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Mutation of Ataxia–Telangiectasia Mutated is Associated with Dysfunctional Glutathione Homeostasis in Cerebellar Astroglia

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Abstract

Astroglial dysfunction plays an important role in neurodegenerative diseases otherwise attributed to neuronal loss of function. Here we focus on the role of astroglia in ataxia–telangiectasia (A–T), a disease caused by mutations in the ataxia–telangiectasia mutated (*ATM*) gene. A hallmark of A–T pathology is progressive loss of cerebellar neurons, but the mechanisms that impact neuronal survival are unclear. We now provide a possible mechanism by which A–T astroglia affect the survival of cerebellar neurons. As astroglial functions are difficult to study in an *in vivo* setting, particularly in the cerebellum where these cells are intertwined with the far more numerous neurons, we conducted *in vitro* coculture experiments that allow for the generation and pharmacological manipulation of purified cell populations. Our analyses revealed that cerebellar astroglia isolated from *Atm* mutant mice show decreased expression of the cystine/glutamate exchanger subunit xCT, glutathione (GSH) reductase, and glutathione-S-transferase. We also found decreased levels of intercellular and secreted GSH in A–T astroglia. Metabolic labeling of L-cystine, the major precursor for GSH, revealed that a key component of the defect in A–T astroglia is an impaired ability to import this rate-limiting precursor for the production of GSH. This impairment resulted in suboptimal extracellular GSH supply, which in turn impaired survival of cerebellar neurons. We show that by circumventing the xCT-dependent import of L-cystine through addition of *N*-acetyl-L-cysteine (NAC) as an alternative cysteine source, we were able to restore GSH levels in A–T mutant astroglia providing a possible future avenue for targeted therapeutic intervention.

Keywords

ataxia–telangiectasia; astroglia; xCT; glutathione; neuronal survival; cerebellum; ataxia–telangiectasia mutated

Introduction

Ataxia–telangiectasia (A–T) is an autosomal recessive disease caused by mutations in the ataxia–telangiectasia mutated (*ATM*) gene. Symptoms of A–T include altered DNA damage repair, immunodeficiency, increased cancer incidence, sterility, and muscle atrophy (Barlow et al. 1998; Boder and Sedgwick 1958; Chun and Gatti 2004; Eisen et al. 1965; Gotoff et al. 1967; Kwast and Ignatowicz 1990). Although cerebellar atrophy is most prominent, patients exhibit degenerative changes of various severities in various brain regions (Habek et al. 2008; Oba et al. 2010; Tavani et al. 2003; Verhagen et al. 2012).

While the mechanisms underlying the widespread degenerations in A–T are unclear, an increased oxidative burden seems to be a contributor (Barlow et al. 1999; Chen et al. 2003; Guo et al. 2010; Hayashi et al. 2012; Kamsler et al. 2001; Kim and Wong 2009; Kuang et al. 2012; Ludwig et al. 2013; Quick and Dugan 2001; Reichenbach et al. 2002; Rotman and Shiloh 1997; Tchirkov and Lansdorp 2003; Yang et al. 2014; Ziv et al. 2005). It has been suggested that the high oxidative burden could be due to accumulation of reactive oxidant species (ROS) from mitochondrial dysfunction (Ambrose et al. 2007; Valentin-Vega et al. 2012), a “byproduct” of defective DNA repair (Dar et al. 1997) or/and a defect in the cellular antioxidant response (for review, see Ambrose and Gatti 2013).

An increased oxidative burden is particularly challenging for the brain that requires an effective anti-oxidant response to prevent damage to neurons and glia. While impaired ATM signaling might render neurons themselves more susceptible to oxidative damage in A–T brains, (Camins et al. 2007; Carlessi et al. 2014; Folch et al. 2012), astroglia play an important role in protecting neurons from oxidative insults (Davies et al. 2011; Hamby and Sofroniew 2010; Lazzarini et al. 2013; Proschel et al. 2014; Tanaka et al. 1999) and astroglial dysfunction leads can enhance neuronal pathologies (David et al. 2009; Davies et al. 2008; Heinemann et al. 2012; Myer et al. 2006; Sofroniew 2005; Spence et al. 2011; Zhang et al. 2013). In A–T, studies have primarily focused on neurons but astroglia isolated from the A–T cerebellum grow slowly, become senescent, show increased spontaneous DNA synthesis and express a number of markers of oxidative and endoplasmic reticulum (ER) stress (Gosink et al. 1999; Kim and Wong 2009; Liu et al. 2005). While astroglial numbers in human A–T cerebella appear normal (despite loss of neurons) (Amromin et al. 1979; Boder 1985), the astroglia near degenerating Purkinje cells show increases in ERK1/2 phosphorylation (Liu et al. 2005). This raises the question of whether oxidative stress coupled with suboptimal antioxidant support in A–T astroglia might contribute to a microenvironment that cannot sustain neuronal health. One mechanism through which astroglia contribute to the microenvironment is through secretion of reduced glutathione (GSH), which is used to maintain neuronal GSH levels (Dringen et al. 2000; Sagara et al. 1996; Wang and Cynader 2000). Healthy astroglia will increase GSH secretion in response

