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Chronic expression of H-ferritin in dopaminergic midbrain neurons results in an age-related expansion of the labile iron pool and subsequent neurodegeneration: implications for Parkinson's disease

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Abstract

While ferritin elevation within dopaminergic (DA) neurons of the substantia nigra (SN) is protective against neurodegeneration elicited by two toxin models of Parkinson's disease (PD), MPTP and paraquat, in young animals, its prolonged elevation results in a selective age-related neurodegeneration. A similar age-related neurodegeneration has been reported in iron regulatory protein 2-deficient (IRP2 −/−) mice coinciding with increased ferritin levels within degenerating neurons. This has been speculated to be due to subsequent reductions in the labile iron pool (LIP) needed for the synthesis of iron-sulfur-containing enzymes. In order to assess whether LIP reduction is responsible for age related-neurodegeneration in our ferritin transgenics, we examined LIP levels in ferritin-expressing transgenics with increasing age. While LIP levels were reduced within DA SN nerve terminals isolated from young ferritin transgenics compared to wildtype littermate controls, they were found to be increased in older transgenic animals at the age at which selective neurodegeneration is first noted. Furthermore, administration of the bioavailable iron chelator, clioquinol (CQ), to older mice was found to protect against both increased LIP and subsequent dopaminergic neurodegeneration. This suggests that age-related neurodegeneration in these mice is likely due to increased iron availability rather than its reduction. This may have important implications for PD and other related neurodegenerative conditions in which iron and ferritin have been implicated.

Keywords

iron; ferritin; Parkinson's disease; labile iron pool; synaptosomes; neurodegeneration

1. Introduction

Iron is essential for life due to its requirement in several basic cellular functions. Conversely, iron is potentially toxic since it can participate in redox reactions that lead to generation of

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reactive oxygen species (ROS). Cells therefore strive to maintain a transient pool of readily available iron, known as the labile iron pool (LIP), within a narrow range, providing enough iron for cellular function while limiting any excess that can participate in toxic reactions (Eisenstein, 2000; Schneider and Leibold, 2000). Excess intracellular iron not utilized for metabolic purposes is normally stored in the cytosol within the iron–binding protein ferritin. Fully-assembled ferritin consists of 24 subunits of H- and L-subunits that form nanocages capable of storing up to 4500 molecules of iron. The H-subunit harbors ferroxidase activity used to catalyse oxidation of ferrous to ferric iron for entry into the ferritin cavity. The L-subunit catalyzes ferrihydrite formation promoting iron storage. Thus, ferritin stores iron in a nontoxic, bioavailable form (Arosio and Levi, 2002). Iron may be mobilized from ferritin by oxidative stress, following localized protein unfolding, or ferritin degradation within lysosomes. Ferritin itself can also undergo degradation by the proteasome following either iron depletion or ferritin oxidation (Galaris and Pantopoulos, 2008).

In Parkinson's disease (PD), iron levels in the SN, the brain region that undergoes preferential neurodegeneration, are reported to be elevated (Riederer et al., 1992, Youdim et al., 1993, Gerlach et al., 1997, Bush, 2000, Schenck et al., 2004). This appears to occur within the dopaminergic neurons that are preferentially affected in this condition (Carmona et al., 2008); (Oakley et al., 2007); (Ortega et al., 2007). Iron can interact with hydrogen peroxide, a major by-product of dopamine oxidation, leading to production of highly reactive hydroxyl radicals via the Fenton reaction (Comporti, 2002). This in turn may contribute to subsequent dopaminergic neurodegeneration.

