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Fluoxetine Ameliorates Behavioral and Neuropathological Deficits in a Transgenic Model Mouse of α -synucleinopathy

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Abstract

The term α -synucleinopathies refers to a group of age-related neurological disorders including Parkinson's disease (PD), Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA) that display an abnormal accumulation of alpha-synuclein (α -syn). In contrast to the neuronal α -syn accumulation observed in PD and DLB, MSA is characterized by a widespread oligodendrocytic α -syn accumulation. Transgenic mice expressing human α -syn under the oligodendrocyte-specific myelin basic protein promoter (MBP1-h α syn tg mice) model many of the behavioral and neuropathological alterations observed in MSA.

Fluoxetine, a selective serotonin reuptake inhibitor, has been shown to be protective in toxin-induced models of PD, however its effects in an *in vivo* transgenic model of α -synucleinopathy remain unclear. In this context, this study examined the effect of fluoxetine in the MBP1-h α syn tg mice, a model of MSA.

Fluoxetine administration ameliorated motor deficits in the MBP1-h α syn tg mice, with a concomitant decrease in neurodegenerative pathology in the basal ganglia, neocortex and hippocampus. Fluoxetine administration also increased levels of the neurotrophic factors, GDNF (glial-derived neurotrophic factor) and BDNF (brain-derived neurotrophic factor) in the MBP1-h α syn tg mice compared to vehicle-treated tg mice. This fluoxetine-induced increase in GDNF and BDNF protein levels was accompanied by activation of the ERK signaling pathway. The effects of fluoxetine administration on myelin and serotonin markers were also examined.

Collectively these results indicate that fluoxetine may represent a novel therapeutic intervention for MSA and other neurodegenerative disorders.

INTRODUCTION

The term α -synucleinopathies collectively refers to a recently recognized group of heterogeneous neurodegenerative disorders with parkinsonism, autonomic dysfunction and

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non-motor deficits (Marti, et al., 2003). In Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) alpha-synuclein (α -syn) tends to accumulate mostly in neurons while in Multiple System Atrophy (MSA) there is extensive accumulation of α -syn in oligodendroglial cells and to a lesser extent in neurons (Lantos and Papp, 1994, Wakabayashi and Takahashi, 2006, Yoshida, 2007). The characteristic oligodendrocytic neuropathological lesions are known as glial cytoplasmic inclusions (GCI) (Arima, et al., 1998, Tu, et al., 1998, Wakabayashi, et al., 1998, Wakabayashi, et al., 1998). In MSA, the characteristic motor, autonomic and non-motor deficits are not responsive to conventional anti-parkinsonian treatments and currently there are no therapies available for MSA. Despite the primarily oligodendrocytic accumulation of α -syn, MSA patients also display considerable neuronal loss in the striatum, cerebellum, brainstem and cortex, accompanied by astrogliosis, microgliosis and myelin loss (Wakabayashi and Takahashi, 2006, Yoshida, 2007). Comparable neurodegenerative alterations have been observed in transgenic (tg) mice over expressing human α -syn (h- α -syn) under the control of an oligodendrocytic-specific promoter (MBP-myelin basic protein) (Shults, et al., 2005); these tg mice develop oligodendrocytic accumulations of α -syn and display behavioral deficits associated with MSA including motor abnormalities and olfactory alterations (Ubhi, et al., 2010). Similarly, tg mice over expressing h- α -syn under the proteolipid protein (PLP) (Kahle, et al., 2002) and the 2,' 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter (Yazawa, et al., 2005) develop MSA-like pathology.

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is a commonly prescribed anti-depressant. In addition to its activity on the serotonergic system, fluoxetine has also been reported to have pro-proliferative activity (Chang, et al., 2010, Clark, et al., 2006, Wang, et al., 2011) and to effect levels of neurotrophic factors (NTFs) such as glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (Allaman, et al., 2011, Chang, et al., 2010, Mercier, et al., 2004). A number of recent studies have also reported that fluoxetine can protect against 6-OHDA (6-hydroxydopamine) (Suzuki, et al., 2010) and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Chung, et al., 2011) induced damage in toxin-induced models of PD. However, the effects of fluoxetine in an *in vivo* transgenic models of α -synucleinopathy remain unclear, in this context, this study examined the effect of fluoxetine in the MBP1-h α -syn tg mice, a model of MSA (Shults, et al., 2005).

We show that fluoxetine administration ameliorated motor behavioral and neuropathological deficits in the MBP1-h α -syn tg mice and that this amelioration was associated with increased levels of the NTFs GDNF and BDNF and activation of the ERK signaling pathway in the fluoxetine-treated mice.

These results support the hypothesis that, in addition to its antidepressant activity, fluoxetine may have a neuroprotective function in the MBP1-h α -syn tg mice, which may be mediated via the elevation of NTF levels. These results suggest that fluoxetine may have a therapeutic role in the treatment of α -synucleinopathies such as MSA.

MATERIALS AND METHODS

Animals and Fluoxetine 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); specifically, this study used the MBP1 line. The MBP-h α -syn line 1 mice (MBP1-h α -syn tg mice) were chosen for this study as they express an intermediate level of α -syn expression compared with the other lines; they are able to be assessed behaviorally and are breedable. In contrast, other higher expressing lines are less viable and more aggressive, making them less suitable for this

study. The MBP1-h α syn tg mice have previously been shown to accumulate α -syn in oligodendrocytes from 3 months of age and to display neuropathological alterations including myelin loss and astrogliosis and behavioral deficits (Shults, et al., 2005). These mice have also been shown to display alteration in specific NTFs (Ubhi, et al., 2010).

A total of 24 mice (6 month old), were used for this study and received either 18mg/kg of fluoxetine by gavage daily for 28 days (MBP1-h α syn tg mice, n=6 and age-matched non-tg mice, n=6) or saline daily for 28 days (MBP1-h α syn tg mice, n=6 and age-matched non-tg mice, n=6).

Motor behavior assessment using the Round Beam Test

The Round Beam test allows the assessment of gait and balance impairments through distance traveled in an allotted amount of time over a round beam placed horizontally. As previously described (Ubhi, et al., 2010), three consecutive trials, 1min each, are run in one day. The total forward distance traveled is recorded, as are the number of foot slippages. Speed on the beam is calculated as 'distance traveled/time taken' and errors on the beam are calculated as 'foot slips/distance traveled'.