to oxidative stress, presumably to provide additional support to the neurons, which are less capable of importing cystine, the bioavailable form of cysteine and rate limiting precursor for GSH biosynthesis (Gegg et al. 2005; Sagara et al. 1993; Shih et al. 2006). Defects in GSH homeostasis, particularly on a chronic level, will thus have an impact on the microenvironment and seem to be an important factor to consider in the context of A–T.

As GSH homeostasis and astroglia function are difficult to study in an *in vivo* setting, particularly in the cerebellum where astroglia are intertwined with the far more numerous neurons (Azevedo et al. 2009; Buffo and Rossi 2013; Lent et al. 2012; Selvadurai and Mason 2011) (thus preventing a distinction between astroglia-specific and neuron-specific defects), we conducted *in vitro* coculture experiments that allow for the generation and pharmacological manipulation of purified cell populations. Cerebellar astroglia isolated from *Atm*^{tm1Awb mut/mut} (here abbreviated with A–T) mice (Barlow et al. 1996) decreased expression of the cystine/glutamate exchanger subunit xCT and of GSH reductase. We also found decreased levels of intercellular and secreted GSH in A–T astroglia. Through metabolic labeling of L-cystine, the major precursor for GSH, we were able to determine that a key component of the defect in A–T astroglia is an impaired ability to import L-cystine for the production of GSH. This impairment results in suboptimal extracellular GSH supply, which in turn impairs survival of cerebellar neurons. We show that by circumventing the xCT-dependent import of L-cystine through addition of *N*-acetyl-L-cysteine (NAC), we were able to increase GSH levels in A–T mutant astroglia.

Materials and Methods

Animal Husbandry and Human Tissue

129S6/SvEvTac-*Atm*^{tm1Awb/J} mice (referred here as A–T animals, Barlow et al. 1996) were purchased from Jackson laboratories and maintained by intercrossing heterozygous animals and renewed every third generation. These mice carry a truncation mutation of the *Atm* gene. Homozygous *Atm*^{tm1Awb} mice display some hallmarks of A–T, including growth retardation, infertility, defects in T lymphocyte maturation, sensitivity to gamma-irradiation, and a very high tumor burden that leads to lethality between 2 and 4 months of age. Animals do not develop the severe cerebellar degeneration seen in humans prior to the onset of lymphoma but show some degree of cerebellar dysfunction by 2 months of age (Barlow et al. 1996). Cells were generated from the second litter of proven breeder dams. All animals were maintained in accordance with University of Rochester Medical Center animal care standards. Postmortem tissue from a 16-year-old A–T patient and control brain tissue matched for age, sex, race, and postmortem interval were obtained from the NICHD Brain and Tissue Bank of Developmental Disorders at the University of Maryland, Baltimore, MD.

Cell Isolation

Astroglia—Cerebella of P0–P5 littermates from heterozygous breedings were dissected and processed separately while genotyping was performed (Barlow et al. 1996). Tissues were treated with collagenase and papain, seeded in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS on poly-L-lysine (PLL) coated

culture flasks. Once the genotype was determined, cells were passaged and culture were established from pooled A–T or WT littermates.

Neurons—Cerebellar granular neurons (CGNs) and Purkinje neurons (PNs) were isolated from A–T and WT mice from P6–P7 and P0–P1 mice, respectively, according a modified protocol by Baptista et al. (1994). Tissues were treated with collagenase and papain and cell suspensions were purified using a 60%, 35% Percoll gradient. 1×10^4 CGNs per 15-mm well and plated on astroglial monolayers.

Cell Culture Conditions and Media

Astroglia culture conditions—Subconfluent passage one cultures were allowed to reach a density at which time they were treated with 10 μ M AraC to select for postmitotic cells and to eliminate micorglia and macrophages. Confluent postmitotic astroglial monolayers used in all the experiments were maintained in 150 μ L of minimal media (high-glucose DMEM + 1% FBS) per cm^2 and used within 4 weeks from the time of dissection. Monolayers were >97% GFAP positive and negative for β -III tubulin, Ox42, a marker for inflammatory cells (Jeong et al. 2013), and galactocerebroside (GalC) (as described below). As all cultures (WT and A–T) contained similar low levels of unlabeled cells, we did not further investigate the identify of these cells, which might represent fibroblasts, endothelial cells, or astroglia with GFAP expression below the detection limit in labeled monolayer cultures. Astroglial-conditioned media (ACM) is defined as minimal media that was collected from astroglial cultures after a 24-h incubation.

