

Iron Accumulation and Dysregulation in the Putamen in Fragile X-Associated Tremor/Ataxia Syndrome

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ABSTRACT: Background: Fragile X-associated tremor/ataxia syndrome is an adult-onset disorder associated with premutation alleles of the *FMR1* gene. This disorder is characterized by progressive action tremor, gait ataxia, and cognitive decline. Fragile X-associated tremor/ataxia syndrome pathology includes dystrophic white matter and intranuclear inclusions in neurons and astrocytes. We previously demonstrated that the transport of iron into the brain is altered in fragile X-associated tremor/ataxia syndrome; therefore, we also expect an alteration of iron metabolism in brain areas related to motor control. Iron is essential for cell metabolism, but uncomplexed iron leads to oxidative stress and contributes to the development of neurodegenerative diseases. We investigated a potential iron modification in the putamen - a structure that participates in motor learning and performance - in fragile X-associated tremor/ataxia syndrome.

Methods: We used samples of putamen obtained from 9 fragile X-associated tremor/ataxia syndrome and 9 control cases to study iron localization using Perl's method, and iron-binding proteins using immunostaining.

Results: We found increased iron deposition in neuronal and glial cells in the putamen in fragile X-associated tremor/ataxia syndrome. We also found a generalized decrease in the amount of the iron-binding proteins transferrin and ceruloplasmin, and decreased number of neurons and glial cells that contained ceruloplasmin. However, we found increased levels of iron, transferrin, and ceruloplasmin in microglial cells, indicating an attempt by the immune system to remove the excess iron.

Conclusions: Overall, found a deficit in proteins that eliminate extra iron from the cells with a concomitant increase in the deposit of cellular iron in the putamen in Fragile X-associated tremor/ataxia syndrome. © 2017 International Parkinson and Movement Disorder Society

Key Words: fragile X; repeat-expansion disorder; CGG; dementia; FXTAS; neurodegeneration; motor disorder

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Relevant conflicts of interest/financial disclosures: P.J.H. holds patents for assays of CGG-repeat expansion and for FMRP ELISA; he is also in nonremunerative collaborations with Pacific Biosciences, Inc., and Roche Diagnostics. Authors otherwise declare no conflict of interest.

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder associated with premutation alleles (55-200 CGG repeats) of the *FMR1* gene; larger expansions (>200 CGG repeats; full mutation) give rise to fragile X syndrome (FXS), the most common inherited form of cognitive impairment. Carriers of premutation alleles are common in the general population, with an estimated frequency as high as 1 in 130 females and 1 in 250 males.^{1,2} However, only about 40% of male carriers and 14% of female carriers will eventually develop FXTAS.^{3,4} FXTAS is characterized by progressive action tremor, gait ataxia, cognitive decline, parkinsonism, neuropathy, and autonomic dysfunction.⁵ Central

nervous system (CNS) pathology includes dystrophic white matter and intranuclear inclusions in neurons and astrocytes.⁶⁻⁸ Although neurological symptoms of FXTAS have only been observed in adults, it is now clear that children carrying premutation alleles may also have forms of clinical involvement that include anxiety, attention deficit hyperactivity disorder, and autism spectrum disorders related to the premutation.^{9,10} In addition, premutation CGG-repeat expansions in the mouse *Fmr1* gene have been shown to alter embryonic neocortical development.¹¹

Premutation carriers display a form of gene dysregulation that is quite distinct from the gene silencing observed with FXS, manifested by substantially increased levels of *FMR1* mRNA, and normal or moderately decreased levels of FMRP. The extent of this altered expression is a function of the size of the CGG-repeat expansion within the premutation range, with larger CGG-repeat expansions associated with higher levels of mRNA and lower levels of protein.¹² Evidence from both human and animal studies implicates a direct toxic gain-of-function of the premutation CGG (pre-CGG) repeat-containing *FMR1* mRNA.¹²⁻¹⁵ Consistent with this hypothesis, the characteristic intranuclear inclusions found in neuronal and glial cells of FXTAS cases have been demonstrated to contain *FMR1* mRNA but not FMRP.^{16,17} Interestingly, FMRpolyG peptides produced by out-of-phase translation are observed in the nucleus;¹⁸ however, their functional significance is not known.

