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Antidepressants reduce neuroinflammatory responses and astroglial alpha-synuclein accumulation in a transgenic mouse model of Multiple System Atrophy

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Abstract

Multiple system atrophy (MSA) is a neurodegenerative disease characterized by the pathological accumulation of alpha-synuclein (α -syn) within oligodendroglial cells. This accumulation is accompanied by neuroinflammation with astrogliosis and microgliosis, that leads to neuronal death and subsequent parkinsonism and dysautonomia. Antidepressants have been explored as neuroprotective agents as they normalize neurotrophic factor levels, increase neurogenesis and reduce neurodegeneration, but their anti-inflammatory properties have not been fully characterized. We analyzed the anti-inflammatory profiles of three different antidepressants (fluoxetine, olanzapine and amitriptyline) in the MBP1-h α -syn transgenic (tg) mouse model of MSA. We observed that antidepressant treatment decreased the number of α -syn-positive cells in the basal ganglia of 11-month old tg animals. This reduction was accompanied with a similar decrease in the colocalization of α -syn with astrocyte markers in this brain structure. Consistent with these results, antidepressants reduced astrogliosis in the hippocampus and basal ganglia of the MBP1-h α -syn tg mice, and modulated the expression levels of key cytokines that were dysregulated in the tg mouse model, such as IL-1 β . *In vitro* experiments in the astroglial cell line C6 confirmed that antidepressants inhibited NF- κ B translocation to the nucleus and reduced IL-1 β protein levels. We conclude that the anti-inflammatory properties of antidepressants in the MBP1-h α -syn tg mouse model of MSA might be related to their ability to inhibit α -syn propagation from oligodendrocytes to astroglia and to regulate transcription factors involved in cytokine expression. Our results suggest that antidepressants might be of interest as anti-inflammatory and α -syn-reducing agents for MSA and other α -synucleinopathies.

Keywords

astroglia; cytokine; chemokine; anti-inflammation; neurodegenerative disease

Introduction

Alpha-synucleinopathies are a group of neurodegenerative diseases characterized by the abnormal accumulation of alpha-synuclein (α -syn) within brain cells (Farrer et al. 1999; Spillantini 1999; Takeda et al. 1998; Wakabayashi et al. 1998a). Alpha-synucleinopathies can be classified according to the cell type in which α -syn accumulates: within neurons as Lewy bodies, or within glial cells as glial cytoplasmic inclusions (Spillantini et al. 1998). Multiple system atrophy (MSA) is a α -synucleinopathy characterized by the accumulation of α -syn aggregates within oligodendroglial cells (Dickson et al. 1999; Papp et al. 1989; Spillantini 1999; Wakabayashi et al. 1998b), which is associated to neuronal loss in the striatum, cerebellum, brainstem and cortex, and that is accompanied by astrogliosis and microgliosis (Wakabayashi and Takahashi 2006; Yoshida 2007). MSA is characterized by autonomic failure and motor impairment, parkinsonism, cerebellar ataxia and pyramidal signs (Gilman et al. 2008). 80% of MSA patients present parkinsonian features (MSA-P subtype), reflecting striato-nigral neurodegeneration, while the other 20% present cerebellar ataxia (MSA-C subtype), which is a consequence of olivo-pontocerebellar atrophy (Gilman et al. 2008). To date, there is no effective treatment (preventive or therapeutic) for MSA. For that reason, several animal models have been developed for the study of the molecular mechanisms involved in the neurodegeneration observed in MSA (Fillon and Kahle 2005; Yazawa et al. 2005) and for the development of potential new treatments. Transgenic (tg) mice that over-express α -syn under the control of oligodendrocytic-specific promoters are the most widespread models of MSA, as none of the neurotoxin models reproduces the specific oligodendroglial pathology of MSA that is crucial for the mechanisms involved in degeneration (Stefanova et al. 2005). Among the tg models, the MBP-h α -syn tg mice develop oligodendroglial accumulation of human α -syn (h α -syn) by means of the promoter of the Myelin basic protein (MBP) gene (Shults et al. 2005). These animals display motor abnormalities and olfactory alterations associated with MSA (Ubhi et al. 2010), together with decreased dendritic density and loss of dopaminergic fibers in the basal ganglia (Shults et al. 2005).

In the MSA brain, as well as in the tg mouse line MBP1-h α -syn, α -syn accumulation induces neurodegeneration, which is also accompanied by neuroinflammation (Sekiyama et al. 2012; Shults et al. 2005). Astrogliosis has been observed in the olivo-pontocerebellar, striato-nigral and autonomic systems, as well as corticospinal tracts of MSA patients (Watanabe et al. 2002). Microglial activation is in part determined by oligodendroglial α -syn accumulation in specific neuroanatomic systems affected in MSA (Ishizawa et al. 2004). Microglial cells stimulated by a combination of α -syn and interferon- γ (IFN- γ) are neurotoxic *in vitro*, and this effect is greater in the presence of mutant α -syn forms that are associated with familial Parkinson's disease (PD) (Klegeris et al. 2008). Activation of microglia produces cytokines, such as interleukin-1 α (IL-1 α), IL-1 β , IL-6, tumor necrosis factor- α (TNF- α), chemokines such as IL-8, as well as inflammatory markers such as intercellular-adhesion molecule-1 (ICAM-1), all of which are known to contribute to tissue injuries (Wyss-Coray and Mucke 2002). Polymorphisms in the genes for IL-1 α (Combarros et al. 2003), IL-1 β (Nishimura et al. 2002), IL-8 (Infante et al. 2005), and ICAM-1 (Infante et al. 2005) are statistically associated with an increased risk of suffering MSA. It is also

important to note that α -syn accumulation is not restricted to neurons or oligodendrocytes, but it has also been observed within astroglial cells in MSA (Wenning and Jellinger 2005), dementia with Lewy bodies (DLB) and PD (Braak et al. 2007; Lee et al. 2010b). Furthermore, astroglial α -syn cytoplasmic inclusions (Lee et al. 2010b), astrogliosis and microgliosis (Shults et al. 2005; Watson et al. 2012) have also been detected in α -syn tg mice, but the mechanisms through which astrocytes are activated in the brain of tg mice are still elusive. One possible mechanism would be direct stimulation of astrocytes by α -syn derived from neurons or oligodendrocytes. Supporting this hypothesis, available data suggest that α -syn may be released from neurons via a non-classical secretory pathway to the extracellular environment (Jang et al. 2010; Lee et al. 2005), and its inflammatory effects might be related to its propagation to neighboring cells (Lee et al. 2010b; Lema Tomé et al. 2012).

Antidepressants have been frequently used for the treatment of the mood changes associated with certain neurodegenerative diseases, but preclinical data suggest that antidepressants might also modulate the progression of the disease (Aboukhatwa et al. 2010; Paumier et al. 2012). These effects include increase in neurogenesis (Malberg et al. 2000), improvement in learning and memory (Knegtering et al. 1994; Valluzzi and Chan 2007; Yau et al. 2002), and elevation in the levels of neurotrophic factors and phosphorylated CREB (Balu et al. 2008; Hisaoka et al. 2008; Hisaoka et al. 2001; Ubhi et al. 2012). Several mechanisms have been proposed to explain how antidepressants exert their neuroprotective actions, such as augmentation of antioxidant defenses (Behr et al. 2012; Kotan et al. 2011), BDNF up-regulation (Balu et al. 2008; Shimizu et al. 2003; Ubhi et al. 2012) and noradrenergic regulation (Cohen et al. 1980; Laifenfeld et al. 2005). Psychotropic medications are usually prescribed for the treatment of the depression associated with PD (Menza et al. 2009; Richard et al. 2012), but their neuroprotective effects are less understood. In the MBP1-h α -syn tg mouse model, the antidepressant fluoxetine restores BDNF and GDNF to physiological levels (Ubhi et al. 2012), and this up-regulation has been proposed to play a key role in its neuroprotective functions.

