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Genetic or Pharmacological Iron Chelation Prevents MPTP-Induced Neurotoxicity In Vivo: A Novel Therapy for Parkinson's Disease

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Summary

Studies on postmortem brains from Parkinson's patients reveal elevated iron in the substantia nigra (SN). Selective cell death in this brain region is associated with oxidative stress, which may be exacerbated by the presence of excess iron. Whether iron plays a causative role in cell death, however, is controversial. Here, we explore the effects of iron chelation via either transgenic expression of the iron binding protein ferritin or oral administration of the bioavailable metal chelator clioquinol (CQ) on susceptibility to the Parkinson's-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP). Reduction in reactive iron by either genetic or pharmacological means was found to be well tolerated in animals in our studies and to result in protection against the toxin, suggesting that iron chelation may be an effective therapy for prevention and treatment of the disease.

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Introduction

Iron levels in the substantia nigra (SN), the dopamine-containing region of the brain that undergoes selective degeneration in Parkinson's disease (PD), have been reported to be elevated in patients with the disorder (Sofic et al., 1988, 1991; Dexter et al., 1987, 1989; Youdim et al., 1993; Gerlach et al., 1994; Yantiri and Andersen, 1999; Griffiths et al., 1999; Andersen, 2001). Accessible ferrous iron (Fe^{2+}) can react with hydrogen peroxide (H_2O_2) produced during oxidative deamination of dopamine to generate hydroxyl radicals (·OH) that can damage proteins, nucleic acids, and membrane phospholipids, leading to cellular degeneration (Beal, 1992; Gutteridge, 1992). However, whether the increase in SN iron is a causal factor in the disease or a consequence itself of neuronal degeneration is controversial (Adams and Odunze, 1991; Berg et al., 2001; Thompson et al., 2001). Experiments were therefore undertaken to test whether iron is causally involved in cellular degeneration associated with toxin-induced parkinsonism by assessing whether iron chelation can act to protect against dopaminergic cell loss.

In the first set of experiments, susceptibility of transgenic mice expressing the ferritin heavy subunit (H ferritin) within dopaminergic SN neurons to the PD-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was assessed. Ferritin, the primary nonheme iron storage molecule in the body, can sequester up to 4500 atoms of ferric (Fe^{3+}) iron as an oxyhydroxide (Harrison and Arosio, 1996). Ferritin is believed to keep iron in a nonreactive form where it cannot promote redox reactions and therefore could be a key component for protecting tissues against iron-catalyzed oxidative damage (Jellinger, 1999). The ferroxidase activity of H ferritin converts harmful labile ferrous iron to less soluble, unreactive ferric iron, while the light subunit (L ferritin) stablizes the ferritin-iron complex, promoting long-term iron storage (Harrison and Arosio, 1996; Rucker et al., 1996). In the second set of experiments, mice were orally pretreated for 8 weeks with the antibiotic 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol or CQ) and assessed for the ability of the compound to protect against MPTP-induced toxicity. Another antibiotic compound, minocycline, has previously been demonstrated to protect against MPTP toxicity, likely due to its ability to decrease nitric oxide-mediated apoptosis (Du et al., 2001). CQ's mechanism of action, however, is likely different. It has been shown to chelate both ferrous and ferric iron (Kidani et al., 1974) and to decrease brain iron levels in normal mice (Yassin et al., 2000). Its oral administration was reported to inhibit β-amyloid accumulation in an Alzheimer's disease (AD) transgenic mouse model via its actions as a metal chelator (Huang et al., 1999; Bush, 2000; Cherny et al., 2001). In addition, in a recently reported small two-year double-blind human phase II clinical trial, it has been reported to be well tolerated in patients and to delay the course of the disease by 9 months (Masters, 2002). Neither ferritin expression nor oral CQ treatment appear to elicit any

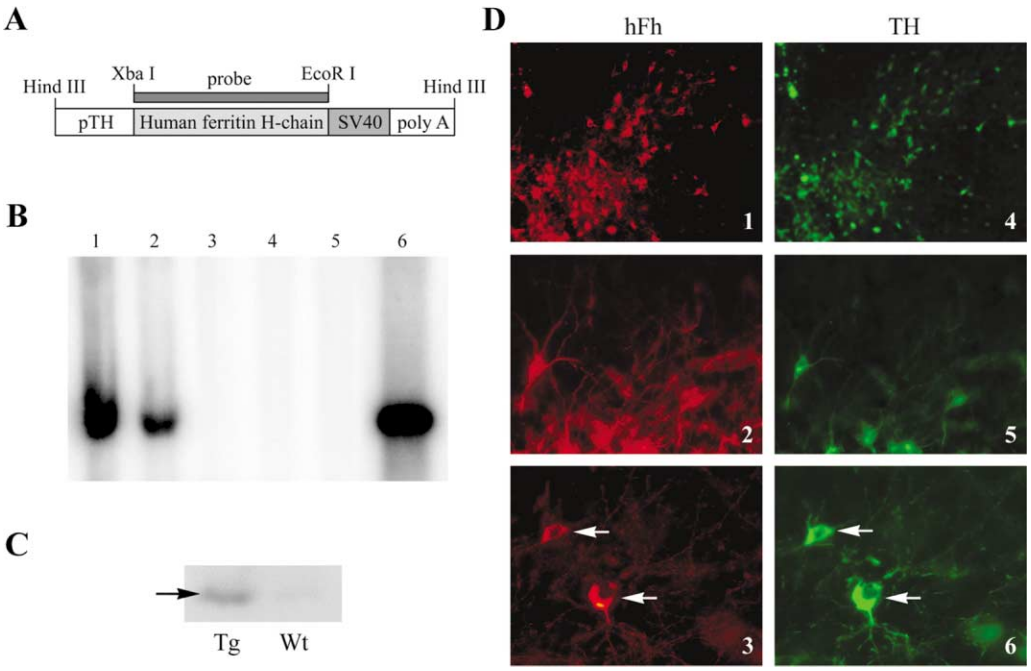

Figure 1. Creation of pTH-Ferritin Transgenics

(A) Schematic of pTH-ferritin construct used for creation of ferritin transgenics and Xba I-EcoRI probe used for Southern analysis. Abbreviations: pTH, 4.8 kb 5′ rat tyrosine hydroxylase promoter region; human ferritin H-chain, human ferritin heavy chain 2.6 kb genomic fragment; SV40/poly A, 900 bp 3′ large T antigen SV40 splice/polyadenylation sequences; probe, ^{32}P-labeled Xba I/EcoRI cDNA fragment of ferritin H-chain.

