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FK506 reduces neuroinflammation and dopaminergic neurodegeneration in an α-synuclein-based rat model for Parkinson’s disease

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Abstract

Alpha-synuclein (α-synuclein) is considered a key player in Parkinson’s disease (PD), but the exact relationship between α-synuclein aggregation and dopaminergic (DA) neurodegeneration remains unresolved. There is increasing evidence that neuroinflammatory processes are closely linked to DA cell death, but whether the inflammatory process is causally involved in PD or rather reflects secondary consequences of nigrostriatal pathway injury is still under debate. We evaluated the therapeutic effect of the immunophilin ligand FK506 in a rAAV2/7 α-synuclein overexpression rat model. Treatment with FK506 significantly increased the survival of dopaminergic neurons in a dose-dependent manner. No reduction in α-synuclein aggregation was apparent in this time window, but FK506 significantly lowered the infiltration of both T helper and cytotoxic T cells and the number and subtype of microglia/ macrophages. These data suggest that the anti-inflammatory properties of FK506 decrease neurodegeneration in this α-synuclein-based PD model, pointing to a causal role of neuroinflammation in the pathogenesis of PD.
**Introduction**

PD is a slowly progressing movement disorder and is characterized by a progressive loss of dopaminergic neurons (DN) in the substantia nigra pars compacta (SNpc) along with the accumulation of aggregated forms of the protein α-synuclein in nigral neurons as well as in other brain regions. The loss of dopaminergic innervation leads to depletion of striatal dopamine, resulting in the progressive decline of movement control (Ma et al., 1997). Currently, most treatments of PD are focused on the symptomatic improvement of motor symptoms related to the loss of the DN in the SNpc. These symptomatic treatments significantly improve quality of life and life expectancy. Still, the development of therapeutic approaches that slow or halt the disease progression remains crucial. Mounting evidence suggests that both innate and adaptive immune responses could contribute to dopaminergic neurodegeneration and are potentially linked to disease progression (Braak et al., 2007). However, the contribution of the different subsets of immune cells as well the trigger by which these cells get activated, is still very unclear.

FK506, also named tacrolimus, has been proposed to exert neuroprotective and neuroregenerative effects in animal models of PD (Gold et al., 2002, Poulter et al., 2004). Administration of FK506 has previously been described to reduce dopamine depletion in the striatum of MPTP treated mice (Kitamura et al., 1994) and to delay ibotenic acid induced neurodegeneration in the globus pallidus of rats (Wright et al., 2008). We have demonstrated that inhibition by FK506 reduces α-synuclein aggregation and associated neuronal cell death in cell culture and in a mouse PD model (Gerard et al., 2010).

FK506 is a well-known immunosuppressive drug that is mainly used post-transplantation to decrease the activity of the recipient’s immunity. The effects of FK506 within the immune system are well characterized. FK506 binds to the immunophilin FKBP (FK506 binding protein). The FK506-FKBP complex interacts with and inhibits calcineurin, a
calcium/calmodulin dependent phosphatase, resulting in both the suppression of a T-lymphocyte signal transduction pathway and IL2 transcription (Liu et al., 1991, Liu et al., 1992). The mode of action for its neuroprotective effects remains to be elucidated. Different types of action have been described to FK506, inhibition of apoptosis and necrosis, attenuation of leucocyte accumulation, reduction of microglia activation. In addition, our group has showed a direct link between FKBP12 and α-synuclein aggregation (Gerard et al., 2008, Gerard et al., 2010, Deleersnijder et al., 2011).

We developed a robust rat model for PD by injection of adeno-associated viral vectors (rAAV2/7) encoding α-synuclein into the SN resulting in reproducible nigrostriatal pathology (Van der Perren A. et al., 2014). This α-synuclein rat model shows improved face and predictive validity. It therefore offers new opportunities for both preclinical testing of potential treatments and understanding the disease mechanism.

