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Alpha-synuclein deficient mice are resistant to toxin-induced multiple system atrophy

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Abstract

Multiple systems atrophy (MSA) is a neurodegenerative disorder characterized by oligodendrocytic accumulations of alpha-synuclein (α syn). Oxidative stress is a key mechanism proposed to underlie MSA pathology. To address the role of α syn modifications, over and above general oxidative modifications, this study examined the effects of 3-nitropropionic acid (3NP) administration, a technique used to model MSA, in knock-out mice lacking α syn (α synKO). Although susceptible to 3NP-induced oxidative stress, α synKO mice display reduced neuronal loss and dendritic pathology. The α synKO mice are resistant to 3NP-induced motor deficits and display attenuated loss of tyrosine hydroxylase and dopamine transporter striatal immunoreactivity. The results suggest that deficits in MSA are not due to general oxidative protein modification but in addition may be related to specific α syn modifications.

Keywords

alpha-synucleinopathy; neurodegeneration; oxidative stress

Introduction

Multiple system atrophy (MSA) is a sporadic neuro degenerative disease of the central nervous system and neuronal loss predominantly in the basal ganglia, brainstem, and cerebellum is a key feature of MSA [1,2]. Pathologically, MSA is characterized primarily by alpha-synuclein (α syn)-positive glial cytoplasmic inclusions, though neuronal α syn inclusions have also been reported [2].

Administration of 3-nitropropionic acid (3-NP), a mitochondrial complex II inhibitor mimics MSA by aggravating nigrostriatal and olivopontocerebellar degeneration in transgenic mice expressing α syn under the oligodendrocytic proteolipid promoter [1], highlighting the role of mitochondrial dysfunction and oxidative stress in MSA pathology. Oxidative stress has also been associated with increased risk of MSA [2-4] and has been reported to posttranslationally modify α syn and enhance α syn fibrillogenesis and aggregation [5].

When evaluating oxidative stress, the relative importance of α syn modifications over a background of general protein oxidation must be determined to assess whether deficits are a direct result of the α syn or merely attributable to general protein modifications.

This study examined the effect of 3NP administration on α syn-deficient knock-out mice (α synKO) compared with those overexpressing human α syn under the oligodendrocytic-specific myelin basic protein (MBP) promoter (MBP- α syn Tg mice). The results show that while 3NP administration induced protein oxidation in the both the α synKO and MBP- α syn Tg mice, the neuropathological and behavioral deficits in MBP- α syn Tg mice were absent in the α synKO mice suggesting that, rather than a generalized response to protein oxidation, 3NP-induced deficits in the MBP- α syn Tg mice [6] are linked to the presence of α syn.

Materials and methods

Animals and treatment

The MBP human α syn transgenic [MBP- α syn Tg mice (MBP1 line)] have been previously described [7], they develop oligodendrocytic α syn inclusions along axonal tracts in the brainstem, basal ganglia, cerebellum, corpus callosum, and neocortex and display myelin loss and astrogliosis [7]. They have been used to model MSA [7–9] and are susceptible to 3NP-induced neurotoxicity [6]. The α synKO were obtained from Jackson Laboratories (ID: 003692, Jackson Laboratories, Maine, USA). A total of 36 mice with a mean age of 8.9 months were used. The mice of each genotypes (MBP- α syn Tg, $n = 12$, α synKO, $n = 12$), and non-transgenic littermates (NTg, $n = 12$) were divided into saline-treated and 3NP-treated groups ($n = 6$ in each group). The 3NP (Sigma, St Louis, Missouri, USA) was dissolved in saline and pH 7.4 was adjusted with 1 M NaOH. The 3NP (or saline) was administered intraperitoneally twice daily over 6 days; 10 mg/kg (days 1 and 2), 20 mg/kg (days 3 and 4), 30 mg/kg (days 5 and 6), total dose 240 mg/kg in 6 days. Behavioral analysis was conducted 3 weeks post-final injection, the mice were sacrificed after behavioral testing.

