Published in final edited form as:

Eur J Neurosci. 2012 January ; 35(1): 10–19. doi:10.1111/j.1460-9568.2011.07933.x.

Fluoxetine rescues impaired hippocampal neurogenesis in a transgenic A53T synuclein mouse model

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Abstract

The accumulation of alpha-synuclein in Lewy bodies and Lewy neurites of different neuronal populations is one of the neuropathological hallmarks in Parkinson disease (PD). Overexpression of human wildtype or mutant alpha-synuclein affects the generation of new neurons in the adult dentate gyrus (DG) of the hippocampus in models of PD. Hippocampal dysfunction with reduced neurogenesis plays an important role in the pathogenesis of depression, an important non-motor symptom in PD. Moreover, effective antidepressant treatment is still an unmet need in PD. The present study explored if impaired hippocampal neurogenesis in the A53T transgenic animal model of PD may be restored by chronic oral application of the selective serotonin reuptake inhibitor (SSRI) fluoxetine. First, we determined the expression pattern of transgenic mutant A53T synuclein in developing DG neurons and showed early expression of the transgene linked to a severely impaired neurogenesis. After chronic fluoxetine treatment we observed an increased adult neurogenesis in the hippocampus of more than 3fold in treated A53T mice compared to controls. The pro-neurogenic effect of chronic fluoxetine application is predominantly related to an increased proliferation of neural precursor cells in the DG, to a lesser extent by induction of differentiation into mature neurons. The analysis of underlying mechanisms revealed an induction of brain derived and glial cells-derived neurotrophic factor levels as a result of fluoxetine treatment. This study underlines the large potential of SSRI dependent mechanisms to stimulate adult hippocampal neurogenesis in alpha-synuclein models and may lead to novel means to improve neuropsychiatric symptoms in PD.

Keywords

Adult neurogenesis; proliferation; α -synuclein; antidepressants; precursor cells

Introduction

The accumulation of misfolded or mutant α -synuclein (α -syn) is one of the major neuropathological hallmarks in Parkinson disease (PD) (Schulz-Schaeffer, 2010). While a

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physiological role of α -syn in developmental plasticity and synaptic remodelling has been proposed (Nemani *et al.*, 2010), α -syn accumulates as intracellular deposits in Lewy bodies and Lewy neurites in PD (Spillantini *et al.*, 1998). Mutations in the human α -syn gene (A53T, A30P, E46K) lead to an early onset of PD (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998; Zarranz *et al.*, 2004). In particular, patients carrying the A53T mutation develop a progressive parkinsonian phenotype including cognitive decline and show a widespread α -syn pathology affecting the hippocampal formation (Duda *et al.*, 2002). Importantly, non-motor symptoms like depression often precede the motor symptoms, are present throughout the disease process, and have a strong impact on quality of life (Tolosa & Poewe, 2009).

In the adult mammalian brain, neurogenesis occurs in two regions: The subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) adjacent to the lateral ventricles harbouring neural stem and precursor cells (Eriksson *et al.*, 1998; Curtis *et al.*, 2007). Adult neurogenesis in the DG involves proliferation of neural stem and precursor cells in the SGZ, the migration of newly generated neuroblasts into the granular cell layer of the DG and the differentiation into mature neurons that functionally integrate into existing circuits (Kuhn *et al.*, 1996; Van Praag *et al.*, 2002). Interestingly, adult neurogenesis is altered by numerous molecular and environmental factors (Mu *et al.*, 2010). Moreover, impairment of adult neurogenesis was described both in patients and animal models of PD (reviewed in Winner *et al.*, 2011). In particular, transgenic mouse models for PD expressing mutant A53T α -syn showed severely impaired adult neurogenesis (Winner *et al.*, 2008; Peng & Andersen, 2011).

Impaired DG neurogenesis is one of the important structural alterations in animal models of depression, efficiently reversed by antidepressants (Santarelli *et al.*, 2003; Encinas *et al.*, 2006; Sahay & Hen, 2007). The effect of imipramine and fluoxetine is dependent on the presence of neurogenic activity and serotonin receptors (Santarelli *et al.*, 2003). Noteworthy, there are effects of fluoxetine on depressive behaviour independent of intact hippocampal neurogenesis (David *et al.*, 2009).

Since hippocampal atrophy occurs in patients with major depression (MacQueen *et al.*, 2003), a direct or indirect link between adult hippocampal neurogenesis and depression is likely (Sahay & Hen, 2007). While depressive symptoms are described in up to 2/3 of all PD patients (Gallagher *et al.*, 2010), the underlying neural circuitries leading to depression in PD are still not fully understood (Politis *et al.*, 2010).

