ACS Chemical Neuroscience

Research Article

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Clioquinol Improves Cognitive, Motor Function, and Microanatomy of the Alpha-Synuclein hA53T Transgenic Mice

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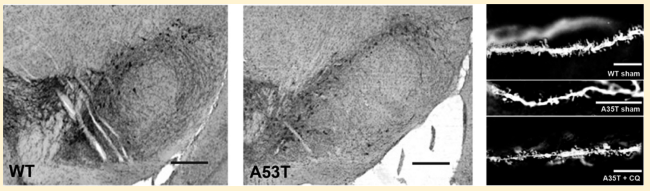
Supporting Information

ABSTRACT: The abnormal accumulation of alpha-synuclein (α-syn) has been linked to a number of neurodegenerative disorders, the most noteworthy of which is Parkinson's disease. Alpha-synuclein itself is not toxic and fulfills various physiological roles in the central nervous system. However, specific types of aggregates have been shown to be toxic, and metals have been linked to the assembly of these toxic aggregates. In this paper, we have characterized a transgenic mouse that overexpresses the A53T mutation of human α-syn, specifically assessing cognition, motor performance, and subtle anatomical markers that have all been observed in synucleinopathies in humans. We hypothesized that treatment with the moderate-affinity metal chelator, clioquinol (CQ), would reduce the interaction between metals and α-syn to subsequently improve the phenotype of the A53T animal model. We showed that CQ prevents an iron−synuclein interaction, the formation of urea-soluble α-syn aggregates, α-syn-related substantia nigra pars compacta cell loss, reduction in dendritic spine density of hippocampal and caudate putamen medium spiny neurons, and the decline in motor and cognitive function. In conclusion, our data suggests that CQ is capable of mitigating the pathological metal/α-syn interactions, suggesting that the modulation of metal ions warrants further study as a therapeutic approach for the synucleinopathies.



KEYWORDS: *Clioquinol, alpha-synuclein, A53T, Parkinson's disease*

Parkinson's disease (PD), Parkinson's disease with dementia (PDD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are all clinically distinct degenerative disorders that are characterized by a unique distribution and abundance of intracellular alpha-synuclein (α-syn) aggregates.[1−4] The evolving literature perspective is that α-syn has a major role in neurotoxicity, that the monomeric form of α-syn is not toxic, and that the polymeric order of this protein determines toxicity; it has even been suggested that insoluble Lewy bodies comprising oligomeric peptides may in fact play a neuroprotective role.[1,5−11]

However, while the initial biochemical events preceding α-syn fibril formation are not well understood, it is clear that increasing either the amount of the protein (e.g., by multiplication of the gene locus) or missense mutations in the *SNCA* gene (such as A53T, A30P, E40k) can promote the fibril reaction and foster the development of parkinsonism.[12−14] It is suggested that a natural tetrameric state of α-syn is in fact the most stable form and that both human cells and mice with the human (h)A53T mutation in fact have a higher abundance of monomeric α-syn; furthermore, this mutated form may be a disease initiator.[15] The overarching theory in the literature is that the principal toxicity is derived from the physical generation of α-syn fibrils and the subsequent formation of intracellular aggregates.

It has been postulated that one of the earliest events in the toxic formation of the α-syn aggregate is brought about by an interaction with metal ions, which initiate structural transformations, aggregation, and then "seeding" of the α-syn oligomer, which then steadily grows in size and forms the distinct inclusions identifiable using standard immunohistochemistry.[16,17] In vitro α-syn interacts with several divalent metal cations to initiate morphological changes in the protein and promote fibrillar and aggregate formation.[17] Of particular interest is its interaction with iron, as there is a selective

Received: September 25, 2015
Revised: October 14, 2015
Published: October 19, 2015

DOI: 10.1021/acschemneuro.5b00253
ACS Chem. Neurosci. 2016, 7, 119−129

increase in iron levels in specific regions of the aging brain,[18,19] which is further exacerbated in PD, particularly in the vulnerable substantia nigra pars compacta (SNc) region.[20,21] Taken together with the reported role of metal dyshomeostasis in the dementia associated with DLB, Alzheimer's disease (AD), and PDD,[22−26] this further supports the hypothesis that iron dysregulation is involved in disease processes that are characterized by pathological protein aggregation.[27,28] Furthermore, α-syn mRNA contains an iron response element that regulates expression of the protein, a feature which is particularly relevant in a cellular environment characterized by increased levels of nigral iron.[29,30] Mechanistically, a dysregulation of iron and α-syn in the SNc may propagate a feed-forward degenerative cascade.[31]

Once manifested, PD is typically associated with motor symptoms and with dementia. Dementia was originally thought to only occur late in the disease in association with the elevation in the number of Lewy bodies.[32] More recently, it has been recognized that patients exhibit a broad variety of symptoms including cognitive decline at an earlier onset not in line with Braak staging.[33] A meta-study indicated that, on average, 24.5% of PD patients have dementia,[34] with the cumulative incidence increasing with age and duration of PD to 90% by 90 years of age.[35] A multivariate analysis also found that the severity of motor symptoms is a significant predictor of dementia, though duration of PD was not.[36] Finally, the hA53T mutation of α-syn appears to be associated with cortical dysfunction and dementia.[14,37]

