Resistance to MPTP-Neurotoxicity in \(\alpha\)-Synuclein Knockout Mice Is Complemented by Human \(\alpha\)-Synuclein and Associated with Increased \(\beta\)-Synuclein and Akt Activation

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**Abstract**

Genetic and biochemical abnormalities of \(\alpha\)-synuclein are associated with the pathogenesis of Parkinson’s disease. In the present study we investigated the *in vivo* interaction of mouse and human \(\alpha\)-synuclein with the potent parkinsonian neurotoxin, MPTP. We find that while lack of mouse \(\alpha\)-synuclein in mice is associated with reduced vulnerability to MPTP, increased levels of human \(\alpha\)-synuclein expression is not associated with obvious changes in the vulnerability of dopaminergic neurons to MPTP. However, expressing human \(\alpha\)-synuclein variants (human wild type or A53T) in the \(\alpha\)-synuclein null mice completely restores the vulnerability of nigral dopaminergic neurons to MPTP. These results indicate that human \(\alpha\)-synuclein can functionally replace mouse \(\alpha\)-synuclein in regard to vulnerability of dopaminergic neurons to MPTP-toxicity. Significantly, \(\alpha\)-synuclein null mice and wild type mice were equally sensitive to neurodegeneration induced by 2’NH2-MPTP, a MPTP analog that is selective for serotoninergic and noradrenergic neurons. These results suggest that effects of \(\alpha\)-synuclein on MPTP like compounds are selective for nigral dopaminergic neurons. Immunoblot analysis of \(\beta\)-synuclein and Akt levels in the mice reveals selective increases in \(\beta\)-synuclein and phosphorylated Akt levels in ventral midbrain, but not in other brain regions, of \(\alpha\)-synuclein null mice, implicating the \(\alpha\)-synuclein-level dependent regulation of \(\alpha\)-synuclein expression in modulation of MPTP-toxicity by \(\alpha\)-synuclein. Together these findings provide new mechanistic insights on the role \(\alpha\)-synuclein in modulating neurodegenerative phenotypes by regulation of Akt-mediated cell survival signaling *in vivo*.

**Introduction**

Parkinson’s disease (PD) is predominantly an idiopathic disorder without cure and with limited symptomatic treatment. PD is marked by a selective and progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) characterized by movement abnormalities including resting tremor, bradykinesia, postural instability and rigidity[1]. Currently, both environmental and genetic factors are implicated in the pathogenesis of PD [2]. The genetic cause for PD was first established with identification of mutations in the gene encoding for the synaptic protein \(\alpha\)-synuclein in several families with the autosomal dominant form of PD [3,4,5,6]. A relationship between \(\alpha\)-synuclein and PD is further suggested by the finding that \(\alpha\)-synuclein is the main structural component of cytoplasmic proteinaceous inclusion bodies (Lewy bodies) and neurites (Lewy neurites) that are characteristic of both sporadic and familial PD cases [7]. Further, transgenic drosophila overexpressing human \(\alpha\)-synuclein demonstrates selective dopaminergic neuronal dysfunction [8] and forced overexpression of human \(\alpha\)-synuclein in rodents show varying degrees of neuronal dysfunction and degeneration [9,10,11,12,13] including dopaminergic degeneration [14,15,16,17,18]. However, a variety of human \(\alpha\)-synuclein transgenic mice with increased \(\alpha\)-synuclein aggregation, motric dysfunction, and neurodegeneration have failed to show overt neurodegeneration of dopaminergic neurons [10,11,12]. Thus, the...
mechanistic basis for α-synuclein abnormalities and dopaminergic degeneration is unresolved.

In addition, the relationship of α-synuclein abnormalities to other in vivo models of PD remains controversial. A widely used animal model of parkinsonism utilizes the selective dopaminergic neurotoxin MPTP that replicates the selective neuronal loss seen in PD [19,20,21]. A potential role for α-synuclein abnormalities in the pathogenesis of MPTP toxicity is suggested from the findings that α-synuclein aggregation occurs in mouse and primate models of MPTP-induced parkinsonism [21,22,23]. However, studies using rodents with increased expression of human α-synuclein have led to conflicting results where some studies show lack of increased sensitivity to MPTP with human α-synuclein expression [24,25] and some studies showing increased sensitivity/abnormality associated MPTP in human α-synuclein transgenic mice [26,27]. The discordance between these studies may be associated with number of factors including differences in MPTP toxicity paradigm used, promoters used, mouse strains, and the age of animals used for the MPTP treatment. Lack of more robust and consistent increase in MPTP toxicity with the overexpression of human α-synuclein is puzzling since several studies show that the expression of endogenous mouse α-synuclein is required to sensitize dopaminergic neurons to MPTP [21,28,29,30,31]. One of the possibilities is that human α-synuclein does not function in the MPTP toxicity pathway in rodents.