Previously, we created transgenic mouse lines in which ferritin levels were selectively elevated within DA SN neurons resulting in an increase in ferritin-bound iron within these cells (Kaur et al., 2003). In young animals, this was found to afford protection against neurodegeneration associated with two widely used animal models of the disease, systemic 1methyl-4-phenyl 2,3,6 tetrahydropyridine (MPTP) (Kaur et al., 2003) and paraquat administration (McCormack et al., 2005). This suggested a direct involvement of iron-catalyzed oxidative stress in subsequent neurodegenerative events associated with these agents. However, prolonged ferritin elevation within the dopaminergic SN neurons was found to lead to a selective age-related neurodegeneration of these cells and to exacerbate MPTP neurotoxicity in older animals (Kaur et al., 2007). Age-related neurodegeneration has also been described to occur in iron regulatory protein 2-deficient (IRP2 −/−) mice; this neurodegeneration has been suggested to be attributable to increased ferritin levels within degenerating neuronal populations in these animals (LaVaute et al., 2001). IRP1+/−, IRP2−/− mice were found to have an even more severe age-related neurodegeneration accompanied by still higher levels of ferritin accumulation (Smith et al., 2004). It has been speculated that iron maintained primarily in a ferritin-bound state may result in a LIP deficiency which could impact on cellular function by reducing the amount of easily available iron needed for the synthesis of important iron-sulfur containing enzymes including those of the mitochondria (Rouault, 2006). In this study, we assessed whether age-related dopaminergic SN neurodegeneration in our transgenic ferritin mice was due to subsequent deficiencies in the LIP in the affected cells as a consequence of prolonged ferritin elevation.

2. Results

In order to assess whether iron availability in our ferritin transgenic mice results in subsequent age-related neurodegeneration, we examined the LIP within DA SN neurons from ferritin-expressing transgenics versus wildtype mice with increasing age in isolated dopaminergic striatal synaptosomes utilizing a novel magnetic bead isolation protocol recently reported by our laboratory (Chinta et al., 2007; Mallajosyula et al., 2008). This method allows selective isolation of terminals emanating from dopaminergic SN neurons thus enabling a wide range

of physiological or biochemical analyses to be carried out specifically in these preparations. Results from these experiments demonstrate that, as expected based on previously published results (Kaur et al., 2003), LIP levels were reduced in the young (2–3 month) ferritin transgenics versus age-matched controls (Fig 1A). However, LIP levels within DA striatal synaptosomes were in contrast found to be increased rather than reduced in older (12 month) transgenic animals, corresponding to the age at which selective neurodegeneration is first noted in these animals (Fig 1B, (Kaur et al., 2007). In order to assess whether accompanying neurodegeneration is the result of prolonged effects of diminished LIP in young mice versus increased LIP in the older animals, we administered the bioavailable iron chelator CQ (30 mg/kg body weight, 3 weeks) to older ferritin transgenics to compare the impact on iron levels and neurodegeneration in these animals versus saline-fed transgenic controls. We previously demonstrated that CQ feeding significantly reduces SN iron levels when administered in this manner to young (2 month old) ferritin transgenics while not significantly affecting wildtype controls (Kaur et al., 2003). CQ administration in older transgenics not only significantly attenuated the age-related increase in LIP (fig 1B) but also attenuated age-related losses in tyrosine hydroxylase-positive (TH+) SN cell numbers (fig 2) in these mice compared to saline-fed ferritin transgenics. In corroboration with CQ-mediated sparing of dopaminergic SN cell numbers, older ferritin transgenic fed CQ also displayed less striatal DA neurite degeneration as assessed by silver staining compared to saline-fed controls (Fig 3). Taken together, these data suggest that it is the increase in LIP levels in the older transgenics which is responsible for subsequent neurodegeneration and, furthermore, iron chelation is an effective method for delaying this age-related neuropathology.