In this study we evaluated the therapeutic effect of FK506 and the role of neuroinflammation in α-synuclein induced neurodegeneration. Treatment with FK506 significantly increased the survival of DN in a dose-dependent way. No reduction in α-synuclein aggregation was apparent in this time window, but FK506 significantly lowered the infiltration of both T helper and cytotoxic T cells and the number of microglia/ macrophages. These data suggest that the anti-inflammatory properties of FK506 decrease the level of neurodegeneration in this α-synuclein-based PD model, pointing to a causal role of neuroinflammation in the pathogenesis of PD.
Materials and methods

Recombinant AAV production and purification

Vector production and purification was performed as previously described (Van der Perren et al., 2011). The plasmids include the constructs for the AAV2/7 serotype, the AAV transfer plasmid encoding the human A53T α-synuclein mutant or eGFP as a control under the control of the ubiquitous CMVie enhanced synapsin1 promoter and the pAdvDeltaF6 adenoviral helper plasmid. Real-time PCR analysis was used for genomic copy determination.

Stereotactic injections

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Bioethical Committee of the KU Leuven (Belgium). Young adult female Wistar rats (Janvier, France) weighing about 200-250 g were housed under a normal 12 h light/dark cycle with free access to pelleted food and tap water. All surgical procedures were performed using aseptic techniques and ketamine (60 mg/kg ip., Ketalar®, Pfizer, Belgium) and medetomidine (0.4 mg/kg, Dormitor®, Pfizer) anaesthesia. Following anaesthesia the rodents were placed in a stereotactic head frame (Stoelting, IL, USA). Injections were performed with a 30-gauge needle and a 10-µl Hamilton syringe. All animals were injected with 3 µl rAAV2/7A53T α-synuclein or rAAV2/7 eGFP as a control (3.0E+11 GC/ml). Stereotactic coordinates used for the SN were anteroposterior (AP) -5.3, lateral (LAT) -2.0, dorsoventral (DV) -7.2, calculated from the dura using bregma as reference. The injection rate was 0.25 µl/min, the needle was left in place for an additional 5 min before being retracted.
Histology

Rats were sacrificed with an overdose of sodium pentobarbital (60 mg/kg, i.p., Nembutal®, Ceva Santé, Belgium) followed by intracardial perfusion with 4% paraformaldehyde in PBS. After postfixation overnight, 50 µm thick coronal brain sections were made with a vibrating microtome (HM 650V, Microm, Germany). IHC was performed on free-floating sections using an antibody against α-synuclein (1:5000, Chemicon 5038). We also used antibodies against TH (1:1000, Chemicon 152), Mac1 (CD11b, 1:500, Serotec), CD68 (1:500, Millipore), MHCII (1:250, Serotec), CD4 (1:500, Serotec) and CD8 (1:500, Serotec). Sections were pretreated with 3% hydrogen peroxide for 10 min and incubated overnight with primary antibody in 10% normal goat or swine serum (DakoCytomation, Belgium). As secondary antibody we used biotinylated anti-rabbit IgG (1:600 (α-synuclein), 1:300 (other antibodies) DakoCytomation), followed by incubation with streptavidin–horseradish peroxidase complex (1:1000, DakoCytomation). α-synuclein and Mac1, CD68, MHCII and CD8 immunoreactivity were visualized using 3,3-diaminobenzidine (0.4 mg/ml, Sigma-Aldrich) and TH immunoreactivity was visualized using Vector SG (SK-4700, Vector Laboratories, CA) as a chromogen.

Scoring method

The levels of CD68 expression were scored 0–4. Score 0, no positive cells; score 1, few isolated positive cells; score 2, scattered cells; Score 3, staining throughout the SNpc; Score 4, staining throughout the whole SN. The levels of MHCII expression were scored 0–5. Score 0, no positive cells; score 1, few isolated positive cells; score 2, scattered cells; score 3, mild staining throughout the SNpc; score 4 extensive staining throughout the SN pc, score 5,
extensive staining throughout the whole SN. All the analyses were performed by an investigator blind to treatment group.

**Stereological quantification**

The number of TH-positive cells and α-synuclein positive cells in the SN was determined by stereological measurements using the Optical fractionator method in a computerized system as described before (Baekelandt et al., 2002) (StereoInvestigator; MicroBright-Field, Magdeburg, Germany). Every fifth section throughout the entire SN was analyzed, with a total of seven sections for each animal. The coefficients of error, calculated according to the procedure of Schmitz and Hof as estimates of precision (Schmitz et al., 2005) varied between 0.05 and 0.10. We quantified both the injected and non-injected SN (internal control), no cell loss was observed in the non-injected side. The number of CD4/CD8 positive T-cells was determined using the Optical fractionator method. The percentage of Mac1 positive area was defined by densitometry using Image J software. The different types of Mac1 (CD11b) positive microglia were quantified using stereological measurements and depicted as a percentage of the microglia sampled. All the analyses were performed by an investigator blind to different groups.

