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Nigral neurodegeneration triggered by striatal AdIL-1 administration can be exacerbated by systemic IL-1 expression

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ABSTRACT

Neuroinflammation has been proposed as an important component of Parkinson's Disease (PD) aetiology and/or progression. However, the inflammatory components and the mechanisms underlying their effects are only partially known. By injecting an adenovirus expressing IL-1 in the striatum, we provoked progressive neurodegeneration of dopaminergic cells in the substantia nigra, motor symptoms and microglial activation. All these effects were attenuated by an anti-inflammatory treatment. Interestingly, peripheral inflammatory stimuli exacerbated IL-1β induced neurodegeneration and the central inflammatory reaction.
These data provide evidence that central, chronic IL-1β expression can trigger and systemic IL-1β exacerbate nigral neurodegeneration and highlight the functional relevance of this cytokine in PD.

1. Introduction

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SN). Genetic components have been linked to familial PD (Lees et al., 2009). The aetiology of idiopathic PD is poorly understood. However, increasing evidence suggested that neuroinflammation could be involved in the pathogenesis and progress of PD (reviewed in Hirsch and Hunot, 2009). Indeed, PD has been associated with head trauma suggesting the involvement of an inflammatory component in the disease (Herrera et al., 2005). Infections by influenza virus, Helicobacter pylori, HIV or even candidiasis and their underlying inflammation have also been suggested, but not proven, as etiological agents of PD (Arai et al., 2006; Epp and Mravec, 2006; Jang et al., 2009). Specially, a case supporting the correlation between a major increase in parkinsonian symptoms and the influenza pandemic of 1918 has been widely discussed (Isgreen et al., 1976; Marttila and Rinne, 1976; Ravenholt and Foege, 1982; Takahashi and Yamada, 1999).

A main and reproducible pathophysiological hallmark of PD in patient and animal models is the presence of microglial activation, that occurs in response to neuronal damage (Cicchetti et al., 2002; Depino et al., 2003; Hirsch et al., 2003; Hirsch and Hunot, 2009; Hunot et al., 1999; McGeer et al., 1988; Mogi and Nagatsu, 1999; Orr et al., 2002; Vila et al., 2001; Whitton, 2007). Animal models of PD using 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), 6-Hydroxydopamine (6-OHDA), and rotenone, induces microglial activation and pro-inflammatory cytokine production (Arai et al., 2006; Block and Hong, 2005; Hald and Lotharius, 2005; Herrera et al., 2005; Kim and de Vellis, 2005; Minghetti, 2005; Sawada et al., 2006; Vila et al., 2001). Microglial cells can be primed but systemic infections or inflammation can switch their phenotype to release neurotoxic molecules that causes exacerbation of neurodegeneration and symptoms, rather than being involved in the etiology of the disease (Perry et al., 2007; Qin et al., 2007). An exacerbating effect of IL-1β on the on-going neurodegeneration in the SN after striatal administration of 6-OHDA was described (Godoy et al., 2008). Thus, pro-inflammatory cytokines could contribute to the exacerbation of disease progression and/or its etiology. However, the mechanisms and the components of inflammation that act on the disease are still unknown. In particular, IL-1β has been shown to be involved in disease progression and/or as trigger of neurodegeneration in the SN if it was expressed chronically in the SN (Ferrari et al., 2006; Godoy et al., 2008).

Anti-inflammatory treatments appear to have neuroprotective effects on models of PD, reducing dopaminergic neuronal loss (Gao et al., 2003a,b). In the SN it has been described that inhibition of microglia activation, reduce the dopaminergic neuronal loss (Castano et al., 2002; Kurkowska-Jastrzebska et al., 2004). The anti-inflammatory steroid dexamethasone (DXM) acts on the inflammatory process regulating the transcription of many inflammatory molecules (Arimoto and Bing, 2003; Godoy et al., 2008; Kurkowska-Jastrzebska et al., 2004; Unlap and Jope, 1995). However, these data are controversial because there are evidences demonstrating that DXM fail to protect dopaminergic neurons in an MPTP model (Aubin, 1998). Minocycline, a tetracycline derivate, with anti-inflammatory and anti-apoptotic properties also exhibited neuroprotective effects in MPTP, 6-OHDA and LPS models of nigral neurodegeneration (Du et al., 2001; He et al., 2001; Wu et al., 2002). Moreover, the administration of an IL-1β neutralizing

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antibody has neuroprotective effects on LPS induced neurodegeneration (Arai et al., 2006).

The aim of this work was to study the effect of a unique component of the inflammatory process, IL-1β, in the nigrostriatal system, and evaluate its involvement in triggering neurodegeneration in the SN and/or exacerbating disease. Taking advantage of the capacity of adenoviral vectors to express transgenes retrogradelly, we injected adenovectors expressing IL-1b in the striatum and analyzed the effects of this cytokine in the SN. Further, we analysed the effects of anti-inflammatory drugs and systemic inflammation on the phenomena induced by the chronic expression of IL-1β. We found that central IL-1β could produce a progressive neurodegeneration in the SN. This effect was accompanied by motor symptoms, showing a possible role of chronic IL-1β expression on triggering nigral neurodegeneration. Importantly, systemic IL-1β expression could exacerbate central IL-1β effects, indicating that peripheral inflammation could induce the exacerbation of nigral neurodegeneration. Finally, anti-inflammatory DXM treatment could abolish all IL-1β mediated effects, showing a potent inhibitory effect on the IL-1β mediated neurodegenerative process.

2. Materials and methods

2.1. Vectors

We used replication-deficient adenoviral vectors expressing human IL-1β and βGal as control. Adenoviral vectors were generated, quality controlled and used as already described (Ferrari et al., 2004; Kolb et al., 2001).

2.2. Animals

Adult male Wistar rats (250 g–300 g), housed under controlled temperature (22 °C ± 2 °C), and artificially lit under a 12 h cycle period and with water and food *ad libitum*. All animal procedures were performed according to the regulations for the use of laboratory animals of the National Institute of Health, USA. Animal experiments were approved by the IACUC of the Institute Leloir Foundation.

2.3. Injections

For central injections, the animals were anesthetized with ketamine chlorhydrate (80 mg/kg) and xylazine (8 mg/kg). The adenoviruses were administered with a 50 μm tipped finely-drawn glass capillary, stereotactically implanted in the left striatum (bregma, −1 mm; lateral, + 3 mm; ventral, −4.5 mm) (Paxinos and Watson, 1986). Coordinates chosen are similar to those used for 6-OHDA inoculation in a model of progressive neurodegeneration widely used (Sauer and Oertel, 1994). Injections of 1 μl of adenoviral vectors or vehicle were infused over 5 min and kept in place for additional 2 min before removal. All surgery procedures took place during the morning to avoid effects of circadian variations in cytokines expression. Human IL-1β and β-galactosidase expressing adenoviral vectors were diluted in sterile 10 mM Tris–HCl, 1 mM $MgCl_2$ (pH = 7.8) and administered at a dose of 5.10^7 viral particles/rat. The animals were killed at 14, 21 and 28 days post-surgery.

Anti inflammatory treatment was performed with dexamethasone (DXM). The DXM group received a daily intraperitoneal dose of DXM (10^{-8} M, Bruber Lab, Argentina) beginning just after Ad IL-1β or Adβgal central injection.

