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Neuroprotective and neurodegenerative effects of the chronic expression of tumor necrosis factor α in the nigrostriatal dopaminergic circuit of adult mice

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ABSTRACT

Tumor necrosis factor (TNF)- α , a pro-inflammatory cytokine, has been implicated in both neuronal death and survival in Parkinson's disease (PD). The substantia nigra (SN), a CNS region affected in PD, is particularly susceptible to inflammatory insults and possesses the highest density of microglial cells, but the effects of inflammation and in particular TNF- α on neuronal survival in this region remains controversial. Using adenoviral vectors, the CRE/loxP system and hypomorphic mice, we achieved chronic expression of two levels of TNF- α in the SN of adult mice. Chronic low expression of TNF- α levels reduced the nigrostriatal neurodegeneration mediated by intrastratial 6-hydroxydopamine administration. Protective effects of low TNF- α level could be mediated by TNF-R1, GDNF, and IGF-1 in the SN and SOD activity in the striatum (ST). On the contrary, chronic expression of high levels of TNF- α induced progressive neuronal loss (63% at 20 days and 75% at 100 days). This effect was accompanied by gliosis and an inflammatory infiltrate composed almost exclusively by monocytes/macrophages. The finding that chronic high TNF- α had a slow and progressive neurodegenerative effect in the SN provides an animal model of PD mediated by the chronic expression of a single cytokine. In addition, it supports the view that cytokines are not detrimental or beneficial by themselves, i.e., their level and time of expression among other factors can determine its final effect on CNS damage or protection. These data support the view that new anti-parkinsonian treatments based on anti-inflammatory therapies should consider these dual effects of cytokines on their design.

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Introduction

Parkinson's disease (PD) is a neurodegenerative, slowly progressive disorder, mainly characterized by the selective loss of dopaminergic neurons of the substantia nigra (SN) (Hirsch and Hunot, 2009), affecting the 1–2% of the general population over the age of 65 (Wilms et al., 2007). Genetic basis has been described in less than 10% of the patients (Farrer, 2006), but the aetiology of idiopathic PD remains to be elucidated. Despite intensive research, the causes of the neuronal death are poorly understood. It has been suggested that neuroinflammation, oxidative stress, protein degradation, mitochondrial dysfunction, disturbed autophagy and aging are associated with this pathology (Hirsch et al., 2003; Hirsch and Hunot, 2000, 2009; Ling et al., 2004; McGeer and McGeer, 2008; Nagatsu et al., 2000; Olanow, 2007; Tansey et al., 2008, 2007; Whitton, 2007; Wilms et al., 2007).

Neuroinflammation, characterized by microglial activation and production of pro-inflammatory cytokines and reactive oxygen species, has been also associated with PD. Interestingly, the SN is the brain region with the highest density of microglial cells (Lawson et al., 1990) and it has a differential susceptibility than other regions of the brain to inflammation. For instance, the injection of LPS, a cytokine inducer, in the SN produces a reduction in the number of tyrosine hydroxylase-positive (TH+) neurons (Castano et al., 1998; Herrera et al., 2000; Iravani et al., 2002; Kim et al., 2000; Ling et al., 2002).

Pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) are key mediators of the inflammatory response. In particular, TNF- α was related to cases of cerebral malaria, HIV-1 associated myelopathy and Multiple sclerosis (MS) (Gelbard et al., 1993; Schobitz et al., 1994). Interestingly, it has been showed that TNF- α is also expressed in the healthy CNS (Sternberg, 1997) and TNF- α does not seem to induce its own expression in the CNS (Blond et al., 2002). CSF and postmortem brains of PD patients display elevated levels of TNF- α as do animals treated with the dopaminergic neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) used to model nigral degeneration in nonhuman primates

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and rodents (Barcia et al., 2005; Boka et al., 1994; Hunot et al., 1999; Mogi et al., 2000; Nagatsu and Sawada, 2005; Sriram et al., 2002). However, the functional role of TNF- α in this pathology is still unclear.

Toxin-mediated neurodegeneration in animal models of PD showed contradictory results on TNF- α action (Rousselet et al., 2002; Sriram et al., 2002). Sriram et al. (2002) showed that TNF- α is involved in the neurodegenerative process during the first 48 h of MPTP injection. Another study found decreased levels of TH and striatal dopamine in the double knock-out for TNF- α receptors (TNF-R1 and TNF-R2) than in wild-type mice (Rousselet et al., 2002), showing a protective effect of TNF- α on dopaminergic neurons. Similar ambiguous results have been described in rats intra-striatally injected with the neurotoxin 6-OHDA. The continuous neutralization of soluble TNF- α by the infusion of a blocker in the SN during the first two weeks after the lesion showed a neurodegenerative effect of TNF- α . However, this effect was not observed if the blocker were infused in the ST (McCoy et al., 2006). In contrast, Gemma et al. (2007) demonstrated that early inhibition of TNF- α increases the 6-OHDA-induced striatal neurodegeneration. However, late inhibition of TNF- α was detrimental for dopaminergic striatal terminals (Gemma et al., 2007). In addition, Castano et al. (2002) showed that the acute administration of TNF- α has no effect on the viability of neurons in the SN. On the contrary, several groups have showed that TNF- α has detrimental effects on dopaminergic neurons (Aloe and Fiore, 1997; Carvey et al., 2005; Clarke and Branton, 2002; De Lella Ezcurra et al.; Gayle et al., 2002; McGuire et al., 2001; Tansey and Goldberg). In conclusion, the conditions that determine whether TNF- α is protective or detrimental are still controversial. No doubt that the type of receptor preferentially used, the signal transduction pathways involved or the molecules induced related to an univocal effect of TNF- α are key to understanding these apparently contradictory effects.

There is a no consensus on the role of endogenous TNF- α in the CNS. The lack of a system permitting the long-term expression of a given cytokine in the SN of adult animals at different concentrations in a time-controlled manner precluded the elucidation of the role of the chronic expression of TNF- α in neuronal survival *in vivo*. In order to understand the functional role of chronic TNF- α on dopaminergic neurons in the SN, we utilized a combination of adenoviral vectors, the CRE/loxP system and hypomorphic mice to express constitutive and up-regulated levels of TNF- α in the SN of adult mice. This system allowed the study of different levels of TNF- α in the SN in a time-controlled manner. We provide evidence that TNF- α effects on the SN are concentration-dependent; e.g. at low levels were neuroprotective while at high levels, were toxic. This approach allowed us to clarify the functional role of TNF- α in the SN according to its level of expression, conceal previous observations and support the concept of the dual role of cytokines on PD.

Materials and methods

K-in construction

We cloned 5' sequences of the engrailed-1 gene upstream of murine TNF- α cDNA and electroporated 1.5×10^7 embryonic stem (ES) cells with 30–60 μ g of this construct using a gene pulser (Bio-Rad) at 25 μ F, 0.4 kV, 400 Ω , 0.9 ms. ES cells grown on neo-resistant feeder layers with ganciclovir were selected, and positive clones were tested by Southern blotting. These cells were injected into CD1 (Swiss) blastocysts, which were transferred into anesthetized pseudo-pregnant foster mothers. Founder animals were back-crossed against C57Bl/6 mice. All animal procedures were performed according to the rules and standards of the German animal protection law and the regulations for the use of laboratory animals of the National Institutes of Health, U.S.A. Experiments were approved by the Ethical Committee of the Institute Leloir Foundation.