This study investigates the effect of fluoxetine on adult hippocampal neurogenesis in a transgenic A53T α -syn animal model and tests the hypothesis whether oral chronic application is able to restore impaired DG neurogenesis. To address possible underlying mechanisms the effect of fluoxetine on levels of neurotrophic factors is further analyzed.

Materials and methods

Experiment I

Transgenic mice expressing human A53T mutant α -syn under the control of the platelet-derived growth factor β (PDGF- β)-promoter were bred from the same background and genotyped as previously described (Hashimoto *et al.*, 2003). To determine the expression pattern of mutant A53T synuclein in developing and mature neurons in the DG, 5 mice transgenic for the human A53T mutant α -syn and 6 non-transgenic (non-tg) littermates at the age of 4–5 months received daily injections with the thymidine analogue bromodeoxyuridine (BrdU) intraperitoneally (i.p.) on 5 consecutive days (50 mg/kg body weight). One month later, animals were deeply anaesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Brains were removed, postfixed overnight in 4% paraformaldehyde/PBS at 4 °C and stored in 30 % sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C. One animal in the A53T a-syn group was excluded due to a severe hydrocephalus.

Experiment II

Transgenic A53T a-syn animals and non-tg controls (see above) at the age of 4–5 months were divided into four weight-, age- and gender-matched groups that received either fluoxetine or control by oral gavage 1×/day over 33 consecutive days: the treatment groups received fluoxetine 18mg/kg dissolved in 0.5% carboxymethylcellulose daily (non-tg Fluox, n=9, A53T Fluox, n=12) and the control groups were treated with 0.5% carboxymethylcellulose only (non-tg Co, n=9; A53T Co; n=14). Animals were kept in normal light-dark cycle of 12h and had free access to food and water. In order to label dividing cells, BrdU was administered daily on days 6–10 of drug administration by i.p. injections (50 mg/kg body weight). At the end of the treatment period, animals were deeply anaesthetized and transcardially perfused as described above. Brains were removed, postfixed and stored accordingly. All experiments were carried out in accordance with the National Institutes of Health guidelines for the humane treatment of animals and were approved by the local governmental commission for animal health.

Tissue processing

The hemispheres of the forebrains were divided and one hemisphere was cut into 25-µm coronal sections using a sliding microtome on dry ice. Sections were stored at 4°C in cryoprotectant solution (25% ethylene glycol, 25% glycerol in 0.1M phosphate buffer, pH 7.4).

Immunostaining

Immunohistochemistry was performed as previously described (Kohl *et al.*, 2010). For BrdU and proliferating cell nuclear antigen (PCNA) stainings, tissue was pre-treated with formamide and HCl to denature DNA. Free-floating sections in Tris-buffered saline (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) were treated with 0.6 % H₂O₂, blocked in 3 % donkey serum and 0.1 % Triton-X100 and incubated with the following primary antibodies: BrdU, rat monoclonal (1:500, AbD Serotec, Oxford, UK), PCNA, mouse monoclonal (1:500), sex determining region Y (SRY)-box 2, goat polyclonal (Sox2; 1:500) and doublecortin, goat polyclonal (DCX; 1:250; all Santa Cruz Biotechnology, Santa Cruz, CA, USA), neuronal nuclei, mouse monoclonal (NeuN; 1:500, Millipore, Billerica, MA, USA), glial fibrillary acidic protein (GFAP; 1:500, Dako, Fort Collins, USA) and human alpha-synuclein, rat monoclonal (1:20, clone 15G7; Enzo Life Science, Farmingdale, NY, USA).

Secondary antibodies were donkey-derived, species-specific and conjugated with Alexa-488, Alexa-568, Alexa-660 (all 1:1000, Invitrogen, Carlsbad, CA, USA) or biotin (1:500, Dianova, Hamburg, Germany). For immunohistochemistry, tissue was treated with avidin-biotin-peroxidase complex (1:100) and 3,3'-diaminobenzidine substrate kit (both Vector Laboratories, Burlingame, CA, USA) to generate a black coloured product. For all antibodies, control stainings omitting the primary antibody produced no signal.

Microscopy and quantification

All morphological analyses were conducted on blind-coded slides. Every sixth section (240-µm interval) of one hemisphere was selected from each animal and processed for immunohistochemistry. To analyze the number of BrdU, PCNA and DCX positive cells in the DG, the number of immunopositive cells was determined using the counting procedure described by Williams and Rakic (Williams & Rakic, 1988). Labeled cells which intersected

the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of the counting frame were not counted. Total numbers of labeled cells within the DG were counted exhaustively after determining the area of the DG. In addition, the number of cells expressing Sox2 exclusively present in the SGZ of the DG was determined in a representative subset of mice (n=6 from each group). Again, every sixth section was selected for manual counting and the same exclusion criteria were applied. Results were multiplied by 6 to obtain an estimate of the total immunopositive cell numbers. All extrapolations were calculated for one hemisphere and should be doubled to represent the total brain values. All counting procedures and measurements of reference volumes were conducted on a light/fluorescence microscope (Zeiss AxioImager M2, Göttingen, Germany) equipped with a semi-automatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, VT, USA) as previously described (Kohl *et al.*, 2010). In addition, all brightfield images were obtained using this microscope.