However, recent studies have shown that, in addition to their restorative effects on neurotrophic factor levels, antidepressants might also be protective by alleviating neuroinflammation (Hashioka 2011; Hashioka et al. 2009). The goal of this study was to determine the anti-inflammatory properties of three different antidepressants in the MBP1-h α -syn tg mouse model of MSA. We observed that antidepressant treatment reduced the number of α -syn-positive cells in the basal ganglia of MBP1-h α -syn tg mice. This reduction was associated with an equivalent reduction in the number of α -syn-positive astrocytes, suggesting that antidepressants inhibit α -syn propagation and/or induce α -syn degradation by astroglial cells. Accumulation of α -syn in astrocytes was accompanied by an increase in pro-inflammatory cytokine levels and a reduction in anti-inflammatory cytokine levels, and some of these changes were reduced or reverted by antidepressant treatment. *In vitro* results from an astroglial cell line confirmed that antidepressants inhibit NF- κ B translocation to the nucleus and reduce IL-1 β protein levels, thus identifying inflammatory pathways potentially involved in the progression of the MSA pathology that might be targeted by antidepressant treatment.

Materials and Methods

Animals and antidepressant administration

Mice expressing human α -syn under the control of the MBP promoter (MBP-h α -syn tg) were generated as previously described (Shults et al. 2005). In this study we used the MBP1 line, as they express an intermediate level of α -syn expression compared to the other lines and they are more viable and less aggressive.

A total of 44 10-month old mice were used in this study. MBP1-h α -syn tg mice and their non-tg littermates were treated for 5 weeks with either vehicle (0.5% methocellulose), fluoxetine (18 mg/kg), olanzapine (5 mg/kg) or amitriptyline (20 mg/kg). Vehicle or antidepressant solutions were administered via gavage 5 times a week in a 5 ml/kg volume. Solutions were made fresh weekly.

Tissue processing

After antidepressant treatment, mice were sacrificed under anesthesia following NIH guidelines for the human treatment of animals, and brains were removed. The right hemibrain was fixed by immersion in 4% paraformaldehyde in PBS pH 7.4 and serially sectioned at 40 μ m with a Vibratome apparatus (Leica, Deerfield, IL) for subsequent analysis. The left hemibrain was kept at -80°C for biochemical analysis, and further processed for either real time PCR or protein analysis.

Immunohistochemistry

Vibratome sections were immunolabeled overnight with antibodies against α -syn (Millipore, 1:200), Glial fibrillary acidic protein (GFAP) (Millipore, 1:500), Iba1 (Wako, 1:2000), doublecortin (DCX) (Santa Cruz, 1:250), Proliferating cell nuclear antigen (PCNA) (Santa Cruz, 1:100), or Bromodeoxyuridine (BrdU) (Accu-Specs, 1:200), followed by incubation with species-appropriate secondary antibodies (Vector Laboratories). Sections were reacted with 3,3'-diaminobenzidine (Vector Laboratories) and imaged on an Olympus BX41 brightfield digital microscope. A minimum of 100 cells was counted per animal, and cell counts are expressed as the average number of positive cells per field (230 μ m $\times$ 184 μ m). Quantification of GFAP and Iba1 staining was performed by obtaining optical density measurements using the Image Quant 1.43 program (NIH) and corrected against background signal levels.

For colocalization analysis, sections were first immunolabeled overnight with an antibody against human α -syn (Syn 211) (Sigma, 1:2500), followed by incubation with its species-appropriate secondary antibody and detection with the Tyramide Signal AmplificationTM-Direct (Red) system (1:100) (Perkin Elmer). Sections were then immunolabeled overnight with an antibody anti S-100 (Sigma, 1:200), followed by incubation with its species-appropriate FITC-labeled secondary antibody (Vector Laboratories). Sections were transferred to SuperFrost slides (Fisher Scientific) and mounted under glass coverslips with anti-fading media (Invitrogen) previous to analysis with a MRC1024 laser scanning confocal microscope (BioRad).

Immunoblotting and ELISA assay

Protein homogenates were prepared from the mouse posterior hemibrain. Briefly, frozen samples were sonicated in homogenization buffer (HEPES 1 mM, benzamidine 5 mM, 2-mercaptoethanol 2 mM, EDTA 3 mM, MgSO₄ 0.5 mM, NaN₃ 0.05%, protease inhibitor cocktail set III 1:100, phosphatase inhibitor cocktail set II 1:100) and centrifuged at 100000 rpm for 1 h (Ultracentrifuge, Beckman Coulter) to obtain cytosolic (soluble) and particulate (insoluble, membrane bound) fractions.

20 µg of protein per animal, from the cytosolic or particulate fractions of mouse brain homogenates, were loaded onto 4-12% Bis-Tris SDS-PAGE gels (Invitrogen), transferred onto Immobilon membranes, washed with PBS, and blocked with bovine serum albumin. After overnight incubation with an antibody against total α-syn (Millipore) or NF-κB p65 (C-20, Santa Cruz), membranes were incubated in HRP-linked secondary antibody (American Qualex), reacted with ECL Western blotting substrate (Perkin Elmer) and developed in a VersaDoc gel-imaging machine (BioRad). An anti-actin antibody was used to confirm equal loading (Millipore). Immunoblotting images were analyzed using Quantity One software (BioRad). Levels of human α-syn were determined in mouse brain homogenates by an ELISA assay (Invitrogen) according to the manufacturer's protocol. 10 µg of protein were used per animal per reaction, and concentration of α-syn was calculated by extrapolating from a human α-syn standard curve.

RNA extraction and real time PCR

Total RNA was extracted from the mouse anterior hemibrain using a Qiagen RNeasy kit and following the instructions of the manufacturer. RNA concentration was determined and 0.5 µg of RNA per sample were used for reverse transcription to cDNA using a High capacity cDNA reverse transcription kit (Applied Biosystems). cDNA solutions were diluted 1:10 in ultrapure water and 4 µl of this dilution were used per reaction. Real time PCR (qPCR) was performed using Fast SYBR Master Mix and primers for human α-syn and mouse beta actin as internal control (Desplats et al. 2012b). qPCR reactions were run in an StepOnePlus Real-Time PCR system (Applied Biosystems) and Ct calculations were made using StepOne software (Applied Biosystems).

Mouse cytokine array

400 µg of protein from the cytosolic fraction of brain tissue homogenates were used for analyzing relative levels of 40 different mouse cytokines using a Mouse cytokine panel array (R&D systems) following the instructions of the supplier. Briefly, tissue homogenates were diluted and mixed with a cocktail of biotinylated detection antibodies. The mixtures were then incubated with nitrocellulose membranes where capture antibodies are spotted in duplicate for each cytokine. Streptavidin-HRP and chemiluminiscent detection reagents were then added sequentially, and the binding of the detection antibody was detected in a VersaDoc gel-imaging machine (BioRad) and quantified using Quantity One software (BioRad).

CG-4 cell culture and human α -syn propagation assay

CG-4 cells were cultured as previously described (Louis et al. 1992). Briefly, cells were grown in serum free culture medium consisting in 70% v/v DMEM with 10% v/v N1 supplement (Sigma) plus 10 ng/ml biotin (Sigma), and 30% conditioned DMEM media from B104 cells. Cells were grown in plates precoated with 0.1 mg/ml poly-L-ornithine. CG-4 cells were transduced with lentiviruses expressing human α -syn (LV-h α -syn) or control vector (LV-Bobi) (Desplats et al. 2012a) using a MOI = 40, and the supernatant was recovered after three days. We used ELISA analysis for measuring the amount of human α -syn released by CG-4 cells (donor cells), and then this supernatant was used for incubating C6 cells (acceptor cells) for 24 h. Uptake of human α -syn from the conditioned supernatant was measured by ELISA analysis in whole cell protein extracts.