3. Discussion

In this present study, we demonstrate that while DA LIP levels are reduced in younger ferritin transgenics (verifying previously published results, Kaur et al., 2003), they become elevated with age. Iron within the young ferritin transgenic brains may be kept in a more non-reactive form due to a relatively high ferritin-to-iron ratio compared to age-matched controls however, with age, increasing brain iron levels may lead to increases in ferritin-bound iron levels (Kaur et al., 2007). It has been demonstrated that ferric iron stored within the ferritin core can be easily reduced by cytotoxic byproducts of dopamine oxidation within dopaminergic neurons including superoxide and 6-hydroxydopamine (6-OHDA), allowing its release from ferritin as ferrous iron (Thomas and Aust, 1986; Monteiro and Winterbourn, 1989; Kienzl et al., 1995; Linert et al., 1996; Double et al., 1998; Comporti, 2002). In the case of the older ferritin transgenics, heavily iron-loaded ferritin could create a pool of easily releasable iron. Ferritin subunits are normally synthesized within the neuronal cell body while mature, assembled heteropolymers are found primarily within axons. Iron-loaded ferritin localized within striatal axons of the older ferritin transgenics could be degraded within lysosomes, releasing iron and elevating the LIP (Rouault, 2001). The normal increase in age-related autophagy (Ward, 2002) in the presence of increased ferritin-bound iron levels in the older transgenics may also contribute to the increased LIP we observed in the older ferritin transgenic mice; lysosomal markers have recently been demonstrated to be present within nerve terminals (Vance et al., 2006). Another possible cause of increased DA LIP is an elevation in levels of H ferritin-rich heteropolymers (or even H-ferritin homopolymers) that are less efficient at long-term chelation of iron. Neuroferritinopathy, a dominantly inherited movement disorder, is characterized by deposition of iron and ferritin in the brain caused by mutations in the ferritin L-chain resulting in the formation of unstable ferritin shells resulting in an increase in intracellular iron availability and oxidative damage (Vidal et al., 2004; Levi et al., 2005; Mancuso et al., 2005). It is important to note in this context that levels of H but not L ferritin have been reported to be elevated in the PD SN (Koziorowski et al., 2007). Furthermore, higher H/L ratios have been suggested to correlate with increased iron turnover and higher risk for oxidative stress (Friedman et al., 2006). Increased H ferritin levels coupled with age-related brain increases in

iron, oxidative stress and autophagy may all contribute to DA LIP increase and subsequent neurodegeneration of this brain region. Studies are ongoing to pinpoint the exact mechanism (s) involved in this process.

Accompanying neurodegeneration noted in the older ferritin transgenics could be a consequence of prolonged effects of LIP deficiency in the young animals or it may be due to the age-related increase in the LIP in the affected neurons. Administration of the iron chelator CQ for a 3 week period in older ferritin transgenics results in a significant decrease in LIP elevation in nerve terminals emanating from dopaminergic SN neurons as well as an attenuation of corresponding age-dependent cell loss and axonal neurodegeneration in these animals. This suggests that neurodegeneration is due to increased rather than decreased LIP levels. This may have important implications for PD and other related neurodegenerative conditions in which iron and ferritin have been implicated and suggests that iron chelation may be an affective therapy for these disorders.

4. Experimental procedures

4.1 Animals

Construction and characterization of the ferritin transgenics used in this study have been previously described (Kaur et al., 2003). Animals were bred in-house and housed according to standard animal care protocols, fed Harlan Teklad 7912 irradiated chow ad libitum, kept on a 12 hr light/dark cycle, and maintained in a pathogen-free environment in the Buck Institute Vivarium. All animal experiments were approved by local IACUC review and conducted according to current NIH policies on the use of animals in research. Young animals used in the study were 2–3 months old and older animals were 12–14 months old. For iron chelation studies, CQ was suspended in saline and delivered via oral gavage at a daily dosage of 30 mg/kg as previously described (Kaur et al., 2003) for a period of 3 weeks; controls received vehicle alone.

4.2 Labile iron pool measurements

The LIP was measured in striatal DA nerve terminals (synaptosomes) emanating from DA SN neurons isolated according to our previously published protocol (Chinta et al., 2007, Mallajosyula et al., 2008). Isolated DA ST synaptosomes were first resuspended in Dulbecco's phosphate buffered saline (DPBS). The fluorescent probe calcein, (CAL) which is quenched in the presence of iron, was used to measure the LIP (Epsztejn et al., 1997). Synaptosomes were loaded with acetomethoxy, a non-fluorescent derivative precursor of CAL(CAL-AM). CAL-AM is hydrolysed inside the cells forming fluorescent calcein which is quenched upon binding to iron and formation of CAL-FE. Labile iron is measured based on dequenching of the fluorescence signal by addition of the cell permeable lipophilic iron chelator salicylaldehydeisonicotinoyl hydrazone (SIH, kindly provided by Dr Ponka P, Canada) which binds to iron releasing fluorescent CAL. Calcein AM was added to a concentration of 0.25 μM to the synaptosomal suspension and incubated for 10 min. Synaptosomes were then washed three times with excess of DPBS to get rid of any extra-synaptosomal calcein. Synaptosomal protein was estimated and 10 μg in a volume of 100 μl per well was loaded in triplicate onto 96 well plates for analysis on a fluorescent plate reader. 100 μl of a 100 μM solution of SIH was added to each well and fluorescence measured in the kinetic mode at intervals of 2.5 minutes. Increased fluorescence with time is reflective of the synaptosomal LIP. Fluorescent measurement at different time points in each treatment condition was averaged for triplicate wells and graphed as a change in relative fluorescent units compared to wildtype synaptosomal fractions.