Total Activity and Thigmotaxis

Briefly, as previously described (Ubhi, et al., 2010), total activity was calculated as total beam breaks in a 10-minute period. The test was conducted for 4 trials each day for a period of 4 days. Thigmotaxis was calculated as the % of time spent in the periphery.

Tissue processing

Following NIH guidelines for the humane treatment of animals, under anesthesia mice were sacrificed and brains removed. The right hemisphere was immersion-fixed in 4% paraformaldehyde in PBS pH 7.4 and serially sectioned at 40 μ m with the Vibratome (Leica, Deerfield, IL) for subsequent analysis of neurodegeneration. The left hemisphere was kept at -80°C for biochemical analysis.

Immunohistochemistry

As previously described (Ubhi, et al., 2010), vibratome sections (40 μ m) were immunolabeled overnight with antibodies against GDNF (rabbit polyclonal, 1:250, Abcam), NeuN (mouse monoclonal, 1:1000, Millipore), GFAP (rabbit polyclonal, 1:500, Millipore), TH (rabbit polyclonal, 1:200; Millipore), doublecortin (goat polyclonal, 1:500, Santa Cruz), PCNA (mouse monoclonal, 1:500, Millipore), tryptophan hydroxylase (5HT-OH) (sheep polyclonal, 1:500, Millipore), serotonin (5HT) (mouse monoclonal, 1:500, Millipore) and α -syn (rabbit polyclonal, 1:500; Millipore) followed by incubation with species-appropriate secondary antibodies (1:2000, Vector Laboratories). Sections stained with GDNF, NeuN, GFAP, TH, doublecortin, PCNA and 5HT-OH were reacted with 3,3'-Diaminobenzidine (DAB) and imaged on the Olympus BX54 brightfield digital microscope. Sections stained with 5HT and α -syn were reacted with FITC-tagged secondary antibodies and were analyzed with the laser scanning confocal microscope (LSCM) (MRC1024, BioRad). Sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, CA) and mounted under glass coverslips with anti-fading media (Vector Laboratories). Additional sections were stained with Luxol Fast Blue (LFB) in order to visualize the myelin layers and imaged on the Olympus BX54 brightfield digital microscope.

As previously described (Ubhi, et al., 2010) stereological estimates of density of NeuN and raphe nucleus 5HT-OH immunoreactive cells was conducted with the Stereo-Investigator Software system (MBF Biosciences) for which images were randomly collected utilizing the optical disector method and expressed as cells per unit volume. The levels of TH, GFAP and

GDNF and 5HT-OH fiber immunoreactivity were assessed by obtaining optical density measurements using the Image Quant 1.43 program (NIH), corrected against background levels and are presented as 'corrected optical density' where the background signal has been subtracted from optical density measurements.

Immunoblotting

Briefly as previously described (Ubhi, et al., 2010), twenty micrograms of protein per mouse, from the cytosolic fraction of mouse brain homogenates, was loaded onto 4–12% Bis-Tris (Invitrogen) SDS-PAGE gels, transferred onto Immobilon membranes, washed and blocked in BSA. After an overnight incubation with antibodies against either GDNF (rabbit polyclonal, 1:1000, Abcam), BDNF (rabbit polyclonal, 1:1000, Millipore), total α -syn (rabbit polyclonal, 1:1000, Millipore), PCNA (1:1000, Millipore), phosphorylated ERK (pERK, 1:1000, Millipore) or total ERK (tERK, 1:1000, Millipore), membranes were incubated in appropriate secondary antibodies, reacted with ECL and developed on a VersaDoc gel-imaging machine (Bio-Rad, Hercules, CA). Anti-beta-actin (mouse monoclonal, 1:1000; Sigma) antibody was used to confirm equal loading. For each antibody, lysates from all 24 samples across the experimental groups were analyzed in triplicate, images presented are representative of the replicates however only contain four samples from each groups for the purposes of clarity. Quantitative analyses presented were performed with results from all the samples tested.

Tissue Culture and Fluoxetine Administration

As previously described (Ubhi, et al., 2010), the rat CG4 line (Louis, et al., 1992) was differentiated to an oligodendrocytic lineage by incubation in 70% (DMEM with 10ng/ml biotin and 100ug penicillin) 30% B104 conditioned media at 37°C. Cells were allowed to differentiate for four days without additional feeding and were infected with LV- α -syn (Spencer, et al., 2009) at a multiplicity of infection (MOI) of 30 and left for an additional three days. Following differentiation and infection, cells were treated with 25uM Fluoxetine (LKT Labs) for 24 hours. Cells were then lysed in TNE (20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 5mM 2-mercaptoethanol, 1 $\times$ protease inhibitor cocktail (Calbiochem) and 1 $\times$ phosphatase inhibitor cocktail (Calbiochem) containing sodium orthovanadate) with 1% Triton-X 100 for immunoblot analysis as described above. Appropriate differentiation into an oligodendrocytic lineage was assessed using the oligodendrocytic marker myelin basic protein (MBP, mouse monoclonal, 1:1000, Abcam), the astroglial marker GFAP (rabbit polyclonal, 1:1000, Millipore) and the neuronal marker Tuj1 (mouse monoclonal, 1:2000, Covance). The effect of fluoxetine treatment was assessed using antibodies against GDNF (rabbit polyclonal, 1:1000, Abcam), total α -syn (rabbit polyclonal, 1:1000, Millipore), phosphorylated ERK (mouse monoclonal, pERK, 1:1000, Millipore) or total ERK (mouse monoclonal, tERK, 1:1000, Millipore). Anti-beta-actin (mouse monoclonal, 1:1000; Sigma) antibody was used to confirm equal loading.

Determination of GDNF protein content by ELISA

As previously described (Ubhi, et al., 2010), the concentration of GDNF protein in mouse whole brain homogenate tissue was determined by ELISA (Promega, Madison, WI), briefly the samples were sonicated in a homogenization buffer (20 mM Tris, pH 8, 137 mM NaCl, 1% Nonidet P-40, 1,7 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin and 0.5 mM sodium vanadate) and centrifuged at 100K for 1 hour (Ultracentrifuge, Beckman Coulter) to obtain cytosolic (soluble) and particulate (insoluble, membrane-bound) fractions. The cytosolic fraction was used for ELISA analysis. Levels of GDNF were determined in homogenates by ELISA according to the supplier's protocol.