C6 glioma cell culture and treatment

C6 glioma cells were cultured as recommended by ATCC (ATCC® CCL-107™). Briefly, C6 cells were grown in F-12K medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin/streptomycin. For immunocytochemistry experiments, cells were plated onto poly-L-lysine-coated glass coverslips at a cell density of 0.28×10^5 cells per cm^2 . For protein extraction and western blotting, cells were plated onto poly-L-lysine-coated 12-well plates at a cell density 1.15×10^5 cells per cm^2 . Treatments were performed in OptiMEM medium for 24 hours. Human α -syn seeds were prepared as previously described (Di Giovanni et al. 2010). Briefly, α -syn was dissolved to a final concentration of 140 μM in buffer (Tris 20mM, NaCl 150mM, pH7.6) and then incubated at 37°C for 72 hours in agitation. The aggregation state is then checked by Thioflavine T staining and dynamic light scattering. Long fibrils are then broken in shorter fibrils by ultrasonication on ice. For *in vitro* treatments, the concentrations used were as follows: human α -syn aggregates, 0.5 μM ; fluoxetine, 25 μM ; olanzapine, 25 μM ; amitriptyline, 25 μM ; SN-50, 1 μM ; BAY 11-7082, 5 μM . For immunocytochemistry, cells were fixed in 4% paraformaldehyde, previous to immunolabeling following the same protocol as for tissue sections and using antibodies against total α -syn (Millipore) and NF- κ B p65 (F-6, Santa Cruz, 1:250). For protein analysis, samples were sonicated in homogenization buffer (HEPES 1 mM, benzamidine 5 mM, 2-mercaptoethanol 2 mM, EDTA 3 mM, MgSO_4 0.5 mM, NaN_3 0.05%, protease inhibitor cocktail set III 1:100, phosphatase inhibitor cocktail set II 1:100) and centrifuged at 3000 rpm for 6 min to obtain cytosolic and nuclear fractions. Immunoblotting was performed as described above, using antibodies against NF- κ B p65 (C-20, Santa Cruz), total α -syn (Millipore), IL-1 β (Abcam) and actin (Millipore).

Statistical analysis

Differences between groups (n=5-7) were tested using t-student test, or one and two-way analysis of variance (ANOVA) with Dunnett posthoc test. For *in vitro* assays, all conditions were assayed in duplicate and repeated in at least two separated experiments. All results are expressed as mean $\pm$ SEM.

Results

Antidepressants reduced α -syn load in a transgenic model of MSA

In MSA there is an extensive oligodendroglial accumulation of α -syn, a feature that is recapitulated in the MBP1-h α -syn transgenic (tg) mice (Shults et al. 2005). As previously reported, the tg mice displayed oligodendroglial accumulation of α -syn in frontal cortex, basal ganglia, and corpus callosum (Figure 1A). Colocalization analysis with antibodies against the oligodendroglial marker p25 and human α -syn revealed that all oligodendrocytes in the brain of tg animals over-expressed human α -syn (data not shown). Compared to tg mice, non-tg animals only displayed very weak α -syn reactivity, as a result of endogenous mouse α -syn expression (data not shown). The cell counts of α -syn-positive cells did not change in frontal cortex or hippocampus after antidepressant treatment (Figure 1B, 1C). However, the three antidepressants analyzed significantly reduced the number of α -syn-positive cells in basal ganglia (striatum) (Figure 1E). The number of oligodendrocytes in basal ganglia, measured as the number of p25-positive cells, did not change with any of the treatments both in tg animals (Supplemental Figure 1). These results suggest that all three antidepressants analyzed in this study have the ability of selectively reducing intracellular α -syn accumulation in the basal ganglia of MBP1-h α -syn tg mice.

Immunoblot analysis confirmed the presence of α -syn in soluble (cytosol) and insoluble (particulate) fractions of brain tissue homogenates (Figure 2A). Quantitative analysis of human α -syn levels by ELISA showed a significant reduction in α -syn protein levels in the particulate fraction after antidepressant treatment (Figure 2C). This reduction was statistically significant for mice treated with fluoxetine or olanzapine, but a non-significant reduction was also observed in the amitriptyline-treated mice. mRNA levels of human α -syn were not affected by antidepressant treatment (Figure 2D), therefore protein reduction was not due to inhibition of the transgene expression. These results indicate antidepressants have the ability to significantly reduce α -syn load in the insoluble fraction of MBP1-h α -syn tg mouse brain tissue homogenates, which correspond to aggregated and membrane-associated pool.

Antidepressants reduce astrogliosis in a transgenic model of MSA

In the MBP1-h α -syn tg mice the pathology is characterized by the presence of astrogliosis in the corpus callosum, hippocampus and basal ganglia (Shults et al. 2005), a sign of neuroinflammation that characterizes numerous neurodegenerative diseases (Streit et al. 2004). Immunohistochemical analysis of astrogliosis by GFAP immunoreactivity demonstrated an increase of astrogliosis in the hippocampus of the MBP1-h α -syn tg mice in comparison to the non-tg mice (Figure 3A). No significant changes in astroglial morphology were observed in the corpus callosum of MBP1-h α -syn tg mice, but groups of reactive astrocytes were observed in the basal ganglia of the MBP1-h α -syn tg mice when compared to non-tg mice (Figure 3A). Antidepressant-treated MBP1-h α -syn tg mice displayed GFAP immunoreactivity that was comparable to that of the non-tg mice. Fluoxetine, olanzapine and amitriptyline drastically reduced astrogliosis in the hippocampus of MBP1-h α -syn tg mice (Figure 3A, 3B). Furthermore, reactive astrocytes were not observed in the basal ganglia of MBP1-h α -syn tg mice treated with any of the antidepressants analyzed (Figure

3A, 3D). However, none of the antidepressants induced significant changes on astroglial morphology in corpus callosum (Figure 3C). These results indicate that all three antidepressants used in this study are able to reduce astrogliosis in the hippocampus and basal ganglia of MBP1-h α -syn tg mice, and that astrogliosis in the corpus callosum of these animals is not sensitive to antidepressant treatment.

Microgliosis has been extensively observed in MSA brains (Schwarz et al. 1998; Watanabe et al. 2002), but activated microglia is rarely detected in α -syn tg mice (Sekiyama et al. 2012). Immunohistochemical analysis of non-tg and MBP1-h α -syn tg mice did not reveal significant changes in microglial morphology, detected by Iba1 staining, in hippocampus, corpus callosum or basal ganglia (Figure 4A). Treatment with fluoxetine, olanzapine or amitriptyline did not modify microglial morphology, and optical density quantification confirmed the morphological observations (Figure 4B-D). However, as crosstalk between activated microglia and astrocytes has been reported in neurodegenerative diseases (Saijo and Glass 2011), we cannot dismiss the possibility of microgliosis in earlier or later stages of the MBP1-h α -syn tg mouse pathology.

Antidepressants reduce α -syn accumulation in astrocytes

Prion-like transference of α -syn from neurons to other neurons and to astroglia has been reported (Desplats et al. 2009; Lee et al. 2010b; Lee et al. 2010c), and it has been proposed to be a main pathological event in the progression of MSA (Kisos et al. 2012; Rockenstein et al. 2012). Based in these reports, we hypothesize that propagation of α -syn from oligodendrocytes to astrocytes could be triggering and/or enhancing astrogliosis in the MBP1-h α -syn tg mice. Double labeling of MBP1-h α -syn tg mice brain sections with an anti-h α -syn antibody (Syn 211) and an anti-S-100 antibody (astrocyte marker) showed colocalization of both markers in cells of the basal ganglia and corpus callosum (Figure 5), and in other in brain structures that also express α -syn, such as hippocampus (data not shown). Treatment with fluoxetine, olanzapine or amitriptyline significantly reduced the ratio of colocalization in basal ganglia, measured as the fraction of S-100 positive cells that also presented α -syn staining (Figure 5B). The colocalization ratio between α -syn and S-100 was more variable in corpus callosum than in basal ganglia, and no significant changes were detected after antidepressant treatment in this brain region (Figure 5C). The accumulation of human α -syn within astrocytes and the reduction of its accumulation after antidepressant treatment were both confirmed by colocalization analysis between Syn 211 and the astroglial marker GFAP (Supplemental Figure 2). These results corroborate that human α -syn can accumulate in astroglial cells and suggest that oligodendrocytes have to ability to release human α -syn to the extracellular environment, as it was previously reported in neurons. The results also show that three different antidepressants are able to reduce accumulation of α -syn in astrocytes in basal ganglia, which it would explain the reduction in α -syn-positive cells observed in this structure after antidepressant treatment.