4.3 Stereological SN TH$^+$ cell counts

Immunocytochemistry was performed using antibody against tyrosine hydroxylase (TH, Chemicon #AB152, 1:1000) followed by biotinlabeled secondary antibody and development using DAB (Vector Laboratories) to immunostain dopaminergic neurons throughout the SN pars compacta. TH+ cells were counted stereologically using the optical fractionator method as previously described by our laboratory (Kaur et al., 2003). Sections were cut at a 40 μm thickness and every 4th section was counted using a grid of 100 × 100 μm. Dissector size used was 35 × 35 × 12 μm. TH cell loss was verified via Nissl staining.

4.4 Dual TH immunocytochemistry and silver staining of striatal axons

Following cardiac perfusion with phosphate-buffered saline (PBS) then 4% paraformaldehyde, brains were removed, dehydrated in 30% sucrose and sectioned at 40 μm. Immunocytochemistry was performed using antibody against TH as above to immunostain dopaminergic terminals throughout the striatum. Silver staining was used for detection of degenerating striatal neurites within this population using the FD NeuroSilver Kit (FD Neuro Technologies). TH-positive, silver stained neurites in four separate fields from the striata (n=5) were double-blind counted to quantitate numbers in ferritin transgenics versus between saline fed control animals.

4.5. Statistical analyses

All data are expressed as mean ± SD for the number (n) of independent experiments performed. Statistical analyses of the data were performed using analysis of variance and post hoc Students't testing. Values of $P < 0.05$ were taken as being statistically significant

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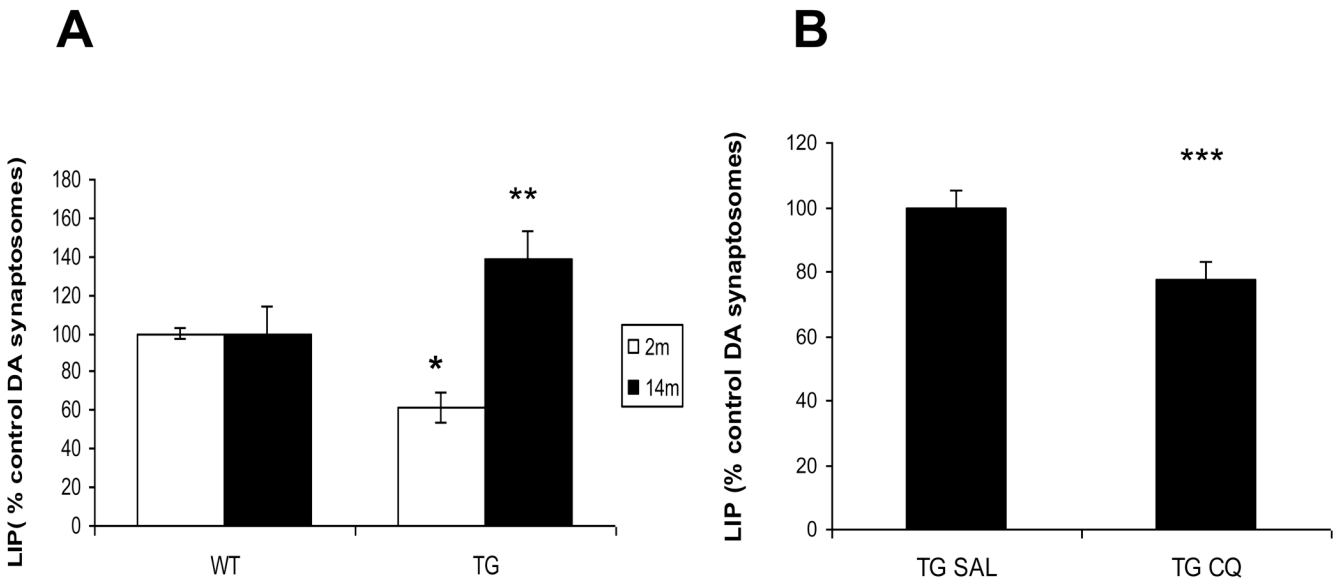