To determine the frequency of neuronal differentiation of newborn cells, every 12th brain section was stained for BrdU and NeuN by immunofluorescence and examined using a confocal laser microscope (Leica TCS-SP2, Bensheim Germany) equipped with a 40× PL APO oil objective (1.25 numeric aperture) and a pinhole setting that corresponded to a focal plane of 2 µm or less. In the DG, all, but not more than 50 BrdU-labeled cells per animal were analyzed for neuronal differentiation. BrdU-positive cells were characterized as solely BrdU-positive (newborn cells) and BrdU+/NeuN+ double-positive cells (newborn neurons), and the percentage of double-positive cells was measured. Moreover, this percentage was multiplied with the total number of newly generated BrdU+ cells to determine the total number of newly generated neurons.

Additional fluorescence images labeling a-syn in combination with DCX, NeuN, GFAP and Sox2 were obtained using a Zeiss confocal scanning laser microscope (LSM 780; 25× and 63× PL APO oil objective, pinhole 1 airy unit).

Immunoblot experiments

Another set of 4–5 months old age and gender-matched transgenic A53T a-syn mice and non-transgenic littermates (n=4 for non-tg Co, non-tg Fluox, A53T Co and A53T Fluox each) were treated with oral fluoxetine or control over 35 days, according to the protocol above. These animals were also kept in normal light-dark cycle of 12h and had free access to food and water. At the end of the treatment period, animals were deeply anaesthetized and sacrificed, brains were removed, and the left hemibrain was kept at –80°C for biochemical analysis.

25 micrograms of protein per mouse were loaded on SDS-PAGE gels (NuPAGE 4–12% Bis-Tris; Invitrogen) and transferred onto polyvinylidene fluoride membranes (Immobilon; Sigma) washed and blocked in 5% bovine serum albumine. Membranes were incubated overnight in primary antibodies against brain derived neurotrophic factor (BDNF; 1:1000; rabbit polyclonal; Abcam), glial cell line-derived neurotrophic factor (GDNF; 1:1000; rabbit polyclonal; Abcam) or human a-syn (1:750; mouse monoclonal; BD Biosciences). After incubation in adequate secondary antibodies (1:5000), membranes were reacted with an enzymatic chemoluminescence system (Novex ECL, Invitrogen) and developed on a VersaDoc gel-imaging machine (Bio-Rad, Hercules, CA). To confirm equal loading, a primary antibody against β-actin (1:5000; Sigma) was used.

Statistical analysis

Statistical analysis was performed using t-test for Experiment I and two-way ANOVA comparisons between the treatment groups of Experiment II with Bonferroni post hoc

analysis (Prism 5; GraphPad Software, San Diego, CA, USA). All data are shown as mean $\pm$ standard error of the mean (S.E.M.). Significance threshold was assumed at $p < 0.05$.

Results

Temporal expression pattern of A53T a-syn during adult neurogenesis in the hippocampus of transgenic mice

To determine the temporal pattern of mutant a-syn expression during proliferation, migration and differentiation of newly generated DG cells, we used an a-syn antibody specific for human a-syn (N-terminus, clone 15G7). We detected intracellular punctuated immunoreactivity for the human transgene abundantly in the hilus of the dentate gyrus, including the SGZ (Fig. 1). We identified specific a-syn signals in progenitor cells of the SGZ expressing Sox2 and in GFAP+/Sox2+ double positive cells. Moreover, immature neuroblasts expressing DCX and mature neurons, labeled with NeuN, show immunoreactivity of a-syn. In non-tg littermates, human a-syn was not present (Fig. 1). This finding suggests that the transgenic A53T mutant a-syn under the PDGF- β promoter is expressed early after birth until a mature neuronal phenotype is obtained.