Iron is essential for many facets of cell metabolism, including DNA synthesis, mitochondrial respiration, synthesis of neurotransmitters, and signal transduction in the CNS. In particular, iron is required for many reactions that modulate CNS function including the synthesis of tyrosine hydroxylase, myelin, and neurotransmitters such as dopamine, norepinephrine, and serotonin.^{19,20} On the other hand, uncomplexed iron reacts with molecular oxygen to generate the reactive oxygen species that lead to oxidative stress^{21,22} and contributes to the development of neurodegenerative diseases. Posttranscriptional regulation of genes involved in iron metabolism is mediated by the interaction of iron regulatory proteins (IRPs) with RNA stem-loop sequence elements known as iron-responsive elements (IREs). Indeed, IREs regulate the expression of iron-binding proteins (IBPs). Processes that alter the transcription and/or translation of IRP/IBPs will result in altered iron metabolism and therefore in the level of oxidative stress, itself a risk factor for neurodegeneration.²³ What is generally unknown about such disorders is whether iron dysregulation is a cause or consequence of the disease.

We have previously demonstrated that the transportation of iron from the circulation into the brain through the spinocerebral fluid and the blood-brain barrier is altered in FXTAS.²⁴ We demonstrated a great increase in iron deposition and an alteration of the

levels of specific IBPs/IRPs within the choroid plexus and brain capillaries.²⁴ Based on the observed altered iron transport into the CNS, we hypothesized that iron levels may be altered within specific brain areas of the FXTAS brain. Because the cerebellum is involved in movement control and the dentate nucleus is the major outflow tract of the cerebellum and because lesions in these structures lead to gait ataxia and action tremor - the 2 core features of FXTAS - we first analyzed iron deposition in the cerebellum. However, only 20%-25% of cases demonstrated a mild increase in iron in the cerebellar cortex and the dentate nuclei of the cerebellum.²⁵ One of the cerebral structures involved in motor control is the striatum, which participates in the control of motor learning, motor performance, and sequences of movement. The striatum is also involved in cognition, emotions, and learning. Here we investigate potential iron dysregulation in the striatum, specifically in the putamen of subjects with FXTAS.

Materials and Methods

Sample Collection

Samples from 9 FXTAS subjects and 9 control subjects were obtained from the FXTAS brain repository at the University of California, Davis, School of Medicine. Additional control tissue was obtained from the Pathology Department at the University of California, Davis, Health System. Control tissue was obtained from subjects who did not have any significant neurological history. Tissue specimens were obtained through consented autopsies with institutional review board approval. FXTAS cases were clinically diagnosed based on the presence of intention tremor, cerebellar ataxia, parkinsonism, memory and executive function deficits, and autonomic dysfunction and confirmed with the posterior presence of intranuclear ubiquitin inclusions in brain cells.

Perl's Staining

A block of cerebellar tissue ($2 \times 2 \times 0.25 \mu\text{m}^2$) containing putamen for each case was immersed in 20% sucrose (Fisher, USA) and embedded in OCT (Fisher). Blocks were cut into 12- μm sections using a cryostat. Sections were left to dry for 10-30 minutes at 37°C before being washed with deionized water. Slides were submerged in a solution containing a 1:1 ratio of 20% hydrochloric acid (Fisher) and 10% potassium ferrocyanide (Fisher) for 20 minutes at room temperature. The presence of blue deposits (iron bound to hemosiderin) was confirmed using a microscope (Nikon Eclipse E200). Slices were counterstained with nuclear fast red (Ricca Chemical Company, USA) for 5 minutes. Slides were washed with water, dehydrated with ethanol, and cleared with xylene before being coverslipped with Permount (Fisher).