Neuronal cocultures—Neurons were cultured in high-glucose DMEM + 1% FBS supplemented with 10 μ g/mL insulin, 10 μ g/mL transferrin, putrescine (0.1 mM), selenium (0.224 mM), progesterone (0.2 mM), pathocyte-4 BSA (0.0286% v/v; ICN Biochemicals, and 5 μ M AraC). Medium was changed every 2 days and cultures were analyzed as indicated.

ACM treatment of CGNs—CGNs were plated in 500 μ L of 10% FBS on PLL-coated 12-mm coverslips. After 3hrs the media was changed to ACM with 5 μ M AraC for 48 hrs followed by daily medium changes using AraC-free ACM. Cells were fixed at 3 DIV. For the GSH add-back experiment, ACM was collected from astroglial cultures as above and supplemented with 250 μ M GSH prior to the addition of the ACM to the CGN cultures.

Preparation of Metabolic Media and Cellular Extracts for Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

ACM for metabolic analysis was obtained by culturing 90 μ L/ cm^2 cystine-free high-glucose DMEM + 1% FBS supplemented with L-cystine (Sigma C7602) or $^{13}\text{C}_6^{15}\text{N}_2$ L-cystine (Cambridge Isotope CNLM-4244) with astroglia for 18 h. *N*-acetylcysteine (NAC) (Sigma A9165) and Trolox C (Sigma 238813) were added to astroglial cultures at a final concentration of 1 mM. 200 μ L of ACM was combined with 200 μ L of ice-cold TBA solution (10mM tributylamine, 15 mM acetic acid in 97:3 [water:methanol]) and then combined with 400 μ L of -80°C MeOH. The astroglial extracts were prepared by aspirating media and quickly adding 50:50 ice-cold TBA solution: MeOH. The samples were

incubated at -80°C for 10 min and the cell extracts were harvested and transferred into 1.5-mL tubes. Extracts and ACM were vortexed and pelleted before the resulting supernatants were analyzed by LC-MS/MS.

Immunocytochemistry

Cultures were fixed with 2% paraformaldehyde and stained with antibodies against GFAP (Dako 1:500), β -III tubulin (Chemicon MAB1637 1:500), GalC (galactocerebroside—in house production), and Ox42 (Chemicon 1:250). 0.2% Triton was used for permeabilization. Nuclei were visualized using DAPI (4',6-diamidino-2-phenylindole).

Quantification of Neuronal Survival and Neurite Outgrowth

For neuronal survival, cells were plated in 150- μL DMEM/F12 with 10% FBS on PLL-coated 96-well plates. After 12 h, the medium was changed to ACM with 5 μM AraC. Medium was changed daily using AraC-free ACM. After 3 DIV, the survival of neurons treated with ACM was quantified by counting calcein AM(+)/PI(−) neurons using a Celigo Adherent Cell Cytometer (Nexcelcom) and normalized to the total number of cells. Neurite length was quantified with Image J software on all neurons that showed a single neurite with a minimum length of 10 μm . At least 100 neurites were counted per coverslip, with at least three coverslips per experimental replicate. Experiments were replicated 3 times.

LC-MS/MS Analysis

Analysis was performed using reversed phase chromatography with an amine-based ion-pairing agent coupled to an electrospray mass spectrometer running in negative mode. The HPLC column used was a Synergy C18 150 $\times$ 2.00 mm column with a 4- μm particle size (Phenomenex). For HPLC, solvent A = TBA solution and solvent B = 100% methanol. The HPLC gradient was as follows: $t = 0$, 0% B; $t = 5$, 0% B; $t = 10$, 20% B; $t = 20$, 20% B; $t = 35$, 65% B; $t = 38$, 95% B; $t = 42$, 95% B, $t = 43$, 0% B; $t = 50$, 0%. LC instrumentation was an LC-20 AD HPLC system (Shimadzu), auto-sampler temperature 4°C , injection volume 20 μL . MS instrumentation was a TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Fisher Scientific). Mass spectrometry parameters were as per (Munger et al. 2006) and metabolite specific mass spectrometry parameters were as per (Bajad et al. 2006). GSH was measured utilizing an MRM scan in negative mode specific for a 306 to 143 transition with collision energy of 17 eV. Labeled GSH was quantified using additional MRM scans specific for the extent of ^{13}C and ^{15}N -labeling. As expected, the dominant labeled GSH isoform that accumulated had +4 m/z ratio, that is, an MRM scan of 310 to 147. The resulting metabolite signal intensities were analyzed by the Xcalibur software from Thermo Electron Corporation. GSH-specific signal intensities were normalized by the protein content of their respective cultures and by the maximum GSH signal for a given run. Normalization to the maximum GSH signal for a given run yields relative differences between samples, and minimizes day-to-day, nonbiologically relevant variation associated with LC-MS/MS analysis.