Antidepressants modulate cytokine and chemokine protein levels in a transgenic model of MSA

Accumulation of α -syn in glial cells has been associated to an increase in the expression of pro-inflammatory cytokines (Lee et al. 2010b), and it is becoming increasingly evident that

neuroinflammation plays a crucial role in the development of neurodegenerative diseases (Frank-Cannon et al. 2009; O'Callaghan et al. 2008). Neuroinflammation is characterized by increased glial activation, pro-inflammatory cytokine production, brain blood barrier permeability and leukocyte invasion (Frank-Cannon et al. 2009; Liesz et al. 2011). In this scenario, astrocytes release pro-inflammatory molecules in response to intra and/or extracellular α -syn, and these molecules activate other glial cells inducing further inflammation that worsens the pathology (Johnstone et al. 1999; Lee et al. 2010a; Lieberman et al. 1989). Some antidepressants have been reported to have anti-inflammatory properties in other models of neurodegenerative diseases (Hashioka et al. 2009). Therefore we investigated the anti-inflammatory properties of fluoxetine, olanzapine and amitriptyline in the MBP1-h α -syn tg mouse model of MSA.

We analyzed the relative expression levels of 40 cytokines and chemokines in the cytosolic (soluble) fraction of non-tg mice and MBP1-h α -syn tg mice treated with vehicle or antidepressants (Table 1) using a mouse cytokine proteomic array, and we observed significant changes in levels of cytokines and chemokines between non-tg and MBP1-h α -syn tg mice. MBP1-h α -syn tg mice showed increased levels of IL-1 β , IL-1 α , IL-13, IL-17, MIP-1 α , G-CSF and IFN- γ , and decreased levels of IL-3, IL-6, IL-10, RANTES, MIP-2, IP-10, I-TAC and TREM-1 when compared to non-tg mice (Table 1). In addition to these statistically significant changes, we also observed smaller, non-significant changes in the relative expression levels of other cytokines and chemokines (Table 1). Due to the detection method used, we cannot rule out the possibility of localized changes that might be functionally significant. These results suggest that overexpression of human α -syn by oligodendrocytes induces neuroinflammation in the MBP1-h α -syn tg mouse, which is characterized by astrogliosis and changes in cytokine and chemokine expression levels.

Fluoxetine, olanzapine and amitriptyline modulated cytokine and chemokine levels in the MBP1-h α -syn tg mice (Table 1). As mentioned above, the MBP1-h α -syn tg mice expressed increased levels of the pro-inflammatory cytokines IL-1 β and IL-17 when compared to the non-tg mice (Figure 6A, 6B). IL-1 β is a pro-inflammatory cytokine that is believed to drive the neuroinflammatory process (Mrak 2009; Rothwell and Luheshi 2000), and that is upregulated in AD, PD, multiple sclerosis, and other neurodegenerative diseases (Griffin et al. 2006; Griffin et al. 1989; Rothwell and Luheshi 2000; Shaftelet et al. 2007). IL-17 is involved in T cell activation, and it is expressed by astrocytes in the ischemic brain (Li et al. 2005; Tzartos et al. 2008; Zimmermann et al. 2013). Fluoxetine and olanzapine, but not amitriptyline, reduced the levels of IL-1 β and IL-17 in the MBP1-h α -syn tg mouse brain (Figure 6A, 6B). Expression levels of IL-1 β were increased in the tg mice by a 153 % when compared to non-tg mice, and fluoxetine and olanzapine reduced these values down to a 50 % increase with respect to non-tg controls (Figure 6A). IL-17 protein levels were increased by a 93 % in the tg mice, and fluoxetine and olanzapine reduced those values to non-tg control values (Figure 6B). Amitriptyline did not modify the expression of those pro-inflammatory cytokines, but instead induced a 200 % increase with respect to non-tg controls. Interestingly, expression of both IL-1 β and IL-17 is regulated by the transcription factor NF- κ B (Awane et al. 1999; Fujisawa et al. 2011). Therefore, we measured NF- κ B p65 levels in nuclear and cytosolic fractions of brain homogenates by immunoblot, and observed that NF- κ B p65 translocation, measured as the ratio between nuclear and cytosolic

NF- κ B p65 levels, was significantly inhibited by antidepressants *in vivo* (Figure 7). This result suggest that inhibition of NF- κ B activity might be mechanistically involved in the modulation of cytokine expression observed in MBP1-h α -syn tg mice treated with antidepressants.

Next, we investigated if the reduction observed in pro-inflammatory cytokines after antidepressant treatment was indeed occurring in astrocytes. For that purpose we immunostained mouse brain sections with antibodies against IL-1 β and GFAP (Supplemental Figure 3), and we observed an increase in astroglial IL-1 β expression in the MBP1-h α -syn tg animals when compared to non-tg mice. Interestingly, fluoxetine and olanzapine induced a reduction in astroglial IL-1 β immunostaining, consistent with the results obtained with the cytokine array.

Conversely, we observed a 40 % decrease on the levels of the anti-inflammatory cytokine IL-10 in the MBP1-h α -syn tg mice with respect to non-tg mice (Figure 6C). Treatment with fluoxetine, olanzapine or amitriptyline increased the levels of IL-10 to the levels detected in the non-tg mice (Figure 6C). Similarly, reduced levels of IL-3, which has trophic factor functions in cholinergic neurons (Kamegai et al. 1990), were also observed in the MBP1-h α -syn tg mice (Figure 6D), and treatment with fluoxetine, olanzapine or amitriptyline normalized the levels of IL-3 to the values observed in non-tg controls (Figure 6C). Interestingly, we did not observe changes in the protein levels of other cytokines, such as GM-CSF or IL-1 α , which are traditionally associated with neuroinflammation (Figure 6E, 6F).

Taken together, these results suggest that the specific pattern of neuroinflammation observed in the MBP1-h α -syn mice is sensitive to modulation by antidepressants, which reduce key pro-inflammatory cytokine levels. However, the results obtained with amitriptyline suggest that this antidepressant may activate both pro- and anti-inflammatory pathways.

Fluoxetine, but not olanzapine or amitriptyline, increased hippocampal neurogenesis in a transgenic model of MSA

It has been reported that neuroinflammation and cytokine levels modulate neurogenesis in tg mouse models of neurodegenerative diseases (Baron et al. 2008; Vallieres et al. 2002). In particular, animal models of α -synucleinopathies show reduced or altered neurogenesis in the hippocampus and subventricular zone (Crews et al. 2008; Winner et al. 2004; Winner et al. 2012; Winner et al. 2008), and antidepressants have been shown to have neurogenetic properties that may account for their behavioral effects (Anacker et al. 2011; Malberg et al. 2000; Santarelli et al. 2003; Wang et al. 2008). Therefore, we examined the effect of the antidepressants fluoxetine, olanzapine and amitriptyline on the hippocampal neurogenesis of non-tg and MBP1-h α -syn tg mice. The MBP1-h α -syn mice did not show significant changes in doublecortin (DCX), PCNA and BrdU staining when compared to non-tg controls at 11 months of age (Supplemental Figure S4). Treatment with fluoxetine resulted in a significant increase in the number of DCX (neuroblasts) and PCNA (proliferation) positive cells in the subgranular zone of the dentate gyrus of both non-tg and MBP1-h α -syn transgenic mice (Supplemental Figure S4B, S4C). Fluoxetine also increased the number of BrdU-positive cells in the hippocampus of non-tg and MBP1-h α -syn transgenic mice (Supplemental Figure

S4D). However, olanzapine and amitriptyline did not increase hippocampal neurogenesis after the 5-week treatment. These results indicate that administration of fluoxetine is more effective than the administration of olanzapine or amitriptyline at increasing the proliferation of neuroblasts and neurogenesis in the time frame and doses used in this study, and that the general anti-inflammatory properties of antidepressants do not correlate with their neurogenetic profile in this tg mouse model of MSA.