(B) Representative Southern blot analysis of genomic tail DNA isolated from pTH-ferritin founders. Lanes 1 and 2, transgenics; lanes 3–5, nontransgenics; lane 6, 2.6 kb Xba I/EcoRI ferritin probe.

(C) Representative Western blot using monoclonal antibody directed against human H ferritin. Abbreviations: Tg, ferritin transgenic; wt, wild-type littermate. Arrow shows expected position of the 21 kDa human ferritin H-chain protein.

(D) Expression of human ferritin protein product in dopaminergic SN neurons verified by representative double immunocytochemistry (ICC) using H ferritin and TH antibodies. Images 1–3, ICC with monoclonal antibody against human H ferritin (hFh dilution, 1:500); images 4–6, ICC with TH antibody (dilution, 1:500). 1 and 4, 10× magnification; 2, 3, 5 and 6, 20× magnification.

apparent adverse general health or behavioral effects, unlike chelators currently used as therapy for iron overload conditions, which can have severe side effects (Porter and Huehns, 1989; Marciani et al., 1991). Results from our studies demonstrate that such in vivo iron chelation protects mice against the toxic effects of the parkinsonian-inducing agent MPTP and suggest that this may be a novel avenue of therapy for the disease.

Results

Transgenic ferritin lines were generated by injection of an 8.3 kb DNA fragment into fertilized mouse embryos containing the rat tyrosine hydroxylase promoter (pTH) driving expression of the human H ferritin gene (Figure 1A). Human ferritin binds iron more tightly than the mouse isoforms, making it a superior iron chelating agent, and monoclonal antibodies are also available that are specific to the human protein (Rucker et al., 1996). In order to prevent iron-induced downregulation of transgenic ferritin RNA translation, the 5′ noncoding region of the gene containing an iron-response element (IRE) was excluded from the construct (Caughman et al., 1988).

Integration of the pTH-ferritin transgene in founder animals was verified by Southern blot analysis (Figure 1B). Expression of the human H ferritin protein in the SN of resulting lines was verified by both Western blot analysis (Figure 1C) and immunocytochemistry (ICC; Figure 1D, 1–3). No changes were observed in endogenous ferritin levels in these animals (data not shown). Double labeling of H ferritin-expressing cells with tyrosine hydroxylase (TH) antibody demonstrated that the transgenic ferritin protein is localized within dopaminergic SN neurons (Figure 1D, 4–6). Adult ferritin transgenics exhibited no overt phenotype, reproduced normally, and displayed no gross alterations in brain size or anatomical features in histologically stained brain sections (data not shown).

Increased iron binding to ferritin would be expected to result in increased conversion of ferrous to ferric iron as it enters the ferritin core and is oxidized to ferrihydrite. Ferric iron's paramagnetic characteristics allow for its visualization by high-field magnetic resonance imaging (MRI); the signal is intensified when iron is bound to ferritin and thereby can be used as a measure of ferritin bound iron (Gilissen et al., 1998; Griffiths et al., 1999). MRI was performed on brains from ferritin transgenics versus nontransgenic littermates and the signal intensity quantified using frontal cortex as an internal control. A 33.7% ± 8.5% increase in signal intensity was observed in transgenic animals versus wild-type controls (Figure 2A; $n = 4$ animals per parameter, $p < 0.01$). Conversely, bioavailable SN ferrous iron levels were found to be

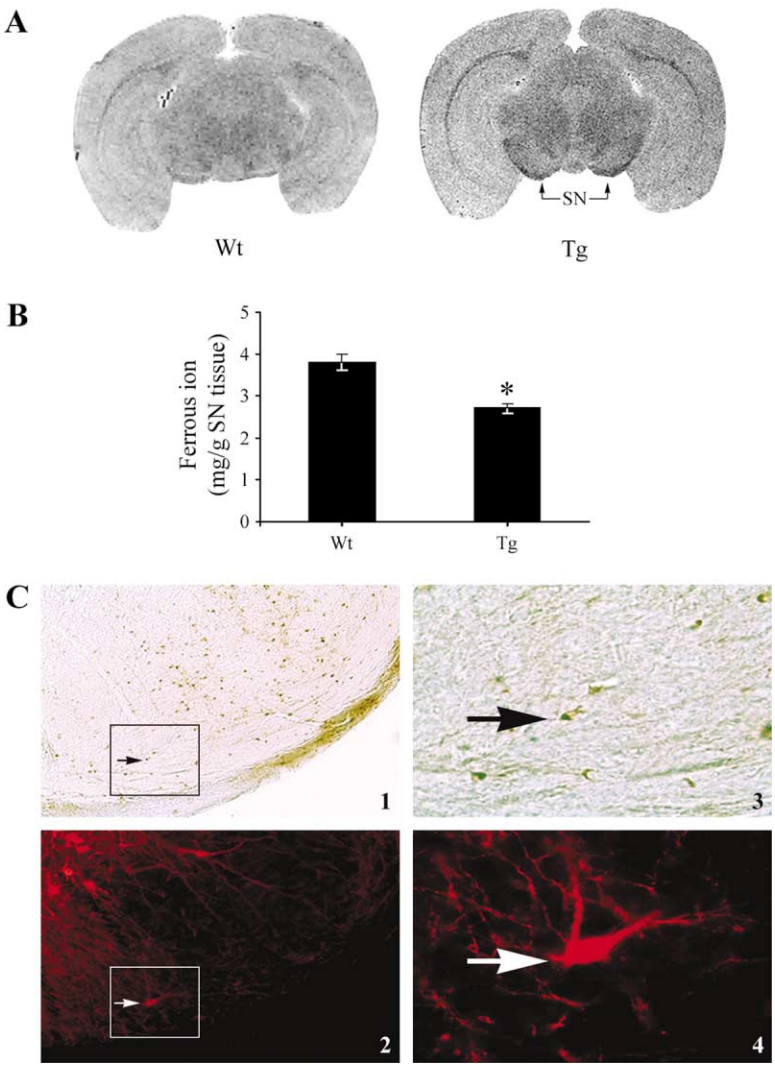

Figure 2. Levels and Localization of Ferric/Ferrous Iron in the SN of pTH-Ferritin Transgenics versus Wild-Type Littermates

(A) Representative MRI analysis of brains from ferritin transgenics versus wild-type animals demonstrating levels of ferritin bound ferric iron; n = 4 for each group. Abbreviations: SN, substantia nigra; wt, wild-type; Tg, transgenic.