2.4. Cylinder test

Forelimb akinesia was assessed using the test previously described (Schallert and Jones, 1993) ($n = 5$–9 per group). This test evaluates the use of the forelimb to support the body against the walls of a cylinder. The rats were put individually in an acrylic cylinder (20 cm × 30 cm). The test was performed between 16:00 h and 19:00 h. The number of wall contacts performed independently with the left and the right forepaw were counted.

2.5. Histology

The animals ($n = 3$–6 per group) were deeply anesthetized and transcardially perfused with heparinized saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH = 7.2). After dissecting the brains, they were placed in the same fixative overnight at 4 °C. Then, the tissues were cryoprotected by immersion in 30% sucrose, frozen in isopentane and serially sectioned in a cryostat (40 μm). The 40 μm sections were used either for cresyl violet staining or for free floating immunohistochemistry.

2.6. Immunohistochemistry

Free-floating sections were incubated in blocking buffer (1% donkey serum, 0.1% Triton in 0.1 M PB), and incubated overnight with primary antibodies. The antibodies used were: anti Tyrosine hydroxylase (TH) for dopaminergic neurons (diluted 1:1000; Chemicon, Temecula, CA) anti ratIL-1ß (1:300; NIBSC, Potters Bar, UK), ED1 (1:200; for macrophages with phagocytic activity; Serotec, Raleigh, NC), anti glial fibrillary acidic protein (GFAP) (1:700; for astrocytes; Dako, Carpinteria, CA). Alternatively, we used MHC II (class II major histocompatibility complex (Serotec, Raleigh, NC) and the biotinylated lectin *Griffonia simplicifolia* ($GSA\text{-}1B_4$, 1:50; Vector Laboratories, Burlingame, CA), for transforming microglial cells (Kaur and Ling, 1991). For immunohistochemical identification of dopaminergic neurons, the sections were incubated with donkey anti rabbit biotin conjugated antibody (Jackson, ImmunoResearch Laboratories Inc., West Grove, PA) followed by Vectastain standard ABC kit (Vector Laboratories, Burlingame, CA) and developed with 3.3′ diaminobenzidine (Sigma, Saint Louis, Missouri). For double labelling immunohistochemistry, the sections were incubated with either indocarbocyanine Cy3 (Cy3) conjugated donkey anti mouse antibody (1:250; Jackson ImmunoResearch Laboratories Inc, West Grove, PA), cyanine Cy2 (Cy2) conjugated donkey anti rabbit antibody (1:250; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) or Cy2 conjugated streptavidin (1:250; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) Digital images were collected in a Zeiss LSM Pascal laser scanning confocal microscope equipped with a krypton–argon laser.

Neutrophils were identified by their characteristic segmented nuclear morphology in Cresyl violet staining as described before (Ferrari et al., 2004, 2006).

2.7. Quantification of TH positive cells

Total number of TH positive cells were counted through the SN at 20× magnification. Every sixth 40 μm thick section was counted. The graph shows the ratio between the TH+ cells in the ipsilateral side related to contralateral side. The sections were counted twice using double-blind procedure.

2.8. Classification of microglial activation

We classified the microglia activation according to Kreutzberg (1996)) and as previously described (Depino et al., 2003; Ferrari et al., 2006).

Stage 1 Resting microglia stage. Rod shaped soma with fine and ramified processes.

Stage 2 Activated ramified microglia. Elongated shaped cell body with long and thicker processes.

Stage 3 Amoeboid microglia. Round shaped body with short, thick and stout processes.
Stage 4 Phagocyte cells. Round shaped cells with vacuolated cytoplasm, no processes can be observed at light microscopy level.

All these cellular types are GSA positive (GSA+), but the stage 4 can also be ED1 positive (ED1+). MHC-II stained all the activation stages but not resting microglia.

2.9. Measurement of IL-1β and other proinflammatory cytokines

The animals ($n = 5$–6 per group) were decapitated and their brains were quickly removed and both the injected and non-injected striatum and the SN were dissected, snap-frozen in liquid nitrogen and stored at −80 °C. Tissue was processed as described previously (Ferrari et al., 2006). Commercially available rat IL-1β kit (R&D, Minneapolis, MN) were used according to the manufacture instructions. The sensitivity of these kits was 65 pg/ml for rIL-1β.

2.10. Systemic stimulus-Adenovirus expressing IL-1β

The adenoviruses expressing IL-1β or βgalactosidase were injected intravenously (i.v) at doses of $1.4.10^9$ particles/μl. The adenovectors were diluted in 300 ml of sterile 10 mM Tris–HCl, 1 mM MgCl2n (pH = 7.8) followed by 300 ml of saline solution. The adenoviruses were administrated in the periphery at day 17 after the injection of the adenovirus in the striatum to achieve its maximum systemic expression at 21 days after central injection. The animals were perfused 28 days after the central injection. The injections were performed during 2 p.m. to 4 p.m. Peripheral treatment was checked by counting the number of leukocyte in blood at day zero and day 4 after the i.v. injections.

2.11. Statistical analysis

Results are expressed as mean ± SEM in the different treatment groups.

All experiments were analyzed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) as post-hoc test, except for Fig. 6A, B and I where two tail unpaired *T* test was performed. To make sure parametric statistical analysis could be carried out, all variables were tested for normality and variance homogeneity with Kolmogorov–Smirnov and Levene test, respectively.

The level of statistical significance was set at $p<0.05$. For clarity, statistical analyses of each test are addressed in each figure. All statistical tests were performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, Illinois).

3. Results

3.1. IL-1β expression in the nigroestriatal system

We exploited the property of adenoviral vectors to allow transgene expression to target brain regions retrogradelly connected from the injection site. By X-gal staining after Adβgal injection in the striatum, we could detect transgene expression all along the SN (around 1.72 mm along the rostro-caudal axis) (data not shown).

The administration of 5.10^7 adenoviral particles/rat striatum induced an endogenous expression of rat (r)IL-1β in both the striatum and the SN. The striatal expression of rIL-1 started at 14 days (76.70 pgIL-1β/mg protein), exhibited a peak at 21 days post injection (125 pgIL-1β/mg protein, but decreased after 28 days (68.49 pgIL-1β/mg protein) (Fig. 1A). The SN showed a peak of IL-1β expression at 21 days post injection (78.04 pgIL-1β/mg protein) that decreased to 35.57 pgIL-1β/mg protein 28 days after the injection (Fig. 1B). Statistical differences