Adenoviral construct

Adbgal was kindly provided by Dr. J. Mallet of Hospital Pitie Salpêtrière, Paris, France (Le Gal La Salle et al., 1993). A nuclear localization signal was added to CRE cDNA (pBS185 plasmid Gibco BRL, Gaithersburg, Maryland) by PCR (Kanegae et al., 1995), and the new construct was sequenced and cloned between a cytomegalovirus promoter and the simian virus 40 polyadenylation signal, downstream of the adenoviral encapsidation signal and upstream of the adenoviral pIX gene. This plasmid, together with the left arm of ClaI-digested and purified Ad5, was transfected into HEK293 cells (ATCC, Manassas, Virginia). Correct recombination was verified by restriction digest of the purified viral DNA obtained by HIRT and further confirmed by Southern blotting. The CRE protein was detected as a band of 32 kDa by Western blots of lysates of HeLa cells (ATCC) that had been transduced with AdCRE. Adenoviral vectors were purified by plaque-formation under agar. Stocks were obtained from large-scale preparations in HEK293 cells by double cesium chloride gradients and were quantified by plaque assay. The final titers were 1.5×10^{10} pfu/ μ l for Adbgal and 1×10^{10} pfu/ μ l for AdCRE. Stocks had less than 1 ng/ml of endotoxin, as assayed with E-TOXATE® Reagents (Sigma, St. Louis, Missouri). Viral stocks were free of auto-replicative particles, as assessed by PCR and by transduction of non-transcomplementary HeLa cells (Lochmuller et al., 1994).

Animal surgery

Animals were housed under controlled temperature ($22 \pm 2^\circ\text{C}$), with a 12 hour cycle period and food and water *ad libitum*. Eight to twelve week old mice, were anesthetized with intramuscular injections of ketamine (150 mg/kg) plus xilacine (15 mg/kg). One single stereotaxic injection of 4 μ g of 6-hydroxydopamine (ICN Biomedicals Inc., Aurora, Ohio), diluted in 2 μ l of PBS containing 0.02% ascorbic acid, or vehicle were infused at a rate of 0.5 μ l/min in the left striatum, in order to produce a progressive degeneration of the nigrostriatal dopaminergic system. The striatal coordinates were 0.4 mm anterior, 1.8 mm lateral from *bregma*, and 3 mm from *dura* (Franklin and Paxinos, 1997).

Adenoviral vectors were injected at 1×10^8 pfu/ μ l in PBS containing 10% glycerol. The SN coordinates were 3.1 mm posterior, 1.3 mm lateral from *bregma*, and 4.5 mm ventral from *dura* (Franklin and Paxinos, 1997). The injections were performed with glass micropipettes having a diameter of 50–70 μ m (Bell et al., 1996), and they were left in place for 5 min before being slowly retracted.

PCR, RT-PCR and real-time RT-PCR

Genomic DNA was extracted from SN samples by standard methods (Ausubel et al., 1995). CRE-mediated recombination was detected with the primers depicted in Table 1. Following an initial denaturation for 4 min at 94°C , the samples were amplified for 35 cycles including denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 3 min. Under these conditions, a 970 bp fragment after excision of the Neomycin-resistance cassette (Neo) was amplified. We did not detect the non-recombinant band of 2970 bp, which included the Neomycine-resistant cassette under our amplification conditions.

Total RNA was purified from specific brain regions with TRIzol reagent (GIBCO BRL), and 5 μ g of total RNA were reverse-transcribed with SuperScript II Reverse Transcriptase (GIBCO BRL). A 570 bp fragment of transgenic TNF- α mRNA was detected using the primers in Table 1 and a PCR protocol identical to that showed above, except that 38 cycles of amplification were performed. Using similar conditions, we amplified a GAPDH mRNA fragment using the primers in Table 1. All products were separated by gel electrophoresis and visualized by ethidium bromide staining.

Table 1
Primers and probes.

	Primer sense	Primer antisense	Probe
TNF (genomic)	CATATGCACCACCATCAAGG	CATCTGGAGCACACAAGAGC	
TNF (transgen)	GAGATTGCTCCACCAGAGC	GGGAGTAGACAAAGGTACAAC	
GAPDH	GATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT	
TNF (total)	CACATCTCCCTCCAGAAAA	AGTGCCTCTTCTGCCAGTTC	56-FAM/AGCACAGAAAGCATGATCCG/36-TAMTph/
β -actin	GATCATGTCTCTCTGAGC	CTGCTTGCTGATCCACATCTG	56-FAM/CCTGGCCTCACTGTCCACCTTCC/36-TAMTph
TNF-R1	GCCTACCTCTCCGCTTGCA	GAGCAGAGGGTCAGTCCCT	
IGF-1	TGGATGCTCTTCAGTTTCGTG	GCAACACTCCAGAATGC	

After sacrifice of the animals, the SN was quickly extracted; total RNA was purified with TRIzol reagent (Sigma) following the manufacturer's specifications for small samples and DNase-treated. Each treatment was pooled ($n=3$ or 4 animals). cDNA was synthesized using oligo-dT_{12–18} with Superscript II Reverse Transcriptase (Gibco). PCR analysis was performed in a total volume of 25 μ l, containing sense and antisense primers (0.4 μ M of each), PCR Buffer (Invitrogen), dNTPs (0.2 mM), BSA (100 μ g/ml), Fluorescein (10 nM) and Platinum Taq DNA Polymerase (Invitrogen) 1 U. The primers and probes used to amplify β -actin and TNF- α are in Table 1. Each sample was tested by triplicate and two independent experiments were done. The results were expressed as the ratio of specific amplicon against β -actin.

The levels of TNF-R1 and IGF-1 cDNA were also tested from the same samples under the following conditions: specific sense and antisense primers (0.4 μ M), SybrGreen (0.16 $\times$), PCR Buffer 1 $\times$, dNTPs (0.2 mM), MgCl₂ (0.3 μ M), BSA (100 μ g/ml), Fluorescein (10 nM) and Platinum Taq DNA Polymerase (Invitrogen) 1 U. For both amplicons, the following program was used: 10 min 95 °C, 40 cycles of 45 s 95 °C, 1 min to 55 °C (data collection and real-time analysis was performed in this step), followed by 30 s to 72 °C. A melting curve was performed to ensure the quality of the amplification.

The sense and antisense primers for TNF-R1 and IGF-1 amplification are presented in Table 1. Each sample was tested by triplicate and two independent experiments were done. The results were expressed as the ratio of specific amplicon against β -actin.

Tissue processing and immunohistochemistry

Animals were anesthetized with ketamine/xilazine as described above and perfused through the aorta with 4% p-formaldehyde in PBS (Sigma). Brains were removed, post-fixed for 4 h at 4 °C, cryoprotected in 30% sucrose, and frozen in dry ice-cold iso-pentane (J. T. Baker, Phillipsburg, New Jersey). Serial 30 μ m coronal cryosections through the SN and striatum were obtained and permeabilized with 0.5% Triton X-100. Slides were stained with cresyl violet to determine the presence of inflammatory cells in the SN as described (Ferrari et al., 2004). Antibodies used for immunohistochemistry were a rabbit polyclonal antibody against tyrosine hydroxylase (TH) (Chemicon, Temecula, California; 1:500) or mouse monoclonal anti-tyrosine hydroxylase (Sigma, St. Louis, Missouri) for dopaminergic neurons; a mouse monoclonal antibody against CRE recombinase (Chemicon; 1/1000); and a rabbit polyclonal antibody against TNF- α (Endogen, Woburn, Massachusetts). Secondary antibodies were biotin-conjugated donkey anti-rabbit, Cy2-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-mouse antibodies (Jackson Laboratories, West Grove, Philadelphia; 1:250). The avidin-biotin complex method was used for TH immunohistochemistry (ABC, Vector Laboratories, Burlingame, California), and the DAKO® Catalyzed Signal Amplification (CSA) System was used for TNF- α (DAKO, Carpinteria, California). The final products were visualized with 3,3'-diaminobenzidine (DAB), and X-gal staining was performed as described Revah et al. (1996). DAB and X-gal stained sections were analyzed under bright-field microscopy (Olympus BX60, Japan), and images were captured with a digital camera (Cool

Snap-Procolor, Media Cybernetics, Silver Spring, Maryland) linked to an image analysis system (Image Pro-Plus, Media Cybernetics).