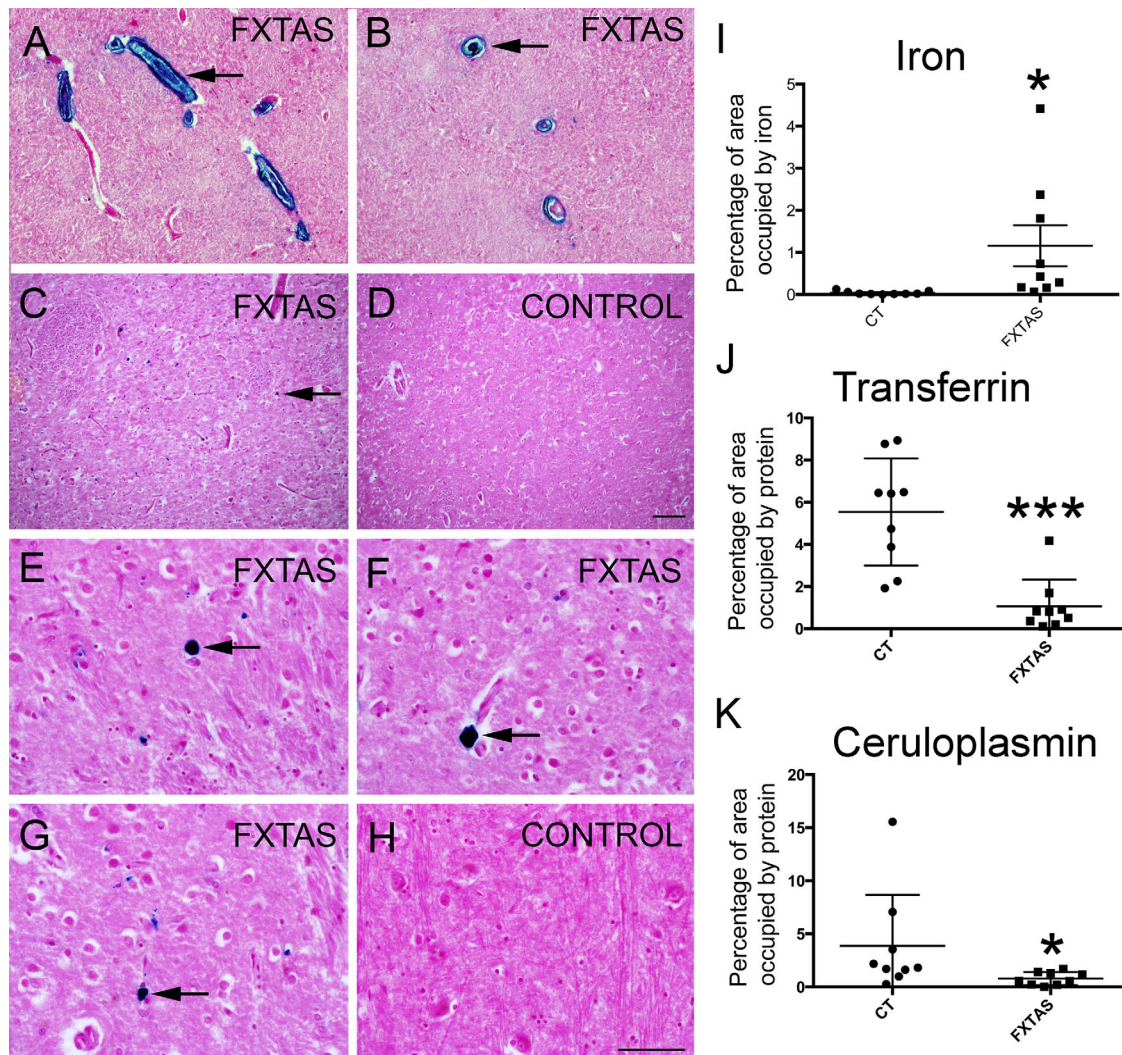


FIG. 1. Iron bound to hemosiderin (blue) accumulated in the putamen in FXTAS. Iron (blue) in sagittal (A) and coronal (B) sections of capillaries showed a great amount of iron in the walls and within the capillaries. Iron was present in the putamen parenchyma in FXTAS (C, E–G) but almost none was present in control tissue (D, H). Arrows point to iron deposits. Tissue is costained with nuclear fast red. (I–K) The amount of iron was increased, whereas the amount of Tf and Cp was decreased in the putamen in FXTAS. Iron: blue, Perl's method; Tf and Cp: brown, immunostaining. Tf, transferrin; Cp, ceruloplasmin. Scale bar: A–D, 200 μ m; E–H, 100 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

Analysis

A 0.5-cm² area of the putamen was imaged using a Keyence BZ9000 microscope. Each 0.5-cm² image was a merge of 36 single 20 \times images. Analysis was conducted using the NIH program ImageJ. The desired blue color (iron) was measured, and the percentage of area occupied by iron was compared between FXTAS and control subjects using *t*-test analysis. A *P* value of 0.05 was used for statistical significance.

Results

Iron Accumulates in the Putamen in FXTAS

We used random samples of putamen obtained from 9 FXTAS and 9 control cases to study iron localization. We first attempted to detect ferrous