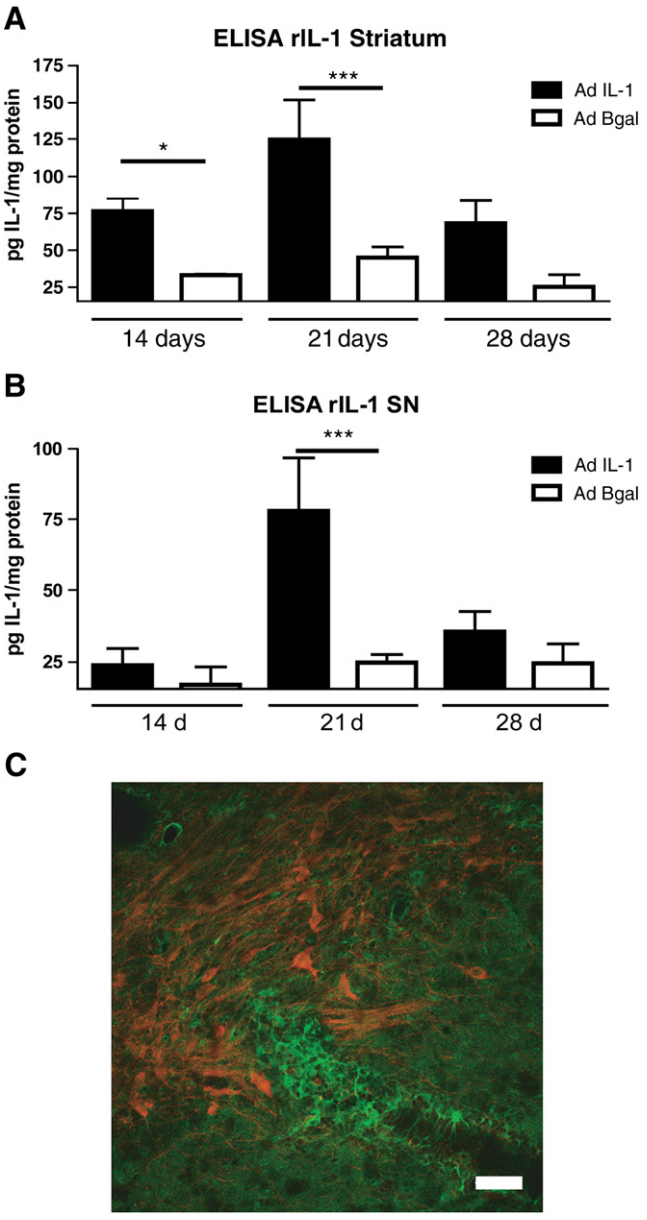


Fig. 1. IL-1β expression in the nigrostriatal system. A. Time course of IL-1β expression in the striatum at 14, 21 and 28 days after the adenovector injection. Two-way ANOVA was followed by LSD post-test. *$p<0.05$ AdIL-1 compared to Adβgal at 14 and 28 days. ***$p<0.001$ AdIL-1 compared to Adβgal at 21 days. Error bars represent SEM. B. IL-1β expression in the SN at 14, 21 and 28 days after adenovector injection. Two-way ANOVA was followed by LSD post-test. ***$p<0.001$ AdIL-1 compared to Adβgal at 21 days. Error bars represent SEM. ($N = 5$–6 per group). C. Immunofluorescence against rIL-1β (green) in the SN. The cells exhibited ramified morphology and numerous processes IL-1β+ were also observed. TH+ cells in red. Scale bar: 50 μm.

in IL-1β expression in the SN were only observed at 21 days post injection. Adβgal-injected animals had basal, non induced IL-1β expression at all time points studied after the adenovirus injection either in striatum or SN (Fig. 1A–B).

Immunohistochemical analysis confirmed the presence of ramified rIL-1β positive cells (rIL-1β +) in the SN of injected animals only at 21 days (Fig. 1C).

3.2. Partial and progressive dopaminergic cell loss in the SN after chronic IL-1β expression in the striatum

The retrograde activity exhibited by the adenovirus expressing IL-1β in the striatum induced also a chronic expression of IL-1β in the SN. This

IL-1β expression in the SN induced a progressive loss of dopaminergic tyrosine hydroxylase-positive (TH +) cells in the ipsilateral SN in comparison with control animals injected with the adenovector expressing β-galactosidase (Fig. 2A). No statistical differences in TH cell number were detected at 14 days with respect to animals injected with Adβgal. However, the number of TH + cells decreased 29.5% with respect to the contralateral hemisphere at 21 days after AdIL-1 inoculation ($p<0.001$ when compared AdIL-1β vs. Adβgal at 21 and 28 days post injection). The neurodegenerative effect of IL-1β expression in the SN was maintained till the last day of analysis (28 days post AdIL-1 injection) (Fig. 2A).

3.3. Motor behaviour deficits after chronic IL-1β expression

We used the cylinder test to assess forelimb akinesia induced by the inflammatory stimulus in the striatum as it was previously described (Schallert and Jones, 1993). The chronic expression of IL-1β in the SN decreased wall contacts at 21 and 28 days after the AdIL-1 injection ($p<0.01$ compared AdIL-1 between left and right paws at 21 and 28 days after adenovector injection) (Fig. 2B). No motor abnormality can be observed at 14 days after the injection. No statistical differences can be observed in the animals injected with the control adenovirus (Fig. 2B).

3.4. Inflammatory infiltrate in the SN after chronic IL-1β expression

The inflammatory processes that occurred in the striatum as a result of the chronic expression of IL-1β was similar to the one described previously after chronic expression of IL-1β in other area of the striatum (Ferrari et al., 2004). The retrograde expression after striatal adenovirus administration induced inflammatory infiltrate that was almost exclusively located in the SN. Occasionally, some animals exhibited vasodilatation with macrophages in the ventral tegmental area (VTA). At 14 days after the injection of the adenovirus, no evident inflammatory infiltrate could be observed, but 21 days after the injection, the SN exhibited an extensive inflammatory area (Fig. 2C). Most of the inflammatory infiltrated were composed of macrophages. The infiltrate can be observed either in the blood vessels or in the parenchyma (Fig. 2C). Blood vessels were vasodilatated and filled with marginated macrophages and scarce neutrophils. At 28 days after the injection, the inflammation was resolved and scarce inflammatory infiltrate and vasodilatation could be observed (Fig. 2D).