Dehay et al.[18] recently outlined three lines of enquiry to be followed that target α-syn pathology. These were the following: (i) increasing clearance of the protein from the brain; (ii) targeting post-translational modifications to the α-syn protein; and, as we describe here, (iii) preventing α-syn aggregation. There are few effective treatments for cognitive disorders in synucleinopathies, with symptomatic therapies using cholinesterase inhibitors having modest efficacy for improving cognition and behavior in DLB patients.[38−40] The dopamine agonist rotigotine has also shown promise in preliminary trials of atypical PD with α-syn pathologies,[41] though the precise mechanism of action of these drugs are not clear. Devos et al.[42] described incredibly promising results with the iron chelator deferiprone, which certainly gives weight to the hypothesis that addressing aberrant iron concentration may slow disease progress by reducing iron-mediated α-syn interactions. Clioquinol (CQ) is an iodinated 8-hyxroyquinoline (8-HQ) that has shown efficacy in arresting metal-mediated neurotoxicity in animal models of parkinsonism.[43] This mechanism may involve the redox-silencing of reactive Fe^{2+},[28] or the prevention of fibrillization of the peptide in a similar manner to ß-amyloid, which itself may involve metal ions[44] or act in another otherwise independent manner.[45,46] Regardless of its precise mechanism of action, the potential of CQ and other 8-HQ molecules demand further study in the search for an effective PD treatment.

In this report, we examined cognitive, behavioral, and anatomical markers in a transgenic mouse model that overexpresses the hA53T mutation of α-syn. We hypothesized that the overexpression of this mutant form of α-syn would result in the metal-mediated formation of toxic aggregates and that treatment with CQ would reduce the accumulation of α-syn and subsequently improve the disease phenotype of these animals.

RESULTS

Effect of Sustained CQ Administration on Motor Function and Nigral Dopaminergic Cell Numbers. Wild-type (WT) mice and transgenic hA53T (hereafter A53T) α-syn mice underwent chronic treatment with CQ for 5 months from 3 to 8 months of age (see Methods for CQ administration protocols), which is prior to the reported onset of motor symptoms for this mouse genotype.[47] As there is a complex underlying anatomical and pharmacological basis for linking motor activity and function of the dopaminergic system,[48−50] we employed an open field test (OFT) to investigate this phenomenon. At 8 months of age the untreated A53T mice showed a decreased time spent in movement compared to both untreated and CQ-treated WT controls (two-way ANOVA with Tukey post hoc test; genotype $F(1, 36) = 9.979$, $p_{CQ} < 0.001$; $p_{untreated} < 0.01$; Figure 1A); the origin of which is presumed to

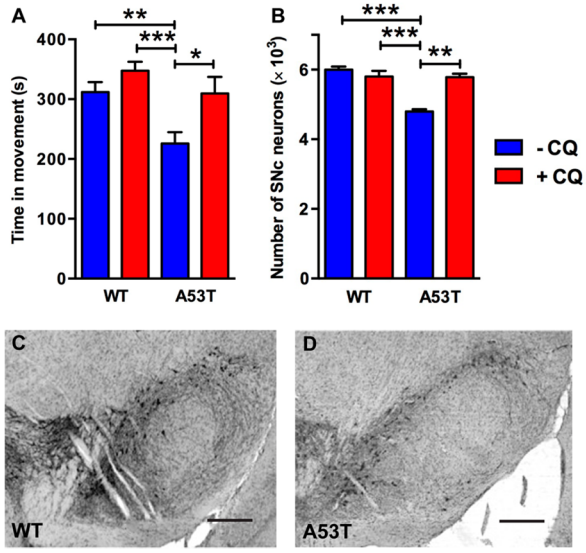


Figure 1. Effect of CQ administration on movement and SNc neuron number. (A) Wild type and α-syn A53T transgenic mice were treated with CQ daily from 3 to 8 months of age. The A53T mice ($n = 10$) showed a decreased time spent in movement (akinesia) compared to WT mice ($n = 13$), which was prevented by a 5-month treatment regime with CQ ($n = 8$). CQ did not alter time spent in movement in the WT animals ($n = 9$). (B) The number of SNc neurons was decreased in 8-month-old A53T mice ($n = 4$); a decrease that was arrested by CQ treatment ($n = 4$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C,D) Representative photomicrographs taken at the third nerve demonstrate decreased DA (tyrosine hydroxylase-positive) cells in the SNc of 8-month-old A53T mice compared to WT. Scale bar = 500 μm.

be a subtle dysfunction of the dopaminergic system. The decrease in time spent in movement was prevented by 5 months of CQ-treatment in the transgenic A53T animals compared to untreated transgenic mice (two-way ANOVA; treatment $F(1, 36) = 9.214$; $p < 0.05$); with treatment having no effect in the WT animals.

To investigate whether the change in motor function could be related to a deficit in the neuronal number, the total number of cells in the SNc was counted by stereology (Figure 1B−D). The number of SNc neurons in untreated WT animals was consistent with previous reports in other strains[51−54] (6001 ± 88; $n_{WT} = 8$). Untreated 8-month old transgenic A53T mice showed a 36% decrease in cell numbers as compared to WT