**Behavioral testing**

The cylinder test was used to quantify forelimb use. Contacts made by each forepaw with the wall of 20-cm-wide clear glass cylinder were scored from the videotapes by an observer blinded to the animal’s identity. A total of 20 contacts were recorded for each animal. The number of impaired forelimb contacts was expressed as a percentage of total forelimb contacts. Non-lesioned control rats should score around 50% in this test.
FK506 treatment: administration and measurement of FK506 concentrations in the blood and CSF

The FK506 treated animals were daily injected in the lateral tail vein with 1mg/kg FK506 (Astellas amp. inf., stock conc. 5 mg/ml), starting 1 day after rAAV2/7 α-synuclein injection. The stock concentration was dissolved in saline to a work concentration of 0.25 mg/ml. For IV injection the animals were anesthetized with isoflurane as described previously (Deroose et al., 2006). The placebo treated animals were injected with the same solution as the FK506 treated animals without the active compound (10 mg/ml Cremophor RH60, 32 mg/ml EtOH in saline). To analyse the bioavailability and the presence of FK506 in the brain, a separate group of Wistar rats (n=3-5) were injected IV for three days (to obtain steady-state concentrations) with different concentrations of FK506 (0.5- 1.0- 1.5- 2.0 and 3.0 mg/kg) (stock concentration 0.25 mg/ml for 0.5 – 1.0 and 1.5 mg/kg FK506 and 0.5 mg/ml for 2 and 3 mg/kg FK506). One hour after the last IV injection blood (tail vein) and CSF (cistern magna) samples were taken to determine peak values. The animals were injected for 5 more days and 24 hours after the last IV injection blood and CSF samples were taken to determine trough values.

FK506 whole blood concentrations were measured using a commercially available liquid chromatography tandem mass-spectrometry kit and validated according to NCCLS and FDA guidelines (MassTrak Immunosuppresants Kit, Waters, Zellik, Belgium) (Napoli et al., 2010). A description of this method was recently published (de Jonge et al., 2012). The samples were injected on an Alliance 2695 HPLC and the detection was performed using a Quattro micro API tandem mass spectrometer (Waters, Zellik, Belgium) (for detailed information see supporting information). Because of lower concentrations of FK506 in cerebrospinal fluid
(CSF) samples, a second sample preparation and a higher sensitive mass spectrometry was needed. In brief, 50 µl zinc sulfate (0.1 M) was added to 50 µl CSF sample in a polypropylene tube (1.5ml). After vortex-mixing, 150 µl internal standard solution (ascomycin dissolved in acetonitrile, final ascomycin concentration 0.5 ng/ml) was added. The samples were vortex-mixed for 20 seconds and centrifuged at 16000 g, 24 °C for 2 min. The supernatant was then transferred and 20 µl was injected onto the Acquity UPLC with a high-end tandem mass spectrometry Xevo TQS (Waters, Zellik, Belgium). Chromatographic separation was performed on a Acquity UPLC BEH 300 C4 column (1.7µm, 2,1x100mm, Waters, Zellik, Belgium) with a flow rate of 0.6 ml/min. The mobile phase, was a gradient with 2 mM ammonium acetate and 0.1% formic acid in both Milli-Q water (A) and methanol (B). The gradient was as follows: after an initial conditioning of 0.5 minute of 50% B, the gradient increased to 100% B in 1 minute; then immediately returning over 1 minute to 50% B and finally equilibrating at 50% B for 2 minutes before the next injection.

For both whole blood and CSF samples, the ionization was achieved using electrospray in the positive ionization mode and the mass spectrometer was operated in multiple-reaction monitoring mode (MRM). The MRM transitions used for quantification were \( m/z \ 821 \rightarrow 768 \) for FK506 and \( 809 \rightarrow 756 \) for ascomycin. Analyte was quantified by use of peak area ratios of analyte over internal standard using non-weighted linear regression.