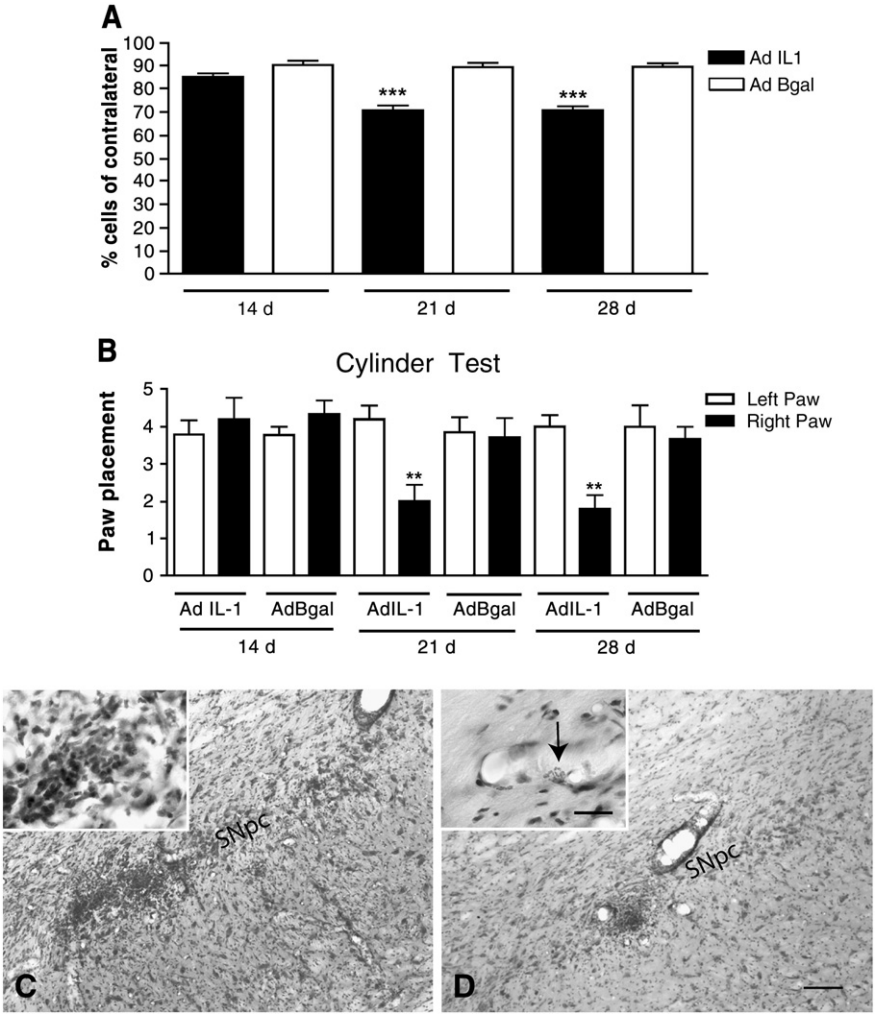


Fig. 2. A. Percentage of dopaminergic cells loss in the SN at different time points after the injection of the adenovirus expressing IL-1β in the striatum. Quantitation of TH positive cells as a percentage of cells in the ipsilateral hemisphere vs. the contralateral one. Two-way ANOVA was followed by LSD post-test. ***$p<0.001$ AdIL-1 compared to the corresponding vehicle at 21 and 28 days. B. Motor behavioural deficits after the injection of IL-1β expressing adenovirus evidenced by cylinder test. The number of first paw placement on the surface of a cylinder was counted for each experimental group. Three-way ANOVA was followed by LSD post-test. **$p<0.01$ when comparing AdIL-1 between left and right paws at 21 and 28 days after adenovector injection. C–D. Representative pictures showing the inflammation in the SN either 21 days after the injection (C) or 28 days post injection (D) staining with Cresyl violet. C. The inflammatory infiltrate were observed through the whole SN and vasodilatation is also evident 21 days post injection. Inset. Higher magnification showing a great amount of macrophages within the lesion. D. At 28 days after the injection scarce inflammatory infiltrate was distributed only in few sections. Inset. Higher magnification showing the presence of scarce neutrophils (arrow) within the blood vessels. Scale bar: 100 μm. Insets. 25 μm.

As expected, no inflammatory infiltrate could be observed with the Adβgal at any time point studied (data not shown).

3.5. Glial activation in the SN

Microglial activation in the SN was evident from day 14 to 28 after the injection of the AdIL-1 in the striatum. At 14 days after injection, most GSA+ cells showed stages 2 and 3 activation (data not shown). However, at 21 days after the injection, numerous GSA+ cells at stage 4 of activation could be observed in the SN surrounding TH+ cells. Some GSA+ cells at stages 2 and 3 of activation could also be observed near the SN (Fig. 3B). This activation decreased at 28 days after the injection when GSA+ cells at stages 2 and 3 of activation were located within the SN. No cells at stage 4 could be observed at this time point

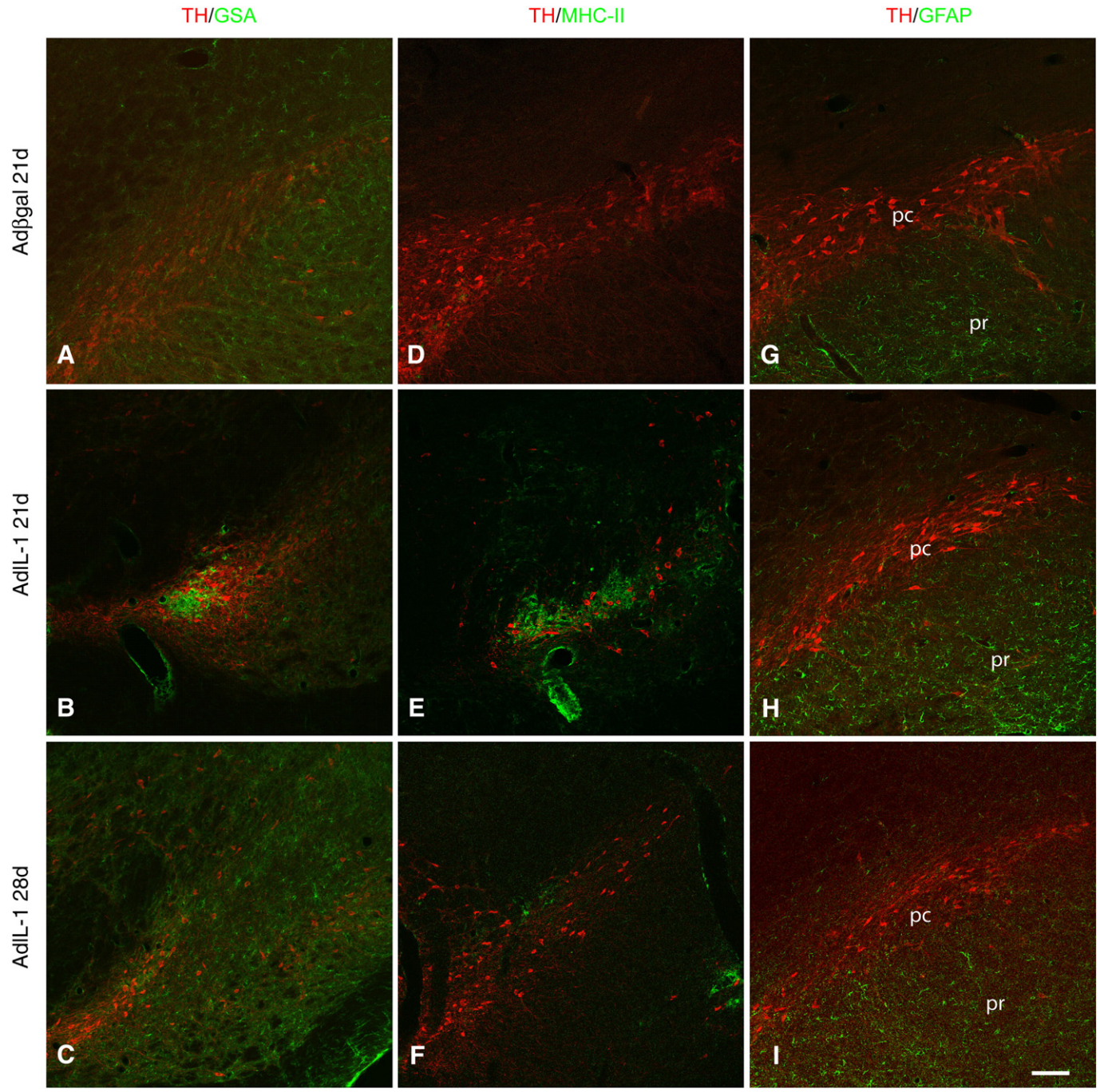


Fig. 3. Glial activation in the SN after the injection of the AdIl-1 in the striatum. A–C. Activation of microglial cells demonstrated by GSA staining. A. The animals injected with the Adβgal showed no GSA label (green) at 21 days after the injection. B. 21 days after the injection of the adenovirus expressing IL-1β, most of the GSA+ cells were stage 4 and can be observed within the SN as demonstrated by TH+ cells (red). C. The microglial activation decreased after 28 days, were GSA+ cells were at stages 2–3. D–F. Expression of the major histocompatibility complex class two (MHC-II) (green) in the SN, identified by TH+ cells (red). D. The animals injected with the adenovirus control showed no MHC-II expression at 21 days after the injection. E. 21 days after the injection of AdIL-1 numerous MHC-II+ cells were distributed along the SN. These MHC-II + cells were stage 4, however, some cells at stages 2 and 3 can also be observed. F. 28 days after the adenovector injection the number of MHC-II cells was decreased and most of label cells were at stages 2 and 3. G–I. Astroglial activation in the SN after the injection of AdIL-1 in the striatum. G. GFAP label (green) can only be observed in the SN *pars reticulata (pr)* when the animals were injected with the control adenovector. H. The SN of the animals injected with the AdIL-1, the GFAP+ label were located in both SN *pars reticulate(pr)* y SN *pars compacta* (*pc*) after 21 days. I. However, the GFAP label remains only in the SN pars reticulate after 28 days. Scale bar: 100 μm.