DOI: 10.1021/acschemneuro.5b00253
ACS Chem. Neurosci. 2016, 7, 119−129

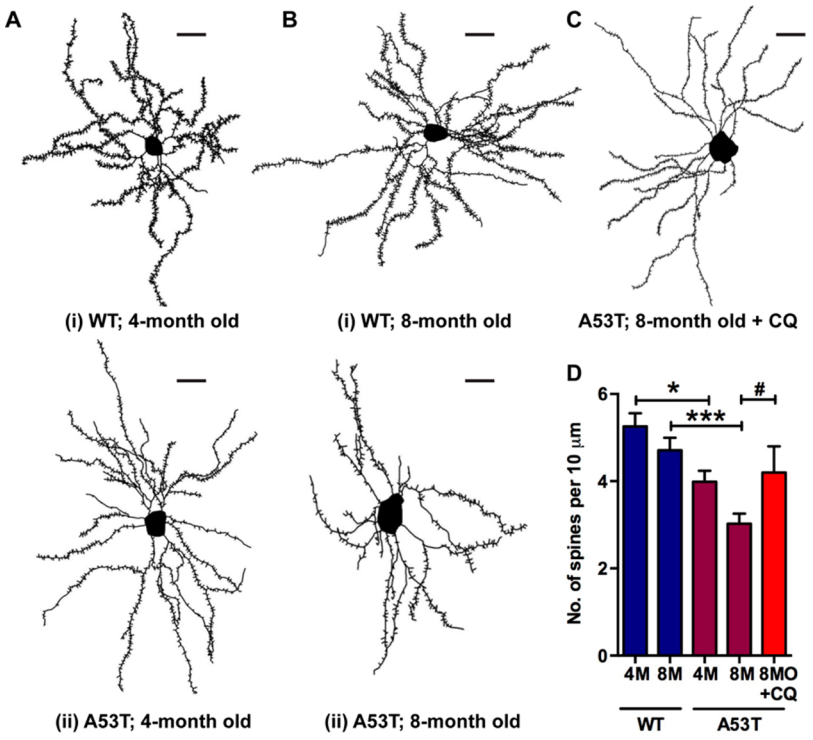


Figure 2. Caudate putamen medium spiny neuron spine densities. (A–C) Tracings of Golgi stained MSN from the dorsal tier of the CPU were analyzed for spine densities in 4- and 8-month old WT and A53T transgenic mice. Scale bar = 25 μm (D) Eight-month-old A53T MSNs (n = 13 animals per group) had approximately half the number of spines compared to age-matched WT controls neurons (n = 9 per group). A significant difference was also observed in the four-month-old A53T (n = 9 per group) compared to age-matched WT (n = 9 per group). Statistical comparisons were made using a two-way ANOVA with Tukey's *post hoc* multiple comparisons test. * $p < 0.05$; *** $p < 0.001$. A Student's two-tailed t-test revealed a mild yet significant restoration of dendritic spine density in 8-month-old A53T mice given a sustained dose of CQ. # $p < 0.05$.

(4797 ± 67, n_{A53T} = 5; two-way ANOVA; genotype $F(1, 11)$ = 25.48; $p < 0.001$), consistent with our previous reports of decreased nigral cell counts in this strain.[55,56] Daily CQ administration for 5 months spanning 3 to 8 months of age definitively prevented the nigral cell loss in mice expressing mutant α-syn (5787 ± 99, n_{A53T} = 4; two-way ANOVA; treatment $F(1, 11) = 10.70$, $p < 0.01$).

Effect of Sustained CQ Dosage on Medium Spiny Neuron Spine Density in the Caudate Putamen. Medium spiny neurons (MSNs) in the caudate putamen (CPU) are innervated by projections from SNc neurons originating in the midbrain, and they are susceptible to damage from dopamine-targeting neurotoxins[57] and atrophy in late-stage idiopathic PD.[58] We examined MSNs from the dorsal tier of the CPU for dendritic spine density, a reduction in which represents further disruption of the dopaminergic pathway (Figure 2A–C). Both 8- and 4-month old transgenic A53T mice had a reduced number of spines compared to age matched WT controls (n = 9 per group; two-way ANOVA; genotype $F(1, 36) = 7.957$; $p < 0.01$; age $F(1, 36) = 30.45$; $p < 0.001$; Figure 2D). The 5-month CQ treatment regime prevented the decline in spine density compared to untreated 8-month-old A53T animals (Student's two-tailed t-test; $t = 2.134$ df = 19; $p < 0.05$; Figure 2D). These data indicate that accumulation of the mutant form of human α-syn has an effect on spine density in transgenic mice that occurs prior to neuron loss (at 4 months of age), and this may disrupt the basal ganglia neuronal circuits; additionally, this demonstrates that CQ administration can partially rescue spine density.

Influence of Acute CQ Administration on Cognitive Function. Mice expressing the human A53T α-syn mutation exhibit age-dependent α-syn inclusions that correlate with spinal-cord-type motor impairment[47] and cognitive deficiencies.[55] In this study, the Morris water maze (MWM) test of memory showed that 8-month-old A53T transgenic mice did not learn the location of the platform as readily as did WT controls from day 3 to the conclusion of the learning component of the study on day 6 (Figure 3A; Student's two-tailed t-test; df = 15; day 3 $p < 0.01$; day 4 $p < 0.05$; day 5 and 6 $p < 0.001$). A probe trial was conducted on day 7, showing that WT animals spent a significantly longer time in the correct quadrant of the MWM (Figure 3B; $p < 0.05$). The velocity of movement simultaneously measured on day 7 was not significantly different, though showed a decreasing trend in the A53T mice (Student's t-test; $p = 0.06$; Supporting Information Figure S1). The ability to perform the MWM in part depends on the ability of both groups to move equally well; therefore, the nonsignificant decrease in the velocity could be physiologically important. Therefore, CQ was not tested in the MWM. Instead, Y-maze and novel object recognition tests were performed to further assess the cognitive deficit, as these tests are not as dependent on ambulatory speed. We had previously shown that short-term treatment with CQ and other 8-hydroxyquinolines are capable of rapidly (within 11 days) reversing dendritic spine density in transgenic AD models.[59,60] To investigate whether CQ was similarly effective in rescuing a cognitive phenotype related to a decrease in dendritic spine density, we conducted Y-maze testing of 8-month-old animals treated with CQ for 28 days. The Y-maze data supported the MWM results, showing that A53T mice were cognitively impaired and that short-term treatment with CQ rescued this deficit, evidenced by an increase in time spent in the novel arm