Unbiased stereological quantification of tyrosine hydroxylase-positive neurons and total neurons

The number of tyrosine hydroxylase-positive (TH+) neurons was assessed by using an unbiased optical fractionator designed by the Stereo Investigator analysis software (MicroBrightField Inc., USA), combined with a Nikon Eclipse E600 microscope and a CX900 video camera (MicroBrightField Inc., USA). Cell counting was performed in the SN pars compacta every fifth 30 μ m section along the entire SN. The SN pars compacta was delineated using a 2 $\times$ objective and a systematic sampling of TH+ neurons on the outlined areas were counted with a 100 $\times$ oil-immersion objective (numeric aperture 1.30). Counts were taken at predetermined intervals ($x=200$, $y=200$), and a counting frame (60 $\times$ 60 μ m) was superimposed on the live image of the tissue sections. Section thickness was determined every fourth measurement by focusing on the top of the section, zeroing the z-axis and focusing on the bottom of the section. The media thickness was 10 μ m and the dissector height was set at 7 μ m with a 1.5 μ m top guard zone.

The results in naive animals were expressed as the estimated number of TH+ neurons in the right hemisphere. In treated animals, we showed the percent of neurons remaining on the ipsilateral side compared to those on the intact contralateral side. Values are expressed as the median $\pm$ S.E.M. of all animals in each group.

In K-in animals injected with adenovectors, TH immunohistochemistry was counterstained with Nissl and neurons were counted after 100 days of injection within the same parameters as described for TH+ cells.

Quantification of TH in the striatum

After ascorbic acid or 6-OHDA injection, the TH+ striatal area was automatically determined by the image analysis software as the percentage of TH+ area with more than 80% of the TH intensity in the injected side compared to the total striatal area in the same hemisphere. 100% of the TH-intensity was set as the media intensity of the contralateral striatum. After 100 days of Adbgal or AdCRE injection in K-in animals, the striatal TH+ terminals were quantified as the percentage of optical density in the injected hemisphere compared to the contralateral side. The measurements were performed automatically every sixth section between 0.1 mm to 1.18 mm from bregma (Franklin and Paxinos, 1997) with the Image Pro plus software.

Detection of astroglial and microglial activation

Double immunofluorescence was performed to detect astrocyte and microglial activation. Brain sections were incubated for 1 h in blocking solution. Then, they were incubated overnight with an antibody against TH (to locate SN) and a rabbit polyclonal anti-GFAP (Chemicon, 1/700) or biotinylated-tomato lectin (Vector; 1/100) for astrocytes and microglia, respectively. Tomato lectin staining was

used since it gave more reproducible and reliable results than other microglial markers tested (e.g., *Griffonia simplicifolia* (GSA-1B4), F4/80, Mac-1 or CD11b). After rinsing, sections were incubated for 2 h in PB containing anti-rabbit IgG conjugated with Cy3 (1/200, Jackson Laboratories) and anti-mouse conjugated with Cy3 for TH and GFAP detection or anti-rabbit conjugated with Cy3 and Cy2-labeled streptavidin (1/250, Jackson Laboratories) for TH and microglial cell detection as secondary antibodies. After careful rinsing, sections were mounted in Mowiol (Calbiochem) and observed on a Zeiss LSM 510 confocal laser scanning microscope.

To classify microglial activation we adapted the microglial activation classification according to Kreutzberg (1996). This is: stage 1: resting microglial cells, rod-shaped cell body with fine ramified processes. Stages 2–3: activated microglia, elongated or amoeboid cell bodies with thick processes. Stage 4: phagocytic cells, round-shaped cell body, no processes can be observed at light microscopy level.

Detection of glial cell line-derived neurotrophic factor (GDNF) by immunofluorescence

Serial 30 μ m coronal cryosections through the SN were permeabilized with 0.1% Triton X-100 and blocked for 1 h. Then, sections were incubated with polyclonal antibody against TH and anti-sheep GDNF (Chemicon, 1/250). After rinsing, sections were incubated with Cy3-conjugated donkey anti-rabbit (Jackson Laboratories, West Grove, Philadelphia; 1:250) and Cy2-conjugated donkey anti-sheep (Jackson Laboratories, West Grove, Philadelphia; 1:250). Double-labeled immunofluorescence images were analyzed by confocal laser microscopy (Carl Zeiss, Germany).

Measurement of nitric oxide synthase (NOS) activity

NOS activity on naive control littermates and K-in mice was measured by NADPH diaphorase staining (Ramos et al, 2002). Briefly, free floating sections were incubated for 90 min in 300 μ l phosphate buffer containing NBT (0.2 mg/ml), beta-NADPH (1 mg/ml) and 0.3% Triton X-100 at 37 °C protected from light. After that, slices were mounted on PBS: glycerol (1:3). The total number of NADPH diaphorase+ cells in the SN and in the striatum was counted from 0.3 mm to 1.2 mm in the anterior–posterior plane. Results were expressed as total number of NOS+ cells in the SN or ST.

Protein extraction

After decapitation, the brains were quickly removed and regions enriched in the SN and ST were dissected. For whole-cell protein extraction, the tissue was homogenized with protein extraction buffer (10 mM Tris pH: 7.4, 10 mM NaCl, 3 mM $MgCl_2$, DTT 10 mM, 0.2% NP-40) plus protease and phosphatase inhibitor cocktail. The homogenate was centrifuged at 10,000 rpm for 15 min and the supernatant was used for this study. Assays to determine the protein concentration were performed by comparison with a known concentration of bovine serum albumin using the Bradford Method.

Western blot

A lysate equivalent to 50 μ g of protein from each brain was run on a 10% SDS-PAGE at 120 V together with a size marker. The protein on the gel was subsequently transferred to a nitrocellulose membrane for 1 h at 90 V. After the transfer, the membrane was blocked in 5% powdered non-fat milk in TBS with 0.1% Tween for 1 h. Then, it was incubated with rabbit anti-MnSOD antibody (1/2500) overnight at 4 °C. After washing, membranes were treated with horseradish peroxidase-conjugated secondary antibodies and then with ECLplus reagent. The same membranes were used for beta-actin immunodetection and the result was expressed as the ratio MnSOD/ β -actin.

Total SOD activity measurement

The total SOD activity was measured by using a spectrophotometric method. Freshly extracted protein (250 μ g) was incubated with EDTA (4 mM), methionine (13 mM), riboflavin (130 μ M) and nitro blue tetrazolium (NBT) (150 μ M) freshly prepared in 50 μ M phosphate buffer, pH: 7.8. The total SOD activity was measured as the inhibition of NBT reduction at 570 nm in duplicates. The results were expressed as the percentage of difference between control without sample (100%) and each sample.