Reduced hippocampal neurogenesis in naïve A53T a-syn mice

To assess the level of impaired neurogenesis we analyzed adult hippocampal neurogenesis in naïve A53T a-syn animals and wildtype littermates. We observed a significant reduction of BrdU+ cells in the DG of transgenic mice by 43 % compared to controls (692 ± 59.9 in non-tg vs. 396 ± 31.2 in A53T; $p = 0.011$, $t_9 = 3.19$; Fig. 2 F). The analysis of differentiation revealed no significant changes in the percentage of BrdU+ cells double labeled for NeuN as a mature neuronal marker; ($p = 0.323$, $t_9 = 1.03$). Thus, the calculated number of newly generated neurons was significantly reduced by 40% in A53T a-syn mice compared to wildtype littermates (450 ± 46 in non-tg vs. 274 ± 20 in A53T; $p = 0.010$, $t_9 = 3.25$; Fig 2 J). To further analyze the underlying cellular mechanism contributing to the decrease in newborn cells, we analyzed cell proliferation in the SGZ of the DG by using PCNA as a marker. PCNA is a cell cycle marker, which is synthesized in early G1 and S phases of the cell cycle (Ino & Chiba, 2000). We observed significantly reduced numbers of PCNA+ cells by 28% in A53T a-syn mice (1032 ± 108 in non-tg vs. 734 ± 41 in A53T; $p = 0.041$, $t_9 = 2.39$; Fig 2 C). Additionally, we detected a reduced number of DCX labeled immature neuroblasts in the DG, however without reaching a significant level ($p = 0.112$, $t_9 = 1.76$). These results suggest that mutant A53T a-syn under the PDGF- β promoter expressed early in the development of newly generated DG neurons, impairs adult DG neurogenesis predominantly due to a reduced proliferation of neural stem and precursor cells.

Chronic fluoxetine treatment leads to an increased DG proliferation in A53T a-syn and non-tg mice

To test the hypothesis whether the impaired hippocampal neurogenesis in transgenic A53T a-syn mice may be reversed, we investigated the effect of chronic oral treatment with fluoxetine, which enhances hippocampal neurogenesis predominantly by increasing proliferation of stem and precursor cells in the DG (Malberg *et al.*, 2000). First, we quantified cell proliferation in the DG of fluoxetine (Fluox) and control (Co) treated A53T a-syn mice and non-tg animals. Using the cell cycle marker PCNA, we detected a strong, about 2.5-fold increase of proliferating cells in non-tg Fluox mice compared to non-tg Co animals (Table 1, Fig. 3 E). Confirming the results from *Experiment I*, Co-treated transgenic A53T a-syn mice showed a reduced proliferation compared to non-tg mice (by 39%). More importantly, fluoxetine fully reversed the proliferative deficit in A53T animals compared to Co-treatment, showing a 4-fold increase of proliferation. It does not only reach the level of

non-tg Fluox mice, but exceeds the baseline rate of neurogenesis in Co treated non-tg mice ($F_{1,43}=38.55$, $p<0.0001$ for treatment; $F_{1,43}=0.56$, $p=0.460$ for genotype; Table 1, Fig 3 E).

Fluoxetine increased the number of Sox2 expressing cells in the SGZ of the DG in A53T and non-tg mice

To further elucidate which cell population in the DG is affected by fluoxetine treatment we quantified cells expressing the progenitor cell marker Sox2 in the SGZ of the DG. Interestingly, the number of Sox2 labeled cells was significantly higher in non-tg Co animals compared to A53T Co mice. Moreover, there was a robust effect of fluoxetine treatment on the number of Sox2 expressing cells in the SGZ in both non-tg and A53T mice, increased by 30% and 93%, respectively ($F_{1,23}=83.40$, $p<0.0001$ for treatment; $F_{1,23}=4.78$, $p=0.041$ for genotype; Table 1, Fig 3).

Strong increase of newly generated DG neurons in A53T mutant mice by chronically administered fluoxetine

Proliferating cells in the SGZ of the DG differentiate further into immature neuroblasts expressing the marker DCX. We observed that fluoxetine results in a strong increase in the number of neuroblasts in non-tg mice by a factor of 2.8, in transgenic A53T a-syn even 3.5, respectively ($F_{1,43}=10.44$; $p<0.0001$ for treatment; $F_{1,43}=1.26$, $p=0.268$ for genotype; Table 1, Fig 4 E). The proportional increase of DCX labeled neuroblasts almost matches the increase in PCNA labeled cells.

Newborn cells in the DG labeled with BrdU 4 weeks after the last injection represent the surviving population of newly generated cells. Quantification of these cells in fluoxetine treated non-tg mice revealed a doubling of the number of BrdU+ cells compared to Co-treated non-tg mice. Interestingly, the number of surviving new cells in the DG of A53T a-syn mice increased by the factor 3.6 reaching the level of fluoxetine treated non-tg mice ($F_{1,43}=31.31$, $p<0.0001$ for treatment; $F_{1,43}=0.64$, $p=0.427$ for genotype; Table 1, Fig 4 J). The absolute amount of newly generated cells in the DG of Co treated transgenic and non-tg mice was similar to the naïve animals in *Experiment 1* indicating that long-term Co treatment did not interfere with adult hippocampal neurogenesis.

