the brain hemisphere exposed to MPTP only as compared with saline animals (control; set to 100%) (Figure 4.5 A, B).

Treatment with both RA^+-NPs and MPTP triggered a significant increase of Pitx3 mRNA levels in the ipsilateral *SN* and of Nurr1 mRNA levels in ipsilateral striatum (290.9±67% and 245.6±53% of MPTP, respectively) as compared to MPTP (set to 100%; Figure 4.5 C, D). However, in the same conditions there was an increase in Nurr1 mRNA levels in the ipsilateral *SN* but no statistical difference was observed when compared to MPTP (130.5±23.2% of MPTP) (Figure 4.5 C).These data suggest that RA^+-NPs enhance the mRNA expression of both Nurr1 and Pitx3 in the striatum and *SN*, respectively, as compared with MPTP-exposed cells. Even if this data suggest that RA^+-NPs can promote protection of dopaminergic neurons by increasing the expression of transcription factors involved in their survival and maintenance, future immunostaining studies should be performed to disclose whether the Pitx3 and Nurr1 increased expression is found specifically in the nuclei of dopaminergic neurons and if a clear recovery in the percentage of TH^+ neurons occurs in the *SN* of these animals. The blank plus MPTP experimental group was not carried out due to the limited availability of mice with a substantially advanced age.