Statistical analysis

Results are presented as mean $\pm$ SEM. Comparisons between K-in mice and control littermates or K-in mice injected with adenovirus were performed using T test, T test with Welch's correction or non parametric Mann–Whitney test, where appropriate. Comparisons were performed using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests, where appropriate. Data were tested for normality (Shapiro–Wilks test) and variance homogeneity (Levene test) to use parametric statistical analysis. The level of statistical significance was set at $P < 0.05$.

Results

Generation of hypomorphic mice with a murine TNF- α transgene under the control of the endogenous engrailed-1 promoter

In order to study the effects of the long-term expression of TNF- α on neuronal survival in the SN, we constructed hypomorphic mice by Knock-in (K-in) technology in which the expression of TNF- α mRNA is under the control of the endogenous engrailed-1 promoter (Fig. 1A), which directs the expression of genes to the SN and the pons in adult mice (Davis and Joyner, 1988). We also inserted a neomycin resistance-expression (Neo) cassette flanked by loxP sequences downstream of the TNF- α coding sequence to reduce its level of expression. By Southern blot analysis of ES cells and adult mice, we observed targeted insertion of the construct after homologous recombination (Fig. 1B). RT-PCR using primers that recognize TNF- α and engrailed 5' non-coding sequences showed a specific band for transgenic TNF- α mRNA in the SN, but not in the hippocampus, of untreated K-in animals (Supplementary Fig. 1D, SN vs. hip from K-in saline).

TNF- α and β -actin expression were determined by Real Time RT-PCR using specific Taqman probes. K-in naive mice expressed a 4.72-fold increased TNF- α mRNA compared to control littermates (wt) ($***P < 0.001$, Mann–Whitney test, $n = 2$ pools of three animals each, Fig. 1C). We did not detect protein encoded by the transgene by ELISA or immunohistochemistry in untreated animals (data not shown).

Targeted and efficient delivery of CRE recombinase into the SN of mice using adenoviral vectors up-regulates TNF- α expression in this region

We sought to up-regulate basal TNF- α levels by deleting the interfering Neo cassette via CRE-mediated loxP recombination. To express CRE in the SN, we generated a replication-deficient adenoviral vector expressing a nuclear form of CRE recombinase under the control of the cytomegalovirus promoter (AdCRE). We found that a single injection of 2×10^8 AdCRE or adenovector expressing bacterial β -galactosidase (Adbgal) transduced $89.6 \pm 4.1\%$ and $88.0 \pm 4.0\%$ TH+ cells in the SN (Supplementary Figs. 1A, B and C). Transgene expression lasted for at least 100 days (data not shown).

The expected CRE-mediated deletion of the floxed Neo cassette was observed 7 days after adenoviral injection in the SN of K-in but not in wt mice transduced with AdCRE, and not in K-in mice injected with Adbgal or saline (Fig. 1D). DNA recombination in these mice was paralleled by an up-regulation of TNF- α expression in the SN, at both

Fig. 1. Generation of K-in mice carrying a TNF- α transgene under the control of the endogenous *engrailed-1* promoter. A) Scheme showing the sites for homologous recombination between the targeting construct and the engrailed locus. B) Southern blot showing correct transgene insertion in ES cells and K-in animals. Arrows indicate primers used to detect genomic recombination. Arrowheads show primers used to detect transgenic TNF- α mRNA by RT-PCR. 5'IS: probe used to detect the correct insertion of the transgene in B. en: band corresponding to the wild-type gene locus. Asterisk indicates the targeted gene locus. wt: wild-type ES cell clone; PGK: phosphoglycerate kinase promoter; TK: thymidine kinase; Eng: engrailed; 3VI: Embryonic stem cell clone; k-in: K-in mice. C) TNF- α mRNA expression levels were quantified by real-time RT-PCR in the SN of adult wild-type and K-in adult mice. K-in mice showed increased expression of TNF- α mRNA levels by 4.72 folds in the SN (** $P < 0.001$, Mann-Whitney test, 2 pooled samples of 3 animals each). D) Detection of CRE-mediated recombination in the SN genomic DNA from the SN of control littermates (wt) or K-in (K-in) mice was extracted and amplified by PCR. The expected band of recombined DNA after excision of the Neo cassette is shown. GAPDH amplification was used as a control of the amount of DNA subjected to PCR. C, control PCR without the addition of DNA; M, molecular weight ladder; Sal: K-in mice injected with saline. E) TNF- α expression levels analyzed by real-time RT-PCR in the SN of K-in mice injected with Adbgal or AdCRE. AdCRE administration up-regulated TNF- α mRNA levels by 7.2 folds in the SN (** $P < 0.001$, Mann-Whitney test). F–G) Immunohistochemical determination of TNF- α . Representative sections of the SN of K-in mice 20 days after injection with Adbgal (F) or AdCRE (G) are shown. A higher magnification of a neuron expressing TNF- α is shown in the inset. Scale bars in F and G: 200 μ m.

7 and 20 days after AdCRE but not Adbgal injection (Supplementary Fig. 1D). By real-time RT-PCR we observed that CRE-mediated recombination leads to a 7.2-fold increase in TNF- α levels in K-in mice compared to those injected with control vector 20 days after treatment (* $P < 0.05$, unpaired two-tailed T test with Welch's correction, $n = 2$ and 6 pools of Adbgal- and AdCRE-injected K-in mice, respectively (Fig. 1E). Up-regulation of TNF- α selectively in the SN of K-in animals injected with AdCRE was confirmed by immunohistochemistry using an antibody against TNF- α (Figs. 1F and G and data not shown). By both RT-PCR and immunohistochemistry, we did not observe TNF- α over-expression in a control region (the hippocampus), in K-in animals injected with Adbgal, or in wt mice injected with AdCRE (Figs. 1E, F and G). These results show that we

successfully generated a model in which the effects of the chronic expression of different levels of TNF- α on the viability of dopaminergic neurons in the SN can be studied.

Constitutive low expression of TNF- α did not affect per se the viability of dopaminergic neurons of SN

In order to characterize a possible effect of TNF- α on the viability of the dopaminergic neurons, we counted the number of TH+ cells in the SN by immunohistochemistry using unbiased stereology. We observed that constitutive low expression of TNF- α did not affect the viability of adult dopaminergic neurons in the SN (5424 ± 765.3 and 5077 ± 390.4 , for wt and K-in naive animals, respectively, $P = 0.7$,

Fig. 2. Tissue response to constitutive low expression of TNF- α . A–B) Representative pictures of an immunohistochemistry against TH in the SN of naive control littermates (A) or K-in (B) adult mice. C) Total number of dopaminergic neurons estimated by counting TH-positive cells in the SN after an immunohistochemistry against TH using unbiased stereology software. D and E) Representative Nissl staining sections of the SN of adult naive control littermates (D) or k-in (E) mice. F and G) Immunohistochemistry against GFAP (green) and TH (red). Astrocyte reactivity was increased in k-in (G) naive animals compared to control littermates (F) in the SN *pars reticulata*. H and I) Immunohistochemistry against TH (red) and Tomato lectin (green). Representative pictures of the control littermates (H) and K-in (I) SN in adult naive animals. A higher magnification of microglia is showed in the h (control littermates) and i (K-in). Scale bars: panels A and B: 200 μ m, panels D–I: 50 μ m.

two-tailed T test, $n=4$ for each group, Figs. 2A–C). Double immunofluorescence against TH and DAT in the SN revealed complete co-localization of both markers (data not shown); showing that constitutive expression of TNF- α did not affect the TH expression.