iron (Fe²⁺) using Turnbull's staining; however, we did not obtain visible staining. This may indicate that Fe²⁺ is not accumulated in FXTAS; however, the lack of staining is most likely a consequence of the oxidation of Fe²⁺ to Fe³⁺ during tissue preparation and histochemical analysis.²⁶ We next used Perl's method, which detects ferric iron (Fe³⁺) bound to hemosiderin.²⁷ We found intracellular iron deposits in the putamen in most control and FXTAS subjects (Fig. 1) and observed deposition of iron in blood vessels in the putamen of FXTAS but not in control cases (Fig. 1A,B). The fractional area of putamen parenchyma occupied by iron deposits in FXTAS cases was approximately 29-fold greater than in controls (1.16% \pm 0.4% [standard error of the mean] in FXTAS, 0.04% \pm 0.1% in control, *P* = 0.03; Fig. 1I).

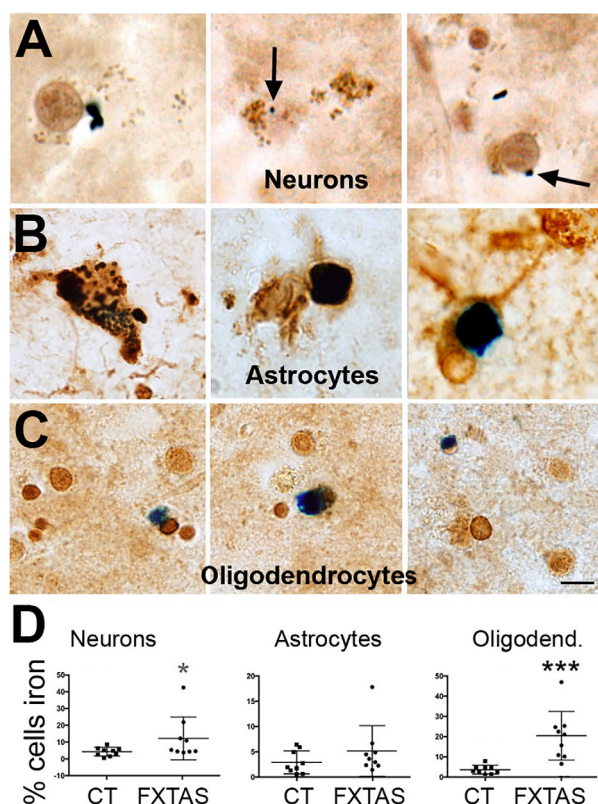


FIG. 2. The number of oligodendrocytes containing iron drastically increased, whereas the number of neurons containing iron marginally increased in FXTAS. However, the number of astrocytes containing iron was similar in FXTAS and control cases. (A–C) Representative cell-types (A: neurons; B: astrocytes; C: oligodendrocytes) containing iron in FXTAS. (D) Number of cells containing iron for each control and FXTAS case included in this study. Iron: blue, Perl's method. CT, control. Calibration bar: 5 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