Behavioral testing

Motor behavior was assessed using the Pole test [10]. The animals were placed upwards on a vertical wooden pole 50 cm long (1 cm in diameter). Time taken to descend the pole (T-total) was measured. Mice received three training trials and five test trials on the same day with a resting period between trials.

Tissue processing

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

Immunohistochemistry

Immunohistochemistry was conducted on 40 μ m vibratome sections, immunolabeled overnight with antibodies against the dendritic marker microtubule-associated protein-2 (MAP2, 1:50, Chemicon, Temecula, California, USA), the neuronal marker NeuN (1:1000, Chemicon), the astroglial marker glial fibrillary acidic protein (GFAP, 1:500, Millipore, Temecula, California, USA), the dopaminergic marker tyrosine hydroxylase (TH, 1:200, Chemicon), the dopamine transporter (DAT, 1:500, Chemicon) and α syn (1:500, Chemicon) followed by incubation with species-appropriate secondary antibodies (1:2000, Vector

Laboratories). The NeuN, α syn, and DAT immunolabeled sections were reacted with diaminobenzidine tetrahydrochloride containing 0.001% H_2O_2 , whereas tetrahydrochloride (TH) immunolabeled sections were reacted with diaminobenzidine containing 0.001% H_2O_2 and nickel chloride for visualization. The MAP2 and GFAP were incubated with fluorochrome-labeled secondary antibodies. The sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, California, USA) and mounted under glass coverslips with antifading media (Vector Laboratories). The sections were analyzed with the laser scanning confocal microscope (MRC1024, BioRad, Hercules, California, USA).

Stereological analysis

An unbiased stereological estimation of striatal α syn and NeuN immunoreactive cells was performed using the disector method with the Stereo-Investigator system (MBS Technology) and expressed as cells per unit volume.

Oxyblot analysis of protein oxidation

Level of protein oxidation in brain homogenates from MBP-h α syn Tg mice, α synKO mice, and NTg mice were analyzed using the Oxyblot Protein Oxidation Detection Kit (Chemicon) as per manufacturer's instructions. The samples were transferred to an Immobilon membrane, which was blocked with 3% bovine serum albumin in phosphate-buffered solution with 0.01% Tween-20 (0.05%), incubated with the anti-2,4-dinitrophenylhydrazine antibody, followed by incubation with secondary antibody, then reacted with chemi-luminescence (ECL, Pierce) and developed on the VersaDoc gel-imaging machine (BioRad).

Analysis of mitochondrial complex II activity

In a 96-well plate, sample wells contained 195 μl of Tris- SO_4 , 0.1 M, pH 7.4, Horse heart cytochrome c (Sigma type II) 1.6 mg/ml, sodium cyanide, 1 mM, 2.5 μl of sodium succinate, 20%, pH 7.5 and 2 μl of the protein sample (30 μg , from detergent insoluble, membrane fraction). Reference wells contained all the above plus 2 μl of sodium malonate 20%, pH 7.5. The samples were left at room temperature for 5 min then absorbance was measured at 550 nm (DTX 880, Beckman Coulter, Brea, California, USA).

Western blot analysis

The levels of total α syn and nitrosylated proteins were determined by immunoblot analysis. Tissue was processed as described earlier [7], protein (20 μg) was loaded in 4–12% Bis-Tris (Invitrogen) SDS-PAGE gels, transferred onto Immobilon membranes, blocked in 3% bovine serum albumin (Fisher Scientific) in phosphate-buffered solution with 0.01% Tween-20 (Sigma), incubated with antibodies against total α syn (1:1000; Chemicon) and (Syn211, 1:1000, Sigma) and nitro-tyrosine (1:1000, Chemicon). After overnight incubation, the membranes were incubated in secondary antibodies, reacted with ECL (Pierce), and developed on a VersaDoc gel-imaging machine (BioRad). Anti- β -actin (1:1000; Sigma) antibody was used to confirm equal loading.