The quantification of whole blood concentrations of FK506 was achieved, by using the calibrators (covering a linear range from 0 to 30 ng/ml) and controls (low, medium and high concentration) of the MassTrak Immunosuppressant kit. The analytical performance of the kit was validated by successful participation of our laboratory in the International Tacrolimus Proficiency Testing Scheme provided by Analytical Services International Ltd. (London, UK). Calibration curves for the quantification of FK506 concentrations in CSF were prepared by spiking FK506 on artificial CSF
(http://www.alzet.com/products/guide_to_use/cfs_preparation.html). The calibration curve was linear over the range of 0.075-20 ng/ml. The limit of detection and the lower limit of quantification were 0.05 and 0.15 ng/ml respectively. The inter-assay (n=5 in triplicate) and intra-assay (n=4 in duplicate) were below 9%. The recovery of FK506 in rat CSF was 88% (n=4).

**Results**

FK506 reduces A53T α-synuclein induced neurodegeneration *in vivo*.

Different neuroprotective and neuroregenerative properties have been attributed to immunophilin ligands such as FK506, but the exact mechanism remains unclear (Gold et al., 2002, Poulter et al., 2004). Since we have previously implicated FKBP12 as a facilitator of α-synuclein aggregation and neurotoxicity (Gerard et al., 2010, Deleersnijder et al., 2011), we hypothesized that FK506 could reduce α-synuclein induced dopaminergic neurodegeneration in our rat model. Therefore, we administered FK506 (1mg/kg) daily for four weeks in the A53T α-synuclein rAAV2/7 rat model (Fig. 1a). As the bioavailability of FK506 by oral delivery can vary (Iwasaki, 2007), we administered FK506 intravenously (IV). Liquid chromatography tandem mass-spectrometry (LCMS) confirmed elevated blood levels of FK506 at all time points (4 days 2.86 ± 0.61 ng/ml, 15 days 2.11 ± 0.69 ng/ml, 29 days 2.40 ± 0.93 ng/ml) compared to placebo treated animals (Fig. 1b). All animals tolerated the daily IV injection of FK506 well, without obvious physical symptoms except for mild diarrhoea. Quantification of the number of TH immunoreactive nigral neurones at 29 days p.i. demonstrated a more than 2-fold higher survival of DN in the rats treated with FK506 compared to placebo controls (Fig. 1c, d). To prove that we observed real neuronal death we additionally performed a vesicular monoamine transporter 2 (VMAT2) and a Nissl staining. Both indicated clear dopaminergic cell death in the SN (data not shown).
To assess the functional effect of the FK506 treatment, the rats were subjected to the cylinder test at three different time points (4 days, 15 days and 29 days p.i.). We noted improvement in the FK506 treated group (34 +/- 10%) compared to the placebo group (18 +/- 16%), but this difference did not reach statistical significance (Fig. 1e).

Based on these results, we decided to perform a dose-response study in order to evaluate the therapeutic window of FK506. First, we tested a dose of 2 mg/kg and 3 mg/kg FK506 in A53T synuclein rAAV2/7 injected rats, but these higher doses of FK506 were not well tolerated. The animals displayed an overall decrease in spontaneous movements (Fig. 2a), severe weight loss over time and increased mortality rate. Therefore, we decided to lower the range to 0.5 - 1.0 and 1.5 mg/kg FK506. Administration of these lower doses in A53T synuclein rAAV2/7 injected rats for 29 days was well tolerated. To test the bioavailability of FK506 in vivo and the presence of FK506 in the brain we measured peak and trough levels in blood and CSF samples after treatment with the different doses of FK506 (Fig. 2b, c). These values were in line with the administered dose range with peak values in the blood between 38 and 388 ng/ml, and in the CSF between 0.36 and 4.5 ng/ml. In rats that received 1.0 mg/kg FK506 the median ratio of 24h trough FK506 concentrations in blood to that in CSF was 10.70 (range: 6.8-57.1). For FK506 doses of 0.5 and 1.5 mg/kg, similar median blood/CSF concentration ratios were observed. Analysis of the number of surviving dopaminergic neurons revealed that animals treated with 1.0 mg/kg FK506 again displayed significant neuroprotection, confirming the results of the first experiment. Rats treated with 1.5 mg/kg FK506 also presented a significant higher dopaminergic cell survival compared to the placebo treated animals, whereas no difference was seen in the 0.5 mg/kg treated animals (Fig. 2d, e). In terms of motor behavior, again, rats that received 1.0 mg/kg FK506 showed a non-significant improvement in the use of the lesioned paw (39 +/- 11%) compared to the placebo
group (29 +/- 12%), while, as expected, no differences were observed in the 0.5 mg/kg group. Interestingly, no behavioral improvement was observed in the animals treated with 1.5 mg/kg (Fig. 2f). Because of the generalized locomotor impairment observed in the animals treated with 2 and 3 mg/kg FK506, we also tested spontaneous movements in the lower dose groups. This revealed that the 1.5 mg/kg FK506 group was considerably slower in forepaw use, which might explain the negative results in the cylinder test (Fig. 2g).