RNA Extraction and Quantification of mRNA by Real Time-PCR (RT-PCR) analysis

Total RNA was extracted from MBP-h_αsyn tg mice and non-tg mice using the RNeasy kit (QIAGEN) as per manufacturer's instructions. All the samples were treated with DNase I to eliminate genomic DNA contamination. RNA quantification was determined by spectrophotometer readings. The ratio of OD260/OD280 was used to evaluate the purity of the nucleic acid samples and the quality of the extracted total RNA was determined using agarose gel electrophoresis.

For cDNA synthesis, 1 µg total RNA was reverse transcribed using iScript cDNA Synthesis kit (BioRad). RT-PCR experiments were performed using the iQ5 Detection System (BioRad). Amplification was performed on a cDNA amount equivalent to 25 ng total RNA with 1× iQ SYBRGreen Supermix (BioRad) containing dNTPs, MgCl₂, Taq DNA polymerase, and forward and reverse primers. PCR reactions were performed on three independent sets of template. Experimental samples and no-template controls were all run in triplicate. The PCR cycling parameters were: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 94 °C for 15 s, 60 °C for 1 min. Finally, a dissociation protocol was also performed at the end of each run to verify the presence of a single product with the appropriate melting point temperature for each amplicon. The amount of studied cDNA in each sample was calculated by the comparative threshold cycle method and expressed using mouse actin as an internal control. RT-PCR primers for GDNF (forward): CGA TAT TGC AGC GGT TCC TG, GDNF (reverse): CTG CAA CAT GCC TGG CCT AC, BDNF (forward): TCA TAC TTC GGT TGC ATG AAG G and BDNF (reverse): AGA CCT CTC GAA CCT GCC C.

Statistical Analysis

Differences between groups were tested using one and two-way analysis of variance (ANOVA) with Fisher PLSD posthoc tests. All results are expressed as mean ± SEM.

RESULTS

Fluoxetine ameliorates motor behavioral deficits in a transgenic model of MSA

MSA, particularly the parkinsonian subtype, is characterized by motor abnormalities such as tremor, rigidity and gait and limb ataxia; many of these features are replicated in our MBP1-h_αsyn tg mice (Shults, et al., 2005). In order to investigate the effects of fluoxetine on motor behavior in the MBP1-h_αsyn tg mice, saline and fluoxetine-treated mice were examined on the Round Beam test to examine gait and balance impairments and in the Open Field test to assess effects on spontaneous motor behavior.

Saline-treated MBP1-h_αsyn tg mice were slower at traversing the beam in comparison to the saline-treated non-tg mice (Figure 1A) indicative of gait and balance deficits. Fluoxetine significantly increased the speed with which the MBP1-h_αsyn tg mice were able to travel the beam (Figure 1A), with the time taken by fluoxetine-treated MBP1-h_αsyn tg mice to travel the beam being comparable to that of the saline-treated non-tg mice. Fluoxetine had no significant effect on the speed of the non-tg mice. Whilst the error-rate (foot slips/10cm) did not differ significantly between saline-treated MBP1-h_αsyn tg and non-tg mice, fluoxetine was able to significantly decrease the number of errors made by both the MBP1-h_αsyn tg (treated=0.047±0.007 vs untreated 0.72±0.035) and non-tg (treated=0.051±0.013 vs untreated 0.92±0.02) mice in comparison to their saline-treated littermates (Figure 1B, p<0.05). Fluoxetine had no effect on levels of total spontaneous activity or thigmotaxis in either group (Figure 1C, D), indicating that its effect on motor behavior is not a reflection of non-specific hyperactivity. These results indicate that fluoxetine is able to ameliorate motor

deficits in the MBP1-h_{asyn} tg mice by improving the gait, balance and motor coordination of these mice.

Fluoxetine ameliorates neurodegenerative pathology in a transgenic model of MSA

In MSA patients there is widespread neurodegeneration (Lantos and Papp, 1994, Wakabayashi and Takahashi, 2006), which is recapitulated in the MBP1-h_{asyn} tg mice (Shults, et al., 2005). Consistent with previous reports (Shults, et al., 2005, Ubhi, et al., 2010), analysis of NeuN immunoreactivity in the saline-treated MBP1-h_{asyn} tg demonstrated decreased neuronal density in the frontal cortex, hippocampus (most notable in the CA3 subfield) (Figure 2A, C, E, F) and basal ganglia (Figure 2G, I, K) in comparison to the saline-treated non-tg mice ($p < 0.05$). In contrast, treatment with fluoxetine restored the levels of neuronal density in the frontal cortex and hippocampus of the MBP1-h_{asyn} tg mice in comparison to saline-treated MBP1-h_{asyn} tg mice (Figure 2C–F, $p < 0.05$). In line with the improved motor behavior, fluoxetine treatment restored neuronal counts in the basal ganglia of the MBP1-h_{asyn} tg mice compared to saline-treated MBP1-h_{asyn} tg mice (Figure 2I–K, $p < 0.05$). Fluoxetine had no effect on NeuN stereological cell counts in the non-tg mice (Figure 2A, B, E, F) and fluoxetine-treated MBP1-h_{asyn} tg mice displayed levels of NeuN immunoreactivity comparable to those observed in the non-tg mice (Figure 2A, B, D, E, F).

In MSA the parkinsonian features have been related to the loss of dopaminergic input to the basal ganglia (Halliday, 2007). Similarly in the saline-treated MBP1-h_{asyn} tg mice there is a loss of tyrosine hydroxylase (TH) immunoreactive fibers in the caudo-putamen region in comparison to the saline-treated non-tg mice (Figure 2L, N, P, $p < 0.05$). Fluoxetine treatment restored TH immunoreactivity in the MBP1-h_{asyn} tg mice to levels comparable with saline-treated non-tg mice (Figure 2L, O, P, $p < 0.05$). Fluoxetine had no effect on basal ganglia TH levels in the non-tg mice (Figure 2L, M, P) or on levels of TH in the substantia nigra of MBP1-h_{asyn} or non-tg mice (data not shown).