DOI: 10.1021/acschemneuro.5b00253
ACS Chem. Neurosci. 2016, 7, 119–129

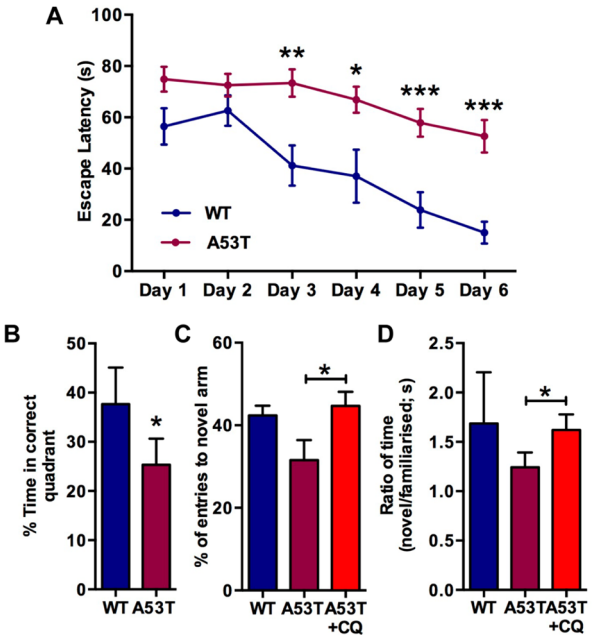


Figure 3. Cognitive performance is impaired in A53T transgenic mice and improves after acute CQ treatment. (A) 8-month-old α-syn A53T mice (n = 9) did not learn to escape the Morris water maze (MWM) as quickly as did age matched WT controls (n = 7). Dunnett's *post hoc* multiple comparisons testing indicated significance between the genotypes from day 3 until the experiment's conclusion on day 6. (B) A probe trial with the MWM was conducted at day 7 and showed that WT mice spent significantly more time in the quadrant where the platform is normally situated, indicating recollection of its location. (C) As the A53T phenotype also features a motor deficit (see Supporting Information Figure S1), we used a Y-maze to confirm that A53T mice were cognitively impaired, with a decrease in novel arm entries from 42% in WT to 32% in the 8-month-old animals A53T mice in the Y-maze. Acute administration of CQ in food for 28 days restored the performance (n = 5 per group). (D) A novel/familiar object recognition test also showed a 30% improvement in CQ treated animals versus untreated A53T mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

of the maze (Figure 3C; one-way ANOVA with Dunnett's *post hoc* test against untreated A53T; treatment $F(2, 12)$; $p < 0.05$). Similarly, a novel object recognition test[60] also demonstrated a 30% recovery in disease phenotype in the short term CQ treated A53T mice (Figure 3D; one-way ANOVA; treatment $F(2, 12)$; $p < 0.05$).

Influence of Acute Administration of CQ on Neuronal Morphology. Neuronal dendrite and spine density in the hippocampus is thought to correlate with hippocampal-dependent learning and memory in mice.[61] To investigate the relationship between memory and neuronal structure, CA1 hippocampal neurons were Golgi stained, and basal and apical dendritic spine numbers were quantified (Figure 4). We found that 4-month-old A53T mice had approximately half the number of basal spines compared to age-matched WT mice (two-way ANOVA; $F_{\text{treatment}}$ (1, 330) = 2.716; $p_{\text{treatment}}$ = 0.1003 genotype; $F_{\text{genotype}}(1, 330)$ = 245.5; $p_{\text{genotype}} < 0.001$; n = 6 per group) that were partially rescued by 28 days of CQ treatment (A53T + CQ = 8.07 ± 0.30 per 10 μm^2; n_{neurons} = 24; sham-treated A53T mice =6.80 ± 0.31 per 10 μm^2; n_{neurons} = 21; $p < 0.05$; Figure 4A). Apical spines were not affected by CQ treatments, though again were near halved in the A53T mutants (two-way ANOVA $F_{\text{treatment}}(1, 331)$ = 1.588, $p_{\text{treatment}}$ = 0.2084; $F_{\text{genotype}}(1, 331)$ = 159.8 $p_{\text{genotype}} < 0.001$; n = 6 per group; Figure 4B). Clioquinol-treatment of WT animals did not result in any change in the density of apical or basal dendritic spines. It is interesting to note that CQ did not rescue the density of CA1 apical spines; this is in contrast to the effects of the 8-HQ PBT2 in transgenic AD models, where, in addition to an increase in basal spine density, there was also a restoration of apical spine density.[59]

Biochemical Properties of hA53T Mutant α-Syn. We used a range of biochemical assays to assess the effect of CQ treatment on the expression and propensity to aggregate of the mutant α-syn peptide from the A53T transgenic mice. Western blot analysis was carried out using the antihuman α-syn monoclonal antibody LB509, which remains sensitive to the A53T mutation.[62] Although chronic CQ treatment did not alter the amount of membrane-bound and soluble monomeric α-syn (solubilized using SDS; Student's two-tailed t-test; t = 0.560; df = 13; p = 0.5; Figure 5A), it did show a decrease in the disease-associated urea-soluble component (often referred to as the "insoluble fraction";[63] t = 4.97 df = 13 $p < 0.001$; Figure 5B), indicating that CQ is involved in either solubilization of α-syn aggregates in vivo or helps prevent their formation.