In this report, we have attempted to more fully document the in vivo relationship between α-synuclein expression and vulnerability of dopaminergic neurons to MPTP. We used multiple transgenic lines at multiple conditions to confirm that increased expression of human α-synuclein is not associated with robust increase in the susceptibility of the human α-synuclein transgenic mice to MPTP toxicity. However, human α-synuclein is able to restore the MPTP-sensitivity of the dopaminergic neurons lacking mouse α-synuclein expression, showing that human α-synuclein can functionally replace mouse α-synuclein in regard to vulnerability of dopaminergic neurons to MPTP-toxicity. Significantly, the mouse α-synuclein null mice are not protected from neurodegeneration induced by 2′NH₂-MPTP, the MPTP analog with selective toxicity to central noradrenergic and serotonergic neurons [32,33]. This indicates that the MPTP resistance phenotype of mouse α-synuclein null mice is selective for dopaminergic neurons and that alterations in the general metabolism and/or trafficking of MPP+ in the synaptic terminal are not involved in MPTP resistant phenotype of the mouse α-synuclein null mice. We also find that the levels of β-synuclein and phosphorylated Akt is inversely correlated with α-synuclein expression in the nigro-striatal system and increased β-synuclein and phosphorylated Akt levels correlate with the protection from MPTP toxicity. Thus, we propose that regulation of β-synuclein and Akt levels by α-synuclein expression is an important contributor to regulation of MPTP-toxicity by α-synuclein.

Results

Increased expression of human α-synuclein does not increase vulnerability of dopaminergic neurons to MPTP-intoxication

Acute MPTP paradigm. Eleven to thirteen week old transgenic mice generated using the mouse prion promoter, overexpressing wild type (line I2-2) or mutant A53T (line N2-5) or mutant A30P (line T3) human α-synuclein, and non transgenic littermates received either an acute paradigm of MPTP (18 mg MPTP/kg free base X4, every two hours) or saline. One week following treatment, HPLC with electrochemical detection demonstrates a profound reduction in total striatal dopamine following an acute paradigm of MPTP-treated mice compared to saline controls (Fig. 1A) (ANOVA, p<0.05). Analysis of striatal levels of dopamine and its metabolite DOPAC showed no differences in the losses between any of the transgenic lines and non-transgenic cohorts following MPTP administration. MPTP treatment also results in increased striatal dopamine turnover (DOPAC/DA) which is not statistically different in any of the transgenic lines compared to non-transgenic animals (data not shown). Stereologic counts of total neurons (Nissl positive) and TH-immunopositive neurons in substantia nigra show that MPTP treatment results in a significant decrease in total and TH-immunopositive neurons of the SNpc in all lines of mice (Fig. 1B) (ANOVA, p<0.05). However, no differences in susceptibility to MPTP are observed between transgenic versus non-transgenic mice.

Sub-acute MPTP paradigm. It is suggested that the paradigm of MPTP delivery may have an effect upon mechanisms of neuronal death. Sub-acute MPTP paradigms may cause apoptotic mechanisms of neuronal death [34,35,36] not seen in acute paradigms, which causes necrotic cell death [37]. Thus, we treated eleven to thirteen weeks old transgenic mice, expressing the A53T mutant human α-synuclein (line N2-5), and non-transgenic littermates with a 5 day sub-acute MPTP paradigm (30 mg MPTP/kg free base once daily for five days). Stereologic neuronal counts were performed 2 weeks following the final MPTP dose (Fig. 2A). Although, a significant loss of total and TH-immunopositive neurons were seen following MPTP administration compared to saline controls (ANOVA, p<0.05), no difference was seen among transgenic and non-transgenic lines. Identical results were obtained with the A30P mutant human α-synuclein transgenic mice treated with the sub-acute MPTP paradigm (data not shown).

Effect of age on the susceptibility of dopaminergic neurons to MPTP. Because aging is associated with increased vulnerability to MPTP-intoxication [38,39] and increased vulnerability to α-synuclein dependent neurodegeneration [11], we examined if age had a synergistic effect with over expression of mutant human α-synuclein on the susceptibility to nigral dopaminergic neurons following MPTP-intoxication. Transgenic and non-transgenic mice from the N2-5(A53T) line were treated with an acute MPTP paradigm (15 mg MPTP/kg free base X 4, every 2 hours) at 12–14 months of age. A lower than usual dose of MPTP was used because of increased sensitivity of aged mice to MPTP. Again, despite the significant loss of SNpc neurons in MPTP treated mice (ANOVA, p<0.05), no increase in susceptibility is observed in transgenic overexpressors versus non-transgenic cohorts (Fig. 2B).

Effect of high expression levels of A53T in mice on the susceptibility of dopaminergic neurons to MPTP. To be certain that overexpression of human α-synuclein does not affect MPTP susceptibility, we also examined the MPTP susceptibility of the A53T transgenic mice expressing very high levels of A53T human α-synuclein. Transgenic mice from line G2-3(A53T) express the transgene at approximately six times the level of endogenous mouse α-synuclein and develop a fatal neurodegenerative disease with an average life span of ~12 months [11]. To investigate if this higher level of expression had a synergistic effect with MPTP on neuronal toxicity, 6 month old transgenic and non-transgenic littermates were treated with acute MPTP paradigm (18 mg MPTP/kg free base X 4, every 2 hours) and numbers of neurons in SNpc were determined (Fig. 2C). Analysis of cell counts show that MPTP treatment of A53T transgenic mice (65%) may result in a slightly lower number of
dopaminergic neurons than with the non-transgenic littermates (70.4%) (Fig. 2C). Specifically, the difference in number of TH-immunopositive neurons between the MPTP treated non-transgenic mice and A53T transgenic mice are significant when the ANOVA analysis performed with the Neuman-Keuls-post-test ($p_{\leq 0.05}$). However, the differences are not significant when ANOVA analysis with Tukey-Kramer posthoc test was applied. Collectively, these results indicate that the increased expression of human $\alpha$-synuclein (either wild type or mutant) do not significantly affect MPTP susceptibility and, at best, very high levels of A53T mutant human $\alpha$-synuclein is required to slightly increase the vulnerability of dopaminergic neurons to MPTP-toxicity.