Immunoblotting

Protein extracts from astroglial monolayers and human tissue were lysed in modified radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Following quantification using the DC Protein Assay (Bio-Rad 5000111), 10 µg of protein from each of the lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes and probed for β -actin (42 kDa; Santa Cruz sc-47773), glutathione reductase (GR, 58 kDa; Abcam, ab16801), glutathione-S-transferase- μ (GST- μ , 25 kDa; Alpha Diagnostics, GSTM11-S), γ -glutamyl cysteine ligase catalytic subunit (γ -GCS, 73 kDa) (Neomarkers PAb RB-1697-P1), glutathione peroxidase 1 (GPx1, 22kDa; Abcam ab16798), GSH synthetase (GSS, 52 kDa; Santa Cruz sc-28966), multidrug resistance protein 1 (MRP1, 190 kDa; Santa Cruz sc-53130), and xCT (35 kDa; Abcam ab37185). For signal detection, membranes were incubated with WesternBright ECL Western blotting detection kit (Advansta K-12045) as per manufacturer's recommendations and imaged with BioBlot BXR film (Laboratory Product Sales BX810). For analysis, films were scanned and analyzed in ImageJ (Schneider et al. 2012). Experimental proteins were first normalized to their respective β -actin expression levels, followed by comparison to the wild-type values.

qPCR Analysis

RNA was isolated from the cerebellar astrocytes isolated from WT and A-T mice using a Nucleospin RNA kit (Macherry-Nagel 740955) as per manufacturer's protocol. Five hundred nanograms of RNA from each sample was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814) with the addition of RNasin Plus RNase inhibitor (Promega N261B). For PCR analysis of the cDNA, samples were diluted 1:5 in nuclease-free water (Life Technologies AM9937) and 1 µL was transferred to 5 µL PCR reactions containing LightCycler 480 Probes Master mix (Roche 04707494001) and Taqman Gene Expression assays. Probes were as follows; GR (Mm00833903), GST μ (Mm00833915), γ GCS (Mm00802655), GPx1 (Mm00656767), GSS (Mm00515065), MRP1 (Mm00456156), and xCT (Mm00442530). Samples were multiplexed with GAPDH (Life Technologies 4352339E) and expression levels were calculated using the C_t method as per the manufacturer's recommendation when using target assays with amplification efficiencies very close to 1 (Applied Biosystems Taqman Gene Expression Assays Technical note 127AP05-03).

Results

Mutation of *Atm* in Astroglia Negatively Impacts Their Ability to Support Mutant and Wild-Type Neurons

To test whether loss of A-T function affects PN and CGN survival, we seeded A-T neurons on astroglial monolayers from wild type (WT) or A-T mice. The survival of A-T PNs was reduced by 42% when grown on A-T astroglia as compared with WT control astroglia (Fig. 1A). A similar impact of neuronal survival was found for CGNs, the major synaptic input of PNs, with a 34% decrease when A-T CGNs were grown on A-T astroglia (Fig. 1B). The survival impairment was not specific to A-T neurons as we also observed decreased WT neuron survival when grown on A-T astroglia (data not shown).

Like neuronal survival, neurite outgrowth (a widely used means of assessing CGN health (Al-Ali et al. 2004; Loh et al. 2008; Sharma et al. 2011) was affected by the presence of A–T astroglia. As shown in Fig. 1C (left panel), A–T CGNs (anti- β -III tubulin, red) extended longer neurites when cocultured with WT astroglia (anti-GFAP labeling of astroglia right panel) than growth on A–T astroglial monolayers (Fig. 1D). When quantified, we found shorter neurites in both A–T (Fig. 1E) and WT (Fig. 1F) CGNs grown on A–T astroglia (dark bar) as compared to WT astroglia (light bars).

To determine whether the effects of A–T cerebellar astroglia on neuronal survival and neurite outgrowth required cell contact, CGNs were cultured for 3 days in the presence of cerebellar ACM harvested from WT (black bar) or A–T (hatched bar) cells (as we expected a higher rate of cell death of A–T neurons compared to WT cells we increased the initial plating density of the A–T neurons by 50%). As shown in Fig. 2A, B, cultures of A–T and WT CGNs exposed to WT ACM had up to 10 times more neurons when compared to minimal medium, confirming the survival supporting activity of ACM from WT astroglia. When cells were exposed for 3 days to A–T ACM we observed increased survival when compared to minimal medium, but 7–10 times lower than the number of neurons seen in WT ACM conditions. The CGNs that survived in A–T ACM also showed less process extension as compared to neurons grown in the presence of WT ACM (Fig. 2C–E).