(B) Bioavailable SN ferrous iron levels, n = 5 for each group. *$p < 0.01$ versus wild-type.

(C) Localization of SN ferric iron within dopaminergic neurons in the ferritin transgenics as verified by double staining of Perls-positive SN cells with TH antibody. 1, 4× magnification of Perls staining in a representative section of the SN region of a ferritin transgenic mouse; 2, 10× magnification of boxed region in panel 1 highlighting position of a TH^+ dopaminergic SN neuron in this brain area (arrow); 3, Perls staining of the dopaminergic neuron highlighted in panel 2; 4, 40× magnification of the dopaminergic neuron shown in panel 2 demonstrating TH positivity.

decreased by 22% ± 9.8% in the ferritin transgenic SN (Figure 2B; wt = 3.8 ± 0.20 μg/g SN tissue, Tg = 2.7 ± 0.13 μg/g SN tissue, n = 4 animals per parameter, *$p < 0.01$), presumably due to its conversion to ferric during oxidation and storage in ferritin. To assess whether the increased ferric iron colocalized with dopaminergic SN neurons, Perls staining was performed in conjunction with immunocytochemistry using an antibody specific for TH (Figure 2C). Perls staining revealed that, in agreement with previous reports (Benkovic and Connor, 1993; Connor et al., 1994; Cheepsunthorn et al., 1998), ferric iron is predominantly localized within SN cells with the appearance of oligodendrocytes in wild-type animals (data not shown). The numbers of ferric iron-positive cells were increased in the transgenic SN and were found to be localized within cell bodies and neuritic processes of TH-positive SN cells (Figure 2C). Estimation of numbers of Perls-positive SN cells demonstrated a 22.4% ± 4.7% increase in the transgenic animals ($p < 0.01$); these cells displayed the correct size, morphology, and TH-positive expression of dopaminergic neurons.

Systemic administration of the neurotoxin MPTP produces a clinical syndrome strikingly similar to PD (Tetrud and Langston, 1989; Chiueh and Rauhala, 1998). Animals treated with MPTP exhibit several of the major hallmarks of PD, including a substantial decrease in numbers of dopaminergic SN neurons. The damaging effect of MPTP administration also mirrors the disease in that oxidative stress appears to play a major role in ensuing neurodegeneration (Yong et al., 1986; Cassarino et al., 1999), including a decrease in glutathione (GSH) levels, as has been reported to occur early in the course of PD (Perry et al., 1982; Sian et al., 1994; Hung and Lee, 1998; Desole et al., 1993; Lan and Jiang, 1997). Both MPTP-induced increases in reactive oxygen species (ROS) and decreases in GSH levels were both found to be prevented in the ferritin transgenics (Figures 3A and 3B). Acute MPTP administration (4 × 20 mg/kg body weight, every 2 hr) resulted in a 56% ± 4% increase in ROS levels in wild-type SN (n = 6 animals, *$p < 0.01$), while no significant change was detected in transgenic animals (95% ± 4.0%, n = 8 animals, **$p > 0.05$). A 2.4% ± 0.3% decrease in SN GSH was observed at 2 hr and a 10.0% ± 0.05% at 8 hr, respectively, after the final MPTP injection in wild-type mice (n = 6, *$p < 0.01$), while no significant change was observed in the ferritin

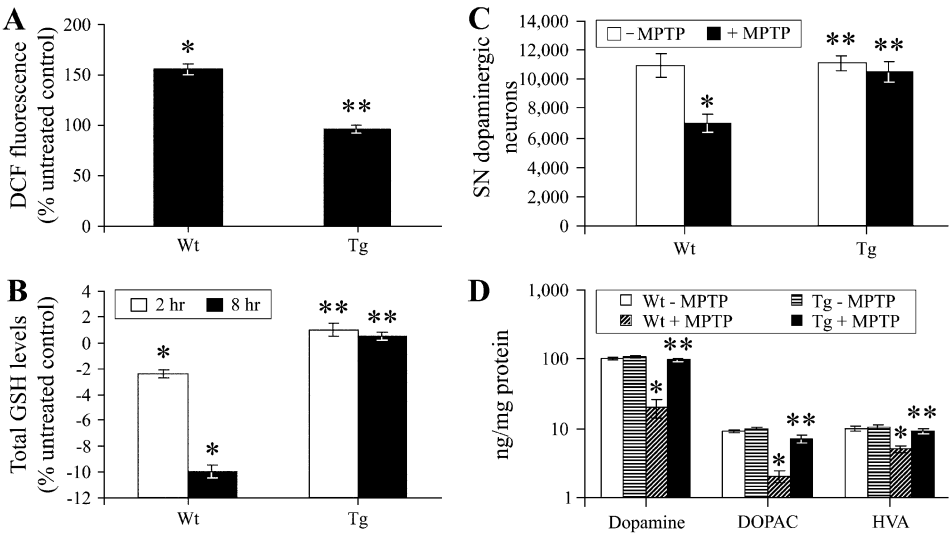

Figure 3. Effects of Acute MPTP Administration on Levels of Oxidative Stress, Dopaminergic SN Neuronal Cell Number and Striatal (ST) Dopamine and Its Metabolites in pTH-Ferritin Transgenics versus Wild-Type Littermates

(A) Percentage change in ROS levels in SN tissue 8 hr following acute MPTP administration, *$p < 0.01$, untreated versus MPTP-treated wt; **$p > 0.05$, MPTP-treated Tg versus wt.