Human $\alpha$-synuclein complements MPTP-resistance phenotype but not the developmental reduction of dopaminergic neurons in $\alpha$-synuclein null mice

The lack of increased MPTP susceptibility with the human $\alpha$-synuclein transgenic mice is surprising since decreased expression of mouse $\alpha$-synuclein is associated with significant protection from MPTP-intoxication [21,29,30,31,40]. One possibility is that human and mouse $\alpha$-synuclein function differently regarding MPTP-susceptibility in mice.

To test the above possibility, we examined whether expression of human $\alpha$-synuclein could restore the susceptibility of dopaminergic neurons to MPTP toxicity in mouse $\alpha$-synuclein null mice. To test whether human $\alpha$-synuclein expression can complement the MPTP-resistant phenotype of the mouse $\alpha$-synuclein null mice, human $\alpha$-synuclein transgenic mice (WT and A53T) were successively crossed to the mouse $\alpha$-synuclein null mice [41] to generate human $\alpha$-synuclein transgenic mice lacking mouse $\alpha$-synuclein expression (Fig. 3A). The resulting mice were subjected to acute MPTP intoxication and nigrostriatal dopaminergic neurotoxicity was studied by performing stereologic neuronal counts of SNpc and quantitation of striatal dopamine and its metabolites 1 week after MPTP (20 mg MPTP/kg free base X4, every 2 hours) in 6–8 week old male mice. Consistent with previous reports [21,28,30], the $\alpha$-synuclein null mice are protected against MPTP-induced neurodegeneration at both striatum and SNpc (Fig. 3B, C). MPTP-induced reductions in striatal metabolites of dopamine, DOPAC and HVA and also increased dopamine turnover (DOPAC+HVA/DA) are blocked in $\alpha$-synuclein null mice (data not shown). However, mice expressing human $\alpha$-synuclein (wild type and A53T) on the mouse $\alpha$-synuclein null background show significant sensitivity to MPTP intoxication.
α-Synuclein Dependent Regulation of β-Synuclein

A. N2-5 line 3 mo (30 mg MPTP/kg once daily for 5 days)

B. N2-5 line 12-14 mo (15 mg MPTP/kg x4, every 2h)

C. G2-3 line 6 mo (18 mg MPTP/kg x4, every 2h)
Figure 2. Effects of sub acute MPTP regimen, aging, and transgene expression level on the MPTP sensitivity of nigral dopaminergic neurons in human A53T α-synuclein transgenic mice. A. Stereologic cell counts of total and TH-immunopositive neurons of SNpc in non transgenic and human A53T transgenic mice (line N2-S) after acute MPTP (15 mg MPTP/kg free base X4, every 2 h) and sub acute MPTP (30 mg MPTP/kg free base once a day for 5 days). Although sub acute MPTP caused significant reductions in total and TH-positive neurons no significant differences were seen among transgenic and non transgenic mice receiving the same treatments (saline or MPTP). B. TH-positive and total neuronal counts in SNpc of 12-14 month old non transgenic and human A53T transgenic mice (line N2-S) after acute MPTP (15 mg MPTP/kg free base X4, every 2 h) and sub acute MPTP (30 mg MPTP/kg free base once a day for 5 days). Although sub acute MPTP caused significant reductions in total and TH-positive neurons no significant differences were seen among transgenic and non transgenic mice receiving the same treatments (saline or MPTP). C. Cell counts of TH-positive and total neurons in high expressig lines of human A53T transgenic mice (G2-3 line, -6-fold) 7 days after acute MPTP intoxication (18 mg MPTP/kg free base X4, every 2 h). MPTP-intoxication resulted in a significant reduction of total and TH-positive neuronal counts in non transgenic and high transgenic mice compared to saline treatments. A moderate increase in the vulnerability was observed in A53T transgenic mice. Data represent mean ± SEM. *p<0.05; statistical significance versus saline controls using two way ANOVA, n = 5-6 per group, n.s., not significant. #, significant using Neuman-Keuls post-test (p<0.05) but not with the Tukey-Kramer post test.

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(FIG. 3B-E). The loss of striatal dopamine and nigral dopaminergic neurons following MPTP treatment in human α-synuclein/mouse α-synuclein null mice were comparable to that seen with the wild type animals (FIG. 3D,E). Furthermore, human α-synuclein/mouse α-synuclein null mice were equally sensitive as the wild type mice on the levels of dopamine metabolites following MPTP (data not shown).

The finding that MPTP-neurotoxicity resistant phenotype of the mouse α-synuclein null mice is completely reversed by the expression of human α-synuclein transgenes show that the lack of robust enhancement in MPTP neurotoxicity in human α-synuclein transgenic mice is not because human α-synuclein cannot function in the MPTP toxicity pathway. A likely possibility is that the level of mouse α-synuclein is saturating and the additional α-synuclein expression does not significantly increase the effects of α-synuclein on susceptibility to MPTP neurotoxicity.