The formation of α-syn fibrils in the presence of Fe(III) was monitored by thioflavin (ThT) fluorescence. Consistent with previous reports,[64] iron increased the rate and amount of aggregated α-syn, whereas CQ reduced iron-mediated fibrillization (Figure 5C). These data support the hypothesis that CQ may intervene in metal-mediated fibril formation, as with ß-amyloid aggregation.[65]

DISCUSSION

In this project, we examined behavioral (cognitive and motor function), microanatomical and biochemical markers in a transgenic mouse expressing the human A53T mutation in α-syn; a model that recapitulates a number of the cardinal features of human synucleinopathies. We hypothesized that treatment with CQ would reduce the formation of insoluble α-syn aggregates to impact upon cell survival, cognition, motor performance, and disrupted neuroanatomy. We showed that the presence of abundant insoluble (urea soluble) α-syn is related to SNc cell loss, reduced hippocampal and CPU MSN dendritic spine density and impairments in both motor and cognitive behaviors. CQ slows the progressive cell loss, improves open field behavior, modestly improves hippocampal spine density and improves short-term memory. We have confirmed our hypothesis that the overexpression of the human A53T missense mutation of α-syn results in the metal-mediated loss of nigral neurons and have further extended this by demonstrating a more widespread disruption in microanatomy in the CPU and hippocampus with corresponding behavioral correlates. CQ could mitigate these effects by reducing fibril formation, either through preventing metal-mediated aggregation of α-syn or by inhibiting the self-assembly of toxic α-syn species.

There are some limitations to the A53T mouse as a model of parkinsonism. As reported, cell loss in the SNc was 36% in the transgenic mice, which is less marked than in idiopathic PD, synucleinopathies, or neurotoxin animal models.[66] The magnitude of the cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) intoxication models more closely matches the magnitude of loss observed in humans.[67] However, there is no perfect animal model of PD that recapitulates *all*

DOI: 10.1021/acschemneuro.5b00253
ACS Chem. Neurosci. 2016, 7, 119−129

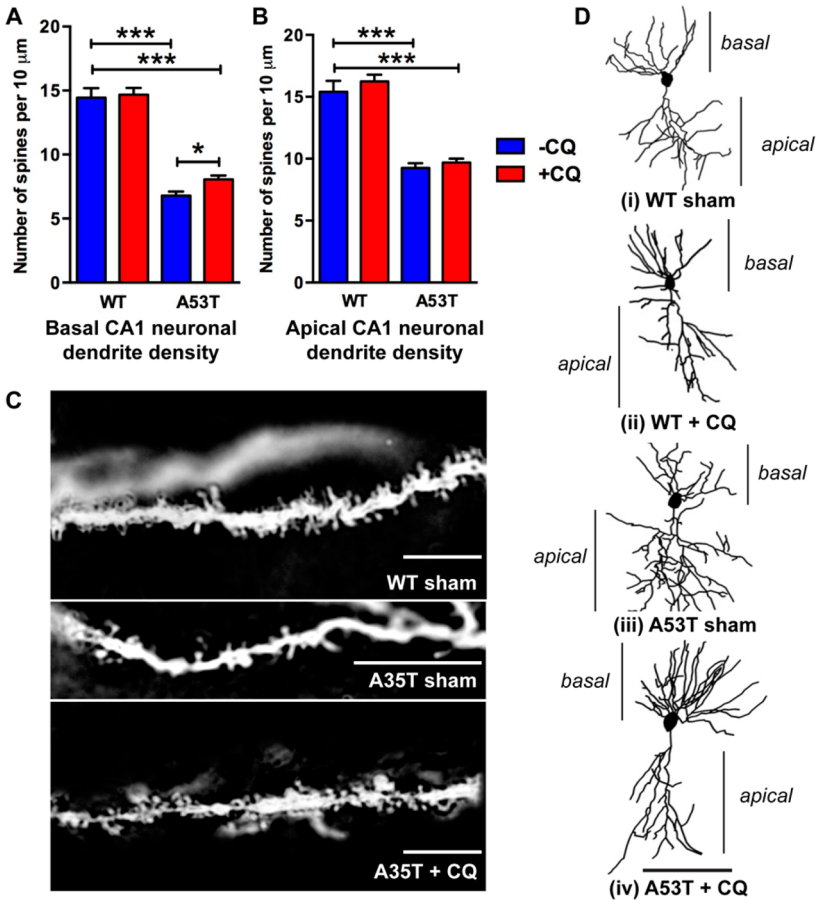


Figure 4. Golgi staining measured CA1 hippocampal neuron basal and apical dendrite spine density. (A, B) The 5-month-old A53T mice had approximately half the number of apical and basal spines than age-matched WT controls. Three weeks of CQ treatment significantly increased the density of spines on the basal dendrites in the A53T, though this increase was still significantly lower than WT controls. No significant difference was observed in the density of apical or basal spines from WT compared to WT mice treated with CQ, and apical dendrite spine density in the A53T mice was not significantly altered by CQ treatment. (C) Representative micrographs of basal dendrites from 5-month-old WT, sham-treated A53T and CQ-treated A53T animals for 3 weeks shows the increase in spine density in the treated animals. Scale bar = 10 μm) (D) Representative reconstructed whole CA1 neurons from each group (i–iv) also demonstrated the differential dendrite densities. Scale bar = 100 μm.

pathological features of the disease;[68] the A53T model also features pathology in the spinal cord that is atypical of idiopathic PD.[47] Regardless, the progressive cell loss in the SNc is a valuable tool for studying how CQ (or other compounds) can impact upon the gradual pathology caused by the interaction between α-syn and iron during the progression of PD.