Statistical methods

The group differences were tested using one and two factor analysis of variance with Fisher's protected least significance difference post hoc tests. Additional preliminary analysis between control and treated groups was by unpaired, two-tailed, Student's *t*-test. All results are expressed as mean $\pm$ SEM.

Results

Induction of oxidative stress α synKO and MBP-h α syn Tg mice after 3NP administration

An Oxyblot assay conducted to assess the oxidative effects of 3NP administration (Fig. 1a, quantified in Fig. 1b) showed increased levels of protein oxidation in all groups (Fig. 1a). The levels of protein oxidation in saline-treated MBP-h α syn Tg mice were significantly higher than both the saline-treated α synKO and NTg, whereas those in the saline-treated α synKO mice were significantly lower than the saline-treated NTg mice (Fig. 1a, quantified in Fig. 1b). To assess the activity of 3NP, an analysis of mitochondrial complex II activity was conducted (Fig. 1c) and reduced activity was observed in all lines (Fig. 1c).

Characterization of α syn expression in α synKO and MBP-h α syn Tg mice after 3NP administration

The analysis of total α syn levels was conducted using two antibodies, the first (Chemicon) showed an increase in total α syn level in the MBP-h α syn Tg mice in comparison to NTg mice, as reported earlier [7] (Fig. 1d, analyzed in Fig. 1e). The second antibody (Syn211, specific for human α syn) also showed high levels of α syn expression in the MBP-h α syn Tg mice (Fig. 1d, analyzed in Fig. 1f). No α syn expression was observed in the α synKO mice (Fig. 1d). 3NP administration had no effect on levels of total α syn in the MBP-h α syn Tg mice [6]. Analysis of nitro-tyrosine expression (a marker of general protein oxidation and nitration) across the lines showed an increase upon 3NP administration in all of the groups (Fig. 1d, analyzed in Fig. 1g). The saline-treated MBP-h α syn Tg mice displayed higher levels of nitro-tyrosine in comparison to saline-treated NTg and α synKO mice whereas the saline-treated NTg and α synKO mice expressed similar levels of nitro-tyrosine expression.

Behavioral analysis of α synKO and MBP-h α syn Tg mice after 3NP administration

The pole test was conducted to assess the impact of 3NP administration upon motor behavior (Fig. 1h). Consistent with earlier reports, the saline-treated MBP-h α syn mice took much longer to descend the pole in comparison to NTg mice [9], indicative of motor deficit, 3NP administration significantly increased this time (Fig. 1h) [8]. In contrast, T-total for saline-treated α synKO did not differ from the saline-treated NTg; 3NP did not have a significant effect. 3NP administration increased the time taken by NTg mice to descend the pole, but this increase was not to the same degree as seen in the MBP-h α syn (Fig. 1h). These results show that while 3NP administration can induce and exacerbate motor deficits in the NTg and MBP-h α syn Tg mice, α synKO mice appear resistant to 3NP-induced deficits.

Neuropathological correlates of 3NP administration in α synKO and MBP-h α syn Tg mice

Immunohistochemical analysis of the striatum was conducted with antibodies against human α syn, TH, and DAT (Fig. 2). Consistent with earlier reports [6,7] saline-treated MBP-h α syn Tg mice displayed significant oligodendrocytic h α syn immunoreactivity in comparison to saline-treated NTg mice (Figs 2a and e, analyzed in Fig. 2g), 3NP administration did not significantly increase level of total human α syn in the MBP-h α syn Tg mice. No h α syn immunoreactivity was detected in saline-treated or 3NP-treated NTg or α synKO mice (Figs 2a–d, analyzed in Fig. 2g).

Immunohistochemical analysis TH and DAT showed a significant decrease in levels of both in the saline-treated MBP-h α syn Tg mice in comparison with both saline-treated NTg and α synKO mice (Figs 2h, j and l, analyzed in Fig. 2n). The 3NP administration significantly reduced levels of TH and DAT immunoreactivity in both the NTg and MBP-h α syn Tg mice (Figs 2h, i, l and m, analyzed in Fig. 2n). In contrast 3NP had no effect on levels of TH and DAT immunoreactivity in α synKO mice, both saline-treated and 3NP-treated α synKO mice displayed comparable levels of TH and DAT immunoreactivity to saline-treated NTg mice.