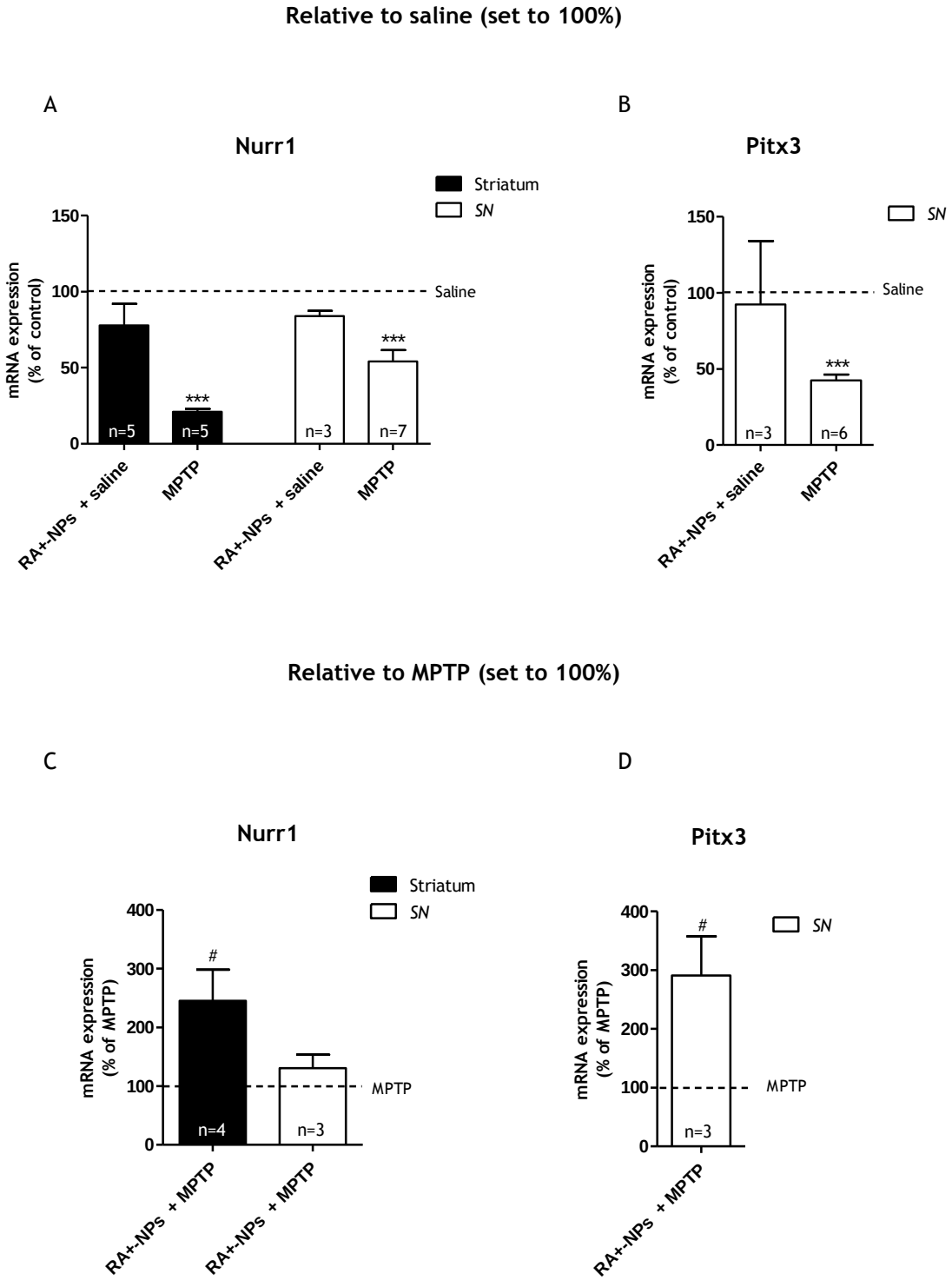


Figure 4.5 RA$^+$-NPs induce Nurr1 and Pitx3 mRNA expression in MPTP-treated old mice. Old mice received RA$^+$-NPs by stereotaxic injections. MPTP or saline (0.9% NaCl) were administered intraperitoneally three days after stereotaxic injections. Seven days after MPTP or saline injections, *SN* and striatum regions were collected for measurement of Nurr1 and Pitx3 mRNA expression by qPCR. The expression levels were normalized to the GAPDH housekeeping gene. (A, B) Bar graphs indicate the percentage of Nurr1 and Pitx3 mRNA expression, respectively, expressed as the percentage of control (saline, 0.9% NaCl i.p.). (C, D) Bar graphs indicate the percentage of Nurr1 and Pitx3 mRNA expression, respectively, expressed as percentage of MPTP. Data represent the mean±SEM (n=3—7 mice). Statistical analysis was performed using one way ANOVA followed by Dunnett's test. ***P<0.0001 compared to control and $^{\#}$P<0.05 compared to MPTP.

Chapter 5

Discussion

In this thesis, we investigated the putative neuroprotective role of RA in a mouse model of PD taking advantage of a recently described nanoparticle delivery system. RA regulates multiple biological processes including cell proliferation and differentiation, by virtue of its ability to modulate the rate of transcription of numerous target genes. It was also described that RA has a protective effect against neurodegeneration of dopaminergic neurons in the *SN* (Ulusoy et al., 2011). However, it requires a fine-tuning of concentration window to achieve its results posing difficulties in the delivery of therapeutic doses. The nanoparticle formulation used in this thesis avoids the use of large concentrations of RA and the use of toxic solvents such as DMSO and ensures intracellular transport and controlled release of RA. Previously, it was shown that this RA^+-NPs formulation enhances subventricular zone neurogenesis both *in vitro* and *in vivo* (Maia et al., 2011, Santos et al., 2012). However, there are no studies showing intracellular transport and controlled release of this molecule by a delivery system for dopaminergic neuroprotection in a context of PD.