**FK506 does not significantly affect A53T α-synuclein aggregation at 1 month.**

To understand the mechanism by which FK506 prevents α-synuclein induced degeneration, we first investigated whether FK506 affects α-synuclein aggregation in the brain. As 1 mg/kg FK506 seemed to be the most effective dose, more detailed analysis was first performed in this cohort. Quantification of the total number of α-synuclein positive cells in the SN revealed a significant increase in the animals treated with 1 mg/kg FK506 29 days after injection compared to the placebo treated animals, consistent with the increased survival of dopaminergic neurons (Fig. 3a). When we divided the α-synuclein positive cells into those with and without aggregates, no differences were observed in the ratio of aggregate-positive to aggregate-negative cells (Fig. 3b). When we evaluated animals treated with a higher (1.5mg/kg) or lower dose of FK506 (0.5mg/kg), also no difference in the ratio of aggregate-positive to aggregate-negative cells was detected (Fig. 3c). As FK506 did not seem to have an effect via α-synuclein aggregation we hypothesized that the responsible mechanism was an effect on inflammation.

**FK506 attenuates neuroinflammation**

As FK506 also has strong immunosuppressive and anti-inflammatory properties (Kaminska et al., 2004), we decided to analyse the local microglia population in our A53T α-synuclein...
rAAV2/7 rat model. The microglial cells in the injected SN were visualized by Mac 1 (CD11b) staining, which detects all microglia/macrophages present (pro-inflammatory, anti-inflammatory, infiltrated macrophages and proliferated microglia). The number of Mac 1-positive microglia/macrophages increased in both groups over time (Fig. 4a), but FK506-treated rats showed significantly less Mac 1 positive cells compared to placebo controls at 15 and 29 days post injection (Fig. 4b). Only few microglia were detected in control animals injected with rAAV2/7-eGFP and in the contralateral SN of all rats (Supplemental Fig. 1a).

In addition, we subdivided the microglia into three subtypes (Sanchez-Guajardo et al., 2010): type A cells ‘resting’ microglia, type B cells ‘isolated activated microglia’, type C microglial cells ‘tendency to form clusters’ (Fig. 4c). We detected a predominance of type A microglia in placebo and FK506 treated animals 4 days p.i.. At 15 days p.i. we observed an increase in the percentage of type B microglia in both groups, but this increase was less pronounced in the FK506 treated animals. At 29 days p.i. type C microglia were predominantly present in the placebo group and significantly more abundant than in FK506 treated animals (Fig. 4d).

To correlate morphology to function, we analysed the expression levels of CD68 as a marker of phagocytic microglia/macrophages, and MHC II for antigen presentation ability. The presence of CD68 and MHC II positive cells was assessed by scoring between 1 and 5 (see materials and methods) (Fig. 5a, b). Both markers were almost undetectable in naive animals. We observed an increase in CD68 and MHC II positive microglia over time in both placebo and FK506 treated rats, but no significant differences were detected between the groups (Fig. 5c). Control animals injected with eGFP rAAV2/7 showed a similar infiltration of CD68 and MHC II positive microglia compared to A53T α-synuclein rAAV2/7 injected rats at day 4, but no increase was observed over time (Supplemental Fig. 1b, c).

Since the up-regulation of MHC II in a subset of microglia suggests a possible role of the adaptive immune system, we investigated T cell infiltration into the SN (Fig. 6). The number
of CD4-positive cells significantly increased in both groups over time, but FK506-treated rats showed significantly less CD4 positive cells compared to placebo controls at 29 days p.i. (Fig. 6a, b, c). Further, we detected a delayed infiltration of CD8 positive T cells in FK506 treated rats, reaching a maximum at 29 days p.i., compared to the placebo treated animals which showed a peak infiltration of CD8 positive cells at 15 days p.i. (Fig. 6d, e, f). eGFP rAAV2/7 injected control rats were characterized by a mild CD8 infiltration at 4 days that disappeared over time (Supplemental Fig. 1e).