Transferrin and Ceruloplasmin Levels Are Decreased in the Putamen in FXTAS

To further investigate potential iron dysregulation in the brains of subjects with FXTAS, we performed immunostaining with antibodies against iron-binding proteins. We first analyzed the distribution and amount of Tf, which was observed to be intracellular. We observed a 5-fold decrease in the amount of Tf in the putamen for FXTAS subjects compared with controls ($1.06\% \pm 0.3\%$ in FXTAS, $5.57\% \pm 0.7\%$ in control, $P = 0.0002$; Fig. 1J). Immunostaining with an antibody against ceruloplasmin (Cp) revealed that Cp levels in the putamen were quite heterogeneous across all cases. However, we found that there was a downward trend in the amount of Cp in FXTAS cases compared with control subjects that approached but did not meet significance ($0.78\% \pm 1.9\%$ in FXTAS, $3.85\% \pm 1.5\%$ in control, $P = 0.07$; Fig. 1C).

Number of Cells Containing Detectable Levels of Iron Bound to Hemosiderin Is Substantially Increased in the Putamen in FXTAS Cases

We also determined whether there was differential accumulation of iron in the different types of cells

populating the putamen by costaining tissue with the Perl's method and antibodies against specific cellular markers (NeuN for neurons, S100 for astrocytes, and Sox10 for oligodendrocytes). We found that there were marginally more neurons and substantially more oligodendrocytes containing iron deposition in FXTAS than in control cases (neurons: $12.21\% \pm 4.0\%$, in FXTAS, $4.5\% \pm 0.9\%$ in control, $P = 0.08$, Fig. 2A,D; oligodendrocytes: $20.41\% \pm 3.7\%$ in FXTAS, $3.66\% \pm 0.6\%$ in control, $P = 0.0008$, Fig. 2C,D). The average fraction of astrocytes containing iron was also increased in FXTAS, but this increase did not meet significance (5.1 ± 1.5 in FXTAS, 2.9 ± 0.2 in control, $P = 0.23$; Fig. 2B,D).

Number of Neurons and Glial Cells That Contain Ceruloplasmin, But Not Transferrin, Is Decreased in FXTAS

To assess whether there was differential cellular accumulation of iron-related proteins, we costained tissue with antibodies against specific cellular markers (NeuN for neurons, S100 for astrocytes, and Sox10 for oligodendrocytes) and an antibody against Tf or Cp. We found that all cell types analyzed presented with equal numbers of Tf-expressing cells in FXTAS and control cases, with Tf-expressing astrocytes and neurons accounting for approximately 50% of the total of each cell type (Fig. 3) and Tf-expressing oligodendrocytes for 70% of total oligodendrocytes. By contrast, there was a generalized decrease in the number of cells that contained Cp. Nearly all cells contained Cp in the control cases (neurons, $97.36\% \pm 0.4\%$; astrocytes, $97.73\% \pm 0.6\%$; oligodendrocytes, $98.50\% \pm 0.3\%$); however, the number of cells containing Cp was decreased in FXTAS (neurons, $39.81\% \pm 6.1\%$; Fig. 4A,D; astrocytes, $50.66\% \pm 4.5\%$; Fig. 4B,D; oligodendrocytes: $83.43\% \pm 1.2\%$; all $P > 0.0001$; Fig. 4).

Number of Microglial Cells Containing Iron, Transferrin, and Ceruloplasmin Is Increased in FXTAS

We analyzed the number of microglial cells - immune cells of the CNS - containing iron, Tf, and Cp and found that there was an increase in the number of microglial cells that contained iron and both proteins.