PD is characterized mainly by a progressive and preferential loss of dopaminergic neurons in *SNpc* who projected their terminals to the striatum, resulting in reduced striatal levels of dopamine (Jankovic, 2008). In this way, in the first part of this study, we investigated whether RA^+-NPs protects TH dopaminergic neurons from MPTP-induced lesion by performing TH immunohistostainings on midbrain and striatal sections. We demonstrated that intrastriatal injections with RA^+-NPs before MPTP i.p. administration significantly reduced the loss of TH cells in the ipsilateral *SN* to levels similar to control (saline mice). Moreover this neuroprotective effect mediated by 100 ng/ml RA^+-NPs was more robust than when compared with 10 μM soluble RA. In fact, the amount of RA payload present in 100 ng/ml of RA^+-NPs corresponds to 4 nM of RA (Santos et al., 2012) and this concentration is 2500-fold lower than the concentration of 10 μM. Similarly, there was significant reduction in intensity and area occupied by TH^+ fibers in contralateral striatum of MPTP-treated mice and this reduction was counteracted with the presence of RA^+-NPs (ipsilateral).Our results are in line with previous findings by others showing that RA can be an effective neuroprotective agent for dopaminergic neurons. In fact, a recent report showed that the intranasal delivery of RA can also protects dopaminergic neurons against neurodegeneration in nigrostriatal dopaminergic neurons (Yin et al., 2012). Other recent studies also indicated that RA reduced the density of TUNEL labeling, a marker for apoptosis, in the ischemic cortex after middle cerebral artery occlusion in rats (Shen et al., 2009). Interestingly, RA has also been widely used to trigger stem cells differentiation into dopaminergic neurons that are required for relevant cell based therapies for PD (Cooper et al., 2010).

To disclose whether RA^+-NPs could induce glial reactivity, striatal slices were immunostained against GFAP and CD11b, markers of astrocytes and microglia, respectively. It is known that in the presence of an excitotoxic or inflammatory stimulus, there is an increase of GFAP expression by astrocytes and an alteration of microglia functional state from resting (ramified) to an activated (ameboid) state. Moreover, others authors showed that the administration of several types of biomaterials can trigger an inflammatory reaction in the brain parenchyma (Fournier et al., 2006, Xue et al., 2012). Accordingly, we found that in saline animals, the striatal administration of RA^+-NPs induced a mild increase of GFAP expression and microglia activation (ameboid state), especially in the proximity of the injection site. However, in the presence of a MPTP-lesion, it seems that RA^+-NPS were able to reduce, at least in part, the increased expression and reactivity of both glial markers. This may be due to an anti-inflammatory role of RA already reported by several other authors (Dheen et al., 2005, Xu and Drew, 2006). However, a more robust and careful analysis should be done including, for instance, the measurement of GFAP and CD11b staining intensity or the evaluation of cytokines (Interleukin-1 beta or Tumor Necrosis Factor-alpha) expression.

Although the precise pathogenesis of PD remains largely unknown, there are increasing evidences suggesting that dysfunction of some transcription factors involved in the differentiation and survival of mDA neurons may be responsible for the development of PD (Jankovic et al., 2005, Luk et al., 2013). Amongst them, Nurr1 and Pitx3 are the most extensively studied. Nurr1 is one of the key regulators for development of dopaminergic neurons which is also considered as a crucial regulator for the expression of several genes involved in PD pathology including DAT and TH (Smits et al., 2003). Pitx3, another key factor for the development of dopaminergic neurons, is highly expressed in the *SN* and VTA of midbrain and is maintained throughout adult life in both rodents and humans (Nunes et al., 2003). In a recent study, it was shown that the mRNA expression of both Nurr1 and Pitx3 were significantly decreased under PD conditions, suggesting that both genes were potential susceptibility genes for PD (Liu et al., 2012). Interestingly, in our experiments using adult mice, we also found that MPTP induced a robust decrease of both Nurr1 and Pitx3 mRNAs and that RA^+-NPs were able to counteract, at least in part, this decrease.

The neurotoxin MPTP has been used extensively as a research toll to investigate the various neuroanatomical and biochemical abnormalities characteristic of PD. One finding to emerge from this research is the observation that older mice are much more susceptible to the neurotoxic effects of MPTP than adult mice. This may occur because the MAO-B activity increases with age, so both lethality and neurotoxicity of MPTP are age-dependent (Jarvis and Wagner, 1985). Because the risk of developing PD by itself is associated with advancing age, the finding that the toxic actions of this neurotoxin also increase with age, at least during the first half of the rodent's life span, has generated considerable interest. So, it is important to evaluate the effects of RA^+-NPs in older animals since age is a risk factor of PD.