Significantly, while the human α-synuclein can complement mouse α-synuclein in regard to MPTP neurotoxicity, human α-synuclein does not completely complement all of the phenotypes of mouse α-synuclein null mice. Mouse α-synuclein null mice have significantly fewer TH+ neurons in SNpc (FIG. 3E) due to developmental reduction in the number of TH+ neurons generated [31] Expression of human α-synuclein in mouse α-synuclein null mice did not restore the number of TH+ neurons to the level of non-transgenic mice (FIG. 3E).

Decreased vulnerability of α-synuclein null mice to intoxication is selective for dopaminergic neurons

It’s believed that α-synuclein null mice are protected against MPTP intoxication because lack of α-synuclein expression affects a yet to be defined process that occurs post uptake of MPP+ into the terminal and pre-mitochondrial complex I inhibition [42]. Based on this hypothesis, potential factors that can be affected by the loss of α-synuclein expression and also modulate MPTP toxicity is the activity of the vesicular monoamine transporter 2 (VMAT2) and the ability of MPP+ to access mitochondrial complex-I. To test whether these factors could be involved, we examined whether the serotoninergic and noradrenergic neurons in mouse α-synuclein null mice were resistant to 2’NH2-MPTP neurotoxicity.

Intoxication with 2’NH2-MPTP leads to degeneration of serotoninergic and noradrenergic neurons without affecting dopaminergic neurons (Table 1 and FIG. 4A and B) [32]. The mode of 2’NH2-MPTP toxicity is virtually identical to that of MPTP [33,43,44]. Thus, we reasoned that if α-synuclein modulates a general synaptic trafficking of MPP+, mouse α-synuclein null mice should be resistant to 2’NH2-MPTP toxicity.

Mouse α-synuclein null and the littermate wild type mice were generated by mating two mice that are both heterozygous for the mouse α-synuclein gene. Six to eight week old male mice were treated with 2’NH2-MPTP (15 mg 2’NH2-MPTP/kg X4, every 2 hours) and the levels of serotonin and norepinephrine in the cortex, striatum, and brain stem were determined 14 days following the last 2’NH2-MPTP treatment. Our results show that 2’NH2-MPTP treatment leads to the profound reductions in the serotonin and norepinephrine levels in all brain regions. The magnitudes of reductions in neurotransmitter levels were comparable between wild type and α-synuclein null mice (FIG. 4A, B). Analysis of serotonergic innervations by immunohistochemical analysis confirms the significant loss of cortical serotonergic fibers following 2’NH2-MPTP treatments in both groups of mice (FIG. 4C). Qualitatively, the losses of serotonergic fibers were notably greater in the α-synuclein null mice than in the wild type controls.

The lack of resistance to the 2’NH2-MPTP toxicity in the α-synuclein null mice suggests that MPTP resistance phenotype of mouse α-synuclein null mice is selective for dopaminergic neurons. Further, our results suggest that alterations in the general metabolism and/or trafficking of MPP+ in the synaptic terminal are not involved in MPTP resistant phenotype of the mouse α-synuclein null mice. Even if such a phenomenon does exist, it might be due to yet undefined inherent mechanisms that differ in dopaminergic and serotoninergic/noradrenergic neurons that affect the general metabolism or trafficking of MPP+.

The levels of β-synuclein and phosphorylated Akt are regulated by α-synuclein expression in ventral midbrain

Previously, increased expression of β-synuclein was found in the mouse α-synuclein null and mouse γ-synuclein null mice [31]. Because, β-synuclein has been shown to protect neurons from cell death, potentially via regulation of p38 [43], by reducing α-synuclein protein expression [46,47] and Akt-phosphorylation [48,49], increased levels of β-synuclein in α-synuclein null mice could be a potential factor involved in the protection of dopaminergic neurons from MPTP toxicity.

To further explore this hypothesis, we examined whether the increased levels of β-synuclein is regionally selective and whether the increase in β-synuclein levels in α-synuclein null mice could be reversed by expression of human α-synuclein. To determine whether β-synuclein levels are increased in brains of α-synuclein null mice, SDS-soluble proteins from cortex and ventral midbrain were subjected to immunoblotting for β-synuclein (FIG. 5A). Semi-quantitative analysis show that, relative to the β-synuclein levels in wild type mice, the ventral midbrain, but not cortex, shows increased levels of β-synuclein in α-synuclein null mice (FIG. 5B). Significantly, the level of β-synuclein is returned to the wild type levels by expressing human α-synuclein in the α-synuclein null background. Additionally, analysis of SDS soluble proteins in other brain regions like dorsal medial brain stem did not significantly affect β-synuclein levels for all the genotypes in comparison to wild type mice (data not shown).
Figure 3. Human α-synuclein complements MPTP resistant phenotype of mouse α-synuclein null mice. A. Expression of mouse and human α-synuclein in wild type (WT), mouse α-synuclein nulls (KO) and mice expressing wild type human α-synuclein (line I2-2) on a mouse α-synuclein null background (hWT/KO), total proteins isolated from 9 month old striata were subjected to immunoblot analysis for total α-synuclein (Syn-1), human α-synuclein (HuSyn), and GAPDH. B and C. TH-immunostaining staining of striatum (B) and SNpc (C) 7 days following saline or acute MPTP (20 mg/kg free base four times every 2 hour) treatment in wild type (WT), mouse α-Syn nulls (KO) and human α-Syn transgenic [wild type (hWT, line I2-2) and A53T (line N2-5)] on the mouse α-Syn null background (hWT/KO; A53T/KO). Only the KO is protected from MPTP toxicity. D and E. MPTP intoxication results in significant reductions in striatal DA-levels (D) and TH-positive neuronal cells (E) in mice with either mouse or human α-Syn expression. The α-Syn KO mice were completely protected against MPTP. The reduced basal number of DAergic neurons in α-Syn KO mice was not complemented by the human α-Syn expression (E). Data represent mean ± SEM. **p<0.01, versus saline and #p<0.05, versus wild type MPTP, Two way ANOVA, n=5-6, n.s not significant, scale bar: 200 µm.