To further characterize the phenotype of these newly generated cells in the DG, we quantified the amount of BrdU+ cells co-expressing the mature neuronal marker NeuN. We observed a significant shift towards neuronal differentiation of newly generated BrdU+ cells in both fluoxetine treated groups, even more pronounced in the non-tg mice ($F_{1,43}=19.81$, $p<0.0001$ for treatment; $F_{1,43}=3.35$, $p=0.075$ for genotype; Table 1, Fig 4 O). The calculation of absolute amount of new DG neurons showed that chronic oral fluoxetine treatment more than doubled (2.4×) the number of new neurons in non-tg Fluox animals compared to non-tg Co mice. In A53T animals the number of new neurons even increased by 3.4×, completely restoring the A53T a-syn mediated impairment on hippocampal neurogenesis ($F_{1,43}=25.97$, $p<0.0001$ for treatment; $F_{1,43}=0.74$, $p=0.395$ for genotype; Fig. 4 P).

Fluoxetine increases levels of BDNF and GDNF levels in A53T mice and non-tg controls

To investigate underlying mechanisms mediating the effect of fluoxetine on DG neurogenesis we compared the expression levels of the neurotrophic factors BDNF and GDNF in the brains of control and fluoxetine treated non-tg and A53T mice (Fig. 5). The immunoblot analysis of BDNF showed reduced levels in Co treated A53Ts compared to non-tg mice, and a significant increase of BDNF content after chronic fluoxetine treatment in both groups ($F_{1,15}=268.08$, $p<0.0001$ for treatment; $F_{1,15}=8.37$, $p=0.014$ for genotype; Fig. 5 B). While the content of GDNF in control treated A53T mice is reduced compared to

non-tg animals, we observed an increase of GDNF expression after fluoxetine treatment both in non-tg and A53T mice, consistent with the increase of hippocampal cell proliferation and survival ($F_{1,15}=152.26$, $p<0.0001$ for treatment; $F_{1,15}=17.83$, $p=0.001$ for genotype; Fig. 5 D). Finally, we analyzed the content of transgenic a-syn in the treated animals. While we could observe a stable overexpression of A53T a-syn both in A53T Co and Fluox groups, the treatment with fluoxetine did not have an effect on the expression level of a-syn ($F_{1,15}=0.01$, $p=0.923$ for treatment; $F_{1,15}=3776.73$, $p<0.0001$ for genotype; Fig. 5 C).

Discussion

The present study shows that chronic oral fluoxetine treatment is sufficiently able to restore the deficit in adult hippocampal cell proliferation and neurogenesis in the transgenic A53T a-syn mouse model for PD. In detail, fluoxetine enhanced adult hippocampal neurogenesis mainly by increasing the proliferative activity of early neural progenitor cells. Moreover, a significant shift towards neuronal differentiation of surviving cells was observed. In addition, the treatment procedure by gavage had no negative influence on hippocampal neurogenesis as Co treated non-tg and A53T mice showed similar numbers of newly generated neurons compared to naïve mice. Moreover, we could show that fluoxetine significantly increases the levels of the neurotrophic factors BDNF and GDNF both in non-tg and A53T mice, explaining at least in part the pro-neurogenic effect. The expression of the transgenic a-syn was unaltered after fluoxetine treatment.

The expression of mutant or wildtype a-syn under the control of the PDGF- β promoter has distinct effects on adult neurogenesis in the DG: Overexpression of wildtype a-syn mainly affects the survival of new neurons without impairment of the proliferation of neural stem and precursor cells (Winner *et al.*, 2004). In contrast, the overexpression of mutant A53T a-syn leads to a decreased proliferation of the precursor cell population in the SVZ (Winner *et al.*, 2008) and in the DG (Crews *et al.*, 2008). The expression pattern of the transgene mutant A53T a-syn in early stages of neuronal development is in line with the activity of the PDGF- β promoter used (Masliah *et al.*, 2000). PDGF- β is expressed in embryonic and postnatal stages of neuronal development and in adult neurons (Sasahara *et al.*, 1992). Moreover, we could show the expression of A53T a-syn in immature newly generated cells of the DG, but also in developing DCX positive neuroblasts and in mature neurons. This expression profile corresponds well with the impact of the transgenic A53T a-syn both on proliferation and survival of newly generated DG neurons.