The seminal observation that the dose of α-syn (duplication and triplication of the normal gene) can induce parkinsonism has initiated research into α-syn toxicity.[12,13] There are numerous studies that have correlated the number and location of Lewy bodies with PD progression,[32] and furthermore, the Lewy body burden is also associated with cognitive impairment in PD;[69,70] this feature is not observed in certain familial forms of PD, including mutations to the *parkin* gene, where Lewy body deposition is more sporadic.[71] However, there still remains the open question of which form of α-syn is toxic. There is a growing body of data that suggest that it is the early oligomeric aggregates of α-syn that are toxic, rather than the later mature fibrils, and that the Lewy bodies may in fact be "protective".[1,8,9,72,73] In order to gain insight into the role of α-syn in synucleinopathies and PD in particular, a number of mouse models have been made. However, neurotoxin models tend not to recapitulate Lewy body formation within the SNc, and the acute toxicity is not reflective of the progressive loss of nigral neurons found in PD.[67] Transgenic strains of mice that express human variants of α-syn and associated pathology have thus become integral research tools.[49,74,75] The variations in the phenotype of these various strains of mice have been attributed to differing expression patterns of α-syn.[76] Previous studies on the A53T strain of mouse used in this study displayed a spinal cord behavioral phenotype, with 10% of the mice impaired in a rotarod test at 8 months of age.[47] The proportion of mice with impairment increases with age to include all animals by 16 months of age. However, visual inspection of the nigra did not reveal any loss of neurons, and so in this study we utilized sensitive stereological measures to assess cell numbers. In humans, clinical symptoms of PD are apparent when 50–70% of SNc dopamine neurons are depleted,[77] with an approximate yearly decline of 5–10% in neurons,[77] and 5% loss in [^{18}F]-labeled 3,4-dihydroxyphenylalanine (DOPA) uptake per year.[78] We found an approximately 30% decrease in the number of SNc dopaminergic neurons in the 8-month-old A53T mice but no loss in nigral cell numbers at 4 months of age, suggesting that approximately 6% of the neurons were lost each month between five and eight months of age, assuming the progressive loss pattern is similarly linear. In idiopathic PD, a recent study showed that there is a 50–90% loss of nigral cells at one year

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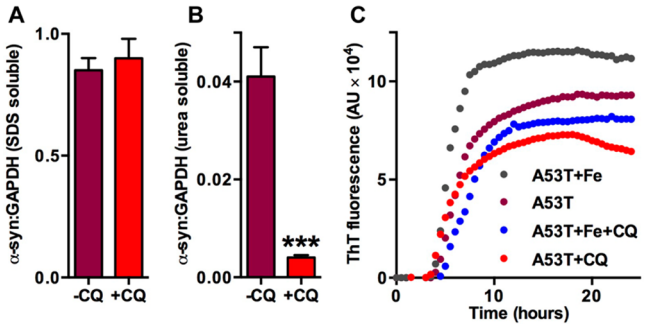

Figure 5. Effect of CQ administration on α-synuclein fractions in A53T mice and in vitro iron-mediated fibrillization. Western Blots were probed with the antihuman α-syn antibody LB509 (Supporting Information Figure S2A,B). (A) There was no difference in the membrane-bound (SDS soluble) fraction of mice treated with CQ for 5 months. (B) Chronic CQ administration decreased the amount of "insoluble" (urea soluble), representative of α-syn aggregates. *** $p < 0.001$. (C) Thioflavin T (ThT) fluorescence (indicative of fibrillization) of synthetic A53T α-syn with and without the presence of CQ and iron (Fe) was monitored over a 24 h period. Fibril formation was demonstrated by an increase in ThT fluorescence at 440 nm, and the data obtained are presented as an average of triplicate readings. Fluorescence was highest when α-syn was incubated with Fe(III) only. Incubation of α-syn with CQ and CQ + Fe(III) produced fluorescence comparable to of α-syn alone.

postdiagnosis,[79] which strongly suggests cell death is occurring many years prior to formal diagnosis. Further, analysis of movement of professional Association Football player Ray Kennedy identified movement disorders at least 10 years before the appearance of unequivocal parkinsonian symptoms and 14 years prior to formal diagnosis.[80] In the mice, the rate of loss of neurons appears much more accelerated than that observed in humans.

Dendritic spines are essential for all synaptic transmission. Spines undergo constant remodeling and are rapidly altered in response to a variety of changing biochemical conditions within the brain. These include depletion in MSN spine numbers in PD,[81] which was recapitulated in the A53T model, and conversely, regeneration of MSN spines in the 6-hydroxydopamine parkinsonian model;[82] dopamine loss from cocaine addiction,[83] and in zinc transporter-3 knockout mouse models that are a suggested model of AD with a memory and synaptic dysfunction phenotype.[84] In post-mortem cortical brain tissue from DLB patients, the accumulation of presynaptic α-syn aggregates are associated with a profound loss of dendritic spines[8] and are considered to be an early marker of pathology. In this study, the reduction in spine number appears to correlate with the accumulation of urea soluble α-syn in older A53T mice. This study showed that the human α-syn A53T transgenic mouse exhibited spine loss in the MSN and the hippocampus and that this loss can be modestly reversed with either chronic or acute treatment with CQ.