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null mice. Finally, α-synuclein level dependent regulation of β-synuclein and p\(\text{Ser}^{473}\) expression in ventral midbrain, but not in cortex, suggest that MPTP-resistant phenotype of α-synuclein
null mice involves neuroprotection by β-synuclein/p\(\text{Ser}^{473}\).

Previous examinations of human α-synuclein expression and the vulnerability to MPTP have been variable. In order to control some of the sources of potential variation, we examined the MPTP-sensitivity of dopaminergic neurons in human α-synuclein transgenic mice as functions of MPTP neurotoxicity paradigms (acute and sub-acute), human α-synuclein variants, human α-synuclein levels, and age. Using two standard paradigms (acute and sub-acute) of MPTP neurotoxicity that results in significant loss of dopaminergic neurons in non-transgenic controls, we observe that wild type and mutant human α-synuclein transgenic mice demonstrate comparable sensitivity to MPTP. However, very high levels of A53T mutant human α-synuclein in transgenic mice may be associated with a modest increase in the MPTP-dependent neurodegeneration. Overall, our results are consistent with the other reports that fail to demonstrate a synergistic effect of human α-synuclein with MPTP [24,25]. However, one study showed that A30P transgenic mice are more sensitive to MPTP neurotoxicity but not to rotenone induced dopaminergic neurodegeneration [27]. A significantly attenuated neurotoxicity in nontransgenic mice (i.e., very little loss of dopaminergic neurons with the dose of MPTP causing ~50% reduction in our study) observed in this study suggest that there may be significant strain background and/or other transgene specific effects [27]. Overall, the general lack of increase in the vulnerability of dopaminergic neurons as a function of increased expression of human α-synuclein suggest that even at high levels of expression, human α-synuclein does not cause generalized stress to dopaminergic neurons that is sufficient to affect the MPTP toxicity paradigm used in this study. However, we can not exclude the possibility that increased expression of human α-synuclein would sensitize the dopaminergic neurons to more chronic toxicity paradigms. A major difference between MPTP-induced parkinsonism and idiopathic PD is time of progression for symptom development, thus it is possible for acute nature of MPTP toxicity paradigm used in this and other studies precludes pathological interaction between α-synuclein and MPTP toxicity [24,25]. This notion is supported by the results showing that the expression of mutant α-synuclein is associated with the increased vulnerability of dopaminergic neurons to chronic application of manganese/paraquat [50].

Although, human α-synuclein and MPTP both can cause neurodegeneration, it is plausible that these effects do not directly interact. Arguments against this line of reasoning, however, include findings of increased α-synuclein levels in mice SNpc following MPTP, increased nitration of α-synuclein in mouse SNpc following MPTP, increased α-synuclein aggregates in SNpc of baboons following MPTP and in vitro studies of α-synuclein toxicity [22,51,52,53]. In particular, a number of in vitro studies show that human α-synuclein overexpression was associated with enhanced cell death following exposure to MPP\(^{+}\), the toxic metabolite of MPTP [54,55]. Finally, studies have shown gene dose dependent reductions in the susceptibility to MPTP in α-synuclein null mice [21,28,29,30,31]. Given these findings, the expectation was that increased levels of α-synuclein would potentiate MPTP neurotoxicity. The lack of enhanced vulnerability to MPTP neurotoxicity with human α-synuclein transgenic mice raised the possibility that human α-synuclein and mouse α-synuclein do not function equally in sensitizing dopaminergic neurons to MPTP toxicity in vivo. However, our results show that human α-synuclein can functionally replace mouse α-synuclein and complements the MPTP resistant phenotype of the mouse α-

| Table 1. Levels of striatal dopamine and its metabolites in wild type and α-synuclein knock out mice following 2'NH\(_2\)-MPTP treatment. |
|---|---|---|---|---|
| Treatment groups | DA [ng/mg] | DOPAC [ng/mg] | HVA [ng/mg] | DOPAC: HVA |
| Saline [WT] | 20.3±2.5 | 1.90±0.06 | 1.74±0.04 | 0.18±0.01 |
| Saline [KO] | 19.4±1.5 | 1.87±0.05 | 1.68±0.05 | 0.18±0.01 |
| 2'NH\(_2\)-MPTP [WT] | 21.2±3.5 | 2.1±0.09 | 1.6±0.05 | 0.17±0.02 |
| 2'NH\(_2\)-MPTP [KO] | 20.5±4.5 | 1.9±0.08 | 1.73±0.08 | 0.17±0.03 |

Striatal levels of dopamine and its metabolites measured after 2 weeks of acute 2'NH\(_2\)-MPTP (15 mg/kg, i.p.) administered four times every two hours in wild type and α-synuclein knock out mice. Data are expressed as mean (n = 5) ± SEM. Statistical analysis was performed by ANOVA, revealing no significant differences among group means. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid, WT, wild type; KO, knock out.