Understanding mechanisms underlying impaired adult neurogenesis is an important prerequisite for the identification of disease mechanisms in neurodegenerative diseases (Winner *et al.*, 2011) and the development of therapeutic strategies for some of the PD associated non-motor symptoms. The pro-neurogenic effect of chronic, but not acutely administered fluoxetine on adult neurogenesis in the DG has been described in naïve rats and mice (Miller *et al.*, 2008; Wang *et al.*, 2008): Fluoxetine predominantly increases the proliferative activity of early progenitor cells in the DG (Encinas *et al.*, 2006). Moreover, a recent study observed an increased differentiation into a neuronal phenotype after fluoxetine treatment (Marlatt *et al.*, 2010). Conflicting results exist from a recent study describing impaired neuronal maturation after oral fluoxetine treatment showing a decreased expression of the neuronal marker calbindin (Kobayashi *et al.*, 2010). Nevertheless, there are species- and strain-specific differences in the response of hippocampal neurogenesis to fluoxetine: different mice strains showed distinct patterns of responsiveness of the DG to the treatment with fluoxetine. Some strains like the A/J and the SWR/J strain even showed no increase of DG neurogenesis after treatment with fluoxetine for 21 days (Miller *et al.*, 2008). In addition, the effect size of fluoxetine on hippocampal neurogenesis appears to be age-dependent: Fluoxetine fails to improve hippocampal neurogenesis in 6 month old mice when

administered i.p. (Couillard-Despres *et al.*, 2009). In this context, it is pharmacologically important that the majority of the previous studies have used i.p. administration for fluoxetine. Our study shows that chronic oral treatment with fluoxetine has a strong impact on hippocampal neurogenesis even in mice of 4–5 months of age with a DBA/BL6 background. Variable resorption after long-term i.p. injections may play an important role in the pharmacokinetics of fluoxetine.

Chronic fluoxetine treatment leads to a stimulation of DG proliferation and neurogenesis in a corticosteroid induced animal model for depression and anxiety (David *et al.*, 2009), and in transgenic animal models for Huntington and Alzheimer disease (Dong *et al.*, 2004; Grote *et al.*, 2005). Moreover, the reduced DG cell proliferation in 6-hydroxy-dopamine (6-OHDA) lesioned rats could be reversed by chronic fluoxetine treatment (Suzuki *et al.*, 2010). Finally, it is important to note that there is limited knowledge about functional effects of super numerous new DG neurons. The massive increase of DG neurogenesis described after epileptic seizures shows an aberrant network (Parent *et al.*, 2006). Just recently, mice with an excessive level of DG neurogenesis by genetically inhibiting apoptosis showed marked improvements in distinct behavioral tasks, such as pattern separation and exploratory behavior (Sahay *et al.*, 2011).

From a clinical perspective, depressive symptoms are observed in 40% up to 70% of PD patients and more importantly often precede motor symptoms (Gallagher *et al.*, 2010). Large randomized controlled trials are lacking in PD patients with depression. While tricyclic antidepressants and SSRIs are widely used, two recent short term clinical studies favour tricyclics over SSRIs to treat symptoms of depression in PD (Devos *et al.*, 2008; Menza *et al.*, 2009).

Concerning the underlying mechanisms of fluoxetine mediated effects on neurogenesis, the presence of serotonergic 5-HT_{1A} receptors is necessary for its effect on DG neurogenesis (Santarelli *et al.*, 2003), while there still exists ambiguity about the causal link between hippocampal neurogenesis and serotonin signalling for the behavioural effects of antidepressants (Sahay & Hen, 2007; Holick *et al.*, 2008). Depletion of the serotonergic input into the hippocampus reduces hippocampal neurogenesis (Ueda *et al.*, 2005), while the effect of long-term stimulation of serotonin receptors results in increased neurogenesis due to 5-HT_{2c} stimulation (Klempin *et al.*, 2010). Serotonergic signaling activates cAMP response element-binding protein (CREB), consecutively upregulating the expression of BDNF, known to be pro-neurogenic (Nibuya *et al.*, 1996). Previous studies have shown that antidepressant treatment stimulates pCREB (Miró *et al.*, 2002) and leads to increased levels of brain BDNF protein and mRNA (De Foubert *et al.*, 2004; Miller *et al.*, 2008). In this study we could show that chronic fluoxetine treatment increases BDNF levels both in non-tg but also in A53T a-syn mice, related at least in part to the strong enhancement of hippocampal neurogenesis. While serum levels of BDNF are reduced in PD patients (Scalzo *et al.*, 2010) and restored by antidepressant treatment using SSRIs (Ricci *et al.*, 2010), we could also detect a reduced BDNF level in the brains of A53T mice compared to controls. Moreover, fluoxetine did not alter the expression levels of the human a-syn in the transgenic A53T mouse model. This is in contrast to a recent *in vitro* study demonstrating interplay of a-syn with protein kinase C leading to CREB-dependent downregulation of BDNF (Yuan *et al.*, 2010). Taken previous findings and this study together, it underlines the importance of the BDNF pathway mediating the effect of fluoxetine.