**Discussion**

In the present study, we show that FK506 reduces neurodegeneration in an improved rAAV2/7-mediated α-synuclein rat model for PD. Our data reveal that the neuroprotective effect of FK506 observed in our α-synuclein model, presumably results from reduced α-synuclein induced neuroinflammation and hints to an important role of the immune response and neuroinflammation in dopaminergic neurodegeneration.

Our rAAV2/7 A53T α-synuclein rat model offers new opportunities for preclinical testing of potential treatments since progressive neurodegeneration, motor impairments and α-synucleinopathy are present. In this study, we questioned whether FK506 could protect against α-synuclein induced neurodegeneration in this rat model. Administration of FK506 has previously been described to reduce DA depletion in the striatum of MPTP treated mice (Kitamura et al., 1994) and to delay ibotenic acid induced neurodegeneration in the globus pallidus of rats (Wright et al., 2008).

In our rat model, the survival rate of DN was significantly higher in rats treated with FK506 (1 mg/kg) compared to placebo controls four weeks after A53T α-synuclein rAAV2/7 injection. Moreover, this neuroprotection resulted in a positive trend towards behavioral
improvement. Taking into account the fast progression of neurodegeneration in our rat model this 2-fold higher survival of DN is substantial. Lowering the dose of FK506 to 0.5 mg/kg abolished the neuroprotective effect, while increasing the dose to 1.5 mg/kg resulted in a higher survival of the dopaminergic neurons. However, higher doses (2-3 mg/kg) were not well tolerated, and even 1.5 mg/kg already affected the general locomotor activity of the animals observed in the cylinder test, suggesting a small therapeutic window of FK506 in A53T α-synuclein rAAV injected rats. In addition, the observed wide range in trough FK506 concentration ratios between blood and CSF, suggests an important variability in penetration of FK506 into the rat brain which is at least partially determined by P-glycoprotein activity in the BBB (Yokogawa et al., 1999).

In an attempt to elucidate the mechanism by which FK506 prevents α-synuclein induced degeneration, we first investigated whether FK506 affects α-synuclein aggregation in the brain. Our group was the first to make a direct link between FKBP12 and α-synuclein aggregation in vitro and in cell culture (Gerard et al., 2008, Gerard et al., 2010, Deleersnijder et al., 2011). We also showed that administration of FK506 reduces α-synuclein aggregation and cell death after LV α-synuclein delivery into mouse striatum (Gerard et al., 2010). However, in this rAAV2/7 based α-synuclein rat model we could not detect a significant effect of FK506 on α-synuclein aggregation. This may be explained by the fact that the aggregation kinetics in our rat model are considerably faster than in the previously used LV mouse model, because of the higher expression levels combined with the use of the aggregation-prone A53T mutant of α-synuclein. Furthermore, due to a higher sensitivity of the dopaminergic neurons of the SN, the extent of neuronal degeneration in our rat model is also significantly higher compared to the degeneration in the mouse striatum. Future experiments using FK506 with a lower dose of rAAV2/7 vector might reveal a potentially more subtle and slower additional effect on α-synuclein aggregation. Furthermore, the
development of new methods and tools to distinguish between different α-synuclein species would allow a more refined analysis.

As FK506 also exhibits strong immunosuppressive effects, it may exert its neuroprotective effect by affecting the microglia/macrophage population through the adaptive immune response. Neuroinflammation and adaptive immunity have frequently been linked to neurodegeneration including PD, although the causal relationship is still under debate (Mosley et al., 2012). Microglia activation and neuroinflammation have been described in various α-synuclein models, but the therapeutic relevance has not yet been demonstrated (Su et al., 2008, Theodore et al., 2008, Su et al., 2009, Gao et al., 2011). Microglia activation profiles observed in our rat model are in line with previous findings (Sanchez-Guajardo et al., 2010), except for some small differences probably due to the degree of neurodegeneration. Overexpression of α-synuclein by means of rAAV2/7 induced an increase in the number of Mac 1 positive microglia/macrophages and phagocytic (CD68+) macrophages, but also influenced the humoral immune response, as both antigen presenting macrophages (MHC II+) as well as T helper cells (CD4+) and cytotoxic T cells (CD8+) were elevated in α-synuclein overexpressing rats. Furthermore, in the presence of high levels of dopaminergic neurodegeneration, microglia showed a predominant clustered macrophagic morphology (type C) which correlates with elevated CD68+ expression (phagocytic marker). Although we cannot prove a direct causal relationship, the presence of type C microglial cells correlated well with conditions of highest dopaminergic cell death. Treatment with FK506 did not induce detectable changes in the number of CD68+ and MHC II+ macrophages but reduced the number of Mac1+ microglia/macrophages. Moreover, a different pattern of microglia morphology was observed in the FK506 group compared to the placebo group. Treatment of FK506 decreased the number of ‘clustered microglia’. This is in line with previous studies
showing that FK506 can suppress the activation of these pro-inflammatory macrophages (Yoshino et al., 2010).