The association between iron and PD is multifaceted and complex; too little iron leads to neurological deficits, yet too much appears to result in neuronal loss.[85] A recent small-scale clinical trial using the iron chelator deferiprone showed extremely promising results in reducing brain iron load and improving motor indicators of PD.[42] Parkinson's disease has long been associated with an increased accumulation of iron in the SN,[86] the mechanism of which is not fully understood. The regulation of iron in the brain is normally tightly controlled,[87] though once disruption of iron metabolism occurs, three independent consequences ensue. First, elevation of iron in the cytosol has been linked to the Fenton reaction causing oxidative stress that is thought to result in progressive neurodegeneration,[88] possibly through interactions with the pro-oxidant DA molecule itself.[29] Thus, modulation of iron through redox silencing could prevent the formation of toxic dopamine products forming through iron-mediated reactions.[89] Second, metals appear to have binding sites on human wild-type α-syn with further alterations in the binding in the known mutant forms (A30P, A53T, E46K).[90,91] These binding sites enable the enhancement of α-syn aggregation and fibril formation in the presence of metals.[17,92] The A53T α-syn mutation has been shown to increase iron-enhanced aggregation and subsequent toxicity in M17 cells.[93] Third, an alteration in the iron level alters the expression of proteins. An increase in iron acts on mRNA translation via iron response elements to alter the expression of proteins such as α-syn and ferritin, both of which are involved in animal models and in the human disease.[31,94,95]

Clioquinol is an orally bioavailable drug with moderate affinity for copper, zinc, and iron. Unlike traditional chelators such as EDTA, CQ does not cause bulk excretion of metals, but rather, it acts as an ionophore/chaperone to redistribute metals from areas of high-abundance to those that may be deficient. Clioquinol has the highest affinity for copper and zinc binding,[96] though it also has affinity for iron.[28] Furthermore, CQ is an antioxidant that potently inhibits metal-mediated hydroxyl radical production.[88] In a previous study, we showed that long-term administration of CQ afforded a 50% decrease in cell loss with 20% reduction in nigral iron compared with MPTP alone.[97] In this study, we demonstrate that CQ has a measurable effect on a parkinsonian model exhibiting a human feature of the disease, further supporting the potential use of 8-HQs in mitigating cell loss in PD by interacting with another key component of the human condition—α-syn oligimerization.

CONCLUSIONS

Our findings support the notion that increased insoluble α-syn species cause SNc cell loss, declines in behavior, reduction in dendritic spines and cognitive deficits. CQ slows the progressive cell loss, improves open field behavior, and modestly improves hippocampal spine density, neuronal morphology, and short-term memory. We suggest that insoluble α-syn is toxic and the formation of the urea soluble α-syn species may be mitigated by the chelation of metals. Furthermore, treatment with CQ prevents the α-syn pathology and behaviors that characterize the human α-syn A53T transgenic mouse line, suggesting that inhibition of α-syn aggregation and the modulation of metal ion homeostasis may represent a novel therapeutic approach for synucleinopathies.

METHODS

Animals. All procedures involving mice conformed to the Australian National Health and Medical Research Council (NHMRC) code of practice for the care and use of animals for scientific purposes and were approved by the Institute Animal Ethics Committee. All experiments were undertaken to reduce the number of animals used and to minimize pain/discomfort. All mice used in this study were confirmed to have the transgene by PCR. The colony of mice with the human α-syn A53T mutation driven by the murine PrP promoter has been well characterized.[98]

DOI: 10.1021/acschemneuro.5b00253
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Clioquinol Administration. Two treatment periods were delivered to the mice; short-term designed to look at the synaptic plasticity involved in cognition (28 days)[60] and chronic administration to investigate the loss of nigral neurons (4−5 months). In the short-term (acute) trials, mice received CQ by oral gavage at a daily dose of 30 mg kg^{-1} for 28 days or a vehicle (standard suspension vehicle) alone without drug. For longer-term trials (4−5 months), CQ was spiked into rodent chow (0.25 g of CQ per kg food; Specialty Feeds, Glen Forest, Western Australia). The mice were weighed weekly, and the average dose across the trial was calculated at 37 mg kg^{-1}. Clioquinol was obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia).

Morris Water Maze. A53T and age-matched WT mice were tested for hippocampal-dependent changes in spatial learning and memory using the MWM protocol.[60] Escape latency to locate the hidden platform during the acquisition trials during the 6 days of training was analyzed, as was the probe trial conducted 24 h after the final acquisition trial.

Y-Maze Test for Spatial Memory and Learning. Mice were subjected to a 2-trial Y-maze test, as previously described.[99] Three identical arms of the maze were randomly designated start arm, novel arm, and other arm. The Y-maze tests were performed after 8 weeks of CQ treatment and consisted of 2 trials separated by a 24-h intertrial interval to assess spatial recognition memory. The first trial (training) was for 10 min, and the mice were allowed to explore only 2 arms (starting arm and other arm). For the second trial (retention), mice were placed back in the maze in the same starting arm, and allowed to explore for 5 min with free access to all 3 arms. By using a ceiling-mounted CCD camera, all trials were analyzed for the number of entries the mice made into each arm. Data were expressed as the percentage of novel arm entries made during the retention trial.

Open Field Testing. The mice were removed from their home cage and placed individually into clear Perspex tracking arenas (Coulbourn TruScan, U.S.A.). The mice were allowed to habituate in the arena for 20 min. The total amount of movements over the next 20 min were recorded and analyzed. The locomotor cell measured the total time in movements in the floor plane by the interruption of a grid of beams. The cumulative time in coordinated motion was aggregated; each movement was defined as a series of successive movements with no rest at a particular coordinates for at least 1 sample interval.