(B) Percentage change in GSH levels in SN tissue 2 hr and 8 hr following acute MPTP administration, *$p < 0.01$, untreated versus MPTP-treated wt at 2 and 8 hr; **$p > 0.05$, MPTP-treated Tg versus wt at 2 and 8 hr.

(C) TH^+ SN cell counts from transgenics versus nontransgenics 7 days following acute MPTP administration, *$p < 0.01$, MPTP-treated versus untreated wt; **$p > 0.05$; untreated and MPTP-treated Tg versus MPTP-treated wt.

(D) ST DA, DOPAC, and HVA content in ferritin transgenic versus wild-type littermates 7 days following acute MPTP, *$p < 0.01$, untreated versus MPTP-treated wt; **$p > 0.05$, MPTP-treated Tg versus wt.

transgenics (+1.0% ± 0.5% at 2 hr and +0.5% ± 0.3% at 8 hr, respectively, n = 8, **$p > 0.05$).

To assess the effects of ferritin expression on acute MPTP-induced dopaminergic SN cell loss, stereological TH^+ cell counts were performed. TH^+ cell numbers in the nontransgenic SN were found to decrease by approximately 30% ± 5.2% following acute MPTP administration (Figure 3C; 10,900 ± 800, saline-treated; 7,000 ± 500, MPTP-treated, n = 5, *$p < 0.01$). In contrast, no decrease was noted following acute MPTP administration in the ferritin transgenics (11,100 ± 600, saline-treated; 10,500 ± 700, MPTP-treated, n = 7, **$p > 0.05$). To confirm the protection against TH^+ SN cell loss in the ferritin transgenic following acute MPTP administration, levels of striatal dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured. Wild-type animals displayed significant depletion of DA, DOPAC, and HVA commensurate with decreased numbers of TH^+ SN neurons (Figure 3D; DA, DOPAC, HVA = 100 ± 3.0, 9.0 ± 0.5, 10.0 ± 0.8 ng/mg protein in saline-treated and 20.0 ± 0.6, 2.0 ± 0.4, 5.0 ± 0.6 ng/mg protein in MPTP-treated, n = 4, *$p < 0.01$). These losses were attenuated in the ferritin transgenics (DA, DOPAC, HVA = 105 ± 3.0, 10.0 ± 0.54, 10.5 ± 0.6 ng/mg protein in saline-treated and 95.0 ± 0.8, 7.0 ± 1.0, 9.0 ± 0.7 ng/mg protein in MPTP-treated, n = 5, **$p > 0.05$ compared to wt). The protective effects of the ferritin transgene could not be explained by decreased conversion of MPTP to MPP^+ following acute administration (transgenic = 143.04 ± 18.02 ng/mg protein MPP^+, wild-type = 109.77 ± 22.03 ng/mg protein MPP^+, n = 4, $p > 0.05$).

To test whether pharmacological iron chelation using a reported well-tolerated bioavailable reagent would have similar protective effects afforded by transgenic expression of an iron-chelating molecule, we examined the effects of the metal-chelating agent CQ on susceptibility to acute MPTP administration. Total SN iron levels were found to be reduced approximately 30% in the CQ-fed versus saline-fed animals (Figures 4A and 4B), well within the reported nontoxic range (Yassin et al., 2000). As with the ferritin transgenics, MPTP-mediated increases in SN oxidative stress and decreases in SN GSH were found to be significantly attenuated following CQ pretreatment (Figures 5A–5C). A 20% ± 3% increase in levels of 4-hydroxynonenol (4-HNE)-protein conjugates, a 15% ± 2% increase in protein carbonyl levels, and a 18% ± 3% decrease in GSH were observed in control SN 24 hr following MPTP injection (n = 5 animals per assay, *$p < 0.01$). However, no significant changes in any of these indices of oxidative stress were found in the CQ-pretreated animals (n = 6 animals, **$p > 0.05$ versus wt). To assess the affects of CQ pretreatment on MPTP-induced dopaminergic SN cell loss, measurements of both striatal dopamine levels and stereological TH^+ cell numbers were performed following acute treatment with MPTP (Figures 5D and 5E). While reductions in striatal dopamine levels in untreated controls were approximately 80% (121.8 ± 25.6 versus 21.4 ± 4.5 mg/g striatal tissue, *$p < 0.01$ versus untreated control), this loss was only 41% in animals pretreated with CQ (147.2 ± 10.6 versus 60.5 ± 8.8 mg/g striatal tissue, **$p < 0.01$ versus Veh/MPTP). No significant difference in striatal dopamine levels was observed between saline

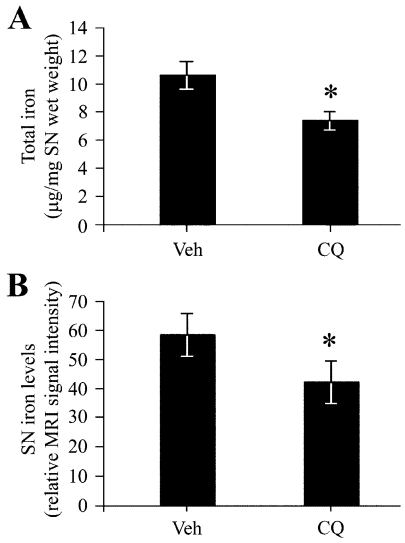

Figure 4. Effects of CQ Pretreatment on Total SN Iron Content

(A) Total SN iron content (μg/mg tissue wet weight) measured via mass spectrometry of vehicle (Veh) versus CQ-fed animals, *$p < 0.01$.