For this reason, in the second part of this study, we also evaluated the effects of RA^+-NPs in Nurr1 and Pitx3 mRNA expression in MPTP-treated old mice by qPCR analysis. The expression levels of Nurr1 in the *SN* and striatum and Pitx3 in the *SN* in MPTP-treated adult mice were significant lower than when compared to control group (saline). This effect mediated by MPTP is more robust in *SN* and striatum of older mice than in young adult mice which is in agreement with the fact of the older mice being more susceptible to effects of MPTP than the younger mice. Our results also showed that RA^+-NPs increased both Nurr1 and Pitx3 mRNA expression in injured *SN* of adult mice but not show increased expression of Nurr1 in striatum. Surprisingly in old mice, there was an increase of Pitx3 in injured *SN* and also an increase of Nurr1 mRNA levels in injured striatum but not in the *SN*. This suggests that Nurr1 and Pitx3 may be RA target genes and, therefore, RA can activate their transcription by binding to RAR-RXR heterodimers. Dopaminergic system is particularly susceptible to the influences of aging, possible as a result of age-related decrease in Nurr1 expression. Nurr1 was found predominantly expressed in dopaminergic neurons and in microglia cells, whereas Pitx3 is expressed selectively in dopaminergic cells present in the ventral *SN*. Therefore, Nurr1 also has a role in inflammation, by suppressing the production of inflammatory mediators by microglia and astrocytes. Therefore, we can hypothesize that the decrease of Nurr1 with age will create a pro-inflammatory milieu responsible for a dopaminergic susceptibility to degeneration (Blasko et al., 2004). To better elucidate these results, it could be relevant to perform double immunostainings for TH/Nurr1 and CD11b/Nurr1 in striatum and *SN* to analyze if this changes occurs in dopaminergic neurons or in microglia. Moreover, as stated before, the increased expression levels of these transcription factors in the RA^+-NPs plus MPTP condition as compared with MPTP-only exposed cells *per se* does not directly correlate with a neuroprotective effect. Future immunostaining studies should be performed to disclose whether the Pitx3 and Nurr1 increased expression found specifically in the nuclei of dopaminergic neurons could correlate with a clear recovery in the percentage of TH^+ neurons in the *SN* of old animals. Adult and old mice were also exposed to both RA^+-NPs and blank NPs in saline animals and as expected, no effect was observed regarding the expression of both transcription factors as compared with the saline contralateral hemisphere (control).

In conclusion, we showed for the first time that RA^+-NPs were able to induce robust neuroprotective effects of *SN* dopaminergic neurons against an acute i.p. MPTP-induced lesion, an effect accompanied by an increase of Pitx3 and Nurr1 mRNA expression.

Chapter 6

Conclusions

In this thesis, we reported for the first time the neuroprotective effect triggered by RA-containing nanoparticles on a MPTP mouse model of PD. In addition, these RA^+-NPs also increased the mRNA expression of Pitx3 and Nurr1, which are transcription factors responsible for dopaminergic neuronal specification and survival. Notably, this formulation offers a significant advantage over soluble RA, either by avoiding the use of solvents like DMSO and by achieving higher neuroprotective effects.

Thus, our results suggest that RA^+-NPs could be a good strategy to boost brain repair in PD and maybe to open new perspectives for the treatment of others neurodegenerative diseases.

6.1 Future Perspectives

In order to strengthen the results presented in this thesis, it would be pertinent to evaluate the effects of RA^+-NPs on nigrostriatal DA levels by the HPLC assay.

It would be also important to quantify the protein levels of both Nurr1 and Pitx3 transcription factors by Western blot or immunostaining. The immunostaining has the advantage of identifying double positive cells for TH/Pitx3 and for TH/Nurr1, and in this way, to evaluate the pattern of expression of these transcription factors specifically in dopaminergic neurons.

It would be relevant to deliver RA^+-NPs into the brain by a less invasive route of administration, such as the intranasal delivery.

Since the RA^+NPs were delivered before the MPTP-induced injury (prophylaxis) it would be also advantageous to administrate these NPs before and/or after the MPTP-induced lesion.