In addition, FK506 treated animals presented a lower number of CD4+ T helper cells 29 days p.i. and a delayed infiltration of cytotoxic T cells 15 days p.i.. These findings could be explained by the direct anti-proliferative effect of FK506 on CD4 or CD8+ T cells (Jones et al., 2006), as well as by the reduced number of microglia (Zawadzka et al., 2005), leading to lowered chemokine secretion and a reduced attractive environment for CD4 and CD8+ T cells. Our findings suggest that FK506 ameliorates DN survival by lowering the infiltration of T cells and the number of Mac 1+ microglia/macrophages, but without any effect on classically activated (CD68+) macrophages or α-synuclein aggregation. Similarly, FK506 has also been shown to mediate neuroprotection through its anti-inflammatory effects in animal models of tauopathy (Yoshiyama et al., 2007).

In summary, we have shown that FK506 provides neuroprotection in a robust A53T α-synuclein rAAV2/7 based rat model, thereby linking neuroinflammation to early progression of synucleinopathy. Since FK506 (tacrolimus) has been administered to kidney transplant patients for a long time, it would be worthwhile to perform meta-analysis studies on the chronic use of tacrolimus and the incidence of PD. Our data support further efforts for pharmacological modulation of neuroinflammation in Parkinson’s disease.

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References


**Legends**

**Fig. 1 FK506 reduces A53T α-synuclein induced neurodegeneration**

(a) Schematic experimental presentation. (b) Measurement of FK506 whole blood concentrations after daily systemic administration of FK506 (1 mg/kg) or placebo in A53T α-synuclein rAAV2/7 injected animals (standard vector dose). (Mean ± s.e.m., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6-8). (c) IHC staining for TH in the SN over time after daily systemic administration of FK506 (1mg/kg) or placebo in A53T α-synuclein rAAV2/7 injected animals. Scale bar = 500 µm. (d) Stereological quantification of the number of TH immunoreactive nigral neurons over time after daily systemic administration of FK506 (1mg/kg) or placebo in A53T α-synuclein rAAV2/7 injected animals. (Mean ± s.e.m., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6). (e) Cylinder test at different time points after daily systemic administration of FK506 (1mg/kg) or placebo in A53T α-synuclein rAAV2/7 injected animals. (Mean ± s.e.m., n= 6-8).

**Fig. 2 Dose-dependent effects of FK506 in the A53T α-synuclein rAAV2/7 rat model**

(a) Time necessary to place 20 forepaws in the cylinder test 29 days after administration of 2 and 3 mg/kg FK506 (Mean ± s.e.m., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6-8). (b) Blood and (c) CSF levels of FK506 were determined 1 h (peak) and 24 h (trough) after administration in Wistar rats. (Mean ± s.e.m., n= 3-5). (d) IHC staining for TH in the SN after daily systemic administration of FK506 (0,5 - 1,0- 1,5 mg/kg) or placebo in A53T α-synuclein rAAV2/7 injected animals. Scale bar = 500 µm. (e) Stereological quantification of the number of TH immunoreactive nigral neurons after systemic administration of different doses of FK506 (0,5 - 1,0-1,5 mg/kg) or placebo for 29 days in A53T α-synuclein rAAV2/7 injected animals (Mean ± s.e.m., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6-8). (f) Cylinder test after systemic administration of different doses of FK506 or placebo for 29 days
in A53T α-synuclein rAAV2/7 injected animals. (Mean ± s.e.m., n= 6-8). (g) Time necessary to place 20 forepaws in the cylinder test 29 days after administration of 0.5, 1.0 and 1.5 mg/kg FK506 (Mean ± s.e.m., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6-8).