Antidepressants inhibit astroglial NF- κ B activation and modulate IL-1 β production in vitro

To confirm the results obtained in the MBP1-ha-syn tg mice, and to test the hypothesis that astroglial α -syn induces cytokine dysregulation and elicits neuroinflammation, we used two *in vitro* models: the oligodendroglial CG-4 cell line (Louis et al. 1992), derived from rat primary cultures of progenitor cells; and the astroglial C6 cell line, originally derived from an N-nitrosomethyl-urea-induced rat brain tumor (Benda et al. 1968). First, we measured the ability of oligodendroglial cells to release human α -syn to the extracellular medium. We infected CG-4 cells with lentiviruses expressing ha-syn (LV-ha-syn) or the empty vector as control (LV-Bobi). After three days of expression, the cell culture supernatant was recovered and the presence of ha-syn measured by ELISA (Figure 8A). We observed a small, albeit significant, release of ha-syn from oligodendrocytes, suggesting that oligodendrocytes may also have the ability to release ha-syn when over-expressed *in vivo*. To further confirm the transmission of ha-syn to astrocytes, we used the oligodendrocyte-conditioned media after three-day of expression of LV-ha-syn or LV-Bobi for treating C6 cells for 24 h. ELISA analysis of whole cell protein extracts revealed a significant accumulation of ha-syn within astrocytes (Figure 8B).

However, as the concentration of ha-syn released by oligodendrocytes was low, we continued exploring the effect of ha-syn on astroglia *in vitro* using recombinant ha-syn. C6 cells were incubated for 24 h with human α -syn aggregates (0.5 μ M) (Figure 8C), alone or together with antidepressants. After 24 h, protein was extracted and separated in cytosolic and nuclear fractions. Western blot analysis of protein extracts revealed that C6 cells incorporated human α -syn from the medium (Figure 8D, 8E). Immunocytochemical analysis against α -syn confirmed internalization of the smaller α -syn aggregates (Figure 8C). Co-incubation of α -syn with antidepressants did not reduce intracellular α -syn levels, but it induced a reduction in the translocation of NF- κ B p65 from the cytosolic to the nuclear fraction (Figure 8D, 8E). This inhibition in NF- κ B p65 translocation was accompanied by a significant inhibition of IL-1 β production with fluoxetine, as well as with the inhibitor BAY 11-7082 (Figure 8E, 8F). Interestingly, amitriptyline did inhibit NF- κ B translocation but it did not decrease IL-1 β production in astroglial cells, suggesting a dual effect of amitriptyline in cytokine regulation. From the *in vivo* and *in vitro* experiments it can be concluded that α -syn can accumulate in astroglial cells, and that antidepressants modulate signaling pathway(s) involved in astroglial cytokine production, such as NF- κ B activation. However, as it follows from the results obtained with amitriptyline, a case-by-case analysis of different antidepressants is necessary, as the same molecule could target simultaneously pro- and anti-inflammatory signaling pathways.

In conclusion, our results show that antidepressants reduce accumulation of human α -syn in astrocytes in the MBP1-h α -syn tg mouse model of MSA, which results in a reduction in the number of α -syn-positive cells in basal ganglia. This reduction in astroglial α -syn accumulation is associated with partial normalization in pro- and anti-inflammatory cytokine levels. The regulatory effect of antidepressants could be mediated at least in part through inhibition of NF- κ B, as confirmed by *in vivo* and *in vitro* experiments. These results highlight the relevance of neuroinflammation in this tg mouse model of MSA and the potential of antidepressants as anti-inflammatory agents for the treatment of neurodegenerative diseases.

Discussion

The present study shows that antidepressants reduce astrogliosis and astroglial α -syn accumulation in the basal ganglia of MBP1-h α -syn tg mice. Furthermore, antidepressants regulate cytokine and chemokine levels in the tg mouse brain, and inhibit NF- κ B signaling in an astroglial cell culture. These results suggest that antidepressants might modulate neuroinflammation in a mouse model of MSA by inhibiting astroglial α -syn accumulation and by regulating the activation of transcription factors that lead to production of inflammatory cytokines. Reduced astrogliosis had been previously reported in the MBP1-h α -syn tg mice after treatment with fluoxetine (Ubhi et al. 2012), but in this manuscript we show that antidepressants also have the ability to inhibit astroglial α -syn accumulation and to regulate inflammatory signaling pathways in this tg model of MSA.

Designing new treatments for neurodegenerative diseases and finding novel neuroprotective effects of the drugs that are already commercialized are aspects of great interest for the development of new therapeutic alternatives. In this sense, antidepressants are good candidates for the treatment of neurodegenerative diseases, as they do not only normalize mood changes, but they also reduce neurodegeneration, increase neurogenesis and restore trophic factor levels (Haynes et al. 2004; Nibuya et al. 1995; Santarelli et al. 2003). Alpha-synucleinopathies are associated with increased α -syn levels that lead to α -syn aggregation and toxicity (Singleton et al. 2003; Tokuda et al. 2010), therefore reduction of α -syn levels could be used as a target for neuroprotection. Alpha-syn oligomers are believed to be the neurotoxic species instead of the fibrillar aggregates (Chen et al. 2009; Winner et al. 2011), and their insertion in the plasma membrane has been proposed as their cytotoxic mechanism (Kayed et al. 2004; Lashuel et al. 2002; Volles et al. 2001). The antidepressants used in this study significantly reduced α -syn protein levels and the number of α -syn-positive cells in the basal ganglia of MBP1-h α -syn tg mice, likely due to an increase in α -syn clearance and/or to a reduction in α -syn accumulation in astrocytes. Antidepressants have been shown to reduce α -syn load in other α -syn tg models; for instance, lithium prevents both α -syn accumulation and neurodegeneration in an animal model of PD (Kim et al. 2011) and it induces the clearance of protein aggregates by autophagy (Sarkar et al. 2005). The reduction of α -syn in the particulate fraction of antidepressant-treated tg mice, together with the reduction in the number of α -syn-positive cells in basal ganglia, suggests that antidepressants might be of use as α -syn-reducing agents.

The three antidepressants used in this study also reduced astrogliosis in the MBP1-h α -syn tg mouse model of MSA. Astrogliosis is observed in numerous neurodegenerative diseases, where it exacerbates behavioral deficits and fuels up the progression of the disease (Maragakis and Rothstein 2006). In the MBP1-h α -syn tg mice, α -syn accumulation and neurodegeneration are accompanied by astrogliosis in the white matter tracts, hippocampus and basal ganglia, and to a lesser extent in the neocortex and brainstem (Shults et al. 2005). In a previous study, fluoxetine treatment significantly reduced GFAP immunoreactivity in the corpus callosum of 6-months old MBP1-h α -syn tg mice in comparison to saline-treated MBP1-h α -syn tg mice (Ubhi et al. 2012), and we observed changes in GFAP immunoreactivity in the hippocampus and basal ganglia of antidepressant-treated 11-months old MBP1-h α -syn tg mice, suggesting that the sensitivity of different brain structures to antidepressant treatment is age-dependent. Furthermore, no significant microglial morphological changes were detected in the MBP1-h α -syn tg mouse model at 11-months of age, suggesting that additional factors might be required to induce neuroinflammation associated with microglia in these animals.

In this study we hypothesize that oligodendrocytes of the MBP1-h α -syn tg mouse model release α -syn to their extracellular environment, and that secreted α -syn is incorporated by astrocytes inducing and/or exacerbating astrogliosis and the release of pro-inflammatory cytokines. Supporting this hypothesis we observed widespread colocalization between a human anti- α -syn antibody and the two different astroglial markers in the MBP1-h α -syn tg mouse brain. In the MBP1-h α -syn tg mouse brain, only oligodendrocytes over-produce human α -syn, which is specifically detected by the antibody Syn 211. If a cell is Syn 211-immunoreactive it follows that it either produces human α -syn, or accumulates human α -syn that is produced by oligodendrocytes. Astroglial α -syn accumulation has been observed in MSA patients (Jellinger and Lantos 2010; Wenning and Jellinger 2005), and it has been suggested that astroglial α -syn aggregates are seeded by neuronal α -syn that propagates to astrocytes in a prion-like fashion (Lee et al. 2010b). However, astroglial α -syn accumulation had not been reported before in tg models of MSA. Our results show that human α -syn accumulates within astrocytes in the MBP1-h α -syn tg mouse brain, that oligodendrocytes have the ability to release human α -syn *in vitro* to the extracellular environment, either actively (exocytosis) or passively, and that astrocytes are capable of taking up extracellular h α -syn. Neurons overexpressing α -syn release α -syn oligomers to the extracellular space by non-traditional exocytosis (Jang et al. 2010; Lee et al. 2005) and astrocytes are able to endocytose and accumulate α -syn of neuronal origin (Fellner et al. 2013; Lee et al. 2010b). Recent studies have focused on α -syn propagation and its relevance in the development of α -synucleinopathies (Angot et al. 2012; Bae et al. 2012; Desplats et al. 2009; Hansen et al. 2011; Lee et al. 2012; Li et al. 2008; Rockenstein et al. 2012). In MSA, oligodendrocytes do not express high levels of α -syn and the α -syn accumulation observed within these cells is also believed to be of neuronal origin (Kisos et al. 2012; Rockenstein et al. 2012). Therefore, cell-to-cell propagation of α -syn is of great relevance in the pathology of MSA and other α -synucleinopathies, and blocking or delaying this process would be of therapeutic relevance for the prevention and/or treatment of the disease.