The neurotrophic factor GDNF has been studied extensively as a potential neuroprotective therapy in PD: GDNF protects dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-OHDA animals models of PD (Bowenkamp *et al.*, 1995; Tomac *et al.*, 1995). Gene therapy approaches to deliver GDNF into the brains of PD rodent

and primate models have shown neuroprotective effects on dopaminergic neurons (Choi-Lundberg *et al.*, 1997; Kordower *et al.*, 2000), leading to clinical studies in human PD patients (Lang *et al.*, 2006). Moreover, intracerebral infusion of GDNF increases cell proliferation in the DG of rats (Chen *et al.*, 2005). Interestingly, fluoxetine has been shown to stimulate GDNF production in vitro (Mercier *et al.*, 2004). The present study indicates that GDNF may also play a role in mediating the effect of fluoxetine on adult hippocampal neurogenesis.

In summary, chronic orally administered fluoxetine overcomes impaired hippocampal neurogenesis in a A53T mutant a-syn mouse model, but even leads to an excessive stimulation of hippocampal neurogenesis by induction of neurotrophic factors, in particular of BDNF and GDNF. Further understanding of SSRI dependent mechanisms may lead to novel approaches to stimulate endogenous generation of new neurons, in particular for treating depressive symptoms in PD.

Acknowledgments

This study was supported by the Bavarian State Ministry of Sciences, Research and the Arts (ForNeuroCell), the German Ministry for Education and Science (BMBF grants 01GN0979 and 01GQ1103), the Bavarian Research Foundation (PIZ-177-10) and the interdisciplinary center for clinical research (IZKF) at the FAU Erlangen-Nuernberg. EM and KU are supported by NIH grants AG 18440, NS 044233, AG 10435, AG 022074 and by the Spencer Family Canadian Donner Foundation.

We would like to thank Dr. Fred H. Gage for stimulating discussions on the study.

Abbreviations

6-OHDA	6-hydroxy-dopamine
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumine
BrdU	bromo-deoxyuridine
CREB	cAMP response element-binding protein
Co	carboxymethylcellulose
DG	dentate gyrus
DCX	doublecortin
EGF	epidermal growth factor
ERK	extracellular-signal regulated kinase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
i.p.	intraperitoneally
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PCNA	proliferating cell nuclear antigen
PD	Parkinson disease
PDGF	platelet-derived growth factor
SGZ	subgranular zone of the dentate gyrus
Sox2	sex determining region Y (SRY)-box 2

SSRI selective serotonin reuptake inhibitor

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Figure 1.

Expression pattern of transgenic mutant alpha-synuclein (green) in the dentate gyrus of transgenic A53T alpha-synuclein (a-syn) mice and non-transgenic littermates. (A–H) Detailed analysis of A53T mutant a-syn expression in developing and mature neurons of the dentate gyrus in A53T mice, showing human a-syn in Sox2+/GFAP+ cells (B), Sox2+ progenitor cells in the SGZ (D), DCX+ neuroblasts (F), and in NeuN+ mature neurons (H). No a-syn expression in non-transgenic controls (A, C, E, G). Scale bar represents 10 μm.

Figure 3. Cell proliferation determined by quantification of PCNA+ cells in the DG of the hippocampus is strongly increased in fluoxetine treated non-transgenic (non-tg Fluox; 2.5fold) and A53T mutant alpha-synuclein mice (A53T Fluox; 4fold) compared to control treated non-transgenic (non-tg Co) and A53T animals (A53T Co). (A–D) Representative images of PCNA staining. (E) Graph depicts the strong effect of fluoxetine on hippocampal cell proliferation. Fluoxetine also affects the progenitor cell population in the SGZ of the DG that expresses the marker Sox2. In A53T mice oral fluoxetine treatment increases the number of Sox2 labelled cells significantly (93%) reaching a comparable level as non-tg animals. (F–I) Representative images of Sox2 staining in the SGZ. (J) Graph showing the effect of fluoxetine on Sox2+ progenitor cells in the SGZ. Scale bar represents 50 μ m. * indicates $p < 0.05$ (2-way ANOVA; Bonferroni post hoc test).