These results show that A–T astroglia are less capable than WT astroglia of supporting neuronal survival and process extension *in vitro* over a 3-day time period and that the observed effects occur independently of cell contact. Surprisingly, the growth of A–T neurons was better in respect of survival and comparable in respect of neurite length to WT neurons when exposed to ACM harvested from WT astroglia, suggesting that the genotype of the astroglia rather than that of the neurons are the defining factors in neuronal growth *in vitro*.

A–T Cerebellar Astroglia and ACM Have Decreased GSH Levels, Impairing Neuronal Support

As A–T is associated with oxidative stress (Kim and Wong 2009; Liu et al. 2005) and astroglia are critical in maintaining normal tissue redox balance (i.e., Liddell et al. 2010), we next determined whether mutant astroglia showed altered redox homeostasis. We quantified the level of GSH in the A–T cerebellar astroglia and in ACM generated by these cells relative to their WT controls using LC-MS/MS analysis. We found a 40% reduction in the levels of intracellular GSH in A–T astroglia compared to controls (Fig. 3A). The decrease in extracellular GSH was even more marked, with ACM produced by A–T astroglia containing only 15% of the GSH levels found in medium conditioned by WT astroglia (Fig. 3B). Using LC-MS/MS, we did not detect appreciable amounts of GSSG. Using an alternative measurement of GSH/GSSG as described by (Tietze 1969), we found that the GSH/GSSG ratio in the cerebellar astroglia was ~30:1 (data not shown). Therefore, the measurement of GSH by LC-MS/MS reflects nearly the total GSH in the astroglia. In contrast, we saw no differences in levels of expression of major neurotrophins like BDNF and GDNF and found no significant difference in A–T astroglia compared to WT astroglia (data not shown).

To test whether the decrease in neuronal support provided by the A–T astroglia might be, at least in part, due to the decreases in the extracellular GSH levels, we supplemented ACM from A–T and WT astroglia with GSH. We observed a significant increase in survival of both A–T (Fig. 3C) and WT (Fig. 3D) neurons following the addition of GSH. Thus, the deficiency in neuronal support by the A–T astroglia can be overcome by GSH supplementation.

A–T Astroglia Show Alterations in Some GSH-Related Proteins

With the indication that decreased GSH production by A–T astroglia contributes to the impaired ability of A–T astroglia to support neurons in culture, we next wanted to understand the cause of disrupted GSH homeostasis which is controlled by synthesis, consumption and export. In order to determine where the disruption in GSH homeostasis occurs, we examined the expression of proteins involved in GSH metabolism; gamma-glutamylcysteine synthetase (γ GCS), glutathione peroxidase 1 (GPx1), glutathione reductase (GR), glutathione synthetase (GSS), glutathione-S-transferase (GST- μ), multidrug resistant protein 1 (MRP1, which transports intracellular GSH to the extracellular space), and the xCT subunit of the cystine/glutamate antiporter, which is an Na⁺-independent heteroexchanger importing 1 cystine molecule into the cell in exchange for one glutamate molecule (Bannai 1986; Shih et al. 2006). While the level of mRNA expression of γ GCS, the rate-limiting enzyme for GSH production, was not changed (protein level slightly increased), the expression of xCT and GR were significantly decreased with respect to both mRNA and protein in A–T cerebellar astroglia (Fig. 4A–C). Protein levels of glutathione-S-transferase were also decreased but mRNA levels remained unchanged. Importantly, the decrease in xCT, GR, and GST- μ protein expression was mirrored in a human A–T tissue sample, where we found a widespread decrease in these same GSH regulatory enzymes including a 40%–50% decrease in these proteins within the cerebellar gray matter of a 16-year-old A–T patient compared to age-matched control brain tissue (Fig. 4D).

Decreased levels of xCT and GR protein in A–T cerebellar astroglia would predict impairments in cystine import and cycling (Seib et al. 2011). To test whether A–T cerebellar astroglia were deficient in cystine import, we incubated A–T and WT astroglia with a stable isotope-labeled L-cystine (¹³C), which is the bioavailable source of cysteine and the limiting precursor in GSH biosynthesis (Beutler 1989). L-cystine can be taken up by the cells via xCT and is incorporated into GSH via a series of enzymatic steps (Fig. 5A). As cultured astroglial cells release up to 10% of their intracellular GSH every hour (Dringen et al. 1997), this GSH must be replenished in order to maintain oxidative homeostasis. Consequently, when cultured in the presence of ¹³C-L-cystine, we expect an accumulation in the ¹³C GSH pool both intracellular and in the ACM over time.