In the MBP1-h_{asyn} tg mice the neurodegenerative pathology is accompanied by astrogliosis in the white matter tracts, hippocampus and basal ganglia (Shults, et al., 2005). Immunohistochemical analysis of levels of astrogliosis by GFAP immunoreactivity demonstrated an increase in astrogliosis in the hippocampus (Figure 3A, C, E, $p < 0.05$) and corpus callosum (Figure 3F, H, J, $p < 0.05$) of the saline-treated MBP1-h_{asyn} tg mice in comparison to the saline-treated non-tg mice. Fluoxetine treatment significantly reduced GFAP immunoreactivity in the corpus callosum of the fluoxetine-treated MBP1-h_{asyn} tg mice in comparison to saline-treated MBP1-h_{asyn} tg mice (Figure 3H–J, $p < 0.05$). Fluoxetine had no effect on GFAP immunoreactivity in the non-tg mice (Figure 3A, B, E, F, G, J) and fluoxetine-treated MBP1-h_{asyn} tg mice displayed GFAP levels comparable to those observed in the non-tg mice (Figure 3A, D, E, F, I, J). Fluoxetine had no effect on GFAP immunoreactivity in the frontal cortex of non-tg or MBP1-h_{asyn} tg mice (Figure 3A–E).

In order to examine the effect of fluoxetine on myelination in the MBP1-h_{asyn} tg mice LFB staining was performed in the corpus callosum and in line with previous studies in these mice (Shults, et al., 2005), the MBP1-h_{asyn} tg mice displayed reduced levels of staining with LFB in the corpus callosum in comparison to non-tg controls (Figure 3K, M, O), indicative of myelin disruption in these mice. Treatment with fluoxetine was able to ameliorate this loss of myelin and returned LFB staining levels in the MBP1-h_{asyn} tg mice to those observed in the control mice (Figure 3M–O). Fluoxetine had no effect on LFB staining in the non-tg mice (Figure 3K, L, O).

Together, these results indicate that the improved behavioral deficits observed in the fluoxetine-treated MBP1-h α syn tg mice may be associated with an amelioration in neurodegenerative pathology as reflected by analysis of NeuN, TH and GFAP and that fluoxetine may be able to ameliorate myelination deficits in the corpus callosum of the MBP1-h α syn tg mice as evidenced by LFB.

Fluoxetine ameliorates deficits in neurogenesis in a transgenic model of MSA

Neurodegeneration has traditionally been viewed in the light of cellular loss in certain neuronal populations in the brain, however emerging research has suggested that in addition to increased cell death there is also a concomitant reduction in levels of neurogenesis (Enciu, et al., 2011, Winner, et al., 2011) such that is possible to view increased cellular loss and reduced neurogenesis as two contributing factors to the neurodegenerative process.

Recent work in mouse models of α -synucleinopathy has reported reduced/altered neurogenesis in the hippocampus and sub-ventricular zone of these animals (Crews, et al., 2008, Marxreiter, et al., 2009, Winner, et al., 2011, Winner, et al., 2004, Winner, et al., 2008). Fluoxetine has been reported to increase levels of neurogenesis (Chang, et al., 2010, Li, et al., 2009, Marcussen, et al., 2008, Perera, et al., 2011, Wang, et al., 2011) therefore we examined the effect of fluoxetine in the hippocampus sub-granular zone of the MBP1-h α syn tg mice. Saline-treated MBP1-h α syn tg mice display significantly reduced levels of doublecortin, a marker of neuroblasts (Figure 4A, C, E, $p < 0.05$) and PCNA, a marker of proliferating cells in the sub-granular zone of the dentate gyrus (Figure 4F, H, J, $p < 0.05$) in comparison to saline-treated non-tg mice, indicating reduced neurogenesis in the tg mice. Fluoxetine treatment resulted in an increase in the number of doublecortin (Figure 4B, D, E, $p < 0.05$) and PCNA (Figure 4G, I, J, $p < 0.05$) immunoreactive cells in the sub-granular zone of both the MBP1-h α syn tg and non-tg mice. Consistent with the immunohistochemical results, immunoblot analysis showed reduced PCNA immunoreactivity in the saline-treated MBP1-h α syn tg mice compared to saline-treated non-tg mice (Figure 4K, L, $p < 0.05$). Fluoxetine treatment resulted in a significant increase in PCNA levels in the MBP1-h α syn tg mice compared to saline-treated and non-tg mice (Figure 4K, L, $p < 0.05$). These results indicate that the MBP1-h α syn tg mice displayed altered levels of neurogenesis in comparison to the non-tg control mice and that administration of fluoxetine is able to ameliorate the deficits in neurogenesis by increasing the proliferation of neuroblasts.

Fluoxetine alters levels of white matter α -syn in a transgenic model of MSA

In MSA there is an extensive oligodendroglial neuropathological accumulation of α -syn, a feature that is recapitulated in the MBP1-h α syn tg mice (Shults, et al., 2005). As previously reported (Shults, et al., 2005, Ubhi, et al., 2010) the saline-treated MBP1-h α syn tg mice displayed oligodendroglial accumulation of α -syn in the frontal cortex (Figure 5A, C and E), basal ganglia (Figure 5F, H and J) and corpus callosum (Figure 5K, M, O). Interestingly, fluoxetine treatment resulted in a decreased accumulation of α -syn in the corpus callosum in treated MBP1-h α syn tg mice compared to saline-treated MBP1-h α syn tg mice (Figure 5M–O, $p < 0.05$), but had no significant effect in the other areas examined. These results indicate that fluoxetine treatment may have a regionally specific effect on oligodendrocytic α -syn load in the white matter of the corpus callosum.

Fluoxetine alters levels of GDNF and BDNF in a transgenic model of MSA

Fluoxetine has been shown to increase levels of NTFs (Allaman, et al., 2011, Hisaoka, et al., 2001, Mercier, et al., 2004), therefore in order obtain a mechanistic understanding of the pathways underlying the neuroprotective effect of fluoxetine in the MBP1-h α syn tg mice we investigated whether fluoxetine treatment could enhance levels of GDNF and BDNF in the MBP1-h α syn tg mice.