In this study, we did not evaluate the percentage of TH^+ cells in the *SN* of old mice due to the limited availability of mice with a substantially advanced age. These experiments are very relevant to prove that this formulation can also protect dopaminergic neurons upon a MPTP-induced lesion in old mice.

Chapter 7

References

Akinc A, Thomas M, Klibanov AM, Langer R (2005) Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med 7:657-663.

Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P, et al. (1993) Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. Proc Natl Acad Sci U S A 90:30-34.

Bastien J, Rochette-Egly C (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 328:1-16.

Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B (2004) How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging Cell 3:169-176.

Breunig M, Bauer S, Goepferich A (2008) Polymers and nanoparticles: intelligent tools for intracellular targeting? Eur J Pharm Biopharm 68:112-128.

Budhu AS, Noy N (2002) Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. Mol Cell Biol 22:2632-2641.

Castro GA, Orefice RL, Vilela JM, Andrade MS, Ferreira LA (2007) Development of a new solid lipid nanoparticle formulation containing retinoic acid for topical treatment of acne. J Microencapsul 24:395-407.

Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. Science 294:1866-1870.

Chen JY, Clifford J, Zusi C, Starrett J, Tortolani D, Ostrowski J, Reczek PR, Chambon P, Gronemeyer H (1996) Two distinct actions of retinoid-receptor ligands. Nature 382:819-822.

Cookson MR (2012) Evolution of neurodegeneration. Curr Biol 22:R753-761.

Cooper O, Hargus G, Deleidi M, Blak A, Osborn T, Marlow E, Lee K, Levy A, Perez-Torres E, Yow A, Isacson O (2010) Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. Mol Cell Neurosci 45:258-266.

Cui M, Aras R, Christian WV, Rappold PM, Hatwar M, Panza J, Jackson-Lewis V, Javitch JA, Ballatori N, Przedborski S, Tieu K (2009) The organic cation transporter-3 is a pivotal modulator of neurodegeneration in the nigrostriatal dopaminergic pathway. Proc Natl Acad Sci U S A 106:8043-8048.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889-909.

Dheen ST, Jun Y, Yan Z, Tay SS, Ling EA (2005) Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia. Glia 50:21-31.

Dickson DW, Fujishiro H, Orr C, DelleDonne A, Josephs KA, Frigerio R, Burnett M, Parisi JE, Klos KJ, Ahlskog JE (2009) Neuropathology of non-motor features of Parkinson disease. Parkinsonism Relat Disord 15 Suppl 3:S1-5.

Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134:921-931.

Fournier E, Passirani C, Colin N, Sagodira S, Menei P, Benoit JP, Montero-Menei CN (2006) The brain tissue response to biodegradable poly(methylidene malonate 2.1.2)-based microspheres in the rat. Biomaterials 27:4963-4974.

Galter D, Buervenich S, Carmine A, Anvret M, Olson L (2003) ALDH1 mRNA: presence in human dopamine neurons and decreases in substantia nigra in Parkinson's disease and in the ventral tegmental area in schizophrenia. Neurobiol Dis 14:637-647.

Gesi M, Soldani P, Giorgi FS, Santinami A, Bonaccorsi I, Fornai F (2000) The role of the locus coeruleus in the development of Parkinson's disease. Neurosci Biobehav Rev 24:655-668.

Giovanni A, Sieber BA, Heikkila RE, Sonsalla PK (1994) Studies on species sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Part 1: Systemic administration. J Pharmacol Exp Ther 270:1000-1007.

Grunblatt E, Zehetmayer S, Jacob CP, Muller T, Jost WH, Riederer P (2010) Pilot study: peripheral biomarkers for diagnosing sporadic Parkinson's disease. J Neural Transm 117:1387-1393.

Jackson-Lewis V, Przedborski S (2007) Protocol for the MPTP mouse model of Parkinson's disease. Nat Protoc 2:141-151.

Jankovic J (2008) Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry 79:368-376.

Jankovic J, Chen S, Le WD (2005) The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. Prog Neurobiol 77:128-138.