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Previous studies [47,48,49] showed that β-synuclein can provide neuroprotection via direct interaction with Akt and enhancing the accumulation of phosphorylated Akt. Thus, we examined whether the levels of pAkt(Ser473) was altered as a function of α-synuclein expression in the lines of transgenic mice. Immunoblot analysis for pAkt(Ser473) and total Akt shows that the level of pAkt(Ser473) is increased in the ventral midbrain but not in cortex (Fig. 5A). As with β-synuclein expression, transgenic expression of human α-synuclein on the α-synuclein null background lead to decrease in pAkt(Ser473) levels (Fig 5B).

Collectively, our results support the hypothesis that the increased expression of β-synuclein in α-synuclein null mice lead to increased level of pAkt(Ser473), contributing to neuroprotection in α-synuclein null mice. The finding that β-synuclein and pAkt(Ser473) levels are selectively increased only in the ventral midbrain of the α-synuclein null mice support our hypothesis that dopaminergic neurons are selectively protected from MPTP-like toxins in α-synuclein null mice.

Discussion

Both α-synuclein and MPTP are believed to cause dopaminergic abnormalities that are relevant to the pathogenesis of PD, and hence we explored the relationship between α-synuclein expression and vulnerability of dopaminergic neurons to MPTP neurotoxicity. We show that the increased expression of human α-synuclein is not associated with increased vulnerability of dopaminergic neurons to MPTP toxicity. The lack of effect is not because human α-synuclein does not function in the MPTP toxicity pathway since human α-synuclein is able to completely restore the MPTP-resistant phenotype of the mouse α-synuclein null mice [21,28,29,30,31]. However, human α-synuclein does not completely replace mouse α-synuclein since the developmental reduction in the dopaminergic neurons is not complemented by human α-synuclein. One plausible explanation to this could be that the human α-synuclein might not be expressed in all cells at similar levels and times as the endogeneous mouse α-synuclein gene. However, we show that the lack of α-synuclein expression is not associated with protection from the 2'NH\(_2\)-MPTP toxicity, indicating that the dopaminergic neurons are selectively protected from toxin-induced neurodegeneration in the mouse α-synuclein null mice.
Figure 4. α-synuclein null mice are not protected against noradrenergic and serotonergic neurotoxin 2′NH2-MPTP. A and B. Cortical (A) and brainstem (B) levels of norepinephrine (NE), serotonin (5-HT) and dopamine (DA) were determined two weeks after saline or acute 2′NH2-MPTP (15 mg/kg X4, every 2 hours) treatment in wild type (WT) and α-synuclein null (KO) mice. 2′NH2-MPTP treatment lead to significant loss of NE and 5-HT but not DA in cortex and brain stem. C. 2′NH2-MPTP treatment resulted in a significant loss of cortical serotonergic fibers both in wild type and α-synuclein null mice. Unlike MPTP, the α-synuclein null mice are not resistant to the neurotoxic insult of serotonergic and noradrenergic neurotoxin 2′NH2-MPTP. Data represent mean ± SEM. ** p<0.05, statistical significance versus saline controls using two way ANOVA, n=6-7 per group, n.s., not significant. Scale bar: 100 μm.

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attenuated mitochondrial toxin induced release of dopamine in a potential effects of neuroprotection from mitochondrial toxin. In addition to the targeting of MPP
not because of alterations in the general synaptic metabolism/ni
Because the expression of nigrostriatal dopaminergic neurons from MPTP-like toxins.
suggest that the lack of synuclein null and mutant Akt/synuclein, which has been shown to affect nigrostriatal dopaminergic neurons [56]. This view is supported by the study showing that synuclein null mice are resistant to other mitochondrial toxins (malonate and 3-nitropropionic acid) that affect nigrostriatal dopaminergic neurons [56]. In this study, it was proposed that attenuated mitochondrial toxin induced release of dopamine in α-synuclein null mice [28] may be an important factor in neuroprotection from mitochondrial toxin. In addition to the potential effects of α-synuclein on dopamine release, our results suggest that the lack of α-synuclein leads to selective protection of nigrostriatal dopaminergic neurons from MPTP-like toxins. Because the expression of β-synuclein, which has been shown to be neuroprotective [43,46,48,49], is increased in the ventral midbrain of α-synuclein null mice (Fig. 5), it seems likely that β-synuclein expression and subsequent activation of Akt are important contributors to the MPTP-resistant phenotype of α-synuclein null and γ-synuclein null mice [31]. This view is consistent with the protection of dopamine neurons from 6-OHDA toxicity by expression of a constitutively active Akt mutant [57]. Moreover, α/β/γ-synuclein-dependent regulation of Akt may provide a mechanistic basis for modulation of dopaminergic neurotransmission by synuclein. Specifically, regulation of Akt signaling modulates dopamine-mediated locomotion in mice, including amphetamine-induced locomotion, and impaired Akt signaling is implicated in schizophrenia [58,59]. Thus, increased activation of Akt in α-synuclein-null mice may account for the impaired amphetamine-induced locomotor response of α-synuclein-null mice [41]. Finally, since increased α-synuclein expression and decreased β-synuclein expression are associated with PD and other α-synucleinopathies [5,56], regulation of Akt activity by synuclein expression may be an important factor in the pathogenesis of human α-synucleinopathies. Notably, other familial PD-linked gene products, parkin [61], DJ-1 [62,63], and PINK1 [64,65] are also associated with Akt signaling, supporting an intriguing notion that alterations in Akt signaling may be a common pathologic factor in PD.