The number of microglial cells that contained iron was more than double in FXTAS when compared with control cases (11.9 ± 2.4 in FXTAS, 4.5 ± 0.9 in control, $P = 0.01$; Fig. 5A,D). In addition, whereas the increase in microglial cells containing Cp greatly increased (7-fold; $66.15\% \pm 2.8\%$ in FXTAS; $9.41\% \pm 2.2$ in control; $P < 0.0001$; Fig. 5C,D), the increase in the number of cells that contained Tf was significant, albeit more modest (1.7-fold;

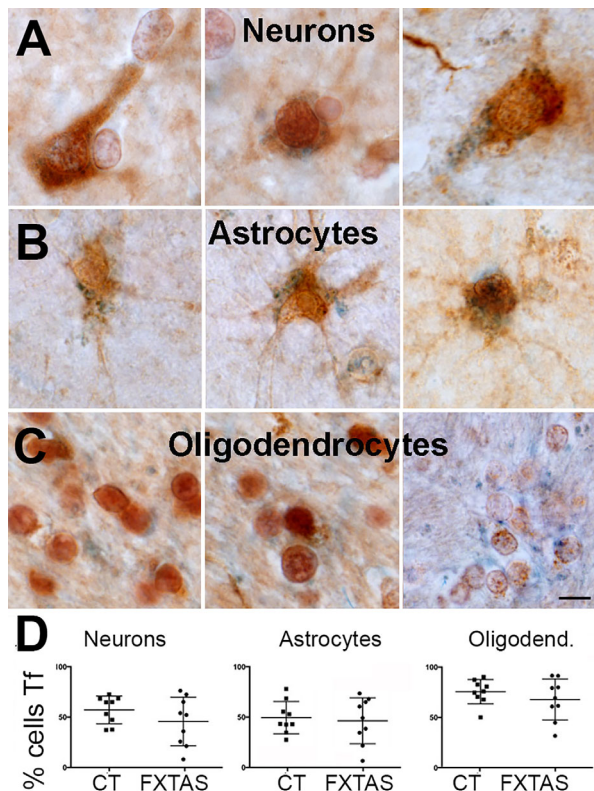


FIG. 3. The number of neurons, astrocytes, and oligodendrocytes containing Tf was similar in FXTAS and control cases. (A–C) Representative cell types (A: neurons; B: astrocytes; C: oligodendrocytes) containing Tf in FXTAS. (D) Number of cells containing Tf for each control and FXTAS case included in this study. Tf: blue. Tf, transferrin; CT, control. Calibration bar: 5 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

28.74% \pm 0.04% in FXTAS, 16.57% \pm 0.01% in control; $P = 0.02$; Fig. 5B,D).

Summarizing, we found a generalized increased iron deposition and an increased number of neuronal and glial cells that accumulate iron in the putamen FXTAS. We also found a generalized decrease in the amount of Tf and Cp present in the putamen together with a decrease in the number of neurons and glial cells that contained Cp. However, we found an increase in the accumulation of iron, Tf, and Cp in microglial cells in the FXTAS putamen. Overall, there is a deficit in proteins that eliminate extra iron from the cells with a concomitant increase in the deposit of cellular iron.

Discussion

Here we have demonstrated excessive deposition of iron bound to hemosiderin in the putamen of patients with FXTAS. Hemosiderin is a degradation product of ferritin and is prominent during conditions of iron overload.²⁸ Alteration of iron metabolism in the central nervous system is known to cause motor impairment and cognitive deficits,²⁹ similar to those present in FXTAS. Iron enters the brain through the blood-