(B) SN iron levels measured via MRI in Veh versus CQ-fed animals, *$p < 0.01$.

versus CQ-fed animals in the absence of MPTP treatment, suggesting that CQ pretreatment alone has no effect (Figure 5D). While SN TH^+ cell numbers in untreated animals were decreased by 46% following acute MPTP administration (15,346 ± 1,471, saline-treated; 8,336 ± 1,093, MPTP-treated, n = 5, *$p < 0.01$), only a 25% decrease was noted in the CQ-fed animals (11,462 ± 915, n = 5, **$p < 0.01$ versus MPTP-treated controls). As with transgenic ferritin expression, the protective effects of CQ pretreatment were not explainable by decreased conversion of MPTP to MPP^+ (CQ-fed = 134.04 ± 8.70 ng/mg protein MPP^+, saline-fed = 138.15 ± 6.63 ng/mg protein MPP^+, n = 5, $p > 0.05$).

In order to examine the effects of CQ pretreatment in a different MPTP toxicity paradigm, we also assessed toxin-induced dopaminergic SN cell loss following a chronic regime of MPTP treatment (5 × 20 mg/kg, 24 hr apart; Figure 5F; Vila et al., 2001). Following chronic treatment with MPTP, SN TH^+ cell numbers in untreated animals were decreased by 31% (10,143 ± 701, saline-treated; 7,010 ± 238, MPTP-treated, n = 6, *$p > 0.01$), but only by 17% in the CQ-pretreated animals (8449 ± 503, n = 5, **$p > 0.01$ versus MPTP-treated controls). No difference in TH^+ cell numbers was observed between vehicle- or CQ-fed animals prior to MPTP treatment (10,143 ± 701, saline-treated; 10,291 ± 660, CQ-fed, n = 6, $p > 0.05$), suggesting the CQ alone had no effect on this parameter.

Finally, we assessed whether either genetic or pharmacological iron chelation provided protection against behavioral motor deficits associated with MPTP treatment (Figure 6). Neither transgenic ferritin expression nor CQ treatment alone was found to result in decreased motor performance as assessed by rotorod (data not shown). However, performance following acute MPTP treatment was found to be decreased, and this MPTP-induced loss in motor activity was significantly attenuated by either transgenic ferritin expression (Figure 6A, 30.86% ± 2.19% decrease in wt; 19.71% ± 1.61% decrease in transgenic versus untreated control, n = 4, $p < 0.01$) or CQ pretreatment (Figure 6B, 36.43% ± 11.7% decrease in vehicle-fed/acute MPTP; 13.55% ± 2.6% decrease in CQ-fed/acute MPTP versus untreated control, n = 4, $p < 0.01$) by 24 hr following the last MPTP dose. In addition, CQ was also found to be protective 96 hr following the chronic MPTP treatment regime (Figure 6C, 24.95% ± 5.97% decrease, vehicle-fed/chronic MPTP; 10.27% ± 3.10% decrease, CQ-fed/chronic MPTP, n = 4, $p < 0.01$).

Discussion

MPTP-induced neurotoxicity has proven in the past to be an invaluable tool for testing drug therapy in experimental parkinsonism as a model for PD (Sedelis et al., 2001; Beal, 2001). MPTP reproduces virtually all symptoms of the disease, including inhibition of mitochondrial complex I activity, decreased GSH and increased oxidative stress levels in the SN, relatively selective neurodegeneration of the dopaminergic nigrostriatal system, striatal dopamine depletion, and motor control deficits, all of which can be reversed by dopamine substitution therapy, the classic PD drug treatment. Its effects were indeed originally discovered in humans as a consequence of inadvertent injection that resulted in an acute parkinsonism. MPTP does not perfectly model the disorder, particularly in terms of the acute nature of onset using this drug and the absence of inclusion bodies in rodents (Betarbet et al., 2002). However, an animal model does not need to recapitulate every feature of the disease in order to be useful in evaluating the potential therapeutic potential of a particular agent.

Elevated levels of brain iron similar to those reported in PD have been shown to result in significantly higher levels of both oxidative stress and dopaminergic cell loss following MPTP administration in vivo, suggesting that elevated iron can contribute to the toxicity of the compound via an oxidative mechanism (Lan and Jiang, 1997). Redox-available iron has been detected in midbrain Lewy bodies in postmortem parkinsonian brains (Castellani et al., 2000), and the oxidation state of iron has been reported to change from ferrous to ferric within SN TH^+ neurons during progression of the disease (Yoshida et al., 2001). Our data demonstrate that chelation of iron via ferritin or CQ in a state that prevents it from participating in oxidative events significantly attenuates toxicity of the parkinsonian-inducing agent MPTP. These results definitively demonstrate the involvement of iron in MPTP-mediated neurodegeneration. These results, in addition, challenge the view that iron accumulation is a late-stage, irreversible event in MPTP toxicity and PD and suggests that iron chelation may be an effective preventative therapy for progressive degeneration associated with the disease.

Transgenic expression of the heavy ferritin subunit was found to prevent dopaminergic SN cell loss associated with MPTP toxicity. The heavy subunit contains catalytic ferroxidase activity, which allows it to detoxify reactive ferrous iron and is the predominant form found