Consistent with previous reports (Ubhi, et al., 2010), the saline-treated MBP1-ha-syn tg mice displayed a significant reduction in the hippocampal levels of GDNF immunoreactivity, in comparison to the saline-treated non-tg mice, (Figure 6A, C, E, $p<0.05$). Fluoxetine treatment resulted in a significant increase in hippocampal levels of GDNF in the treated MBP1-ha-syn tg mice compared to saline-treated MBP1-ha-syn tg mice (Figure 6C–E, $p<0.05$). In agreement with the immunohistochemistry, analysis of GDNF levels by ELISA also demonstrated a robust increase in levels of GDNF following fluoxetine administration (Figure 6F, $p<0.05$). This effect was observed in both the non-tg and MBP1-ha-syn tg mice (Figure 6F).

The immunohistochemical (Figure 6A–E) and ELISA (Figure 6F) results were confirmed by a third independent method, namely immunoblot analysis (Figure 6G). As anticipated, there was a significant reduction in the levels of GDNF (Figure 6G, H, $p<0.05$) and BDNF (Figure 6G, I, $p<0.05$) in the saline-treated MBP1-ha-syn tg mice compared to saline-treated non-tg controls. Treatment with fluoxetine significantly increased GDNF and BDNF levels in the MBP1-ha-syn tg mice and in the non-tg mice in comparison to their saline-treated cohorts (Figure 6G–I, $p<0.05$).

Given that the expression of NTFs triggered by fluoxetine has been reported to involve the ERK signaling pathway (Li, et al., 2010, Li, et al., 2008, Mercier, et al., 2004) we examined the ratio of pERK to tERK as a surrogate marker of pathway activation. We observed significantly increased the levels of pERK to tERK in the fluoxetine-treated MBP1-ha-syn tg and non-tg mice compared to the respective saline groups (Figure 6G, J, $p<0.05$). These results indicate the fluoxetine is able to significantly increase protein levels of GDNF (and BDNF) and activate the ERK signaling pathway in both non-tg and MBP1-ha-syn tg mice.

In order to determine whether the effects of fluoxetine on NTF levels is regulated at the transcriptional level, mRNA levels of GDNF, BDNF and ha-syn in the MBP1-ha-syn tg mice were examined by qPCR (Figure 7). No differences in levels of GDNF or BDNF mRNA were observed between saline-treated non-tg and MBP1-ha-syn tg mice (Figure 7A, B) and fluoxetine-treatment had no effect on levels of GDNF mRNA in these mice (Figure 7A). In contrast, fluoxetine treatment resulted in a significant increase in BDNF mRNA levels in both non-tg and MBP1-ha-syn tg mice (Figure 7B, $p<0.05$). Finally fluoxetine treatment had no detectable effect on levels of ha-syn mRNA in the MBP1-ha-syn tg mice (Figure 7C). Collectively, these results indicate that fluoxetine is capable of promoting the expression of GDNF protein and BDNF protein and mRNA in the MBP1-ha-syn tg mice and suggest that this NTF-enhancement by fluoxetine may have to the ability to ameliorate motor and neuropathological alterations observed in the MBP1-ha-syn tg mice (Shults, et al., 2005).

Fluoxetine has been shown *in vitro* to increase astroglial expression of GDNF and BDNF (Allaman, et al., 2011) however its effect on these NTFs, specifically GDNF, in oligodendrocytes, remains unexamined. To investigate this, and to further verify by an independent method the effects of fluoxetine on oligodendrocytes *in vitro*, the CG4 rat cell line was used. These cells were differentiated to an oligodendrocytic phenotype, infected with LV- α -syn and treated with 25uM fluoxetine for 24 hours. Consistent with the studies in the MBP1-ha-syn tg mice, immunoblot analysis of the cell lysates demonstrated a significant increase in the levels of GDNF in the fluoxetine-treated, LV- α -syn infected differentiated CG4 cells in comparison to LV- α -syn infected differentiated CG4 cells alone (Figure 8A, B, $p<0.05$). The increase in GDNF protein levels was accompanied by increased ERK activation as evidenced by an increased ratio of pERK to tERK (Figure 8A, C, $p<0.05$). Taken together, these studies support the notion that the neurotrophic effects of fluoxetine might be mediated in part by the increased expression of GDNF by oligodendroglial cells.

Effect of Fluoxetine on the serotonergic system in a transgenic model of MSA

In order to examine the serotonergic system in the MBP1-h_{asyn} tg mice we performed immunohistochemistry with an antibody against tryptophan hydroxylase (5HT-OH), an enzyme involved in the synthesis of serotonin, in neurons in the brainstem raphe nucleus and the projecting fibers. Additional analysis of serotonergic terminals in the hippocampus was performed using an antibody against serotonin (5HT).

In comparison to saline-treated non-tg mice, saline-treated MBP1-h_{asyn} tg mice display a decrease in levels of 5HT-OH immunoreactivity in neurons in the brainstem (Figure 9A, C, E), decreased 5HT-OH immunoreactive fibers (Figure F, H, J). Fluoxetine treatment increased 5-HT-OH immunoreactive fibers in the MBP1-h_{asyn} tg mice (Figure 9H–J) but had no effect on brainstem neurons (Figure 9C–E).

Saline-treated MBP1-h_{asyn} tg mice also display a decrease in levels of 5HT immunoreactivity in hippocampal nerve terminals (Figure 9K, M, O) and fluoxetine treatment had no effect on levels of 5HT immunoreactivity in these nerve terminals (Figure 9M–O).

DISCUSSION

The present study sought to investigate the hypothesis that administration of the antidepressant fluoxetine may ameliorate deficits in the MBP1-h_{asyn} tg mice, a tg model of MSA. The results demonstrate that fluoxetine is indeed able to reduce motor behavioral deficits and neurodegenerative pathology in the MBP1-h_{asyn} tg mice. Furthermore, we suggest that the neuroprotective effects of fluoxetine in this model of α -synucleinopathy are mediated by its ability to increase the expression of GDNF and BDNF.

NTFs are altered in a number of neurodegenerative disorders (Siegel and Chauhan, 2000, Zuccato and Cattaneo, 2009) and specific alterations in the levels of GDNF and BDNF have been reported in neurodegenerative disorders including Alzheimer's disease (AD) and PD (Hong, et al., 2008, Peterson and Nutt, 2008, Ramaswamy, et al., 2009, Saragovi, et al., 2009, Siegel and Chauhan, 2000, Yasuhara, et al., 2007, Zuccato and Cattaneo, 2009). Recently we reported a selective reduction in the expression of GDNF in patients with MSA, a feature that is recapitulated in the MBP1-h_{asyn} tg mice (Ubhi, et al., 2010). We have also shown that infusion of GDNF rescued the motor behavioral and neuropathological deficits in these tg mice (Ubhi, et al., 2010).