(Fig. 3C). The animals injected with the adenovector control expressing b-galactosidase exhibited scarce microglia activation; only microglia at stages 2–3 could be observed in one or two section, especially at 14 and 21 days after the injection (Fig. 3A).

MHC-II expression was increased at 21 days after the injection of the adenovirus in the striatum. The MHC-II label was distributed along the SN *pars compacta*. Most of the MHC-II positive cells were at stage 4. However, some cells at stages 2 and 3 could also be observed (Fig. 3E). 28 days after AdIL-1 injection, the MHCII label decreased and some stages 2–3 cells were observed in the SN (Fig. 3F). On the contrary, no MHCII+ cells were seen in the control group at any time point studied (Fig. 4D).

Astroglial activation could also be observed at all time points. GFAP label could be detected in ramified and stellate cells and in stout processes of astroglial cells. At 21 days after the AdIL-1 injection, the GFAP+ cells were located in both the SN *pars compacta* and SN *pars reticulata* (Fig. 3H). However, at 28 days after AdIL-1 injection, the GFAP expression was mainly distributed along the SN *pars reticulata* (Fig. 3I).

3.6. IL-1β expression in the nigrostriatal system decreased after dexamethasone (DXM) treatment

Animals were daily treated with DXM, starting immediately after the central AdIL-1 injection.

The expression of rIL-1β in the SN dramatically decreased after the treatment with DXM in comparison with non-treated animals. The IL-1β content in the SN decreased from 78.04 pg/mg prot or 35.57 pg/mg prot to non-detectable levels either at 21 days ($p<0.001$) or at 28 days ($p<0.05$) after DXM treated animals as determined by ELISA, respectively (Suppl Fig. 1). Animals injected with Adβgal showed no detectable levels of basal or induced IL-1β when they were treated with DXM (Suppl Fig. 1). Therefore, the anti-inflammatory treatment prevented the rIL-1β expression in SN.

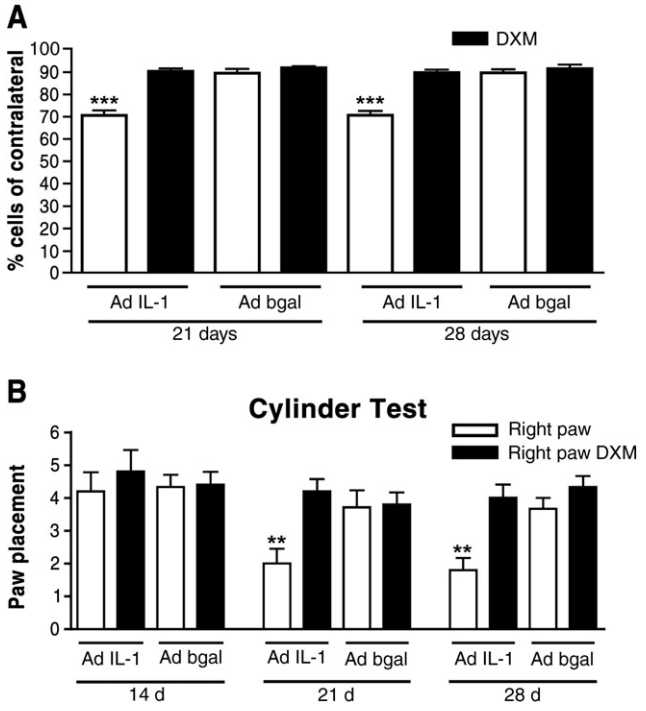


Fig. 4. A. Percentage of dopaminergic cell loss after the treatment with DXM. DXM prevent the neurodegeneration in the SN at both 21 and 28 days. Three way ANOVA followed by LSD post-test was performed. ***$p<0.001$ significant difference between AdIL-1+DXM and AdIL-1−DXM at each time point. ($N=3$–5 per group) B. Motor behavioral deficits after the treatment with DXM in the animals injected with AdIL-1β in the striatum. Performance in the cylinder test after treatment with or without DXM. The absolute number of wall contacts performed with right (contralateral) paw is shown. Three way ANOVA followed by LSD post-test was performed. **$p<0.01$ significant difference between AdIL-1+DXM vs AdIL-1−DXM at each time point. ($N=5$ per group).

3.7. DXM treatment prevented the dopaminergic cell loss in the SN after chronic expression of IL-1 in the striatum

The animals injected with AdIL-1 in the striatum and treated with DXM during 21 or 28 days exhibited similar percentage of dopaminergic cells in the SN compared with those injected with the Adβgal. On the contrary, at 21 days after the injection of the AdIL-1β, the number of dopaminergic cells was 70.50% in non-DXM treated animals. DXM treatment increased the cell number of TH positive cells to 90.20% in this group ($p<0.001$) (Fig. 4A). No differences can be observed in the animals injected with Adβgal. Similar protective effects on survival of dopaminergic cells could be observed at 28 days after central injection when the number of dopaminergic cells was still significantly increased in DXM-treated animals compared to the non-treated group (88.67% to 70.67%, DXM to non-DXM treated animals respectively ($p<0.001$) (Fig. 4A).

3.8. DXM treatment prevented the motor behaviour deficits after chronic IL-1β expression

We further characterized the effects of DXM treatment with the cylinder test to evaluate forelimb akinesia. The chronic expression of IL-1β in the striatum statistically decreased the wall contacts 21 and 28 days but not 14 days after adenoviral (Fig. 4B). This effect was prevented in animals injected with AdIL-1 for 21 and 28 days and treated with DXM for 21 days ($p<0.01$) or 28 days ($p<0.01$). Rats injected with Adβgal showed no statistical differences in the number of paw placement at any time point studied (Fig. 4B).

3.9. DXM treatment diminished microglia activation

Animals injected with the control adenovirus did not show microglial activation at 21 days post injection (Fig. 5A). DXM dramatically reduces the GSA label in the SN 21 days after the injection of the adenovirus expressing IL-1β in the striatum (Fig. 5B). There were no stage 4 GSA+ cells in the SN at this time point. Most of the microglial cells were stages 2–3 (Fig. 5B). At 28 days, no microglial morphological activation (stage 1) could be observed in the DXM-treated animals (Fig. 5C). As it was demonstrated using GSA, no MHC expression could be observed 21 days after the injection with Adβgal (Fig. 5D). Animals injected with AdIL-1b and treated with DXM exhibited MHC-II+ ramified cells at stages 1 and 2 at 21 day (Fig. 5E), and this label disappeared after 28 days (Fig. 5F).