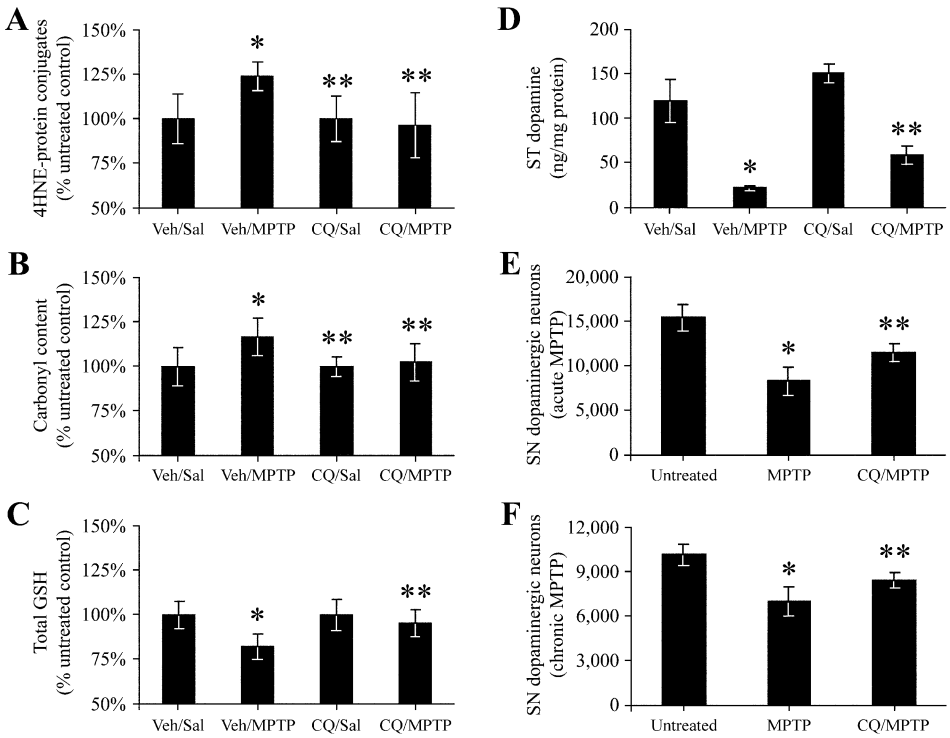

Figure 5. Protective Effects of CQ Pretreatment against MPTP-Mediated Oxidative Stress and Dopaminergic Cell Loss under Either Acute or Chronic MPTP Dosing Regimes

(A and B) Levels of 4-HNE-protein conjugates (A) and protein carbonyl content (B) as assessed by slot blot analysis of SN tissue 24 hr following acute MPTP or saline (Sal) administration in the absence or presence of CQ pretreatment, *$p < 0.01$, Veh/MPTP versus Veh/Sal; **$p > 0.05$, CQ/Sal and CQ/MPTP versus Veh/Sal.

(C) Total SN GSH levels 24 hr following acute MPTP or saline administration ± CQ pretreatment, *$p < 0.01$, Veh/MPTP versus Vehl/Sal; **$p > 0.05$, CQ/Sal and CQ/MPTP versus Veh/Sal.

(D) ST DA, DOPAC, and HVA content in saline versus CQ-fed mice 7 days following acute MPTP, *$p < 0.01$, Veh/MPTP versus Veh/Sal; **$p > 0.05$, CQ/Sal and CQ/MPTP versus Veh/Sal.

(E) TH^+ SN cell counts from saline versus CQ fed 7 days following acute MPTP administration, *$p < 0.01$, MPTP versus untreated wt; **$p < 0.01$ CQ/MPTP versus MPTP-treated wt.

(F) TH^+ SN cell counts from saline versus CQ fed 7 days following chronic MPTP administration, *$p < 0.01$, MPTP versus untreated wt; **$p < 0.01$ CQ/MPTP versus MPTP-treated wt.

in brain neurons (Harrison and Arosio, 1996; Connor et al., 1995; Han et al., 2000). It is rapidly upregulated in response to oxidative stress, and overexpression in vitro results in increased resistance to H_2O_2-mediated insult (Orino et al., 2001; Cozzi et al., 2000), suggesting that it may play an important role as a biological antioxidant by sequestering iron that is normally free to participate in oxidative events. Several recent reports have suggested that diseases of iron overload may have their basis in misregulation of iron storage by ferritin. A dominantly inherited iron overload disease in a Japanese pedigree, for example, was recently attributed to a point mutation in the iron response element (IRE) in the H ferritin gene promoter, which leads to increased binding affinity of the iron regulatory protein (IRP), decreasing H ferritin synthesis and resulting in increased cytoplasmic iron levels (Kato et al., 2001). A mutation in the gene encoding the ferritin light subunit has also recently been reported to cause a dominantly inherited adult-onset basal ganglia disease similar to PD due to a change in its conformation that affects its ability to function as a stabilizer of the ferritin-iron core, resulting in increased iron release, suggesting that iron excess can have serious neurological consequences (Curtis et al., 2001; Connor et al., 2001; Thompson et al., 2001).

Like ferritin, CQ also has metal binding properties, although it appears to act via chelation of both ferrous and ferric iron rather than conversion of available ferrous to bound unreactive ferric iron. It is lipophilic and therefore freely crosses the blood-brain barrier. CQ has recently been shown to inhibit plaque formation and accompanying behavioral declines in an AD transgenic mouse model (Cherny et al., 2001; see commentary by Melov, 2002). We found that CQ given at similar concentrations and time periods found to be effective in the AD mouse studies results in significant attenuation of the neurotoxic effects of MPTP. CQ has been shown to reduce bioavailable brain iron in normal control mice with no apparent adverse health or behavioral effects (current study; Yassin et al., 2000). CQ treatment also does not result in depletion in systemic iron levels that could cause adverse physiological effects (Yassin et al., 2000). This is in contrast to other currently used iron chelators administered to patients with iron overload conditions that have been shown to have toxic side effects at the higher dosages needed to overcome the

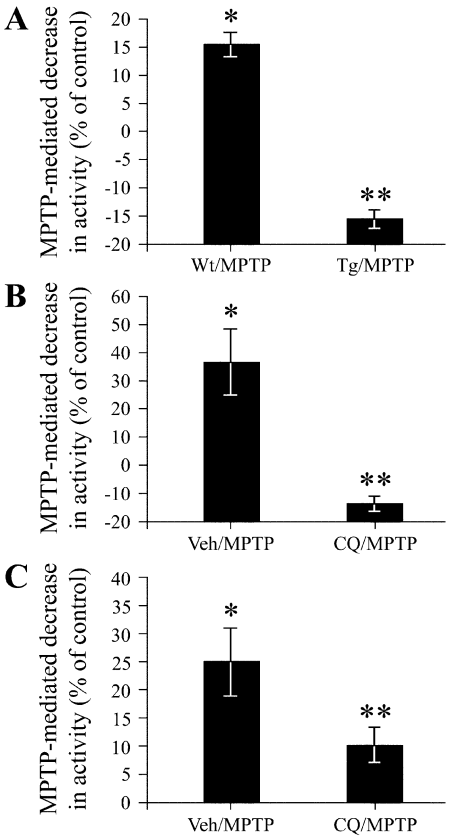

Figure 6. Protective Effects of Transgenic Ferritin Expression and Pharmacological CQ Treatment against Motor Deficits Elicited by Either Acute or Chronic MPTP Treatment

(A) Decreases in motor activity as assessed by rotorod in wt versus ferritin transgenic mice 24 hr following acute MPTP treatment. *$p < 0.01$, MPTP versus untreated wt; **$p > 0.01$, MPTP-treated Tg versus wt.