NTF replacement strategies have been widely investigated in a number of neurodegenerative disorders (Siegel and Chauhan, 2000, Zuccato and Cattaneo, 2009). There has been a great deal of interest in GDNF replacement therapies in relation to PD in particular and many of these have progressed to the clinical trial stage however there have been mixed reports regarding the success of these methods, many of which are highly invasive (Hong, et al., 2008, Nutt, et al., 2003, Peterson and Nutt, 2008, Yasuhara, et al., 2007). Therefore, there is a real need for an alternative method to augment GDNF levels in MSA patients. A non-invasive approach such as fluoxetine administration may fulfill this need and it may indeed prove more efficacious than GDNF replacement alone given that it also affects BDNF levels.

In support of this possibility, we showed that fluoxetine administration is able to recapitulate many of the neuroprotective effects of GDNF infusion observed in our earlier study (Ubhi, et al., 2010) and that it may have a disease-modifying effect on regional levels of α -syn aggregation. The effects of fluoxetine observed in this study are consistent with studies in which fluoxetine has been shown to reduce the loss of dopaminergic neurons in MPTP

(Chung, et al., 2011) and 6-OHDA (Suzuki, et al., 2010) models of PD and LPS induced degeneration of the nigral system (Chung, et al., 2010), moreover our study expands the therapeutic application of fluoxetine to MSA, particularly in relation to its ability to augment levels of GDNF.

The effects of fluoxetine have been widely investigated in astrocytes *in vitro* (Allaman, et al., 2011, Li, et al., 2009, Li, et al., 2010, Li, et al., 2008, Mercier, et al., 2004) and rat C6 glioblastoma cells (Hisaoka, et al., 2001) however the present study is the first to report fluoxetine-induced increases in GDNF in oligodendrocytes, the primary cell type affected in MSA. Whilst the oligodendroglial cell culture data does not exclude the possibility that the effects of fluoxetine might target in the MBP1-hsxn tg mice cells other than oligodendrocytes it does provide important information regarding the ability of fluoxetine to affect this cell type. Moreover, given that both oligodendrocytes and astrocytes have been reported to express GDNF (Du and Dreyfus, 2002, Schaar, et al., 1993, Wilkins, et al., 2003), the most parsimonious explanation for the effect of fluoxetine in the MBP1-hsxn tg mice is that a combinatorial effect on these cell types is responsible for the motor and neuropathological improvements observed.

In addition to reducing the neurodegenerative pathology in the neocortex and nigrostriatal system, treatment with fluoxetine also rescued the alterations in neurogenesis observed in the MBP1-hsxn tg mice. Previous studies have shown that adult neurogenesis is defective in models of α -synucleinopathy (Crews, et al., 2008, Marxreiter, et al., 2009, Winner, et al., 2004, Winner, et al., 2008) and it has been suggested that defects in neurogenesis might be responsible for non-motor deficits in these disorders (Winner, et al., 2004, Winner, et al., 2008). Our findings in the fluoxetine-treated MBP1-hsxn tg mice are in agreement with previous studies showing that fluoxetine potentiates neurogenesis in human stem cells and hippocampal cells (Chang, et al., 2010, Suzuki, et al., 2010) and with studies of neurogenesis in the APPsw (Tg2576) mutant mice model of AD (Dong, et al., 2004) and the Ts65Dn mouse model of Down syndrome (Bianchi, et al., 2010, Clark, et al., 2006).

The mechanisms through which fluoxetine might enhance GDNF and BDNF levels to promote neurogenesis and rescue the neurodegenerative pathology in our tg MSA model remain unclear. However, a number of studies indicate that fluoxetine treatment may enhance transactivation of serotonin receptors including the 5-HT_{2B} and 5-HT_{2C} receptors (Li, et al., 2009, Li, et al., 2010, Li, et al., 2008) resulting in ERK phosphorylation and activation of the ERK signaling pathway (Li, et al., 2009, Li, et al., 2008, Mercier, et al., 2004). This is consistent with the increased pERK/tERK ratio we observe in the fluoxetine-treated mice and in the *in vitro* study. Moreover, increased expression of GDNF by fluoxetine is likely to be ERK-dependent as it is blocked by U0126, a mitogen-activated protein kinase (MAPK)-ERK kinase inhibitor (Hisaoka, et al., 2001, Mercier, et al., 2004). In contrast, fluoxetine-induced BDNF expression is not blocked by U0126, suggestive of an ERK-independent pathway (Mercier, et al., 2004). One possible alternative pathway by which fluoxetine may enhance BDNF expression is via the transactivation of the BDNF receptor TrkB (Rantamaki, et al., 2011, Saarelainen, et al., 2003, Wyneken, et al., 2006) followed by the binding of BDNF to the TrkB receptor resulting in the internalization of the BDNF-TrkB complex which then undergoes retrograde transport from spines to cell bodies where it activates the synthesis of more BDNF (Wyneken, et al., 2006).

The present study is the first to demonstrate alterations in the serotonergic system in the MBP1-hsxn tg mice. We show a decrease in serotonin raphe nucleus brainstem neurons, fibers and nerve terminals in these mice compared to controls, these results are consistent with a previous study in humans (Tada, et al., 2009) and with studies that have shown that BDNF and GDNF may subserve a supporting function for serotonergic neurons (Djalali, et

al., 2005). We show that fluoxetine administration in the MBP1-h α syn tg mice is able to increase 5HT-OH immunoreactive projecting fibers, without effecting numbers of neurons or nerve terminals suggesting that, rather than increasing the actual numbers of serotonergic neurons or terminals, fluoxetine may play a role in increasing the transport of serotonin from the soma to the axons in these mice.