Fig. 3 FK506 does not modulate α-synuclein aggregation within 1 month time frame
(a) Stereological quantification of the number of α-synuclein positive nigral cells after FK506 (1mg/kg) treatment. (Mean ± s.d., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6).
(b) The percentage of aggregate-positive cells in the SN over time after FK506 (1mg/kg) treatment. (Mean ± s.d., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6). (c) The percentage of aggregate-positive cells in the SN 29 days after FK506 (0.5 - 1.0-1.5 mg/kg) treatment (Mean ± s.d., * p<0.05 by ANOVA and Bonferroni post hoc test, n= 6).

Fig. 4 FK506 reduces the number of A53T α-synuclein induced microglia/ macrophages in vivo
(a) IHC staining for Mac 1 demonstrating the presence of microglia at different time points after injection of A53T α-synuclein rAAV2/7 (standard vector dose) with daily placebo or FK506 treatment (1mg/kg). Scale bar = 50 µm. (b) Quantification of the % Mac 1 positive area using Image J. (Mean ± s.d., * p<0.05 versus 4 days for both groups, # p<0.05 versus the placebo treated animals by ANOVA and Tukey post hoc test, n= 3). (c) The three morphologies of microglia are represented: Type A (resting microglia), type B (isolated microglia), type C (microglia in clusters). Scale bar = 70 µm. (d) Quantification of each morphology type is depicted as the average percentage distribution per group as a function of time. (Mean, ** p<0.01 to the other group at same time point by Two way ANOVA and Tukey post hoc test, n= 3).

Fig. 5 FK506 does not affect the number of CD68+ and MHC II+ cells
(a) The levels of CD68 expression were given a score of 0–4. Scale bar = 200 µm. (b) The levels of MHCII expression were given a score of 0–5. Scale bar = 200 µm. (c) An observer blind to the sections identity scored expression levels based on number of positive cells and area covered, as explained in the methods (Mean ± s.d., *p<0.05 versus 4 days, #p < 0.05 versus 15 days by ANOVA and Tukey post hoc test, n.s. not significant, n= 3).

**Fig. 6 FK506 reduces or delays the infiltration of CD4 and CD8+ T cells**

(a) IHC staining for CD4 on placebo treated animals 15 days after rAAV α-synuclein injection. Scale Bar = 30 µm. (b, c) Stereological quantification of the number of CD4 positive cells in the SN in (b) placebo or (c) FK506 (1mg/kg) treated animals over time (Mean ± s.d., *p<0.05 by ANOVA and Tukey post hoc test, #p<0.05 versus the other group at same time point, n = 3-4). (d) IHC staining for CD8 on placebo treated animals 15 days after rAAV α-synuclein injection. Scale Bar = 30 µm. (e, f) Stereological quantification of the number of CD8 positive cells in the SN in (e) placebo or (f) FK506 (1mg/kg) treated animals over time (Mean ± s.d., *p<0.05 by ANOVA and Tukey post hoc test, #p<0.05 versus the other group at same time point, n = 4).

**Supplemental Fig. 1 Stereotactic injection of eGFP rAAV2/7 does not significantly induce activation of microglia/ macrophages or CD8+ T-cells in vivo**

(a) IHC staining for Mac 1 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale bar = 400 µm, NI: non injected side (b) IHC staining for CD68 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale bar = 400 µm (c) IHC staining for MHCII 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale bar = 400 µm. (d) IHC staining for CD8 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale Bar = 50µm.
Fig. 1 FK506 reduces A53T α-synuclein induced neurodegeneration
Fig. 2 Dose-dependent effects of FK506 in the A53T α-synuclein rAAV2/7 rat model
Fig. 3 FK506 does not modulate α-synuclein aggregation within 1 month time frame
Fig. 4 FK506 reduces the number of A53T α-synuclein induced microglia/macrophages in vivo
Fig. 5 FK506 does not affect the number of CD68+ and MHC II+ cells
Fig. 6 FK506 reduces or delays the infiltration of CD4 and CD8+ T cells.
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(a) IHC staining for Mac 1 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale bar = 400 µm, NI: non injected side (b) IHC staining for CD68 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale bar = 400 µm (c) IHC staining for MHCII 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale bar = 400 µm. (d) IHC staining for CD8 4 and/or 29 days after injection of eGFP rAAV2/7 or A53T α-synuclein rAAV2/7. Scale Bar = 50µm.