LC-MS/MS analysis of the cell lysates grown in the presence of ¹³C-L-cystine revealed significantly lower levels of ¹³C GSH synthesized from exogenously supplied ¹³C-L-cystine in the A–T astroglia cell lysates compared to WT controls (Fig. 5B) suggesting either decreased ¹³C-L-cystine import and/or GSH synthesis. To determine whether the exogenously provided ¹³C-L-cystine would also show decreased output of newly synthesized GSH into the culture media, we measured ¹³C-GSH in the ACM generated from

the A-T and WT astroglia. As shown in Fig. 5C, ACM derived from A-T astroglial has a dramatic decrease in ^{13}C GSH, with mutant ACM only containing 13% of the total ^{13}C GSH found in WT ACM. This indicates that the decreased intracellular ^{13}C GSH within the A-T astroglia seen in Fig. 5B was not due increased export of the newly synthesized GSH but instead impaired ^{13}C -L-cystine import and/or GSH synthesis.

A-T Astroglial Production and Secretion of GSH Can Be Enhanced by N-Acetyl-L-Cysteine

We also examined whether A-T astroglia exhibited defects in ability to synthesize GSH. We provided A-T cerebellar astroglia with *N*-acetyl-L-cysteine (NAC). NAC is a cell-permeable GSH prodrug that provides L-cysteine to the cells without requiring import via xCT (Dringen and Hamprecht 1999). NAC can also act extracellularly, reducing L-cystine to L-cysteine, which can then be imported by alternative amino acid transport systems (Beiswanger et al. 1995; Dringen 2000), thereby also bypassing the need for xCT-dependent transport of L-cystine. As NAC can also act as an ROS scavenger that could have an impact on the need of cells to generate intracellular GSH, we also included the water-soluble Vitamin E analog Trolox as an antioxidant control (Davies et al. 1989).

We found that treatment of A-T cerebellar astroglia with NAC significantly increased the intracellular concentration of ^{12}C -GSH (Fig. 6A) and ^{13}C -GSH (Fig. 6B) compared to untreated A-T astroglia, suggesting that mutant cells are indeed able to generate GSH if provided L-cysteine. The commonly accepted mechanism of action for NAC is its deacetylation to cysteine for GSH synthesis. Although NAC could also act intracellularly as a reducing agent to break the disulfide bonds in GSSG, this would not account for the changes in total GSH due to the high GSH:GSSG ratios in astroglia. It is also not likely that NAC is converting GSSG to GSH as an antioxidant as we saw no change in levels of either ^{13}C -GSH or ^{12}C -GSH in the presence of Trolox. We next examined whether the increase in intracellular GSH after NAC treatment could lead to an increase in exported GSH. We found that ACM generated from A-T cerebellar astroglia treated with NAC contained slightly increased levels of ^{12}C -GSH (Fig. 6C) and ^{13}C -GSH (Fig. 6D), while Trolox exposure had again no effect on levels of secreted GSH. These data are consistent with the normal levels of γ -GCS in A-T astroglia (Fig. 4B, C), which predict that if cells receive adequate levels of cystine via an xCT-independent mechanism they are able to increase GSH production.

Discussion

Our studies on cerebellar astroglia isolated from a mouse model of A-T (Barlow et al. 1996) have identified a novel redox defect in these cells. Coculture experiments revealed that the GSH provided by mutant astroglia is suboptimal for neuronal survival *in vitro*. We propose that the cell intrinsic failure of mutant astroglia to support neurite outgrowth and survival of either mutant or WT cerebellar neurons may contribute to the cerebellar pathology in A-T.

It has long been established that GSH provided by astroglia is critical in supporting normal neuronal survival and function (Dringen et al. 2000; Gegg et al. 2005; Lewerenz et al. 2009; Wang and Cynader 2000). We focused our initial analysis on neuronal survival of CGNs and PNs and neurite outgrowth (as a surrogate measure of neuronal health) in CGNs. While this

endpoint is reliable, easy to quantify and relevant to the human pathology that is defined by neuronal cell death, it will be of interest in the future to examine aspects of neuronal function that precede cell death in the context of A–T astroglia. For example, the deficient dendritogenesis in PNs that has been reported in two A–T mutant strains (Chen et al. 2003; Elson et al. 1996) or the nuclear accumulation of histone deacetylase 4 (HDAC4) in A–T mutant PNs that is thought to promote their degeneration (Li et al. 2012), may also be driven, at least in part, by defective astroglial function. It would also be of interest to determine whether the defect in GSH homeostasis observed here is more prominent in Bergmann glial that are closely associated with PNs or is also found in the velate protoplasmic astroglia of the granule cell layer. However, current tissue culture techniques do not allow the specific isolation of these distinct cell populations and reliable methods to measure GSH secreted from these distinct population *in vivo* have not been established.