Constitutive low expression of TNF- α did not produce inflammatory infiltrate or morphological microglia activation

In order to characterize the effects of the low expression of TNF- α in the SN parenchyma we evaluated the inflammatory response by Nissl staining. Constitutive low expression of TNF- α did not produce recruitment of blood cells in adult K-in mice (Figs. 2D and E). In addition, we found that constitutive low expression of TNF- α produced astrocytosis in the SN *pars reticulata* but not in the SN *pars compacta* of adult K-in mice compared with control littermates (Figs. 2F and G). Microglial cells were not activated due to the low expression of TNF- α , as they showed rod soma with ramified processes without signs of morphological activation, analyzed by immunostaining against tomato lectin (Figs. 2H, h and I, i).

Constitutive low expression of TNF- α in the SN is neuroprotective against striatal 6-OHDA injection

To investigate the effect of the constitutive low expression of TNF- α on a model of PD, we progressively killed dopaminergic neurons in the

SN by denervating the striatum (ST) with 6-OHDA and determined the number of TH+ neurons in the entire SN by unbiased stereology (Fig. 3), as well as the TH+ remaining area in the ST (Fig. 4). After 7 days of treatment with 6-OHDA, a comparable neurodegeneration was observed in the SN of control and K-in animals (56.36 ± 8.34 and 61.05 ± 4.93 TH+ cells for wt and K-in injected with 6-OHDA, respectively, $n=4$ for wt and $n=3$ for k-in, Figs. 3A–D and I). However, after 20 days of neurotoxin administration, the number of TH+ neurons was significantly higher in the K-in mice (62.78 ± 9.62 , $n=5$) than in wt mice (33.02 ± 5.31 , $n=5$) ($*P<0.05$; neurotoxin-injected K-in vs. wt, Figs. 3E, F and I). This neuroprotective effect of constitutively expressed TNF- α was transient since it was not detected after 43 days of neurotoxin injection (33.17 ± 4.73 and 38.35 ± 15.64 , for wt and k-in respectively, $n=4$ for each group, Figs. 3G–H and I). Double immunofluorescence against TH and DAT in the SN revealed complete co-localization of both markers (data not shown).

The effect of the constitutive low expression of TNF- α on the dopaminergic striatal terminals was evident at every time studied (Fig. 4I). In wt animals the effect of neurotoxin was evident as soon as 7 days after treatment (Figs. 4A and B, $n=5$, $*P<0.05$ in wt mice injected with vehicle vs. neurotoxin injected, two ways ANOVA, Bonferroni post test). On the contrary, the differences between K-in animals injected with vehicle vs. 6-OHDA observed at these time points were not significant ($P>0.05$). After 20 days of neurotoxin-injection, the K-in mice showed an increased TH+ area ($68.57\% \pm 9.47$) than wt

Fig. 3. Constitutive expression of TNF- α transiently protects dopaminergic neurons against 6-OHDA toxicity. A–H) Representative sections of the SN were immunostained with antibodies against tyrosine hydroxylase after vehicle or 6-OHDA administration. A–D) SN of control littermates (A,C) or K-in (B,D) after 7 days of vehicle (A,B) or 6-OHDA injection. E–F) Ipsilateral SN of control littermates (E) or K-in (F) mice after 20 days of neurotoxin injection. G–H) Ipsilateral SN of control littermates (G) or K-in (H) mice after 43 days of neurotoxin injection. I) Quantitation of the percentage of TH+ neurons in the entire SN on the ipsilateral vs. contralateral sides. K-in: K-in animals; wt: control littermates; veh: vehicle. Scale bar: 50 μ m. * P < 0.05 for control littermates vs. K-in animals 20 days after injection of 6-OHDA treatment.

mice (45.18 ± 6.3) (Figs. 4C and D, $n = 7$, * P < 0.05 two ways ANOVA, Bonferroni post test). At this time point a neuroprotective effect of TNF- α was more evident. We also observed the protective effect of the constitutive level of TNF- α in striatal terminals of K-in after 43 days of neurotoxin injection (47.45 ± 4.49 in k-in mice and 26.73 ± 8.27 in wt) (Figs. 4E–H, $n = 5–6$, * P < 0.05 between control and K-in mice injected with 6-OHDA). The TH+ striatal area in wt and K-in mice injected with 6-OHDA between 20 and 43 days was reduced around 25% of the total area, showing the same kinetics of TH loss in both genotypes. Thus, the rate of decrease in the TH+ area was similar between control littermates and K-in mice injected with 6-OHDA.

Constitutive low expression of TNF- α produced differential molecular response between the SN and the ST

As TNF could exert its actions through two receptors, we quantified by Real-Time RT-PCR the expression of TNF- α Type I and Type II receptor mRNAs. Adult naive K-in mice showed an increased level of TNF- α type I receptor in the SN compared to wt mice (Fig. 5A, * P < 0.05, two-tailed unpaired T test, $n = 2$ pools of 4 SN for each group). The TNF- α receptor Type II expression could not be detected in the SN of wt and K-in mice either by Real-Time RT-PCR or Western blot.

Fig. 4. Neuroprotection of striatal dopaminergic terminals of K-in mice after intrastratial injection of neurotoxin 6-OHDA. A–H) Representative sections of the ST were immunostained with antibodies against tyrosine hydroxylase after vehicle or 6-OHDA administration. A–B) Striatum of control littermates (A) or K-in (B) after 7 days of 6-OHDA injection. C–D) Ipsilateral striatum of control littermates (C) or K-in (D) mice after 20 days of neurotoxin injection. E–H) Ipsilateral SN of control littermates (E, G) or K-in (F, H) mice after 43 days of vehicle (G, H) or neurotoxin (E, F) injection. I) Quantitation of the percentage of TH+ striatal area vs. total striatum area on the ipsilateral side after treatment. K-in: K-in animals; control littermates: control littermates; veh: vehicle. Scale bar: 500 μ m. * $P < 0.05$ for control littermates vs. K-in animals after injection of 6-OHDA. & $P < 0.05$ for control littermates injected with vehicle vs. 6-OHDA after 7 days of treatment.

Since TNF- α has been shown to be neuroprotective via different molecules, we select the ones which may be more relevant for the physiology of the dopaminergic nigrostriatal system.

It has been described that TNF- α could modify IGF-1 expression (Sara et al., 1993). Real-time RT-PCR was performed to evaluate the expression of this molecule. Interestingly, the constitutive low

expression of TNF- α increased the level of IGF-1 mRNA in the SN of K-in animals (Fig. 5B, * $P < 0.05$ one-tail unpaired T test). In addition, there was no IGF-1 detected in the ST (data not shown).