3.10. The systemic and sustained expression of IL-1β after AdIL-1 administration i.v. increased neurodegeneration in the SN

We have expressed IL-1β chronically in the periphery after central administration of the adenovirus expressing IL-1β. Previous results have demonstrated that the peak of the AdIL-1 expression was achieved between 3 and 5 days after the injection (Suppl fig. 2). Therefore, we injected the AdIL-1 i.v. at day 17 in order to get the maximum peripheral expression at day 21, the day when neurodegeneration and motor symptoms start in this model.

Chronic, systemic IL-1β expression exacerbated the neurodegeneration induced by IL-1β expression in the SN at 28 days after the central injection. The percentage of dopaminergic cells loss decreased to 57.40% compared to the animals peripherically injected with the control adenovirus ($p<0.001$) (Fig. 6A).

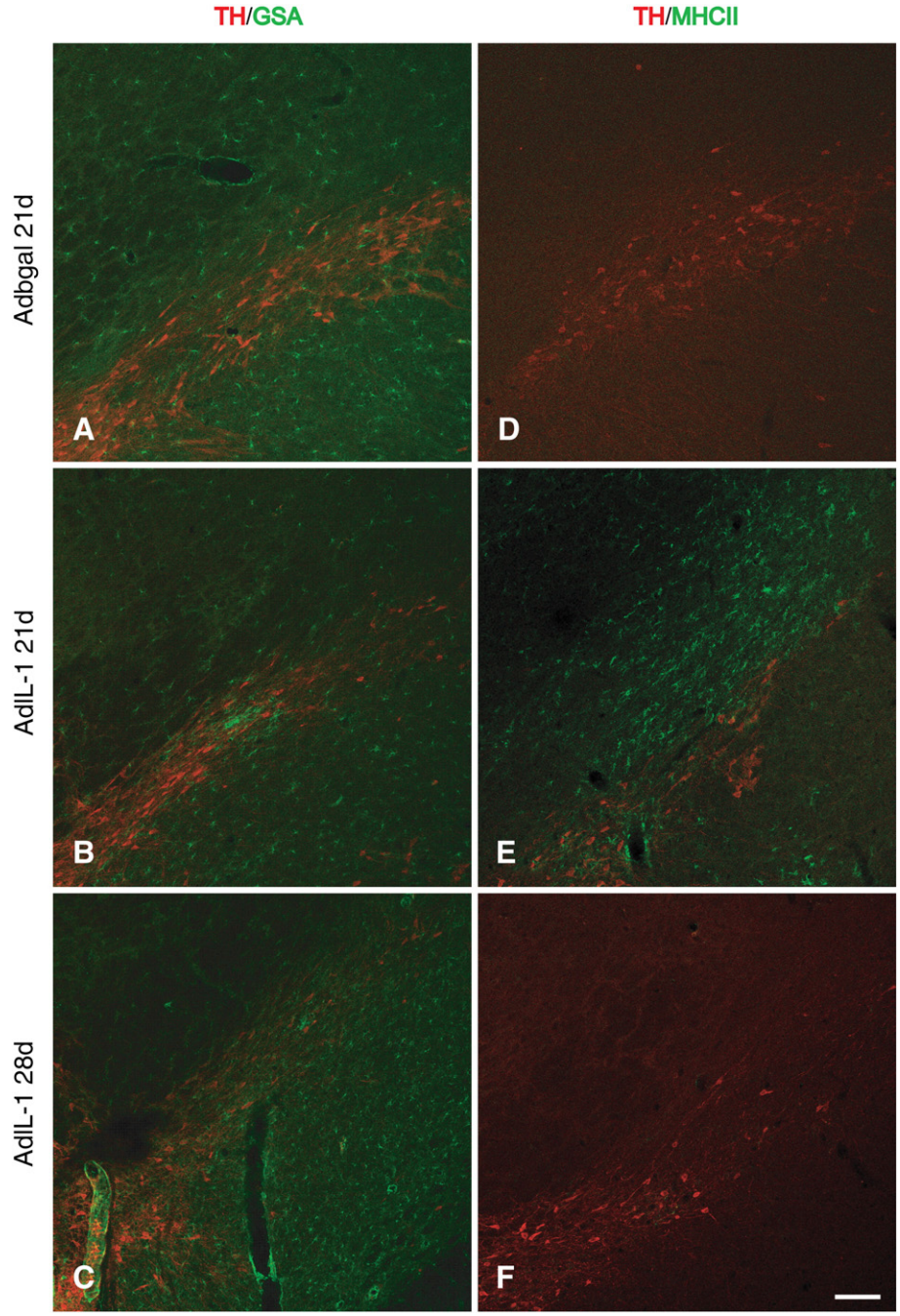


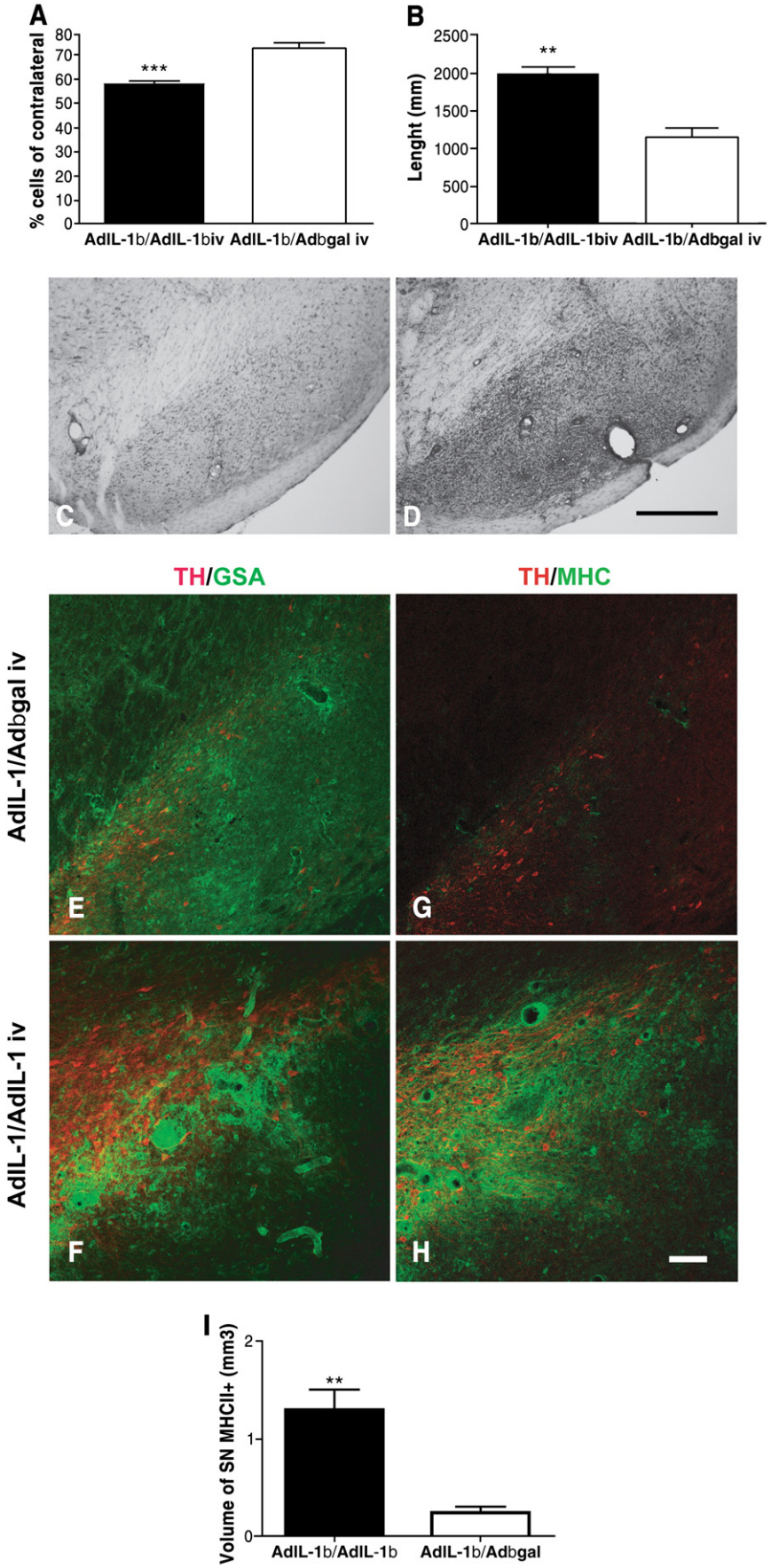
Fig. 5. Microglia activation in the anti-inflammatory treatment with DXM. A–C. Activation of microglia demonstrated by GSA. No microglia activation can be observed in Adβgal treated animals (A). No GSA+ cells at stage 4 were observed in the animals injected with the AdIL-1 with DXM treatment (B) No microglia activation can be observed after 28 days after AdIL-1 injection and treated with DXM (C). D–F. MHC-II expression after the anti-inflammatory treatment. MHC-II+ cells at stages 1 and 2 can be observed at 21 days after the AdIL-1 injection and the DXM treatment (E). No MHC-II + cells were observed either after 21 days of Adβgal injection (D) or 28 days after the AdIL-1 treated with DXM (F). Scale bar: 100 μm.