The *in vitro* analysis of astroglia allowed us to uncover a unique disruption in GSH homeostasis with mutant astroglia showing reduced levels of xCT, GR, and GST- μ but not of γ GCS, GPx1, GSS, or the GSH exporter MRP1. This finding was surprising as these genes are thought to be coordinately regulated by the antioxidant response element (ARE) promoter and are upregulated under oxidative conditions (Lewerenz et al. 2013; Seib et al. 2011). Such a coordinated upregulation is seen in astroglia in Alexander's disease, where stress is associated with the expected increased expression of enzymes involved in GSH homeostasis (Hagemann et al. 2006).

The decrease in xCT in A–T cerebellar astroglia was associated with impaired cystine import and decreased intracellular GSH levels and is consistent with previous findings showing that astroglial cultures prefer cystine for synthesis of GSH (Kranich et al. 1998). The amount of GSH secreted by astroglia directly correlates with intracellular GSH levels (Sagara et al. 1996), and we observed decreases in the amount of secreted GSH despite these cells having normal levels of the GSH exporter MRP1. That the decrease in both intracellular as well as secreted GSH is due to a limited supply of the GSH precursor cysteine, rather than defects in GSH biosynthesis, was supported by the observation that GSH production in A–T astroglia could be substantially increased by providing cysteine via NAC, thus overriding the need for efficient xCT-transported cystine. It would have been interesting to determine whether ACM from NAC-treated mutant astroglia can rescue neurons, but it is not possible to ensure a complete elimination of residual NAC in our cultures, which would rescue neurons irrespective of the composition of the ACM. We also demonstrated that the decreased GSH levels generated by mutant astroglia were at least in part responsible for the neuronal cell death as supplementation of ACM from mutant astroglia with GSH was able to rescue the decreased neuronal survival and neurite outgrowth.

The decrease in xCT in our cerebellar mutant astroglia did not impair the *in vitro* growth of these cells as has been suggested by Shih et al. (2006), who found that cortical astrocytes isolated from xCT loss-of-function mutants did not grow *in vitro* unless β -ME, a reducing agent, was present. The cerebellar astroglia we isolated from A–T animals did not require β -ME and their growth was comparable to WT astroglia. This growth behavior not only allowed us to conduct coculture experiments but also demonstrated that the effects of genetic

loss of xCT on cortical astrocytes does not predict the impact of decreased xCT levels on astroglia from other CNS regions. It is thus perhaps not useful to draw parallels of A–T brain to xCT loss of function models that show a variety of mild to severe phenotypes depending on the specific mutations (De Bundel et al. 2011; Massie et al. 2011; Sato et al. 2005; Shih et al. 2006).

It is curious that ATM mutations, which are associated with the DNA damage response and cell cycle control, could lead to altered expression of the xCT gene. While it is tempting to speculate that the impairment of xCT in A–T mutant astroglia might be due to a deregulation of the Nrf2/ARE pathway, the canonically pathway that controls xCT (Lewerenz et al. 2009), other proteins that are coordinately regulated by this pathway (i.e., GSS, MRP1, or μ GCS Shih et al. 2003) are not altered in A–T mutant cells. In addition, expression of xCT in also changes in response to amino acid deprivation and increased cAMP (Seib et al. 2011) and the presence of interleukin-1 β (Jackman et al. 2010). As ATM serves as a central hub of cellular signaling, phosphorylating more than 700 protein substrates (Matsuoka et al. 2007), this suggests that the deregulation of xCT might be an indirect response to altered upstream signaling cascades.

Irrespective of the precise mechanisms by which loss of ATM affects xCT expression, an obvious question that arises from our studies is whether drug-induced activation of Nrf2 and thus xCT in A–T astroglia could provide a therapeutic avenue to normalize the GSH levels and overcome neuronal death as has been suggested for Parkinson disease (Chen et al. 2009) and amyotrophic lateral sclerosis (Vargas et al. 2008). Upregulation of Nrf2 in cortical primary rat astrocytes was been shown to protect cocultured neurons from oxidative stress-induced cell death (Haskew-Layton et al. 2010). Upregulation of xCT in normal non-neural cells has been shown to provide protection against tumor formation and the potential beneficial role of upregulating xCT in the eye is an intense field of investigation (see for review, Lewerenz et al. 2013). Targeting xCT in the brain is, however, highly problematic due to its dual function and its need to be tightly regulated. This exchanger provides cystine for GSH synthesis by releasing glutamate into the extracellular space and inappropriately increasing xCT might thus lead to excess accumulation of glutamate, which can also be toxic to neurons (Albrecht et al. 2010; Blanc et al. 1998; Hertz et al. 1999; Talantova et al. 2013). Thus, it appears that expression levels of xCT have to be tightly regulated and only an optimal expression level will ensure a healthy balance between increased GSH production during oxidative stress and control of extracellular glutamate.