In vitro studies have confirmed that extracellular α -syn triggers microglial and astroglial activation (Fellner et al. 2013; Zhang et al. 2005). Furthermore, it has been shown that astrocytes are able to incorporate α -syn from the extracellular medium (Lee et al. 2010b), but it is not clear if internalized α -syn could trigger astroglial activation *in vivo*. Although a possible effect of extracellular α -syn cannot be ruled out, here we show that antidepressants induce a reduction in astroglial α -syn accumulation that is concurrent with reduced astroglial activation, while levels of soluble α -syn remained constant. Possible mechanisms used by antidepressants to reduce astroglial α -syn accumulation and modulate cytokine production are outlined in Figure 10. Antidepressants may regulate astroglial cytokine production directly, by modulating transcription factors such as NF- κ B, or indirectly by blocking the propagation of α -syn from oligodendrocytes to astrocytes (Figure 9). In this sense, the reduction in α -syn levels and accumulation in astrocytes could be achieved by inhibiting transgene expression, blocking α -syn release to the extracellular environment or α -syn endocytosis by astrocytes, and/or activating astroglial degradation of α -syn (Figure 9). Inhibition of transgene expression is ruled out in the MBP1-h α -syn tg mice as antidepressants did not alter α -syn mRNA levels. Although therapeutic doses of fluoxetine have been shown to decrease neuronal fatigue by inhibiting vesicle release in hippocampal neurons (Henkel et al. 2010), a larger body of evidence supports increased α -syn degradation, as amitriptyline stimulates autophagic processes in astrocytes and neurons (Zschocke and Rein 2011), and fluoxetine induces Type II autophagic cell death in a resistant lymphoma cell line (Cloonan and Williams 2011). However, more investigation will be required to fully understand the mechanisms involved in the reduction of astroglial α -syn accumulation in the MBP1-h α -syn tg mouse model of MSA.

Our results suggest that astroglial α -syn accumulation could be a critical factor in initiating and/or exacerbating astrogliosis in the MBP1-h α -syn tg animals. After being exposed to an inflammatory stimulus, astrocytes produce cytokines that may be used for self-regulation or for the regulation of other glial cells. This phenomenon creates a feedback loop, allowing glial cells to regulate one another (Zhang et al. 2010). Evidence suggests that α -syn initiates astrogliosis by interacting with astroglia, and that the molecular signals released by activated astrocytes contribute to the maintenance and spreading of gliosis. In order to measure neuroinflammation and to identify a plausible correlation between astrogliosis and changes in the production of pro-inflammatory and anti-inflammatory signaling molecules, we analyzed cytokine expression levels in the MBP1-h α -syn tg mouse brain. Glial cells can produce and respond to many cytokines (such as IL-1, IL-6, IL-10, IFN- γ , TNF- α), colony-stimulating factors and chemokines, and thus play an active role in the development of neurological symptoms in certain disease states (Lobsiger and Cleveland 2007; McGeer and McGeer 1995; Smith et al. 2012). Furthermore, genetic polymorphisms in genes for several cytokines and chemokines, such as IL-8, TNF- α and IL-1 β , have been associated to an increased risk of suffering MSA (Infante et al. 2005; Nishimura et al. 2002; Nishimura et al. 2005), confirming the important role of neuroinflammation in MSA pathology. We observed significant changes in the expression levels of key cytokines in the MBP1-h α -syn tg mice when compared to non-tg littermates, the most relevant being the increase in IL-1 β levels. IL-1 β is a mediator in Lewy body pathologies (Griffin et al. 2006) and a critical inflammatory cytokine that is up-regulated in neurodegenerative diseases such as AD and

PD (Blum-Degen et al. 1995; Mogi et al. 1996; Shaftelet et al. 2008). Genetic polymorphisms in the IL-1 β gene are associated with an increased risk of MSA (Nishimura et al. 2002). Interestingly, we also observed increased levels of IL-17 in brain homogenates of MBP1-h α -syn tg mice compared to non-tg controls. $\gamma\delta$ T cells are the primary source of IL-17 (Lockhart et al. 2006), but IL-17 is also expressed by astrocytes in ischemic brain tissue (Li et al. 2005; Zimmermann et al. 2013), where it stimulates the production and expression of pro-inflammatory cytokines, such as GM-CSF, IL-1 β and TNF- α , by human macrophages (Jovanovic et al. 1998). Expression of both IL-1 β and IL-17 is regulated by a positive feedback loop that involves NF- κ B activation (Awane et al. 1999; Fujisawa et al. 2011), which suggests a co-regulation between both cytokines that is confirmed by the fact that IL-1 β induces IL-17 expression (Lalor et al. 2011). Affected brain areas of patients with MSA show a marked immunoreactivity for nuclear NF- κ B p65, which is almost exclusively localized in activated microglia (Schwarz et al. 1998). It has been reported that microglia can internalize aggregated α -syn, leading to activation of NF- κ B signaling with downstream induction of chemokines (Cao et al. 2012). Our *in vitro* results confirm that astrocytes also have the ability to internalize small human α -syn aggregates, and this internalization resulted in significant NF- κ B activation. However, we cannot rule out the possibility that NF- κ B activation might also be induced by other α -syn species, such as α -syn oligomers. Future studies will clarify the involvement of oligomeric α -syn in astroglial activation in the C6 glioma cell line.

Anti-inflammatory cytokines are immunoregulatory molecules that control the pro-inflammatory cytokine response and play an important role in brain, as they regulate the expression of pro-inflammatory molecules, thus exerting a neuroprotective role. We observed a reduction in the expression levels of the anti-inflammatory cytokine IL-10 in the MBP1-h α -syn tg mouse brain when compared to non-tg animals. IL-10 promotes survival of neurons and glial cells in the brain by blocking the effects of pro-apoptotic cytokines and by promoting expression of cell survival signals (Bachis et al. 2001; Molina-Holgado et al. 2001; Pahan et al. 2000; Strle et al. 2002; Zhou et al. 2009), and its expression is altered during the course of most major diseases in the CNS (Strle et al. 2001). IL-10 also limits inflammation in the brain, by reducing synthesis of pro-inflammatory cytokines, suppressing cytokine receptor expression, and inhibiting receptor activation (Bodhankar et al. 2013; Sawada et al. 1999; Tang-Feldman et al. 2011). MBP1-h α -syn tg mice also expressed reduced levels of IL-3, which has trophic action in central cholinergic neurons (Kamegai et al. 1990) and it exerts a neuroprotective role in cells treated with β -amyloid fibrils (Zambrano et al. 2007). Therefore the reduction of IL-3 levels might be another sign of reduced trophic support level in tg mice. Interestingly, levels of other anti-inflammatory cytokines, such as IL-1ra and IL-13, were increased in the MBP1-h α -syn tg mice when compared to non-tg controls, suggesting that anti-inflammatory compensatory mechanisms might be at play.