Among other molecules associated to the protective actions of TNF- α , MnSOD has been described in several *in vivo* and *in vitro* models (Bruce-Keller et al., 1999; Rogers et al., 2001). For this reason,

Fig. 5. Possible molecular mediators of the neuroprotective effect of constitutive TNF- α expression. Gene transcript analysis of adult naive SN of control littermates and K-in mice. Gene transcript for TNF-R1 (A) and IGF-1 (B) were analyzed by real-time PCR. β -actin gene was used as an internal control. C–E) Representative Western blot against MnSOD and β -actin in the SN (C) and ST (D) of adult naive control littermates and K-in animals. E) Quantification of MnSOD expression levels in the SN and ST in control littermates and K-in animals by Western blot. F) Total SOD activity in the SN and ST of control littermates and K-in animals expressed as percentage of inhibition of the NBT–Riboflavin–Methionine reaction. G–H) Representative sections of double immunofluorescence analysis to detect GDNF (green) and TH (red) on SN of control littermates (G) and K-in (H). * $P < 0.05$, T test. Scale bar: panels G and H: 50 μ m.

we performed a Western blot on total protein lysate of SN and ST to quantify MnSOD levels. The K-in animals showed increased levels of MnSOD in both areas (Figs. 5C–E, ** $P < 0.01$, unpaired T test). Since SOD activity could be modulated independently of the level of protein, we analyze the total SOD activity by spectrophotometry. The total SOD activity was increased in extracts of ST of K-in compared to wt animals (Fig. 5F, * $P < 0.05$, unpaired T test), but not in the SN. These results showed that TNF- α expression produced differential actions in the SN and the ST.

As TNF- α can increase GDNF expression (Appel et al., 1997), we analyzed GDNF expression in the SN by immunohistochemistry. We observed similar GDNF expression in the SN pars compacta of wt and K-in mice. On the contrary, while there was no GDNF expression detected in the SN pars reticulata of wild-type mice, GDNF was detected in that region of K-in mice, reflecting a higher GDNF expression in animals expressing low, constitutive levels of TNF- α (Figs. 5G and H, naive wt and K-in, respectively).

We tested the possibility that the SOD activity could be inhibited by nitrosylation via TNF- α -induced NOS. We performed the NADPH-diaphorase method to evaluate if TNF- α modified the number of cells with NOS activity. We found that the number of NOS+ cells was increased in the SN of K-in animals compared to wt mice (Figs. 6A–C, * $P < 0.05$, unpaired T test). However, there was no difference in the number of NOS+ cells between K-in and wt mice in the ST (Figs. 6D–F). This different modulation of NOS could account for the different regulation of SOD activity in the SN and the ST.

These results also confirmed the synthesis of TNF- α protein, showing specific molecular modulation in the SN and ST of constitutive low levels of TNF- α .

Long-term expression of up-regulated TNF- α in the SN produced chronic monocyte recruitment, astrocytosis and microglia activation

Adenoviral injection *per se* caused a transient inflammatory response in the SN, irrespective of the transgene expressed and the genetic modification of the mice used 4 days after its administration (Figs. 7A and B), as we expected. This innate response to the vector was mainly composed by monocyte/macrophages and neutrophils around the dilated blood vessels (Figs. 7a and b). Apoptotic bodies were also observed in K-in animals injected with both adenovectors (Figs. 7a and b). By 20 days, the inflammatory response resolved in K-in mice injected with Adbgal (Figs. 7C, D, c and d). However, in K-in animals injected with AdCRE an inflammatory infiltrate composed almost exclusively by monocyte/macrophages could be observed mainly around blood vessels at 20 and 100 days post AdCRE injection (Figs. 7D and d). The macrophage infiltration diminished after 100 days of AdCRE injection as well as the vasodilation (Figs. 7D and d).

Astrocytosis was observed in the SN of K-in mice 4 days after any adenovector was injected (Figs. 7G and H). After 20 days of the injection of Adbgal, astrocytes were only activated in the SN pars reticulata, at comparable levels observed in naive K-in mice (Figs. 7I

Fig. 6. Differential NOS activity in the SN vs. ST. A–F) NOS activity was measured by diaphorase staining on serial sections of SN (A–C) and ST (D–F) of naive control littermates (A and D) and K-in (B and E) animals. Panels a, b, d and e represent higher magnifications of the panels with the same letter (in capital). C and F) The graphics show the total number of NOS+ cells in the SN (C) or ST (F) of control littermates and K-in mice. * $P < 0.05$, Mann–Whitney test. Scale bar: panels A, B, D and E: 200 μ m. Panels a, b, d and e: 50 μ m. Arrows represent nNOS positive neurons.

and K). On the contrary, K-in animals injected with AdCRE, which over-express TNF- α , showed astrocytosis in the SN pars reticulata and pars compacta from 4 to 100 days after injection (Figs. 7H, J and L).

Additionally, adenoviral injection produced an unspecific microglial response in K-in animals after 4 days of treatment, while activated microglia in K-in mice injected with Adbgal showed an elongated-shaped cell body with ramified thick processes (Fig. 7M). In those animals injected with AdCRE we also observed amoeboid cell bodies with short processes, showing microglial morphology with features of increased activation (Fig. 7N).

At 20 and 100 days after of adenoviral injection, microglial cells in K-in mice treated with Adbgal showed a resting phenotype (rod-shaped soma with fine and ramified processes), similar to naive animals (Figs. 7O and Q). On the contrary, microglial cells still showed activated morphology (round-shaped body with short processes) in K-in animals 20 days after AdCRE administration (Fig. 7P). 100 days after treatment, the microglia showed a morphological stage 2 and 3 in K-in animals injected with AdCRE (Fig. 7R).

Long-term expression of up-regulated TNF- α in the SN causes progressive neurodegeneration in the nigrostriatal dopaminergic system

To assess the potential effect of the long-term up-regulation of TNF- α on neuronal viability, we injected AdCRE and Adbgal into the SN of K-in mice. By immunohistochemistry, we found that the remaining percentage of TH+ cells was $36.39\% \pm 8.44$ in K-in mice injected with AdCRE and $71.98\% \pm 2.43$ in those injected with Adbgal 20 days after injection ($n = 4$ and 3 , respectively; * $P < 0.005$, T test, comparing Figs. 8A and B and graphics in 8C). At 100 days after AdCRE administration to K-in mice, we observed a higher reduction in TH+ cell number in the SN ($24.26\% \pm 5.73$) as compared with K-in animals

injected with Adbgal ($73.54\% \pm 9.43$) ($n = 5$ and 3 respectively, ** $P < 0.01$, T test; comparing Figs. 8D, E and graphics in F). Signs of neurodegeneration were confirmed in these K-in mice after 100 days of adenovector injection by an independent method (Supplementary Figs. 2A and B). In addition, to determine the specificity of the effects of the up-regulated TNF- α on dopaminergic and not other neurons of the SN, we have counted by Nissl staining the total number of neurons in the SN of K-in mice 100 days after AdCRE injection. First, in the contralateral hemisphere of K-in animals we measured a total of $78.57\% \pm 3.96$ TH-positive neurons over all neurons (Nissl-stained). These data reflects the percentage of all neurons in the SN that were dopaminergic (TH-positive). The percentage of total neurons remaining was 85.79 ± 6.23 and $47.9\% \pm 6.8$ in K-in animals injected with Adbgal or AdCRE, respectively, as determined by Nissl counting ($P < 0.05$, $n = 3$ per group) (Fig. 8G). The number of total vs. TH-positive neurons remaining after Adbgal administration was not statistically significant (85.79 vs. 73.54 ; $P = 0.34$). On the contrary, the number of total vs. TH+ neurons remaining after AdCRE administration was statistically significant (47.9 vs. 24.26% ; * $P < 0.05$). Therefore, there was a higher percentage of TH-neurons than total neurons dying after AdCRE treatment. From these data, we conclude that AdCRE treatment caused dopaminergic cell death preferentially.