3.11. The systemic and sustained expression of IL-1β increased the inflammatory infiltrate in the SN

Chronic IL-1β expression dramatically increased the inflammatory response in the SN at 28 days after the central injection ($p<0.01$) (Fig. 6B). This response is much more extensive than that observed at 28 days after central injection of the adenovirus with no peripheral injection (Fig. 6 B, C, D) (length of the lesion is 1.96 mm when treated with peripheral AdIL-1 compared to 1.18 mm in control animals). The inflammatory infiltrate was distributed along the whole SN and it was composed mostly of macrophages. Vasodilatation and the presence of apoptotic bodies was also evident (Fig. 6D). The animals injected with the Adβgal exhibited a similar inflammatory response to that described for the AdIL-1β centrally injected. In this case, the inflammatory infiltrate was observed in 1 or 2 sections, where mostly macrophages could be observed (Fig. 6C).

3.12. The systemic and sustained expression of IL-1β increased microglial activation

The number of GSA+ cells in the SN dramatically increased in animals injected with AdIL-1β centrally and AdIL-1β peripherally (Fig. 6F). Most of these cells were at stage 4, however cells at stages 2 and 3 were observed in SN and surrounding tissue. Animals


A
% cells of contralateral
80
70
60
50
40
30
20
10
0
***
AdIL-1b/AdIL-1biv
AdIL-1b/Adbgal iv
B
Lenght (mm)
2500
2000
1500
1000
500
0
**
AdIL-1b/AdIL-1biv
AdIL-1b/Adbgal iv
C
D
TH/GSA
TH/MHC
AdIL-1/Adbgal iv
AdIL-1/AdIL-1 iv
E
G
F
H
I
Volume of SN MHCII+ (mm3)
2
1
0
**
AdIL-1b/AdIL-1b
AdIL-1b/Adbgal

peripherally injected with control adenovector showed microglia activation evidenced as the presence of GSA+ cells (Fig. 6E). This activation is much lower than that exhibited in AdIL-1b injected. However, some cells at stage 4 were observed, but most of the sections have stages 2–3 cells (Fig. 6E). The volume of MHC-II positive cells was statistically increased in animals with peripheral AdIL-1β compared to those animals injected with the Adβgal (Fig. 6G–H, I) (1.3 mm^3 in AdIL-1 treated animals and 0.25 mm^3 in Bgal injected animals). Most of these cells were at stage 4 located in the SN, but some MHC-II positive cells at stages 2–3 can also be observed specially in regions that surrounded the SN (Fig. 6G). Animals that received control adenovirus as peripheral stimulus showed scarce MHCII staining (Fig. 6H).

4. Discussion

The present study demonstrated that the chronic expression of IL-1β in the SN induced dopaminergic cell loss and motor dysfunctions, which was impaired by an anti-inflammatory drug and exacerbated by a systemic and sustained expression of IL-1β. These results confirmed previous studies that sustained IL-1β expression is capable of triggering neurodegeneration in the SN (Ferrari et al., 2006). The advantage of this model is that the side effect of the surgery and the adenoviral antigenic load was skipped. This is a main advantage, considering that the SN is very sensitive to inflammation and mechanical injury, which could induce unnecessary noise in the analysis. We also demonstrated that the toxic effect of IL-1β on the SN can be inhibited by using a potent anti-inflammatory agent, DXM. Moreover, we also showed that systemic inflammation triggered by IL-1β can exacerbate the neurodegenerative effect induced by the expression of a unique proinflammatory cytokine, confirming the results obtained previously in a 6-OHDA model (Godoy et al., 2008).

The first evidence of the role of inflammation in PD has been described by McGeer et al. (1988, 2003) who demonstrated evidence of microglial activation in PD patients. Elevated levels of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), IL-1β and IL-6 in the striatum and cerebrospinal fluid where found in PD patients (Mogi et al., 1994; Muller et al., 1998; Nagatsu et al., 2000; Reale et al., 2009) Activated microglia and pro-inflammatory cytokine expression was also found in animal models of PD (Cicchetti et al., 2002; Frank-Cannon et al., 2009; Gao et al., 2002; Kim and Joh, 2006; Long-Smith et al., 2009; Nagatsu et al., 2000; Nagatsu and Sawada, 2005; Orr et al., 2002; Schwab et al., 2009; Tansey and Goldberg, 2009). Pro-inflammatory cytokine expression has been associated with the etiology and both detrimental or beneficial effects in PD patients and animal models of PD leading to controversy (Choi et al., 2009; Gao et al., 2003a; Hirsch et al., 2003; Hirsch and Hunot, 2009; Hohlfeld et al., 2007; McGeer and McGeer, 2008; McGeer et al., 2002; Saura et al., 2003; Teismann and Schulz, 2004; Vazquez-Claverie et al., 2009). However, these apparently contradictory results are always to be expected from pleiotropic and context-dependent molecules such as cytokines. In this regard, it is relevant to find rules that govern unidirectional effects of a given cytokine in PD. Although a wealth of data has been accumulated regarding the functional role of cytokine production in PD, the rules that govern their effects are still unclear.