Fig 1. Labile iron pool measurements

(A) LIP levels measured via calcein dequenching following 10-min SIH treatment in ST DA synaptosomes isolated from 2 month and 14 month old ferritin transgenics (Tg) versus wildtype littermate controls (WT), * $p < 0.001$ compared to 2 month WT; **$p<0.005$ compared to 12 month WT. (B) LIP measured via calcein dequenching following 10-min SIH treatment in ST DA synaptosomes isolated from 14 month old saline fed Tgs versus CQ fed Tgs. *** $p<0.05$ compared to saline controls.

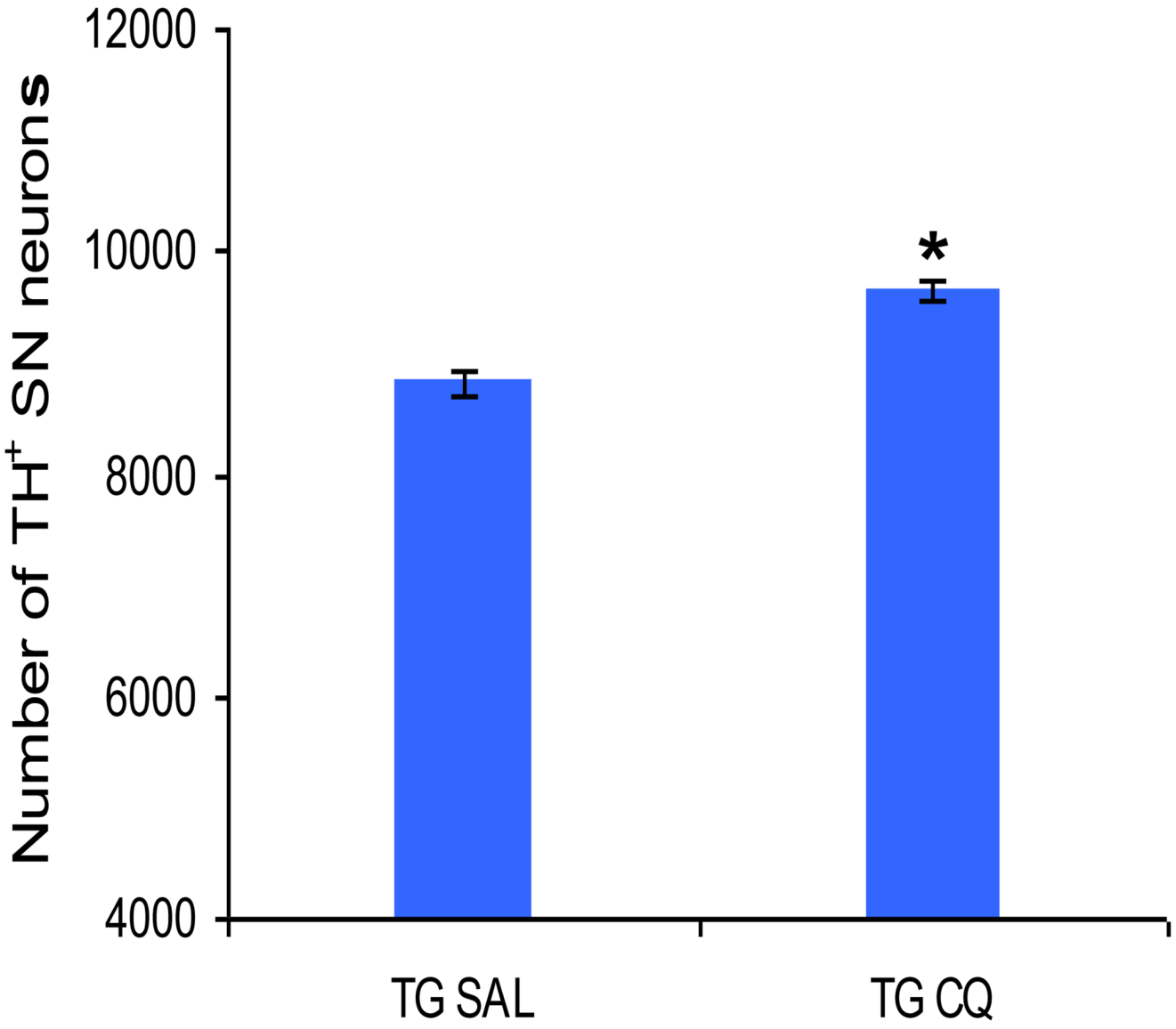

Fig 2. Sterologicalcounts of SN TH$^+$ cell numbers