Figure 4. Strong increase of hippocampal neurogenesis in fluoxetine treated non-transgenic (non-tg) and A53T mutant alpha-synuclein (A53T) mice. (A–E) Chronic oral fluoxetine increases the absolute number of doublecortin (DCX) labelled neuroblasts by 2.8× in non-tg, even stronger in A53T animals by 3.5×. (F–J) Impaired survival of newly generated cells in A53T Co mice is fully restored by fluoxetine treatment in A53T Fluox mice, increasing the number of BrdU+ cells 3.6 fold. Non-tg Fluox mice show a comparable increase, doubling the number of BrdU labelled surviving new cells. (K–P) The generation of new neurons in the DG is impaired in A53T Co mice compared to non-tg Co. This deficit is fully reversed by a strong pro-neurogenic effect of fluoxetine in both groups, depicted in representative images (K–N). (O) The percentage of newly generated cells differentiating into a mature neuronal phenotype is significantly increased in both fluoxetine treated groups, as determined by double-labeling for BrdU and NeuN. (P) Calculation of absolute numbers of

surviving new neurons in the DG revealed significantly more newly generated neurons in non-tg Fluox (2.4 fold) and A53T Fluox mice (3.4 fold) compared to controls. Scale bars represent 50 μm . * indicates $p < 0.05$ (2-way ANOVA; Bonferroni post-hoc test).

Figure 5.

Fluoxetine treatment leads to increased levels of the neurotrophins brain derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) while levels of the transgene alpha-synuclein (a-syn) remain unchanged in non-transgenic (non-tg) and A53T mutant alpha-synuclein (A53T) mice. (A–D) Immunoblot analysis of levels BDNF, GDNF and human a-syn in the cytosolic fraction from whole-brain homogenates from control and fluoxetine treated non-transgenic mice (non-tg Co, non-tg Fluox) and control and fluoxetine treated A53T animals (A53T Co, A53T Fluox). (B) Quantification of BDNF levels showing significant increase in both Fluox groups compared to Co groups. (C) Levels of transgene a-syn are not altered in A53T animals by fluoxetine treatment. (D) Significant higher levels of GDNF in non-tg and A53T mice treated with fluoxetine compared to control treatment.

* indicates $p < 0.05$ (2-way ANOVA; Bonferroni post-hoc test).

Table 1

Adult neurogenesis in the dentate gyrus of fluoxetine treated A53T α -syn mice and non-tg mice (A) Experimental design for non-transgenic (non-tg) and transgenic A53T mutant alpha-synuclein (A53T) daily and orally treated with fluoxetine 18 mg/kg (Fluox) or control (Co) over 33 days. Animals received BrdU injections on days 6–10 and were perfused on day 33. (B) The number of proliferating cells within the dentate gyrus (DG) of transgenic animals (A53T) and non-transgenic controls (non-tg) are presented as the mean number of PCNA positive cells. The number of early progenitor cells is reflected by the quantification of Sox2 expressing cells in the subgranular zone of the DG (SGZ). The amount of neuroblasts is determined by counting cells expressing DCX in the DG. The numbers of surviving newly generated cells is estimated by the mean number of BrdU positive cells. Percentages of new neurons colabeling for BrdU and NeuN (%NeuN + cells) are presented. To determine the number of surviving new neurons after 4 weeks, the absolute number of BrdU+ neurons was calculated for non-tg and A53T mice treated with fluoxetine or control. All numbers are given as mean $\pm$ S.E.M..

(A) Design of Experiment:

(B)	Non-tg Co	Non-tg Fluox	A53T Co	A53T Fluox
Proliferation in the DG				
PCNA	1033 $\pm$ 76	2548 $\pm$ 480 [#]	628 $\pm$ 51	2541 $\pm$ 335 [*]
Progenitor cells in the SGZ of the DG				
Sox2 ($\times 10^3$)	10.63 $\pm$ 0.30	14.17 $\pm$ 0.71 [#]	7.58 $\pm$ 0.35	14.67 $\pm$ 0.80 [*]
Neuroblasts in the DG				
DCX ($\times 10^3$)	3.50 $\pm$ 0.26	9.74 $\pm$ 2.00 [#]	2.29 $\pm$ 0.13	8.38 $\pm$ 1.42 [*]
Neurogenesis in the DG				
BrdU	879 $\pm$ 36	1787 $\pm$ 318 [#]	501 $\pm$ 50	1844 $\pm$ 266 [*]
% BrdU/NeuN	74.3 $\pm$ 1.9	84.9 $\pm$ 2.1	73.2 $\pm$ 1.3	79.2 $\pm$ 2.2
BrdU $\times$ % BrdU/NeuN	656 $\pm$ 37	1551 $\pm$ 297 [#]	425 $\pm$ 52	1457 $\pm$ 249 [*]

[#] represents significant differences of Non-tg Co vs. Non-tg Fluox,

^{*} represents significant difference comparing A53T Co vs. A53T Fluox (2-way ANOVA, Bonferroni post hoc test).