We also show that fluoxetine administration was able to ameliorate the myelin deficits observed in the corpus callosum of MBP1-h α syn tg mice, again these results are in line with the reported involvement of NTFs such as BDNF and GDNF on myelination (Hoke, et al., 2003, Notterpek, 2003, Tada, et al., 2009, Tolwani, et al., 2004, Xiao, et al., 2009, Xiao, et al., 2010, Zhang, et al., 2009). This effect on oligodendrocytes in the corpus callosum is particularly interesting in light of the regional effect of fluoxetine at lowering α -syn immunoreactivity in the corpus callosum, however further, more detailed studies, are necessary in order to fully determine the precise mechanisms underlying this observation. In addition to its effects on serotonin, NTF levels and neurogenesis, fluoxetine is reported to effect inflammatory processes (Kubera, et al., 2004, Roumestan, et al., 2007) and mitochondrial activity (Abdel-Razaq, et al., 2011, Lee, et al., 2010). Further investigation of these alternative pathways is also necessary in order to fully define the mechanisms underlying the neuroprotective effects of fluoxetine.

It is also important to note that our particular interest in fluoxetine was precipitated by previous reports of its neuroprotective activity (Chung, et al., 2010, Chung, et al., 2011, Suzuki, et al., 2010) and that fact that it is currently in a Phase II trial for depressive symptoms in MSA (NCT01146548). However, it is possible that other SSRIs or psychopharmacological agents may have a similar effect in the MBP1-h α syn tg mice or other models of α -synucleinopathy and/or neurodegeneration, for example, paroxetine, another SSRI, has been reported to prevent loss of nigrostriatal dopaminergic neurons by inhibiting brain inflammation and oxidative stress in the MPTP mouse model of PD (Chung, et al., 2010). Indeed, there is a whole body of evidence to suggest a neuroprotective effect of psychopharmacological treatments (Hu, et al., 2011, Lauterbach, et al., 2010, Machado-Vieira, et al., 2009) and many of these drugs have been reported to effect levels of NTFs (Gonzalez-Pinto, et al., 2010, Heberlein, et al., 2010, Hunsberger, et al., 2009, Lee, et al., 2011, Nishimura, et al., 2008, Parikh, et al., 2004, Quiroz, et al., 2010).

Antidepressants and other psychopharmacological agents have traditionally been prescribed to patients with neurodegenerative disorders in order to help them cope with symptoms such as depression and anxiety (Aboukhatwa, et al., 2010). It is only in the last few decades that these drugs have been examined more closely to assess other effects that they may be having (Lauterbach, et al., 2010). The most well documented example of this kind of multifactorial effect involves lithium, a commonly prescribed mood stabilizer treatment for bipolar depression, which has been shown to effect levels of amyloid beta ($A\beta$) and tau in AD. The effects of lithium on $A\beta$ and tau are related to its ability to inhibit the activity of GSK-3 β a key kinase involved in the transcriptional regulation of amyloid precursor protein (APP, the precursor of $A\beta$) and in the phosphorylation of tau. Antipsychotics such as haloperidol and chlorpromazine have also reported to effect levels of $A\beta$ and tau phosphorylation (Lauterbach, et al., 2010).

The effects of psychopharmacological agents are not restricted to AD-related proteins, valproic acid, used to treat certain types of seizures or episodes of mania in patients with bipolar disorder, has been shown to increase α -syn expression and prevent its nuclear translocation (Leng and Chuang, 2006), whilst the antioxidant melatonin has been reported to reduce α -syn aggregation *in vitro* (Ishido, 2007, Klongpanichapak, et al., 2007, Klongpanichapak, et al., 2008). It is clear that drugs traditionally prescribed for disorders

such as depression or schizophrenia may have more pleiotropic effects than previously anticipated and further investigation of these effects is necessary in order to fully appreciate the therapeutic potential of these drugs in neurodegenerative disorders such as MSA.

In conclusion, we demonstrate that the SSRI fluoxetine is able to ameliorate motor behavioral and neuropathological alterations in the MBP1-h_αsyn tg model of MSA, these results suggest that fluoxetine, a well characterized and FDA approved drug, may potentially have a therapeutic application for MSA patients and for other disorders that display alterations in NTF expression.

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Highlights

- We examined the effect of fluoxetine in the MBP1-h α syn tg mice, a model of MSA
- Fluoxetine ameliorated motor deficits in the MBP1-h α syn tg mice.
- Fluoxetine decreased neurodegenerative pathology and increased levels of NTFs
- Fluoxetine promoted proliferation and reduced corpus callosum accumulation of α -syn
- Fluoxetine may represent a novel therapeutic intervention for MSA

Figure 1. Fluoxetine ameliorates motor deficits in the MBP1-hαsyn transgenic model of MSA
 The effect of fluoxetine administration on motor behavior was examined using the Round Beam Test. (A) Speed and (B) Error rate were recorded. (C) Total activity and (D) thigmotaxis were also examined. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test.

Figure 2. Fluoxetine administration ameliorates neuropathological deficits in neuronal density and TH alterations in the MBP1-h α syn transgenic model of MSA

The effect of fluoxetine administration on neuronal density was examined using an antibody against the neuronal marker NeuN. (A–D) NeuN immunoreactivity in the neocortex and hippocampus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-h α syn tg and fluoxetine-treated MBP1-h α syn tg mice respectively. (E) Analysis of neocortical NeuN immunoreactivity across the experimental groups. (F) Analysis of hippocampal NeuN immunoreactivity across the experimental groups. (G–J) NeuN immunoreactivity in the basal ganglia of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-h α syn tg and fluoxetine-treated MBP1-h α syn tg mice respectively. (K) Analysis of basal ganglia NeuN immunoreactivity across the experimental groups. (L–O) The effect of fluoxetine administration on TH level was in the basal ganglia of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-h α syn tg and fluoxetine-treated MBP1-h α syn tg mice respectively. (P) Analysis of TH immunoreactivity in the basal ganglia across the experimental groups. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test. Scale bar (AD) = 200 μ M, (G–J and L–O) = 50 μ M.

Figure 3. Fluoxetine administration does not alter levels of astrogliosis in the MBP1-hαsyn transgenic model of MSA

The effect of fluoxetine administration on astrogliosis was examined using an antibody against the astrocytic marker GFAP. (A–D) GFAP immunoreactivity in the neocortex and hippocampus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (E) Analysis of neocortical GFAP immunoreactivity across the experimental groups. (F–I) GFAP immunoreactivity in the corpus callosum of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (J) Analysis of GFAP immunoreactivity in the corpus callosum across the experimental groups. (K–N) Luxol fast blue (LFB) stained myelin in the corpus callosum of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (O) Analysis of LFB in the corpus callosum across the experimental groups. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test. Scale bar (A–D) = 150 μ M, (F–I) = 50 μ M and (K–N) = 5 μ M.