In summary, our results are consistent with the notion that α-synuclein expression has significant cell biological consequences for dopaminergic neurons and some of these effects, such as vulnerability to MPTP-like toxins and regulation of β-synuclein expression, appear to be specific for dopaminergic neurons. In particular, given the important implications of our finding that α-synuclein expression regulates β-synuclein expression in nigrostriatal system, it will be important to further study cell biological basis for how α-synuclein regulates β-synuclein expression. Further, our studies provide a strong rationale for critical examination of the mechanistic relationship between Akt activation and MPTP toxicity. Finally, given that β-synuclein can attenuate α-synuclein toxicity in vivo [16,47,48,49], it is attractive to propose that regulation of endogenous β-synuclein expression and Akt activation may be a significant factor in the transgenic animal models of α-synucleinopathy and in human α-synucleinopathies.

Materials and Methods

Animals
All mice were housed and treated in strict accordance with the National Institutes of Health Guide for Care and use of Laboratory Animals. Mice were housed in a pathogen free facility, about 4-5

Figure 5. Levels of α-synuclein affects β-synuclein expression and basal Akt phosphorylation in vivo. A. Total tissue extracts from cortex and ventral midbrain of wild type (WT), moSyn-null mouse (KO), and HuSyn transgenic (line I2-2) on moSyn-KO background (KO+) were immunobloted for endogenous β-synuclein, Akt (pSer473 and total) and GAPDH. B and C Semi-quantitative analysis of β-synuclein (B) and pSer473-Akt levels (C). The values are mean ± SEM from 4 animals (*p<0.05, **p<0.01, ANOVA with Newman-Keuls post-test). doi:10.1371/journal.pone.0016706.g005
animals per cage in a temperature controlled room with a 12 hour light/dark cycle and with food and water ad libitum. All procedures were approved by and conformed to the guidelines of the Institutional Animal Care Committee of Johns Hopkins University.

Transgenic lines of mice were generated using the MoPrP promoter as previously described to over express human α-synuclein (WT, line I2-2, ~3-fold increase in total α-synuclein level), A53T mutant human α-synuclein (A53T, line N2-5 and G2-3, ~3-fold and ~6-fold increase, respectively) and A30P mutant human α-synuclein (A30P, line T3 and O2, ~3-fold and 10-fold increase, respectively) [11]. Non transgenic littermate cohorts were also generated for each of these lines to account for potential genetic differences. The transgenic mice used were N3-N5 generation backcrossed to C57/BL6 strain.

Human α-synuclein transgenic mice lacking mouse α-synuclein expression was generated by successive mating of human α-synuclein transgenic mice, congenic for C57BL6 strain (N10), to mice lacking endogenous α-synuclein [41] that were procured from Jackson labs. To generate the cohorts for the MPTP treatment, mice that are double heterozygous for human α-synuclein and mouse α-synuclein were mated to mice heterozygous for mouse α-synuclein. The resulting progenies contained all of the possible genotypes. The resulting male non-transgenic (wild type), mouse α-synuclein null, and human α-synuclein transgenic on mouse α-synuclein null background littermates were used for the current study.

MPTP and 2′NH₂-MPTP treatment in mice

Male mice were used for neurotoxic challenges induced by dopaminergic neurotoxin MPTP and its structural analogue 2′NH₂-MPTP, which is a selective neurotoxin for central serotoninergic and noradrenergic neuronal systems. Two different MPTP paradigms were used in the present study. For the acute paradigm, mice were administered four acute doses of MPTP (20 mg/kg, 18 mg/kg, and 15 mg/kg free base) every two hours four times a day and analyzed 7 days post-treatment. For sub-acute MPTP paradigm, mice were treated with 30 mg/kg free base MPTP daily for a total of 5 days and toxicity analyses performed two weeks after the last MPTP injection. To assess neurotoxicity to central serotoninergic and noradrenergic neuronal system, mice were treated with 2′NH₂-MPTP in an acute paradigm of 15 mg/kg 2′NH₂-MPTP divided into four doses separated by 2 hours and the toxicity analyses were performed two weeks later. Control cohorts of mice received equivalent volumes of saline at the same frequency as the respective paradigms of MPTP or 2′NH₂-MPTP used in the study. All systemic injections were administered through the intraperitoneal route and the neurotoxins were dissolved in normal saline. All procedures involving MPTP and 2′NH₂-MPTP injections in mice were performed according to standard procedures [66].