Novel Object Recognition. The novel object recognition test was used as another measure of cognitive function.[100] Cohorts of five-month-old A53T (-CQ n = 12, + CQ n = 13) mice were treated with 30 mg kg^{-1} CQ or vehicle alone for 28 days. Briefly, the mice are habituated to the chamber (size 38 cm × 27 cm × 12 cm high) for 15 min on 3 successive days (day 1−3), and on day 4, the mice were exposed for 10 min to two identical blocks (1.5 × 1.5 × 1.0 cm). Retention was tested 1 or 24 h after this exposure by replacing one of the familiar objects with a novel object (1.5 × 1.5 × 2.0 cm). The activity around the blocks was filmed for 10 min. The retention of the mice was calculated by the ratio of time spent attending the novel object (seconds) divided by the time spent attending the familiar object (seconds). The aggregate time in attendance of the object is when the mice make contact with mouth, nose, or paw or facing the object and are within 2 mm of the objects.

Rapid Golgi Stain. Following behavioral assessment, the animals were deeply anaesthetized (sodium pentobarbitone 100 mg kg^{-1}) and perfused with PBS. The brain was removed and one hemisphere processed for a rapid Golgi stain (FD Rapid Golgi Stain Kit, MTR Scientific, U.S.A.), the other half processed for stereological estimation of SNc neuron numbers. Tissues were placed in Golgi solutions and stored in the dark at room temperature for 2 weeks. Using a cryostat set at −27 °C, the brains were sectioned at 90 μm and transferred to gelatin-coated slides. Sections were stained, dehydrated, cleared, and coverslipped in mounting medium, then analyzed by microscopy and measurements taken for the number of spines and length of dendrites. Camera Lucida drawings were made with the assistance of a software controlled stage and microscope (Neurolucida, MBF Bioscience, U.S.A.).

Stereological Estimates of Cell Numbers. The brains were immersion fixed in 4% paraformaldehyde for 4 h and then transferred to 30% sucrose in PBS for 2 days. Brains were sectioned in a 1:2 series at 40 μm and stained with a neutral red Nissl stain. The total number of neurons in the SNc was estimated using a fractionator sampling design.[51] Immunohistochemistry, together with a Nissl counterstain, was performed on a parallel series to assess the number of tyrosine hydroxylase (a proxy for DA) positive cells. Counts were made at regular predetermined intervals (x = 140 μm; y = 140 μm). Systematic samples of the area occupied by the nuclei were made from a random starting point. An unbiased counting frame of known area (45 μm × 35 μm) was superimposed on the image of the tissue sections. Stereology was performed with a Leica microscope with a 63× (NA = 1.36) lens and StereoInvestigator software (MBF Bioscience, U.S.A.).

Western Blot for α-Syn. Tissue samples were collected, frozen, and stored at −80 °C for later use. Brain homogenates were prepared by sequential extraction, with the SDS soluble and urea soluble fractions examined by Western blot. Protein concentrations of the initial brain homogenates were estimated using an assay for bicinchoninic acid (BCA) (Pierce Protein Assay Kit, ThermoFisher Scientific, U.S.A.). Western blots were run as described previously[60] with α-syn expression detected with the antihuman α-syn antibody LB509 (Invitrogen, ThermoFisher).

Alpha-Syn Fibrillization Assay. Alpha-synuclein fibril formation in the presence of Fe(III) was monitored by thioflavin T (ThT) fluorescence. Lyophilized recombinant α-syn (14 μM) was prepared in 10 mM of phosphate buffer (PB; pH 7.4) and filtered through a 0.2 μM filter syringe (Minisart RC4 filters, Satorius, Goettingen, Germany). Triplicate α-syn preparations were incubated in the presence of ferric nitrate (14 μM, prepared in 10 mM PB; pH7.4, Sigma-Aldrich, U.S.A.) and CQ (14 μM, prepared in 10 mM PB; pH 7.4) at 37 °C with continual shaking at 1400 rpm (Thermomixer Comfort, Eppendorf, Germany) for 24 h. Every 30 min, 10 μL aliquots were transferred to a black 96 well microtiter plate (Costar, Corning, MA, U.S.A.) with 270 μL ThT working solution (5 μM ThT Sigma-Aldrich, U.S.A.; prepared in 10 mM PB; pH 7.4). Fluorescence intensity was measured on a Flexstation3Microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) using a 440−10 nm/485−10 nm excitation/emission filter set.

Statistical Analysis. Statistical analysis was performed using Prism 6.05e (GraphPad) and the tests used are stated in the Results as necessary. Where appropriate, data are reported as ± standard error of the mean (SEM).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00253.

Morris water maze data (Figure S1) and Western blot analysis (Figure S2) (PDF)

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Funding

P.A.A., A.I.B., and D.I.F. are supported by funds from the National Health and Medical Research Council and the Australian Research Council. The Florey Institute of Neuroscience and Mental Health acknowledge the strong support from the Victorian Government and in particular the funding from the Operational Infrastructure Support Grant.

DOI: 10.1021/acschemneuro.5b00253
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Notes

The authors declare the following competing financial interest(s): P.A.A and D.I.F. are shareholders and scientific consultants to Prana Biotechnology Ltd. A.I.B. is a shareholder in Mesoblast Ltd, Cogstate Ltd, Brighton LLC, Prana Biotechnology Ltd, Eucalyptus LLC, and is a payed consultant for Collaborative Medicinal Discovery LLC and Brighton LLC.

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