brain barrier and the choroid plexus–cerebrospinal fluid barrier. We previously demonstrated an alteration of iron transport into the brain through the choroid plexus–cerebrospinal fluid barrier. In addition, here we demonstrated deposition of iron in blood vessels in FXTAS but not in control tissue. These data suggest that the levels of iron may be altered in the brain parenchyma.²⁴ We initially asked if iron levels were altered in peripheral blood in FXTAS and analyzed peripheral blood in 3 cases of FXTAS but did not find any alteration in iron levels or iron-related proteins (data not published). We next analyzed iron levels in the brain parenchyma. Brain regions affected in FXTAS include the cerebellum, where the number of Purkinje cells is reduced and present with simple and twin intranuclear inclusions,³⁰ and the putamen which is hypotrophic and presents with numerous simple intranuclear inclusions. We previously demonstrated that increased iron deposition in the cerebellar cortex and cerebellar dentate nucleus cannot be used as an FXTAS-distinguishing feature, because whereas some FXTAS cases presented with an increase in iron deposition when compared with control, most did not.²⁵

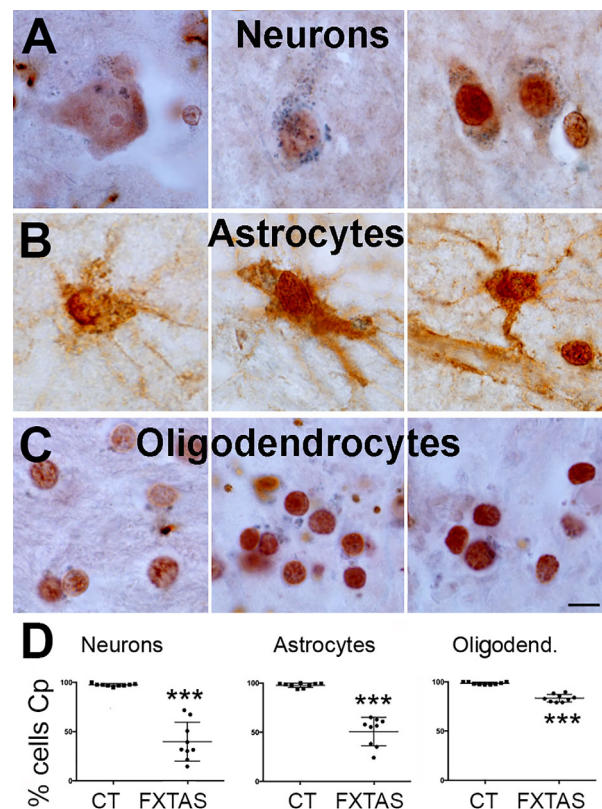


FIG. 4. The number of neurons, astrocytes, and oligodendrocytes containing Cp drastically decreased in FXTAS when compared with control cases. (A–C) Representative cell types (A: neurons; B: astrocytes; C: oligodendrocytes) containing Cp in FXTAS. (D) Number of cells containing Cp for each control and FXTAS case included in this study. Cp: blue. Cp, ceruloplasmin; CT, control. Calibration bar: 5 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