Immunohistochemistry

Immunohistochemical analyses for tyrosine hydroxylase expression was performed using methods previously described [67] using a rabbit anti-tyrosine hydroxylase antibody (Novus Biologicals, Littleton, CO). Serotoninergic axons were visualized by enhanced immunocytochemistry with an anti-serotonin antibody using an established method [68]. Briefly, formaldehyde fixed frozen sections were incubated with the rabbit anti-serotonin antibody (1:12,500, Incstar, Stillwater, MN) followed by the secondary antibody [1:1000 biotin-labeled F(ab)² Fragment goat-anti rabbit antibody [Jackson Immunoresearch, West Grove PA] and visualized by incubation in biotin-streptavidin-HRP (Vector Laboratories, Burlingame, CA) and SigmaFast™ DAB Peroxidase Substrate (Sigma, St. Louis, MO). Sections were mounted on glass slides and dehydrated in graded ethanol, followed by chloroform: ethanol (1:1) and rehydrated. The sections were then incubated in 1.5% silver nitrate solution for 1 h at 56°C, rinsed by running tap water for 20 min, incubated in 0.2% gold chloride at room temperature in dark followed by distilled water rinse for 20 min and incubated in 5% thiosulphate for 5 min at room temperature and washed with distilled water and dehydrated in graded ethanol, cleared in xylene, and mounted with permount.

Stereorologic Cell Counts

Stereorologic methods were employed to determine an unbiased estimate of total neurons and tyrosine hydroxylase (TH) immunopositive and Nissl-stained neurons within SNpc. Briefly, the SNpc of mice were processed for TH immunohistochemistry and Nissl counter stain on every fourth midbrain section throughout the entire extent of SNpc as described previously [67,69]. Neurons were counted using the optical fractionator [70] using a computer-assisted image analysis system, consisting of an Axioshot photomicroscope (Carl Zeiss Vision, Hallbergmoos, Germany) comprising of a Zeiss planapochromat 100X oil objective equipped with a computer-controlled motorized stage, a video camera, and the Stereo Investigator software (MicroBright-Field, Williston, VT). Cell counts were performed throughout the entire extent of the SNpc using a standard mouse [71] atlas as anatomical reference. The total number of TH-positive neurons was calculated using the formula previously described for this method [72].

Measurement of biogenic amines by HPLC with electrochemical detection

To determine the concentration of biogenic amines in discrete brain regions by HPLC with electrochemical detection, mice were sacrificed by decapitation and the brain was quickly removed and discrete brain regions dissected and immediately frozen and stored at -80°C. The tissue was weighed and sonicated in 0.2 ml of 0.1 M perchloric acid containing 0.01% EDTA and 25 μg/ml 3,4-dihydroxybenzylamine (DBHA) (Sigma, St. Louis, MO) as an internal standard. After centrifugation (15,000×g, 10 min, 4°C), 20 μl of the supernatant were injected onto a C-18 reverse phase Spheri 5, RP-18, 4.6 mm ×25 cm catecholamine column (BASi, West Lafayette, IN). The mobile phase consisted of 0.15 M chloroacetic acid, 0.2 mM EDTA, 0.86 mM sodium octyl sulphate, 4% acetonitrile and 2.5% tetrahydrofuran (pH = 5). The flow rate was kept at 1.5 ml/min. Biogenic amines and their metabolites were detected by an electrochemical detector, Prostar ECD Model 370 (Varian, Palo Alto, CA), with the working electrode kept at 0.6 V. Data were collected and processed on a Star Chromatography Workstation 5.52 (Varian) [67].

Immunoblot Analysis

Immunoblot analysis for synuclein and other proteins were determined as previously described [11,73]. For the immunoblot analysis of Akt, same procedure was used except tissue was homogenized in the TNE buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA] containing protease inhibitors (5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin), detergent (0.5% Nonidet P-40, 0.5% Na-Deoxycholate and 1% SDS), and a phosphatase inhibitor cocktail (Sigma).

Briefly, homogenates containing equal amount of protein were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane (BioRad, Hercules, CA), and immunoreacted with an
appropriate primary antibody followed by a HRP-conjugated secondary antibodies (Kirkegaard Perry Labs Inc. MD, USA). The immunoreactive proteins were visualized by incubating the blots in the chemiluminescence substrate (Pierce, Rockford, IL) and detection with ChemiDoc XRS system (Biorad, Hercules, CA). The quantitative analyses of the immunoreactive proteins were performed with the Quanitiy One I-D Analysis software (Biorad, Hercules, CA). Statistical analysis was performed using the ratios of the densitometric value of each band and its corresponding GAPDH loading control within each genotype group.

To detect various synuclein variants, following primary antibodies were used: total α-synuclein (Syn-1 mAb, BD Biosciences Pharmingen, San Jose, CA, 1:2000); human α-synuclein (HuSyn-1 rabbit pAb, 1:20000) [11]; β-synuclein (mAb, Research Diagnostic Inc. Concord, MA 1:500). To detect Akt and phosphorylated Akt levels, following primary antibodies were used: total Akt (pAb), Akt-Ser473 and &Cell Signalling Technology Inc. Danvers, MA) and for loading control GAPDH (Research Diagnostics Inc. Concord, MA, 1:5000) was used.

Statistical Analysis
Data represent mean ± SEM from groups of animals and statistical analysis applied with two-way ANOVA. When F values implied significance at a level p<0.05, Fisher’s post-hoc analysis or Tukey-Kramer multiple comparison tests was applied to determine where the differences among groups arose. All statistical analyses were performed using the Prism software (GraphPad, San Diego, CA).

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Author Contributions
Conceived and designed the experiments: BT ASM VLD MKL. Performed the experiments: BT AS NW YL SAA WS. Analyzed the data: BT AS NW YL SAA WS VLD MKL. Contributed reagents/materials/analysis tools: VLD TMD MKL. Wrote the manuscript: BT TMD MKL.

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