Antidepressants, acting on serotonin, norepinephrine and dopamine receptors, are anti-inflammatory against pro-inflammatory cytokine processes, specifically by regulating IFN- γ (Yuan et al. 2012), IL-10 (Song et al. 2009; Tai et al. 2009), TNF- α (Sadeghi et al. 2011; Song et al. 2009) and IL-6 (Liu et al. 2011). Fluoxetine protects neurons against microglia-

mediated neurotoxicity *in vitro*, by inhibiting microglial NF- κ B activation and the consequent decrease in the production of pro-inflammatory factors (Zhang et al. 2012). Intraperitoneally administered amitriptyline exhibits a marked anti-inflammatory effect on carrageenan-induced paw edema in rats (Sadeghi et al. 2011; Vismari et al. 2010), and four weeks of prolonged administration of amitriptyline induces a significant increase in the secretion of IL-10 (Kubera et al. 2000). Atypical antipsychotics such as olanzapine suppress TNF- α and IL-6, and up-regulate IL-10 (Sugino et al. 2009). Antidepressant treatment modulated cytokine and chemokine production in the MBP1-h α -syn tg model, thus providing some insight into the molecular pathways involved in neuroinflammation in the MBP1-h α -syn mouse model. The three antidepressants analyzed normalized IL-10 levels in the MBP1-h α -syn tg mice, and this effect on IL-10 levels might mediate their ability to block astrogliosis as this anti-inflammatory cytokine attenuates astroglial reactivity (Balasingam and Yong 1996). We also show that fluoxetine and olanzapine reduced IL-1 β and IL-17 levels in the MBP1-h α -syn mouse brain. Both IL-1 β and IL-17 can signal through the TRAF6-IKK signaling pathway (Cao et al. 1996; Schwandner et al. 2000), which results in the activation of NF- κ B (Awane et al. 1999). None of the antidepressants used in this study normalized levels of cytokines that signal through the Jak-STAT pathway, such as G-CSF, IFN- γ and IL-13 (Darnell et al. 1994; Tian et al. 1996; Welham et al. 1995), suggesting that NF- κ B regulation might be mechanistically involved in the anti-inflammatory effect of antidepressants. Supporting this hypothesis, it has been reported that fluoxetine treatment suppresses NF- κ B activity in kainic acid-treated mouse brains, which would explain in part its anti-inflammatory properties in that model (Jin et al. 2009). Fluoxetine also inhibits NF- κ B in intestinal epithelial cells (Koh et al. 2011), and fluoxetine-induced neuroprotection involves inhibition of NF- κ B in microglial primary cell culture (Lim et al. 2009; Zhang et al. 2012). Furthermore, olanzapine has been shown to prevent nuclear translocation of NF- κ B in phencyclidine-treated rats (Wang et al. 2001). The fact that amitriptyline regulated cytokine levels differently than fluoxetine and olanzapine, may be related to its molecular mechanism of action. Amitriptyline is a dual serotonin-norepinephrine reuptake inhibitor (Tatsumi et al. 1997). Although it has been shown that norepinephrine can reduce neuroinflammation (Heneka et al., 2010) and inhibit NF- κ B signaling in astrocytes (Gavrilyuk et al., 2002), in the MBP1-h α -syn tg mice amitriptyline seems to have a dual pro- and anti-inflammatory role by a mechanism yet to be studied.

The results obtained *in vitro* confirmed that antidepressants inhibit astroglial NF- κ B. Although this inhibition did not result in an increase in α -syn clearance, it did correlate with reduced IL-1 β levels in C6 cells treated with fluoxetine or the inhibitor BAY 11-7082, suggesting a causal relationship between NF- κ B inhibition and IL-1 β production in astroglial cells. However, other mechanisms might be affecting cytokine production in this cell line, as it follows from the results obtained with olanzapine and amitriptyline. These results suggest that NF- κ B regulation may be mechanistically involved in the anti-inflammatory properties of antidepressants, and NF- κ B inhibition would be of interest for the treatment of neuroinflammation in MSA.

Finally, it has been proposed that the effects of antidepressants are correlated to their ability to increase neurogenesis (Santarelli et al. 2003). The molecular mechanisms proposed for

antidepressant-induced increase in neurogenesis are numerous; fluoxetine would increase neurogenesis through p21 inhibition and/or BDNF and GDNF regulation (Kohl et al. 2012; Pechnick et al. 2011; Ubhi et al. 2012) and amitriptyline through regulation of the glucocorticoid receptor (Anacker et al. 2011). Neuroinflammation and neurogenesis are deeply interrelated, as cytokines regulate the proliferation of neural precursor cells (Deleyrolle et al. 2006; Turbic et al. 2011; Wang et al. 2007). In this sense, glial cells and the factors they release play a dual role in neurogenesis acting as anti-proliferative or proliferative agents. Mediators released by reactive microglia, such as cytokines and nitric oxide, inhibit adult neurogenesis in inflammatory conditions (Liu et al. 2006; Monje et al. 2003; Vallieres et al. 2002). On the other hand, neurogenesis seems to be induced by microglial cells activated by IL-4 or low level of IFN- γ , which has been associated with increased neuroprotection (Baron et al. 2008; Song et al. 2005; Wong et al. 2004). However, despite reports that olanzapine and amitriptyline also induce neurogenesis (Chadwick et al. 2011; Kodama et al. 2004), only fluoxetine increased hippocampal neurogenesis in both non-tg and MBP1-h α syn tg mice, suggesting that antidepressant-induced cytokine changes are not directly correlated to neurogenesis in this tg mouse model of MSA.

In conclusion, our results confirm that antidepressants have specific anti-inflammatory properties in a MSA tg mouse model, revealing an important role for neuroinflammation in MSA pathology and suggesting a potential use of these drugs as anti-inflammatory agents for the treatment of MSA and related neuropathologies. More research is necessary to understand the molecular mechanisms of neuroinflammation during the progression of the disease, but our results suggest that antidepressants might be of use as anti-inflammatory agents and inhibitors of astroglial α -syn accumulation for the treatment of MSA and other α -synucleinopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Antidepressants reduced the number of α -syn-positive cells in basal ganglia in a transgenic model of MSA

(A) α -syn immunostaining of frontal cortex, hippocampus, corpus callosum and basal ganglia of MBP1-h α -syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. (B) Cell counts per field of α -syn-positive cells in frontal cortex. (C) Cell counts per field of α -syn-positive cells in hippocampus. (D) Cell counts per field of α -syn-positive cells in corpus callosum. (E) Cell counts per field of α -syn immunostaining of basal ganglia. Error bars represent $\pm$ SEM. ** $p < 0.01$. *** $p < 0.001$. Scale bar = 50 μ m.

Figure 2. Antidepressants reduced α-syn levels in a transgenic model of MSA

(A) Western blot analysis of total α-syn in the cytosolic and particulate fractions of protein extracts from non-transgenic and MBP1-hα-syn transgenic mice treated with vehicle or antidepressants. Significant results of three mice per group are shown. (B) ELISA analysis of human α-syn in the cytosolic fraction of non-transgenic and MBP1-hα-syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. (C) ELISA analysis of human α-syn in the particulate fraction of non-transgenic and MBP1-hα-syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. (D) qPCR analysis of human α-syn mRNA levels in the cytosolic fraction of non-transgenic and MBP1-hα-syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. * $p < 0.05$, ** $p < 0.01$.

Figure 3. Antidepressants decreased reactive astrogliosis in a transgenic model of MSA
 (A) GFAP immunostaining of the hippocampus, corpus callosum and basal ganglia of non-transgenic and MBP1-hα-syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. (B) Optical density quantification of GFAP staining in hippocampus. (C) Optical density quantification of GFAP staining in corpus callosum. (D) Optical density quantification of GFAP staining in basal ganglia. Error bars represent $\pm$ SEM. * $p < 0.05$. Scale bar = 50 μm.

Figure 4. Antidepressants did not affect microglial morphology in a transgenic model of MSA (A) Iba1 immunostaining of the hippocampus, corpus callosum and basal ganglia of non-transgenic and MBP1-ha-syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. (B) Optical density quantification of Iba1 staining in hippocampus. (C) Optical density quantification of Iba1 staining in corpus callosum. (D) Optical density quantification of Iba1 staining in basal ganglia. Error bars represent $\pm$ SEM. Scale bar = 50 μm.