Jarvis MF, Wagner GC (1985) Age-dependent effects of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Neuropharmacology 24:581-583.

Katsuki H, Kurimoto E, Takemori S, Kurauchi Y, Hisatsune A, Isohama Y, Izumi Y, Kume T, Shudo K, Akaike A (2009) Retinoic acid receptor stimulation protects midbrain dopaminergic neurons from inflammatory degeneration via BDNF-mediated signaling. J Neurochem 110:707-718.

Kawaguchi R, Yu J, Honda J, Hu J, Whitelegge J, Ping P, Wiita P, Bok D, Sun H (2007) A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 315:820-825.

Kong XY, Cai Z, Pan L, Zhang L, Shu J, Dong YL, Yang N, Li Q, Huang XJ, Zuo PP (2008) Transplantation of human amniotic cells exerts neuroprotection in MPTP-induced Parkinson disease mice. Brain Res 1205:108-115.

Langston JW (1998) Epidemiology versus genetics in Parkinson's disease: progress in resolving an age-old debate. Ann Neurol 44:S45-52.

Le W, Conneely OM, He Y, Jankovic J, Appel SH (1999a) Reduced Nurr1 expression increases the vulnerability of mesencephalic dopamine neurons to MPTP-induced injury. J Neurochem 73:2218-2221.

Le W, Conneely OM, Zou L, He Y, Saucedo-Cardenas O, Jankovic J, Mosier DR, Appel SH (1999b) Selective agenesis of mesencephalic dopaminergic neurons in Nurr1-deficient mice. Exp Neurol 159:451-458.

Liu H, Wei L, Tao Q, Deng H, Ming M, Xu P, Le W (2012) Decreased NURR1 and PITX3 gene expression in Chinese patients with Parkinson's disease. Eur J Neurol 19:870-875.

Luk KC, Rymar VV, van den Munckhof P, Nicolau S, Steriade C, Bifsha P, Drouin J, Sadikot AF (2013) The transcription factor Pitx3 is expressed selectively in midbrain dopaminergic neurons susceptible to neurodegenerative stress. J Neurochem.

Maia J, Santos T, Aday S, Agasse F, Cortes L, Malva JO, Bernardino L, Ferreira L (2011) Controlling the neuronal differentiation of stem cells by the intracellular delivery of retinoic acid-loaded nanoparticles. ACS Nano 5:97-106.

Marletaz F, Holland LZ, Laudet V, Schubert M (2006) Retinoic acid signaling and the evolution of chordates. Int J Biol Sci 2:38-47.

McCaffery P, Drager UC (1994) High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. Proc Natl Acad Sci U S A 91:7772-7776.

Mizuno Y, Sone N, Saitoh T (1987) Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. J Neurochem 48:1787-1793.

Narvekar M, Xue HY, Wong HL (2012) A novel hybrid delivery system: polymer-oil nanostructured carrier for controlled delivery of highly lipophilic drug all-trans-retinoic acid (ATRA). Int J Pharm 436:721-731.

Nicklas WJ, Vyas I, Heikkila RE (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci 36:2503-2508.

Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP (2003) Pitx3 is required for development of substantia nigra dopaminergic neurons. Proc Natl Acad Sci U S A 100:4245-4250.

Peng J, Andersen JK (2011) Mutant alpha-synuclein and aging reduce neurogenesis in the acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Aging Cell 10:255-262.

Paxinos and Franklin (2001) The mouse brain in stereotaxic coordinates. Second Edition, Academic Press.

Perlmann T, Jansson L (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev 9:769-782.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Przedborski S, Vila M (2001) MPTP: a review of its mechanisms of neurotoxicity. Clin Neurosci Res 1:407-418.

Reichmann H, Schneider C, Lohle M (2009) Non-motor features of Parkinson's disease: depression and dementia. Parkinsonism Relat Disord 15 Suppl 3:S87-92.

Ruberte E, Friederich V, Chambon P, Morriss-Kay G (1993) Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. Development 118:267-282.