We also found that the injection of adenoviral vectors per se resulted in a loss of 28% of TH+ cells at 20 and 100 days (see Adbgal, Figs. 8A and D). We have observed that adenoviral inoculation has comparable toxic effects in wt animals administered with Adbgal or AdCRE compared to K-in animals injected with Adbgal after 20 or 100 days (data not shown). These results suggest that the progressive and long-lasting toxic effects of TNF- α up-regulation in K-in mice/AdCRE are not determined by the initial toxic response to the adenoviral inoculation.

Fig. 7. Analysis of the tissue reaction after adenoviral injection. A–F) Inflammatory reaction in the SN by Nissl staining. A and B) Representative sections of the SN of K-in mice injected with Adbgal (A) or AdCRE (B) 4 days after adenovector administration. See the innate response of the tissue to the adenoviral injection. C and D) Representative sections of the SN of K-in animals injected with Adbgal (C) or AdCRE (D) 20 days after adenovector administration. E and F) Representative sections of the SN of K-in animals injected with Adbgal (E) or AdCRE (F) 100 days after adenovector administration. See the mononuclear infiltrate only in AdCRE-injected animals. G–L) Astrocyte reactivity in the SN after the injection of adenovirus. G, I, K) Representative pictures of the SN after injection of Adbgal at 4 (G), 20 (I) and 100 days (K) of treatment. H, J, L) Representative sections of K-in animals injected with AdCRE after 4 (H), 20 (J) and 100 days (L) of treatment. Immunohistochemistry against GFAP (green) and TH (red). M–R) Microglial activation after 4 days (M and N), 20 days (O and P) or 100 days (Q and R) after administration of Adbgal (M, O, Q) or AdCRE (N, P, R). Immunohistochemistry against TH (red) and Tomato lectin (green). Insets: v: blood vessels; *: apoptotic bodies; m: macrophage; n: neutrophil; μ and μ': microglia. Scale bars: Panels A–F: 50 μm, panels a–f: 200 μm, panels G–R: 50 μm.

Long-term up-regulation of $TNF-\alpha$ affects striatal terminals

To determine whether the $TNF-\alpha$ over-expression had any effect in the striatal TH+ density of AdCRE-injected K-in mice after 100 days of adenoviral injection, we measured the striatal TH+ area by immunohistochemistry. We observed that the AdCRE administration produced a net higher reduction in the TH immunostaining in the ST compared with Adbgal injection, obtaining an TH+ density of 25.89 ± 5.49 and

85.53 ± 5.2 for K-in animals injected with AdCRE or Adbgal, respectively (Figs. 9A,B and C, $n = 5$, $**P < 0.01$, Mann–Whitney test).

Discussion

Using a combination of hypomorphic mice, adenoviral vectors and CRE/loxP system, we studied the effects of the chronic expression of $TNF-\alpha$ at three different levels in the SN of adult mice (basal,

Fig. 8. Neurodegeneration of TH+ neurons in the SN of K-in mice after AdCRE injection. A, B, D and E) Representative sections of the ipsilateral SN immunostained with antibodies against tyrosine hydroxylase after 20 days (A,B) or 100 days (D,E) of Adbgal (A,D) or AdCRE (B,E) administration. C) Quantification of the percentage of TH+ neurons in the entire SN on the ipsilateral and contralateral sides was compared 20 days after treatment. F) Quantification of the percentage of TH+ neurons in the entire SN on the ipsilateral and contralateral sides 100 days after treatment. G) Quantification of the total number of Nissl neurons in K-in animals injected after 100 days of adenoviral injection. Values showed are median percentage of neurons $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$ for K-in AdCRE vs. Adbgal injected animals. Scale bars: panels A, B, D and E: 200 μ m.

constitutive low expression and up-regulated levels). We found that constitutive expression of low levels of TNF- α was transiently neuroprotective against 6-OHDA toxicity in the SN and the ST compared to basal levels. These beneficial effects were more evident in the ST than in the SN. On the other hand, we found that the chronic up-regulated expression of TNF- α resulted in a progressive loss of DA neurons in the SN and their terminals, gliosis and monocyte/macrophage recruitment.

Our results indicate that TNF- α levels and the duration of expression are relevant for the final effect of this cytokine in the SN. Low TNF- α levels lead to a neuroprotective effect while up-regulated levels triggered neuronal demise. It is unknown how close this previously unreported neuroprotective effect of TNF in a complex

animal model represents a clinical situation. PD patients do not manifest symptoms of this disease until at least 75% of the neurons required for motor modulation in the SN are dead. Thus, a marginal increase in the percentage of TH+ neurons may result in symptomatic relief (Lang and Lozano, 1998). In any case, this protective property of low TNF expression should encourage the design of anti-TNF- α treatments targeting specifically the toxic effects of TNF. The transient nature of the protective effect on neuronal cell bodies in the SN and on their ST terminals may be related with, at least, two phenomena. One of them might be produced by the death of dopaminergic neurons producing the TNF- α , diminishing the level required for the protective effect of TNF- α . On the other hand, it may be possible that the molecular pathways activated by 6-OHDA produce a neutralization of

Fig. 9. Over-expression of TNF- α produces degeneration of the striatal dopaminergic terminals. A–B) Representative sections of immunohistochemistry against TH in the striatum after 100 days of Adbgal (A) or AdCRE (B) injection in the SN. C) Percentage of TH content in the ST showed as the ratio of ipsilateral/contralateral optical density in K-in animals injected with Adbgal or AdCRE. ** $P < 0.01$ for K-in animals injected with Adbgal vs. AdCRE.

an early protective mechanism of low expression of TNF- α . In order to elucidate the reasons for the transient protective effect further research is warranted.

Interestingly, the acute administration of TNF- α in the SN had no effect on neuronal viability (Castano et al., 2002), suggesting that the chronicity in the expression is key to the effects observed.

McCoy et al. (2006) have proposed a neurodegenerative effect of TNF- α in a model of PD. On the other hand, Gemma et al. (2007) have showed that the inhibition of TNF- α after the intrastriatal injection of 6-OHDA worsened the progression of the neurodegeneration. In contrast, at later time points they showed opposite TNF- α effects. Two other previous reports used TNF receptor knock-out mice to study the effect of TNF- α on the MPTP-model of nigrostriatal degeneration (Rousselet et al., 2002; Sriram et al., 2002). While one group observed neuroprotective effects in mice with both TNF- α receptors ablated (suggestive of a neurodegenerative effect of TNF- α), the other found decreased dopamine levels in the striatum in the same mice, indicating a beneficial effect of TNF- α . Several methodological issues could account for this discrepancy. For example, the doses of MPTP used were different (12.5 mg/kg vs. 60 mg/kg). Interestingly, dissimilar MPTP regimes of administration have been shown to lead to dramatic changes in the mechanisms of cell death (Furuya et al., 2004). Importantly, in both studies, TNF- α up-regulation in the MPTP model is transient (only until 48 h after MPTP administration), but while Sriram et al. performed their analysis between 24–48 h after MPTP administration, Rousselet and colleagues examined the animals 10 days later. In the light of our results, the lack of TNF receptors could have caused an early inhibition of the toxic effect of the up-regulated TNF- α expression in these mice in the first study (Sriram et al., 2002). This effect may not be sustained at later time points, since TNF- α up-regulation is acute and only lasts for 48 h in the model used. Concomitantly, the lack of TNF receptors at a later time point of analysis could be precluding the observation of a beneficial effect of lower TNF- α expression levels in the second study (Rousselet et al., 2002; Sriram et al., 2002). Although these interpretations are speculative and the reasons for their discrepancy remain to be proven, this interpretation fits with our experimental data and the notion of a dual effect of TNF- α on neuronal vitality in the SN.