Figure 4. Fluoxetine ameliorates deficits in neurogenesis in the MBP1-hαsyn transgenic model of MSA

The effect of fluoxetine administration on neurogenesis was examined in the MBP1-hαsyn tg mice using markers of neurogenesis. (A–D) Doublecortin immunoreactivity in the dentate gyrus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively, arrows indicate doublecortin positive cells. (E) Analysis of dentate gyrus doublecortin levels across the experimental groups. (F–I) PCNA immunoreactivity in the subgranular zone of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively, arrows indicate PCNA positive cells. (J) Analysis of dentate gyrus doublecortin levels across the experimental groups. (K) Immunoblot of levels PCNA in saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (L) Analysis of PCNA in saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. Beta-actin was used as loading control. Error bars represent mean ± SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test. Scale bar (A–D) = 100μM, (F–I) = 50μM.

Figure 5. Fluoxetine selectively alters α syn load in the corpus callosum of the MBP1-h α syn transgenic model of MSA

The effect of fluoxetine administration on α syn protein levels was examined using immunohistochemistry and confocal microscopy. (A–D) Confocal images of α syn immunoreactivity in the neocortex and hippocampus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-h α syn tg and fluoxetine-treated MBP1-h α syn tg mice respectively. (E) Analysis of frontal α syn immunoreactivity across the experimental groups. (F–I) Confocal images of α syn immunoreactivity in the basal ganglia of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-h α syn tg and fluoxetine-treated MBP1-h α syn tg mice respectively. (J) Analysis of α syn immunoreactivity in the basal ganglia across the experimental groups. (K–N) Confocal images of α syn immunoreactivity in the corpus callosum of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-h α syn tg and fluoxetine-treated MBP1-h α syn tg mice respectively. (O) Analysis of α syn immunoreactivity in the corpus callosum across the experimental groups. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test. Scale bar (A–D) = 200 μ m, (F–I, and K–N) = 50 μ m.

Figure 6. Fluoxetine enhances GDNF and BDNF protein levels and activates the ERK signaling pathway in the MBP1-hαsyn transgenic model of MSA

The effect of fluoxetine administration on protein levels of GDNF was examined using immunohistochemistry and ELISA. (A–D) GDNF immunoreactivity in the hippocampus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (E) Analysis of hippocampal GDNF immunoreactivity across the experimental groups. (F) GDNF levels in the cytosolic fraction (containing soluble proteins) from whole-brain homogenates from saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test. Scale bar (A–D) = 150 μ m

The effect of fluoxetine administration on GDNF and BDNF protein levels and the ERK signaling pathway was also examined by immunoblot. (G) Immunoblot analysis of levels GDNF, BDNF, phospho-ERK (pERK) and total ERK (tERK) in the cytosolic fraction from whole-brain homogenates from saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (H) Analysis of GDNF levels in saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (I) Analysis of BDNF levels saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. (J) Analysis of ERK activation (taken as a ratio between pERK/tERK) in saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hαsyn tg and fluoxetine-treated MBP1-hαsyn tg mice respectively. Beta-actin was used as loading control. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test.

Figure 7. Differential effect of fluoxetine on GDNF, BDNF and hα-syn mRNA levels in the MBP1-hαsyn transgenic model of MSA

Quantitative-PCR was conducted to examine the effect of fluoxetine treatment on mRNA levels of GDNF, BDNF and hα-syn. (A) GDNF mRNA levels in saline and fluoxetine-treated non-tg and MBP1-hαsyn tg mice. (B) BDNF mRNA levels in saline and fluoxetine-treated non-tg and MBP1-hαsyn tg mice. (C) h-αsyn mRNA levels in saline and fluoxetine-treated non-tg and MBP1-hαsyn tg mice. All results are presented as mean mean $\pm$ SEM of the ratio of the probe to murine actin. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test.

Figure 8. In vitro fluoxetine administration elevates GDNF levels and ERK signaling in an oligodendrocytic cell line

In order to examine the effect of fluoxetine on oligodendroglial levels of GDNF the CG4 rat cell line was differentiated to an oligodendrocytic phenotype, infected with LV- α -syn and treated with 25 μ M fluoxetine for 24 hours. (A) Immunoblot analysis of oligodendroglial differentiation in cell lysates from CG-4 using oligodendroglial, astrocytic and neuronal markers (MBP, GFAP and Tuj-1), levels of GDNF and phospho-ERK (pERK) and total ERK (tERK). Brain homogenate from a non-tg mouse was used as a positive control for antibody signal and beta-actin was used as a loading control. (B) Analysis of GDNF levels in fluoxetine-treated oligodendroglial CG-4 cells. (C) Analysis of ERK activation (taken as a ratio between pERK/tERK) in oligodendroglial CG-4 cells. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test.

Figure 9. Characterization of the serotonergic system in the MBP1-hα-syn transgenic model of MSA and the effects of fluoxetine

The effect of fluoxetine administration on the serotonergic system was examined using immunohistochemistry. (A–D) Representative images of tryptophan hydroxylase (5HT-OH) immunoreactivity in the brainstem raphe nucleus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hα-syn tg and fluoxetine-treated MBP1-hα-syn tg mice respectively. (E) Analysis of 5HT-OH in the raphe nucleus across the experimental groups. (F–I) Representative images of 5HT-OH immunoreactivity in fibers projecting from the raphe nucleus of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hα-syn tg and fluoxetine-treated MBP1-hα-syn tg mice respectively. (J) Analysis of 5HT-OH in projecting fibers across the experimental groups. (K–N) Confocal images of 5HT immunoreactivity in hippocampal nerve terminals of saline-treated non-tg, fluoxetine-treated non-tg, saline-treated MBP1-hα-syn tg and fluoxetine-treated MBP1-hα-syn tg mice respectively. (O) Analysis of 5HT-immunoreactive hippocampal nerve terminals across the experimental groups. Error bars represent mean $\pm$ SEM. * Indicates a significant difference ($p < 0.05$) between the groups indicated by the bar analyzed by one-way ANOVA and Dunnett's post hoc test. Scale bar (A–D) = 20 μ M, (E–H) = 20 μ M and (I–L) = 10 μ M.