Cells were counted at a magnification of 100x using the optical fractionator approach as previously described (Kaur et al 2003), n=5 mice per condition, *p<0.05 compared to saline-fed ferritin Tgs (TG SAL) versus CQ-fed (TG CQ).

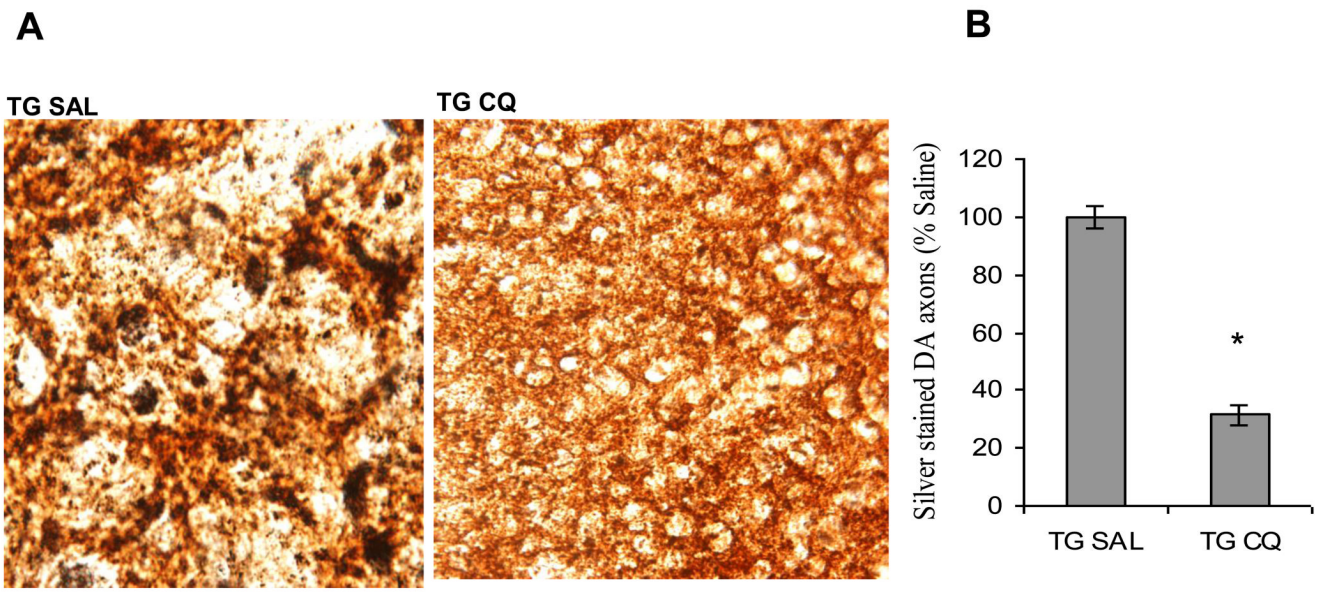

Fig 3. A Representative histograph of striatal neurodegeneration as assessed by silver staining co-localized within tyrosine hydroxylase-positive (TH+) ST DA axons

(A) Data presented at 40 × magnification demonstrating elevation of silver impregnation in SN CQ) ferritin transgenics. (B) Quantification of numbers of silver-stained dopami nergic axons in striatal sections of TG-SAL vs TG CQ, * P< 0.001. An average count from five independent fields on comparable anatomically-matched sections from n = 5 animals is presented.