In addition, a model where TNF- α over-expression in the SN via adenoviral vector inoculation leads to dopaminergic cell death, monocyte infiltration and astrocyte and microglia activation to stage 4 has been recently described (De Lella Ezcurra et al., 2010). The levels of expression of TNF- α in that model were detectable by ELISA, which indicates higher TNF- α expression levels than the ones achieved in the K-in mice injected with AdCRE in our study, where TNF- α reached its highest level of expression. The observation that high TNF- α levels leads to neurodegeneration in the SN in another animal model strengthens the observation that TNF- α over-expression in K-in mice injected with AdCRE leads to similar toxic effects in the SN as described in the present study. In addition, it defines “high” TNF- α expression levels with toxic effects on the SN to levels detectable by immunohistochemistry (our results) or ELISA (above 7.8 pg/mg of protein) (De Lella Ezcurra et al., 2010). In addition, the AdCRE-injected K-in mice provided mainly a dopaminergic cell death.

Importantly, TNF- α has also been shown to have dual effects in animal models of a more typical chronic inflammatory and neurodegenerative disease such as multiple sclerosis (MS). For example, TNF- α accelerated the onset of EAE and induced oligodendrocyte apoptosis, but it was also necessary for the regression of myelin-specific T cell activity at a later time point (Kassiotis et al., 2001). In addition, while this cytokine had an accelerating effect on acute demyelination, it also induced the proliferation of oligodendrocyte progenitors and remyelination (Arnett et al., 2001). The clinical importance of these findings is supported by demonstrations that anti-TNF- α therapies failed to improve, or even worsened, the symptoms of MS patients (1999; van Oosten et al., 1996). This clinical

outcome suggests that the anti-TNF therapy could have diminished the late beneficial effects of TNF- α in these patients. TNF- α levels cannot be monitored and easily regulated in the SN of patients. In view of the analogy with the clinical outcomes of anti-TNF- α therapy in MS, our results put a cautionary note on unspecific anti-TNF- α treatments against PD and suggests it is crucial to understand the molecules mediating univocal (beneficial OR toxic) TNF- α effects or generate TNF- α agonists with univocal effects (Tansey et al., 2008).

Our results suggest several possible mechanisms by which TNF- α may mediate neuronal death or protection in the SN. The neuronal death can be mediated, at least in part, by activated microglia as it has been demonstrated by several groups in mice injected with 6-OHDA (He et al., 2001) or MPTP (Sriram et al., 2006; Wu et al., 2002). The neuroprotective effects of low chronic expression of TNF- α may be mediated by the up-regulation of TNF-R1, GDNF and IGF-1 in the SN. Distinctively, the possible molecular pathway in the ST might be through the induction of SOD activity, which scavenges free radicals.

GDNF, one of the most potent neuroprotective molecules for dopaminergic neurons described to date (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1997; Kordower et al., 2000), can be induced by TNF- α (Appel et al., 1997). GDNF prevents neuronal degeneration of the lesioned nigrostriatal system in vivo (Akerud, 2001; Beck et al., 1995; Bilang-Bleuel et al., 1997; Broome et al., 1999; Choi-Lundberg et al., 1997) and improves motor deficits (Bensadoun et al., 2000; Gash et al., 1996; Kordower et al., 2000). In our model, TNF- α induced GDNF and thus, GDNF is a main candidate to mediate the beneficial effects of TNF- α .

In vitro experiments have shown that TNF- α inhibit survival signals from IGF-1 (Sara et al., 1993), and IGF-1 blocks the effects of TNF- α . In fact, the relation between TNF- α and IGF-1 is termed silencing of survival signals (SOSS) (Venters et al., 2000, 1999). On the other hand, Ezquer et al. (2006) have observed an increase in TNF- α without changes in IGF-1 in the SN after acute hypoxia in neonatal rats. Surprisingly, our results showed an increase on IGF-1 in K-in mice expressing chronic low levels of TNF- α . We conclude that the chronicity and levels of TNF- α expression could explain this unexpected up-regulation of IGF-1, making it another candidate to mediate the neuroprotective effects of TNF- α .

In accordance with our results, an increase on MnSOD mediated by TNF- α has been described (Bruce-Keller et al., 1999; Rogers et al., 2001). The total SOD activity includes Cu/Zn SOD, extracellular SOD and MnSOD activities. SOD activity seems to be inhibited by nitrosylation of their tyrosines by NOS (Bayir et al., 2007). NOS expression was increased only in the SN (where increments in MnSOD expression were not correlated with increased SOD activity) and not in the ST (where elevated MnSOD expression correlated with increased SOD activity). Therefore, our results suggest that NOS expression in the SN could be mediating the lack of correlation between MnSOD expression and SOD activity in the SN. Regional differences, as we observed between SN and ST have been described in other models (Blond et al., 2002; Depino et al., 2005; Liu et al., 2003). In this context, our data support the idea that the increased MnSOD activity (and the lack of NOS expression) could be mediating TNF- α beneficial effects in the ST, while GDNF and IGF-1 could be relevant for the neuroprotective effects of TNF in the SN.

As expected, a mixed infiltrate was observed at early time points (4 days) in all adenoviral vector-injected groups (Cheshenko et al., 2001; Parks and Bramson, 1999). Animals with up-regulated TNF- α in the SN showed an infiltrate composed almost exclusively of monocyte/macrophages at late time points (20 to 100 days). Acute injection of TNF- α in the brain also results in selective monocyte/macrophage recruitment as observed in this study (Blond et al., 2002; Griffin et al., 2004; Schnell et al., 1999). This selective monocyte/macrophage recruitment was associated with a lack of IL-1 induction, which in turn can lead to neutrophil recruitment in the CNS (Blond et al., 2002; Ferrari et al., 2004, 2006). Our findings suggest that even

after 100 days of chronic TNF- α expression, this cytokine-specific pattern of immune cell recruitment is conserved. These data support the view of cytokine-specific cell recruitment in the brain. In addition, TNF- α was able to activate microglial cells only when its expression levels were detectable by immunohistochemistry in K-in mice treated with AdCRE and not when constitutively expressed at low levels in untreated K-in mice. This observation is similar to the one described recently in a different model of nigral neurodegeneration by TNF overexpression (De Lella Ezcurra et al., 2010). This differential activation of microglial cells by high TNF- α levels is another example of a concentration-dependent effect of this cytokine. In addition, it suggests a functional role of these cells independently of other cellular immune components such as neutrophils as has been discussed for other neurodegenerative processes (Emerich et al., 2002).

Our data is in line with the current thinking that cytokines can be deleterious or beneficial for neuronal survival depending on multiple factors. In this work, we demonstrated that one important factor is the concentration of TNF- α in the SN. Although there are numerous examples of concentration-dependent, dual effects of cytokines on immune function in the periphery, this is the first example that a chronic expression of a single cytokine can have such opposite and dramatic effects on dopaminergic neurons of nigrostriatal system analyzed in the same animal model. Thus, a potentially beneficial therapy for PD based on TNF- α or inflammatory regulation should consider the alteration of cytokine levels among the variables affected. Alternatively, a potential beneficial intervention can become independent of the TNF- α levels in the SN if the molecular mechanisms of the protective TNF- α action are targeted, such as GDNF, IGF-1 or antioxidant activity.

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