Analysis of striatal neuronal density and dendritic complexity as evidenced by NeuN and MAP2 immuno-reactivity, respectively (Fig. 3), showed a significant reduction in neuronal number and area of MAP2-immunoreactive neuropil in the saline-treated MBP-h α syn Tg mice in comparison to both saline-treated NTg and α synKO mice (Figs 3a and e, analyzed in Figs 3g, h and l, analyzed in Fig. 3n), this is consistent with published reports [6,7]. 3NP administration had no effect on neuronal density and dendritic complexity in the MBP-h α syn Tg mice (Figs 3e and f, analyzed in Figs 3g, l and m, analyzed in Fig. 3n), but did appear to decrease dendritic complexity in the NTg mice, as evidenced by a decrease in MAP2 immunoreactive neuropil in the NTg mice (Figs 3h and i, analyzed in Fig. 3n). The 3NP administration did not effect neuronal number and dendritic complexity in the α synKO mice, with both saline-treated and 3NP-treated α synKO mice displaying similar levels of NeuN and MAP2 immunoreactivity to saline-treated NTg controls (Figs 3c and d, analyzed in Figs 3g, j and k, analyzed in Fig. 3n).

Analysis of astrogliosis, as evidenced by GFAP immunoreactivity, showed a significant increase in GFAP-immunoreactivity in the saline-treated MBP-h α syn Tg mice in comparison to both saline-treated NTg and α synKO mice (Figs 3o, q and s, analyzed in Fig. 3u). 3NP had no significant effect on GFAP immunoreactivity in the MBP-h α syn Tg mice (Figs 3s and t, analyzed in Fig. 3u) but did increase GFAP immunoreactivity in the NTg mice (Figs 3o and p, analyzed in Fig. 3u). The 3NP did not affect GFAP immunoreactivity in the α synKO mice, with saline-treated and 3NP-treated α synKO mice displaying similar levels to saline-treated NTg controls.

Discussion

This study shows for the first time that, compared with MBP-h α syn Tg mice, α synKO mice are resistant to neuropathological and behavioral deficits associated with 3NP administration. The 3NP resistance of the α synKO mice is consistent with earlier studies where α syn deficient mice have been shown to exhibit reduced susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine [11-13], 6-hydroxydopamine [14,15], and 3NP [16]. Earlier work on α synKO mice has focused on Parkinson's disease-related toxins, dopamine metabolism, and toxin-induced lesion formation in α synKO mice whereas data regarding neuropathological measures such as neuronal density and dendritic complexity were sparse. This study sought to bridge this gap by investigating these mice in relation to MSA and shows that while α synKO mice are susceptible to 3NP-induced mitochondrial complex II activity inhibition and display a global increase in protein oxidation as evidenced by the Oxyblot assay and nitrotyrosine immunoblot results, they do not display the same reduced neuronal density or dendritic loss that is evident in the MBP-h α syn Tg mice upon 3NP administration. In addition, α synKO mice are resistant to motor deficits as evidence by the pole test, consistent with the attenuated loss of striatal dopamine markers TH and DAT, this study is the first to report an attenuation of a disease-related behavioral phenotype in the α synKO mice.

Conclusion

Oxidative stress may underlie disease progression in synucleinopathies such as MSA [17] and emerging data suggests that posttranslational oxidative modifications of α syn are linked to α syn accumulation and inclusion formation [5,18–22]. Results from this study are consistent with this and suggest that, while oxidative stress, resulting from mitochondrial dysfunction, can induce widespread oxidative protein changes, the presence of α syn plays a key role in the neurodegeneration and behavioral deficits observed.

Acknowledgments

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Fig. 1.