Figure 5. Antidepressants reduced colocalization of α -syn and S-100 in basal ganglia in a transgenic model of MSA

(A) Double immunostaining for human α -syn (Tyramide Red, TRed) and S-100 (FITC) in the corpus callosum and basal ganglia of non-transgenic or MBP1-h α -syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. Arrows indicate cells showing colocalization between α -syn and S-100. (B) Quantification of α -syn/S-100 colocalization in basal ganglia, expressed as the ratio of S-100-positive cells that show α -syn staining. (C) Quantification of α -syn/S-100 colocalization in corpus callosum, expressed as the ratio of S-100-positive cells that show α -syn staining. Error bars represent $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$. Scale bar = 10 μ m.

Figure 6. Antidepressants modulated pro-inflammatory and anti-inflammatory cytokine levels in a transgenic model of MSA

Cytokine levels in the cytosolic fraction of non-transgenic or MBP1-hα-syn transgenic mice treated with vehicle or antidepressants were analyzed using a proteomic array. Results are expressed as relative optical density relative to the non-tg control condition. (A) IL-1β. (B) IL-17. (C) IL-10. (D) IL-3. (E). IL-1α (F) GM-CSF. Error bars represent ± SEM. * p<0.05, ** p<0.01, *** p<0.001, when comparing to non-tg control. # p<0.05, ## p<0.01, when comparing to tg treated with vehicle.

Figure 7. Antidepressants inhibited NF-κB activation in a transgenic model of MSA

(A) Western blot analysis of NF-κB in the cytosolic and nuclear fractions of protein extracts from non-tg MBP1-hα-syn transgenic mice treated with vehicle, fluoxetine, olanzapine or amitriptyline. Significant duplicates per group are shown. (B) Densitometric analysis of the levels of the NF-κB p65 immunoreactive band, expressed as the ratio between the nuclear and cytosolic fractions. Error bars represent $\pm$ SEM. * $p < 0.05$.

Figure 8. Antidepressants inhibited NF- κ B activation and modulated IL-1 β levels in an astroglial cell line

(A) ELISA analysis of human α -syn in the cell culture supernatant of CG-4 cells infected with the lentiviral vectors LV-Bobi (empty plasmid, control) or LV-h α -syn for three days. (B) ELISA analysis of human α -syn in whole cell protein extracts of C6 cells incubated for 24 h with the cell culture supernatant of CG-4 cells infected with LV-Bobi or LV-h α -syn. CM, conditioned media. (C) Double immunostaining for α -syn (Tyramide Red, TRed) and NF- κ B p65 (FITC) in control and α -syn-treated (0.5 μ M) C6 glioma cells. (D) Densitometric analysis of the levels of NF- κ B p65, expressed as the ratio between nuclear and cytosolic fractions from control and α -syn-treated C6 cells co-incubated with antidepressants or the NF- κ B inhibitor BAY 11-7082 (BAY). (E) Immunoblot analysis of NF- κ B, IL-1 β and total α -syn in the cytosolic and nuclear fractions of protein extracts from control and α -syn-treated C6 cells co-incubated with antidepressants or the NF- κ B inhibitor BAY 11-7082 (BAY). Significant duplicates per group are shown. (F) Densitometric analysis of the levels of the IL-1 β immunoreactive band in the cytosolic fraction from control and α -syn-treated C6 cells co-incubated with antidepressants or the NF- κ B inhibitor BAY 11-7082 (BAY). Error bars represent $\pm$ SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Scale bar = 50 μ m.

Figure 9. Proposed mechanisms for antidepressant-induced modulation of astroglial cytokine production in a transgenic model of MSA

Our results suggest that (A) in the MBP1-h α -syn transgenic mice, oligodendrocytes express the human α -syn transgene and synthesize high levels of α -syn protein. α -syn oligomerizes (red spheres) and it might be released to the extracellular environment. Astrocytes might incorporate extracellular α -syn and accumulate it intracellularly, leading to an imbalance in the production of cytokines. (B) In the antidepressant-treated MBP1-h α -syn transgenic mice, antidepressants might regulate cytokine production by several mechanisms: increasing oligodendroglial clearance of α -syn, blocking release and/or internalization of α -syn, and/or increasing astroglial clearance of α -syn. The reduction in astroglial α -syn accumulation, together with regulation of molecular mechanisms involving transcription factors such as NF- κ B, might modulate the expression of pro- and anti-inflammatory cytokines, leading to reduced neuroinflammation.

Table 1Changes in cytokine and chemokine levels in a transgenic model of MSA¹.

Target	Alternate name	MBP1-hα-syn tg			
		vehicle	fluoxetine	olanzapine	amitriptyline
IL-1α	IL-1F1	-12.76	+19.17	-19.60	+9.9
IL-1β	IL-1F2	+153.60 ^{**}	+55.80	+49.20	+202.00 ^{**}
IL-1ra	IL-1F3	+40.61 [*]	+69.14 [*]	+68.93 [*]	+104.45 ^{**}
IL-2		-28.41	+9.15	-4.67	+34.99
IL-3		-75.94 [*]	-31.25	-3.80	-30.08
IL-4		-26.86	-39.36	-6.64	-24.11
IL-5		-25.72	-8.63	+24.06	+40.77
IL-6		-40.22 [*]	-15.51	-35.34	+36.38
IL-7		-35.47	+2.54	-27.79	-26.78
IL-10		-42.38 ^{***}	-1.88	+1.20	+30.80
IL-13		+42.54 [*]	+27.49	+55.77 [*]	+10.04
IL-12 p70		-26.25	+12.64	-14.85	+61.28 [*]
IL-16		-19.95	-2.26	-14.48	+45.08 ^{**}
IL-17		+92.90 [*]	-34.63	-3.46	+210.90 ^{**}
IL-23		-16.98	+25.22	+13.38	+25.56
IL-27		+0.36	+3.54	+35.89	+50.27 [*]
I-309	CCL1/TCA-3	-30.78	-6.40	-28.67	-20.09
JE	CCL2/MCP-1	-38.76	-9.16	-30.68	-38.19
MIP-1α	CCL3	+53.45 [*]	+93.79 [*]	+59.73 [*]	+107.85 [*]
MIP-1β	CCL4	-11.57	+9.76	-37.10	+48.53
RANTES	CCL5	-67.79 [*]	-57.53 [*]	-27.66	+233.93 [*]
Eotaxin	CCL11	-30.91	-2.91	-30.03	-4.72
MCP-5	CCL12	-29.53	-3.85	-15.05	-30.52
TARC	CCL17	-16.57	-7.92	-9.39	+62.07 [*]
KC	CXCL1	-37.10	-26.13	-12.73	-39.78
MIP-2	CXCL2	-56.40 [*]	-5.35	-67.51 [*]	+183.23 [*]
MIG	CXCL9	-25.73	+0.37	-44.51	+34.97
IP-10	CXCL10/CRG-2	-87.31 [*]	-87.00 [*]	-57.90 [*]	-60.78 [*]
I-TAC	CXCL11	-50.56 [*]	-24.47	-50.25 [*]	+45.61
SDF-1	CXCL12	-25.54	-27.65	+69.42 [*]	+144.73 [*]
BLC	CXCL13/BCA-1	-15.52	-11.80	-20.42	+68.42 [*]
C5/C5a		+12.12	-24.32	-37.60	+59.51

Target	Alternate name	MBP1-ha-syn tg			
		vehicle	fluoxetine	olanzapine	amitriptyline
G-CSF		+51.83 *	+4.61	+42.42 *	-28.66
GM-CSF		-8.67	+11.00	-15.68	+6.20
M-CSF		+5.96	+5.79	+3.49	+6.53
sICAM-1	CD54	-18.51	+13.26	-1.01	-1.83
IFN-γ		+65.00 *	+84.22 *	+93.82 *	+10.87
TNF-α		-20.00	+8.21	-14.61	+35.46 *
TIMP-1		-22.24	-28.28	-9.63	-30.53
TREM-1		-61.56 *	-50.95 *	-77.43 *	-58.77 *

¹ Cytokine levels in the cytosolic fraction of non-transgenic or MBP1-ha-syn transgenic mice treated with vehicle or antidepressants were analyzed using a proteomic array. Results indicate the percentage of increase (+) or decrease (-) of the signal with respect to non-tg control values.

*
p<0.05

**
p<0.01.

p<0.001.