(B) Decreases in motor activity in Veh versus CQ-fed mice 24 hr following acute MPTP treatment, *$p < 0.01$, MPTP versus saline-treated wt; **$p < 0.01$, MPTP-treated CQ versus wt.

(C) Decreases in motor activity in saline versus CQ-fed mice 96 hr following chronic MPTP mice, *$p < 0.01$, MPTP versus saline-treated wt; **$p < 0.01$, MPTP-treated CQ versus wt.

compounds' low lipid solubility that impair their ability to cross the blood-brain barrier (Porter and Huehns, 1989; Marciani et al., 1991). It should be noted that it cannot be excluded that the protective effects of CQ may be due in part to previously reported CQ-mediated depletions in brain copper levels, as copper can act to facilitate Fe^{2+} toxicity (Cherny et al., 2001); however, copper levels, unlike iron, have not been shown to be elevated in PD. No adverse effects were attributable to CQ administration in our study at a similar dosage (g/kg body weight) to that used in the previous transgenic APP study (Huang et al., 1999; Bush, 2000; Cherny et al., 2001). Results from a recent small two-year double-blind phase II trial assessing the efficacy of CQ as a possible treatment for AD suggest that oral administration of the agent accompanied by B12 supplementation is well tolerated in humans over the period of the trial at similar dosages to those used in both the previous APP transgenic and in our current MPTP study and were, in addition, efficacious in delaying the course of the disease. Acute administration of the compound at significantly higher dosages had previously been associated with a subacute myelo-optic neuropathy (SMON), which was primarily confined to Japan (Masters, 2002; Tsubaki et al., 1971). SMON appears to resemble a subacute accelerated form of B12 deficiency, and CQ has been shown to lower levels of brain and serum vitamin B12 (Yassin et al., 2000). However, a causal relationship between SMON and CQ intake has not been established; for example, CQ was used extensively in Japan for 20 years before the first cases of SMON were reported (Meade, 1975; Nakae et al., 1973; Baumgartner et al., 1979; Clifford Rose and Gawel, 1984). In light of this possibility, the Alzheimer phase II clinical trials (Masters, 2002) were performed with B12 coadministration, and dosages of the drug were kept to a fraction of those dosages used previously that demonstrated neurotoxic effects (e.g., Yoshimura et al., 1992; Tateishi, 2000). The possible toxic effects of chronic CQ administration at these dosages beyond the period of the trial, however, have yet to be assessed.

Increases in reactive brain iron are not specific to PD but are also seen in such diverse neurodegenerative disorders as multiple system atrophy, Huntington's disease, Alzheimer's disease, progressive supranuclear palsy, aceruloplasminemia, and Hallervorden-Spatz (Dexter et al., 1991; Connor et al., 1992; Smith et al., 1997; Gitlin, 1998; Janetzky et al., 1997). Misregulation of iron metabolism resulting in iron accumulation, therefore, may be a general phenomenon contributing to the progression of several neurodegenerative conditions. Brain iron accumulation along with increased ROS production is part of the normal aging process, particularly in the basal ganglia, and this in itself may contribute to the increased age-related susceptibility in a subset of these diseases (Bartzokis et al., 1997; Zecca et al., 2001; Christen, 2000; Thompson et al., 2001). Brain H ferritin levels are known to increase with age, likely as a protective response to increasing iron levels; however, this increase does not appear to occur in either PD or AD brains (Connor et al., 1995; Zecca et al., 2001; Thompson et al., 2001). Although it has been previously speculated that increasing the iron loading of ferritin may increase the risk of free radical damage (Double et al., 1998; Griffiths et al., 1999), our data in contrast suggest that increased ferritin is in fact neuroprotective. Indeed, ferritin has recently been reported to normally be absent in dopaminergic SN neurons, and this may, in combination with other factors such as elevated iron levels, contribute to their susceptibility to oxidative stress (Moos et al., 2000). It is of interest in this regard that SN levels of ferritin in humans have been reported to actually be decreased in PD patients compared to age-matched controls, although this is somewhat controversial (Reiderer et al., 1989; Jellinger et al., 1990; Dexter et al., 1991; Jenner et al., 1992; Mann et al., 1994). Extensive elimination of iron from the brain is not desirable, as it is an abundant brain metal essential for several normal metabolic functions including the synthesis and release of dopamine in the SN (Beard et al., 1993; Glinka et al., 1996; Connor et al., 2001; Thompson et al., 2001). In addition, its deficiency during development has been associated with neurobehavioral dysfunction (Connor et

al., 1995). However, our data suggest that chelators such as ferritin or CQ, which can remove excess iron without apparent interference with its normal functions in the adult nervous system system, may postpone or prevent the progression of such neurological diseases as PD (Gassen and Youdim, 1997).

Experimental Procedures

Mouse Studies

Mice were housed according to standard animal care protocols, fed ad libitum, kept on a 12 hr light/dark cycle, and maintained in a pathogen-free environment in the Buck Institute Vivarium. Animals used for studies were young adults (2–6 months of age). Ferritin transgenic mice were generated via injection of an 8.3 kb Hind III DNA fragment containing 4.8 kb of 5′ upstream sequences from the rat TH gene (Banerjee et al., 1992), 2.6 kb of human genomic ferritin DNA encompassing the 4 coding region exons (Hentze et al., 1986), and 3′ SV40 splice and polyadenylation sequences into fertilized B6D2 mouse embryos to create pTH-ferritin transgenic founder animals. For CQ studies, C57Bl mice were obtained from Jackson Labs and randomized for therapy trials. CQ was suspended in saline and delivered via oral gavage at a daily dosage of 30 mg/kg as previously described for a period of 8 weeks (Cherny et al., 2001); controls received vehicle alone. For acute MPTP studies, mice were treated with either 4 × 20 mg/kg, 2 hr apart or 2 × 20 mg/kg, 12 hr apart of the toxin, and SN or ST samples taken at the times specified for various analyses. For chronic MPTP studies, mice were treated with 5 × 20 mg/kg MPTP, 24 hr apart, and samples taken at specified times for analyses.