Analysis of oxidative stress, alpha-synuclein (α -syn) levels and behavioral deficits upon 3-nitropropionic acid (3NP) administration. Oxyblot analysis of global protein oxidation levels in non-transgenic littermates (NTg), knock-out mice lacking α syn (α synKO), and myelin basic protein (MBP)- α syn Tg mice (a), analyzed in (b). Assessment of mitochondrial complex II (Mito Cmplx II) activity levels after 3NP administration in the NTg, α synKO, and MBP- α syn Tg mice (c). Immunoblot analysis of total α syn levels, using two different antibodies and nitrotyrosine levels 3NP administration in the NTg, α synKO, and MBP- α syn Tg mice (d), quantitative analysis (e–g). Motor behavior was assessed using the pole test (h). Mice were placed at the top of a vertical pole and total time to descend (T-total) was measured. *Significant difference ($P < 0.05$, one-way analysis of variance and post hoc Fisher's test) between saline and 3NP-treated members of the same genotype (saline-treated NTg vs. 3NP-treated NTg mice). **Significant difference ($P < 0.05$, one-way analysis of variance and post hoc Fisher's test) between mice of different genotype (saline-treated NTg vs. saline-treated α synKO mice). Arb., arbitrary.

Fig. 2.

Immunohistochemical analysis of alpha-synuclein (α -syn) and dopamine markers in non-transgenic littermates (NTg), knock-out mice lacking α syn (α synKO), and myelin basic protein (MBP)- α syn Tg mice after 3-nitropropionic acid (3NP) administration. Immunohistochemical analysis was performed to examine the number of α syn immunoreactive cells in the striatum of vehicle-treated and 3NP-treated NTg mice (a and b), α synKO mice (c and d) and MBP- α syn mice (e and f), analyzed in (g). To assess the effect of 3NP administration on dopamine markers immunohistochemical analysis was performed to examine the number of tyrosine hydroxylase (TH) and dopamine transporter (DAT) immunoreactive cells in the striatum of vehicle-treated and 3NP-treated NTg mice (h, i, o, and p, respectively), α synKO mice (j, k, q, and r, respectively) and MBP- α syn mice (l, m, s, and t, respectively), analyzed in (n) and (u). Scale bar = 50 μ M. *Significant difference ($P < 0.05$, one-way analysis of variance and post hoc Fisher's test) between saline and 3NP-treated members of the same genotype (saline-treated NTg vs. 3NP-treated NTg mice). **Significant difference ($P < 0.05$, one-way analysis of variance and post hoc Fisher's test) between mice of different genotype (saline-treated NTg vs. saline-treated α synKO mice).

Fig. 3.

The knock-out mice lacking α syn (α synKO) display attenuated neuropathological deficits after 3-nitropropionic acid (3NP) administration. Immunohistochemical analysis of neuronal marker (NeuN) positive cells in the striatum was conducted in order to investigate the effects of 3NP administration on neuronal density in saline-treated and 3NP treated non-transgenic littermates (NTg) mice (a and b), α synKO mice (c and d) and myelin basic protein (MBP)-h α syn Tg mice (e and f), analyzed (g). Dendritic pathology was assessed by the percentage of microtubule-associated protein-2 (MAP2) immunoreactive area of neutrophil in the striatum of saline-treated and 3NP treated NTg mice (h and i), α synKO mice (j and k) and MBP-h α syn Tg mice (l and m), analyzed in (n). Astrogliosis after 3NP administration was assessed through glial fibrillary acidic protein (GFAP) immunoreactivity in the striatum of saline-treated and 3NP treated NTg mice (o and p), α synKO mice (q and r), and MBP-h α syn Tg mice (s and t), analyzed (u). Scale bar (a–f and o–t) = 50 μ M, scale bar (h–m) = 20 μ M. *Significant difference ($P < 0.05$, one-way analysis of variance and post hoc Fisher's test) between saline and 3NP-treated members of the same genotype (saline-treated NTg vs. 3NP-treated NTg mice). **Significant difference ($P < 0.05$, one-way analysis of variance and post hoc Fisher's test) between mice of different genotype (saline-treated NTg vs. saline-treated α synKO mice). Arb., arbitrary.