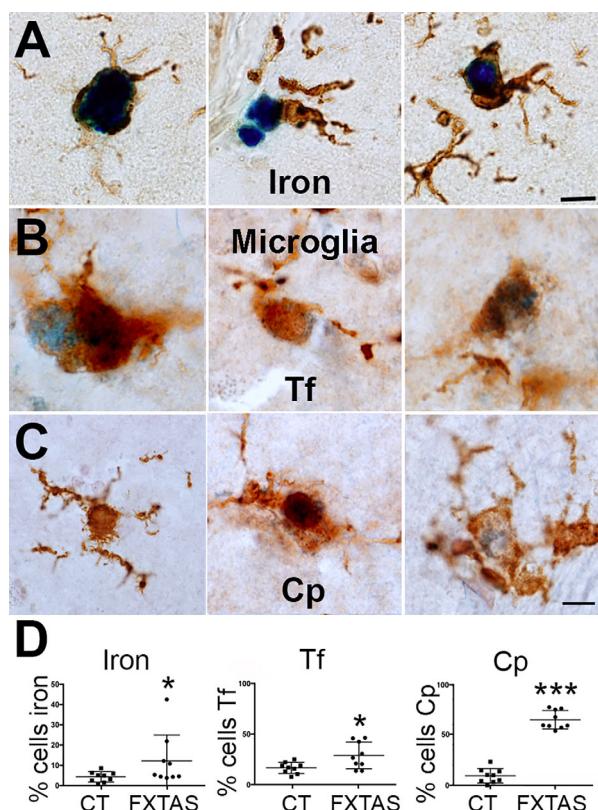


FIG. 5. The number of microglial cells containing iron, Tf, and Cp was increased in the putamen in FXTAS. (A–C) Representative microglial cells containing iron, Tf, and Cp. (D–F) Number of microglial cells containing iron, Tf, and Cp for each control and FXTAS case included in this study. Iron, Tf, and Cp: blue. Tf, transferrin; Cp, ceruloplasmin; CT, control. Calibration bar: 5 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

However, we found substantial iron bound to hemosiderin in the parenchyma of the putamen in FXTAS cases but not in control tissue. Studies of MRI patients with FXTAS rendered evidence of subcortical gray matter degeneration involving the striatum, including symmetric hypointensity in the putamen and caudate in T2-weighted SE images that was present in 4 patients examined, indicative of iron accumulation.^{31,32} Our data are in agreement with previous MRI data.

We do not know if this accumulation of iron in the putamen is a consequence or a cause of the RNA toxicity that occurs in FXTAS, but we hypothesize that most likely this finding is a consequence of RNA toxicity. Processes that alter the transcription and/or translation of IRPs or IBPs will result in altered iron metabolism and increased oxidative stress. Concomitantly, we discovered a decrease in the expression of Cp and Tf. Tf binds to iron and transports it into the cell, whereas Cp regulates the exit of excess iron from the cell.³³ The decrease in the level of Cp and Tf proteins in the cells of the putamen explains the iron accumulation within these cells.

Because iron is one of the major sources of intracellular reactive oxygen species (ROS), accumulation of

nonheme iron is believed to play a major role in degeneration of the basal ganglia, both in normal aging and in neurodegenerative diseases. We found that all the major cell types accumulate iron in the putamen, but neurons and oligodendrocytes are most affected by this alteration, and therefore these cells are going to be subjected to high ROS and consequent degeneration, decreasing the number of neurons within the putamen and the level of fiber myelination. High ROS and increased cell death are in agreement with previous reports demonstrating pronounced atrophy in FXTAS.³¹ White matter degeneration has been described as the most prominent neuropathologic characteristic of FXTAS.^{7,33} The very pronounced accumulation of iron in oligodendrocytes may be the link between FMR1 mRNA gain of function and the white matter pathology characteristic of FXTAS. Microglial cells greatly accumulate iron and iron-binding proteins, indicating the attempt by the immune system to clean the parenchyma of excess iron and unutilized iron-binding proteins and therefore to normalize iron metabolism in FXTAS.

Iron deposition has been linked to other degenerative diseases. Neurodegeneration with brain iron accumulation is a group of syndromes characterized by excess iron accumulation in the globus pallidus and, to a lesser degree, in the substantia nigra. It clinically presents as a neurodegenerative disease with progressive hypo- and/or hyperkinetic movement disorders and a variable degree of pyramidal, cerebellar, peripheral nerve, autonomic, cognitive, and psychiatric involvement and visual dysfunction.^{35,36} Other diseases that present with an increased iron deposition include Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis, restless legs syndrome, and prion diseases.³⁷ Interestingly, parkinsonism and dementia are common in those with FXTAS, and restless legs syndrome is more common in those with the premutation compared with controls.^{2,29} Because of the accumulation of iron in FXTAS, the use of iron-chelating agents such as deferiprone may be adequate for treatment of iron-related motor symptoms.

Overall, we have demonstrated that iron metabolism is altered in the putamen in FXTAS. This alteration has implications for understanding the pathophysiology of FXTAS and for the development of new clinical treatments. ■

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