Southern Blot Analysis

Genomic DNA from ferritin founders was digested with Xba I, separated on a 1% agarose gel, transferred to Hybond (Amersham), and hybridized with a ^{32}P-labeled 2.6 kb Xba I-EcoRI ferritin genomic fragment. Founder animals positive for the transgene were bred out to create lines for analysis; nontransgenic littermates were used as negative controls.

Western/Slot Blot Analyses

SN were dissected and homogenized in 10 mM HEPES-KOH (pH 7.2), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 20 μg/ml leupeptin (Nicholson et al., 1995). Fifteen micrograms of total protein from each sample was either run on a 15% SDS-PAGE gel (BioRad) and transferred to nitrocellulose membrane or directly slotted onto membrane. Membranes were incubated with 10–50 μg/ml primary antibody (heavy chain human ferritin monoclonal, Ramco Laboratories; anti-HNE Michaels adduct rabbit polyclonal, Calbiochem; anti-DNP rabbit polyclonal, Intergen), followed by horseradish peroxidase-conjugated secondary antibody (Vector Laboratories). Autoradiography was performed with enhanced chemiluminescence (Amersham Pharmacia). For 4HNE-protein conjugates and protein carbonyls, relative optical band density were quantified using a ChemiImager 5500 (Alpha Innotech Corporation). Reported values are the results of three independent experiments.

Immunocytochemistry

Animals were cardiac-perfused with phosphate-buffered saline (PBS) followed by 10% formalin, and brains were removed and postfixed for 15 hr followed by 30% sucrose and sectioning at 40 μm on the coronal plane. ICC was performed as previously described (Andersen et al., 1994). Specific primary antibodies were applied and visualized with fluorescence (Streptavidin-Cy3 for red fluorescence and Streptavidin-Cy2 for green fluorescence, Jackson Immunochemicals).

SN Iron Levels by Magnetic Resonance Imaging (MRI)

MRI studies were performed using a Bruker AMX500 11.7 tesla MRI system as previously described (Gilissen et al., 1998). Brains were fixed as described above and MRI performed in the coronal plane. Comparisons of SN hypointensity (dark area) on T2-weighted MR samples encompassing SNc, SNr, and red nucleus were performed (IPLab Spectrum, Scientific Image Processing from Scanalytics, Inc.; Morgan et al., 1999). Intensity was normalized using cortical white matter as control.

Spectrophotometric Analysis of Bioavailable Ferrous Iron

Levels of ferrous iron available to bind ferrozine were determined in dissected SN tissue spectrophotometrically at 578 nm as previously described (Agrawal et al., 2001).

Ferric Iron Histochemistry (Perls)

Coronal sections from brains of adult animals were subjected to formalin fixation and Perls staining using potassium ferrocyanide as previously described (Hill and Switzer, 1984). The percentage area covered by ferric-ferrocyanine product was assessed by Camera Luminace Drawing.

ROS by DCF Fluorescence

Animals were i.p. injected with either 30 mg/kg body weight MPTP or saline. Eight hours after injection, synaptosomal fractions were prepared from the SN and used for DCF analysis (Ali et al., 1992). Fluorescence was monitored on a Turner spectrofluorometer with an excitation wavelength of 448 mm and an emission wavelength of 525 nm. Protein was normalized by the Bradford method.

GSH Levels

Following MPTP or saline injection, GSH levels were measured in the SN by the method of Griffith (1980).

Histology and Neuron Counts

Neuronal counts were performed on TH^+-positive SN neurons using the unbiased dissector method (West, 1993). Fixed coronal brain sections (40 μm) were immunostained with TH antibody (1:500 dilution, Chemicon) and coverslipped in aqueous medium, and TH^+ cells counted from a total of 15–20 sections in each field per brain (i.e., every second section) at a magnification of 100× using the optical fractionator approach.

Striatal Dopamine/DOPAC and MPP^+ Levels

Animals were injected with either 15 mg/kg body weight MPTP or saline every 2 hr for 4 doses. Dopamine, DOPAC, and HVA or MPP^+ from dissected striata were analyzed by HPLC using a 5 μm C-18 reverse phase column and precolumn (Brownlee Labs) followed by electrochemical detection with a glassy carbon electrode (Klivenyi et al., 2000).

SN Iron Levels by Mass Spectrometry

SN was dissected and snap frozen in liquid nitrogen and the wet weight was determined; then, it was lyophilized and the dry weight/tissue was measured. Preweighed lypholized samples were next taken up in 0.1 ml of concentrated nitric acid (Aristar, BDH) and allowed to digest overnight. The samples were then heated to 80°C for 15 min and cooled, and 0.1 ml 30% hydrogen peroxide added. Samples were heated to 70°C for 15 min, cooled, and diluted 1/40 into 1% $HN0_3$ for analysis by inductively coupled plasma mass spectrometry (ICP-MS) using an Ultramass 700 (Varian) in peak-hopping mode with 0.100 AMU spacing, 1 point per peak, 50 scans per replicate, 3 replicates per sample. Preparation blanks processed in a similar manner were used as controls. Plasma flow was 15 l/min with auxiliary flow of 1.5 l/min, RF power was 1.2 kW, and sample was introduced at a flow rate of 0.88 l/min.

Rotorod Performance

Motor activity was measured via rotorod performance utilizing an Accelerated Rota-Rod for mice 7650 (UGO Basil, Italy) with a rod diameter of 3 cm according to the manufacturer's instructions. The mice were allowed to acclimatize for 20 s at the lowest speed, i.e., 4 rpm, and then the rotor was allowed to accelerate to 40 rpm during a period of 5 min. Fall of the animal from the rod was taken as the end point of run. Each animal was tested three times on each test day with an hour of rest between consecutive runs. Animals were tested 1, 2, 3, 4, and 7 days post final MPTP treatment.

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