Concerning aetiology, systemic infections have been suggested as etiological agents of PD (Arai et al., 2006; Epp and Mravec, 2006; Isgreen et al., 1976; Jang et al., 2009; Marttila and Rinne, 1976; Ravenholt and Foege, 1982; Takahashi and Yamada, 1999). The acute injection of a single cytokine such as IL-1β in the SN did not induce neurodegeneration (Castano et al., 2002; Depino et al., 2003). Here we described that long term expression of IL-1β in the SN without additional injury or antigenic load can trigger dopaminergic cells loss when expressed in the SN. The levels of IL-1β expression achieved were within the lower range that produces an inflammatory response when injected in the periphery in rodents and humans (Dinarello, 1997). Thus, our and previous evidence strongly suggest that low levels of IL-1β can elicit neurodegeneration provided that its expression is sustained for several days (Ferrari et al., 2006). A similar conclusion could be drawn from studies performed with TNF-α, another major pro-inflammatory cytokine (De Lella Ezcurra et al., 2009). In contrast, regional differences were described concerning neuronal susceptibility, because chronic IL- β expression either in the striatum or the hippocampus did not induce neurodegeneration in spite of neutrophil infiltration (Ferrari et al., 2004; Shaftel et al., 2007). Thus, we provide evidence that IL-1β expression has to be sustained in time to provoke nigral neurodegeneration and motor symptoms. This observation suggests that sustained IL-1β expression might be considered a risk factor for PD initiation and highlights the susceptibility of the SN to inflammatory stimuli.

In terms of disease progression, toxic and beneficial effects of pro-inflammatory cytokines in the nigrostriatal system have also been observed (Arai et al., 2006; Hirsch and Hunot, 2009; Hirsch et al., 2005; McGeer and McGeer, 2007; Tansey and Goldberg, 2009; Whitton, 2007). Using a new model of neurodegeneration, we confirmed previous work that show that systemic, sustained IL-1β expression can exacerbate disease progression (Godoy et al., 2008). Thus, we provide new evidence that, once the neurodegenerative process has started, systemic inflammation can exacerbate disease progression. Under this view, systemic and sustained inflammation could be regarded as a risk factor for the exacerbation of PD progression. These observations could be extended to other neurodegenerative diseases such as Alzheimer and prion diseases as Perry and colleagues have initially suggested (Perry, 2004; Perry et al., 2007; Qin et al., 2007). In addition, it has been also demonstrated that acute systemic inflammation elicited before or simultaneously to central injuries worsened CNS damage, suggesting that inflammatory stimuli could primed the CNS to increase the toxicity of a second stimuli (Koprich et al., 2008; McColl et al., 2007, 2008; Spencer et al., 2007).

The long term expression of IL-1β in the SN induced strong microglial activation and this activation is previous to statistically significant neurodegeneration. Therefore, temporally, the sustained expression of IL-1β expression induced microglia activation and these events exerted neurodegeneration in the SN. MHC-II increased expression and stage 4 of activation correlated with toxic IL-1β effects that were reverted by DXM treatment. These observations

Fig. 6. Effect of peripheral inflammatory (Ad IL-1b i.v.) stimulus over the ongoing neurodegeneration. A. Dopaminergic cell loss in the SN after a peripheral stimulus. Two tail unpaired *T* test was performed. ***$p<0.001$ AdIL-1/AdIL-1 iv compared to AdIL-1/Adβgal iv. B. Length of the inflammatory infiltrate in the SN. The animals injected with AdIL-1/AdIL-1 i.v. exhibited a statistical increase in the length of the inflammatory response when compared to AdIL-1/Adβgal i.v (two tail unpaired *T* test was performed. **$p<0.01$ comparing AdIL-1/AdIL-1 iv vs AdIL-1/Adβgal iv.). C–D. Inflammatory infiltrate in animals treated with peripheral stimulus visualized by Cresyl violet staining. C. Animals with Adβgal as peripheral stimulus exhibited less inflammatory infiltrate in the SN. D. Animals that received AdIL-1 as peripheral stimulus exhibited an extensive inflammatory region characterized by inflammatory infiltrate (mostly composed of macrophages) and vasodilatation after 28 days. Scale bar: 500 μm. E–H. Glial activation after peripheral inflammation. E–F. Microglia activation in the SN of animals treated with Adβgal peripheral stimulus evidenced with GSA. E. The animals injected with the Adβgal as peripheral stimulus showed microglia activation evidenced as GSA+ cells (green), with cells at stages 3 and 2. Some cells at stage 4 can also be observed. F. The animals injected with the AdIL-1 peripherally exhibited an increased of microglial activation, with most of the cells at stage 4. G–H. Expression of MHC-II (green) in the SN of animals treated with peripheral stimulus. G. The animals that received Adβgal as peripheral stimulus did not express MHC-II in the SN. H. SN of animals treated AdIL-1 as peripheral stimulus exhibited a massive expression of MHC-II, where most of the cells were at stage 4. In addition, cells at stages 3 and 2 can be observed surrounded the SN. Scale bar: 100 μm. I. Volume of MHC-II+ cells in animals treated either with AdIL-1 or Adβgal as peripheral stimulus. Two tail unpaired *T* test was performed **$p<0.01$ AdIL-1/AdIL-1 iv compared to AdIL-1/Adβgal iv. ($N=5$ per group).

tentatively suggest a functional role of this type of activated microglia to the toxic effects of IL-1β observed on nigral neurons. However, microglial activation cannot univocally be related to toxic effects on neurons (Perry et al., 2007) and thus, we conclude that we cannot ascribe a functional role of a define type of microglia (stage 4 or MHC-II) to the effects we observed. Functional experiments should be performed to answer this question.

The treatment with DXM prevents the neurodegeneration in the SN and also diminished the microglia activation, evidenced as lack of GSA+ label and MHC-II expression. DXM is an anti-inflammatory agent that acts reducing the expression of numerous pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α (Kimberlin et al., 1995; Kurkowska-Jastrzebska et al., 2004). It also inhibits the expression of MHC-I and MHC-II (Castano et al., 2002; Kiefer and Kreutzberg 1991). Since IL-1β seems to mediate the deleterious effects on nigral neurons, our data suggest that DXM effects on neurodegeneration could be most likely due to the inhibition of IL-1β expression. In addition, non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, indomethacin, acetylsalicylic acid, celecoxib and salicylic acid have been reported to have neuroprotective effects against dopaminergic neurodegeneration induced either by 1-methyl-4-phenyl-1,2,3,6-tetradropyridine (MPTP) or 6-OHDA (Esposito et al., 2007; Lee et al., 2009; Sairam et al., 2003; Sanchez-Pernaute et al., 2004; Teismann et al., 2003a,b). In summary, we have studied the effects of long term expression of a unique pro-inflammatory cytokine in the nigrostriatal system. This system allowed the dissection of the pro-inflammatory response in order to avoid side effects generated by a multifactorial response. Striatal long term expression of IL-1β induced neurodegeneration and microglia activation in the SN. SN neurodegeneration was diminished by potent anti-inflammatory drug treatment, as DXM. Interestingly, dopaminergic cell loss could be exacerbated by a pro-inflammatory event in the periphery induced by IL-1β expression. Taken together, our data prompt us to suggest that sustained central IL-1β expression may be regarded as a risk factor for PD initiation and long-term systemic IL-1β expression may be considered a risk factor for the exacerbation of PD progression. These univocal, IL-1β mediated effects provide experimental models to identify downstream molecules that might serve as therapeutic targets against PD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2010.02.018.

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