Saijo K, Winner B, Carson CT, Collier JG, Boyer L, Rosenfeld MG, Gage FH, Glass CK (2009) A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. Cell 137:47-59.

Santos T, Ferreira R, Maia J, Agasse F, Xapelli S, Cortes L, Braganca J, Malva JO, Ferreira L, Bernardino L (2012) Polymeric nanoparticles to control the differentiation of neural stem cells in the subventricular zone of the brain. ACS Nano 6:10463-10474.

Schapira AH (2006) Etiology of Parkinson's disease. Neurology 66:S10-23.

Shen H, Luo Y, Kuo CC, Deng X, Chang CF, Harvey BK, Hoffer BJ, Wang Y (2009) 9-Cis-retinoic acid reduces ischemic brain injury in rodents via bone morphogenetic protein. J Neurosci Res 87:545-555.

Smidt MP, Smits SM, Bouwmeester H, Hamers FP, van der Linden AJ, Hellemons AJ, Graw J, Burbach JP (2004) Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. Development 131:1145-1155.

Smits SM, Ponnio T, Conneely OM, Burbach JP, Smidt MP (2003) Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. Eur J Neurosci 18:1731-1738.

Szuts EZ, Harosi FI (1991) Solubility of retinoids in water. Arch Biochem Biophys 287:297-304.

Tetrud JW, Langston JW (1989) MPTP-induced parkinsonism as a model for Parkinson's disease. Acta Neurol Scand Suppl 126:35-40.

Toulouse A, Sullivan AM (2008) Progress in Parkinson's disease-where do we stand? Prog Neurobiol 85:376-392.

Ulusoy GK, Celik T, Kayir H, Gursoy M, Isik AT, Uzbay TI (2011) Effects of pioglitazone and retinoic acid in a rotenone model of Parkinson's disease. Brain Res Bull 85:380-384.

Vasir JK, Labhasetwar V (2007) Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev 59:718-728.

Vila M, Vukosavic S, Jackson-Lewis V, Neystat M, Jakowec M, Przedborski S (2000) Alpha-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP. J Neurochem 74:721-729.

Vogel S, Mendelsohn CL, Mertz JR, Piantedosi R, Waldburger C, Gottesman ME, Blaner WS (2001) Characterization of a new member of the fatty acid-binding protein family that binds all-trans-retinol. J Biol Chem 276:1353-1360.

Volpicelli F, De Gregorio R, Pulcrano S, Perrone-Capano C, di Porzio U, Bellenchi GC (2012) Direct regulation of Pitx3 expression by Nurr1 in culture and in developing mouse midbrain. PLoS One 7:e30661.

von Campenhausen S, Bornschein B, Wick R, Botzel K, Sampaio C, Poewe W, Oertel W, Siebert U, Berger K, Dodel R (2005) Prevalence and incidence of Parkinson's disease in Europe. Eur Neuropsychopharmacol 15:473-490.

Xu J, Drew PD (2006) 9-Cis-retinoic acid suppresses inflammatory responses of microglia and astrocytes. Journal of neuroimmunology 171:135-144.

Xue Y, Wu J, Sun J (2012) Four types of inorganic nanoparticles stimulate the inflammatory reaction in brain microglia and damage neurons in vitro. Toxicol Lett 214:91-98.

Yin LH, Shen H, Diaz-Ruiz O, Backman CM, Bae E, Yu SJ, Wang Y (2012) Early post-treatment with 9-cis retinoic acid reduces neurodegeneration of dopaminergic neurons in a rat model of Parkinson's disease. BMC neuroscience 13:120.

Zhu X, Perry G, Moreira PI, Aliev G, Cash AD, Hirai K, Smith MA (2006) Mitochondrial abnormalities and oxidative imbalance in Alzheimer disease. J Alzheimers Dis 9:147-153.

Zigmond MJ, Berger TW, Grace AA, Stricker EM (1989) Compensatory responses to nigrostriatal bundle injury. Studies with 6-hydroxydopamine in an animal model of parkinsonism. Mol Chem Neuropathol 10:185-200.