While targeting expression of xCT might not be a viable option, our experiments show that GSH supplementation is sufficient to significantly increase the ability of conditioned medium from A–T astroglia to support neuronal survival and neurite outgrowth, thus raising the question of whether delivery of cystine or cysteine to astroglia or neurons through xCT-independent mechanisms might be of value. Treatment of A–T mice with NAC already has shown promising results outside the CNS and resulted in decreased DNA damage, ameliorated premature senescence, decreased chondrocyte hypertrophy, lowered risk of lymphoma, and prevention of bone marrow failure (Ito et al. 2007; Reliene and Schiestl 2008). While the absence of robust neurodegeneration in existing mouse models of A–T has made it difficult to assess the utilization of antioxidant therapies for human A–T *in vivo*, our

findings on isolated A–T astroglia might offer a new cellular tool to evaluate the therapeutic efficacy of different antioxidant approaches to slowing the course of neurological change, and our findings also suggest that NAC or other cysteine precursors might be particularly useful in this regard. It is becoming increasingly apparent that NAC may provide benefit in a variety of neurological conditions, including depression, autism, and treatment of addiction (Asevedo et al. 2014; Dean et al. 2011; Frye and Rossignol 2014; Ghanizadeh and Moghimi-Sarani 2013; Maes et al. 2011)), and there has been interest in trials with antioxidants in treatment of A–T. Unfortunately, it appears that none of these trials have used NAC, which our work suggests might be of particular value in such efforts.

In addition to a decrease in xCT, we also saw a selective decrease in GR and GST- μ . GR facilitates the recycling of GSH from the oxidized form GSSG, and thus, regenerates GSH at the expense of NADPH. Interestingly, inhibition of GR can occur without any alteration in the activity of GPX1 or GST- μ and thus can exacerbate the degree of GSH depletion. While it has been shown that GR activity is dependent on the levels of GSH, our data would indicate the expression of GR might also be responsible for the reduced GSH levels (Barker et al. 1996). In this context, it might be of concern that some antibiotics (e.g., cefotaxime, with a limited ability to cross the blood brain barrier; Dahyot-Fizelier et al. 2013), which might be used in A–T patients due to a high susceptibility to infections, inhibit GR on erythrocytes (Erat and Ciftci 2003) and could also negatively affect CNS GR levels.

Of particular interest is also the decrease in GST- μ . The presence of GST- μ has been shown to delay the onset of Parkinson disease and humans with a null mutation are at higher risk for the development of Alzheimer's disease and Parkinson's disease. Thus, downregulation or loss of GST- μ is itself a risk factor for the development of degenerative diseases (Mazzetti et al. 2015). Factors that affect GST- μ levels might thus be useful for being considered in the context of A–T.

In light of the defects, we found in *in vitro* cultured A–T astroglia, which seem to be mirrored in Western blots from human A–T tissue, it seems surprising that A–T mice show only a mild cerebellar defect and no overt cerebellar degeneration (Lavin 2013), the hallmark of the disease in humans. While it is not clear why mouse models fail to recapitulate the neurodegeneration, one reason might lie in the relatively short lifespan of mice. It is possible that the astrocytic defects create a progressive imbalance between GSH synthesis and glutamate release that overwhelms the system in humans over time but does not reach the threshold of the same dysfunction in short-lived mice. Interestingly a recent study on aged versus newborn *in vitro* grown astrocytes suggests that the normal aging process is associated with an upregulation of xCT (other enzymes were not measured in this study) and an increase in GSH, presumably to combat oxidative damage (Souza et al. 2015). The astrocytes we isolated from young A–T brain were already decreased in xCT levels compared to controls. It is possible that the defect in xCT would have a strong impact on aged animals as A–T astroglia might not be able to upregulate xCT and generate increased GSH levels, thus becoming progressively more vulnerable to oxidative damage. Unfortunately, A–T animal models in oxidative stress sensitive genetic backgrounds die prematurely of cancer (Genik et al. 2014), thus limiting the analysis of the impact of age on the development of cerebellar degeneration.

In summary, our work uncovered a previously unrecognized defect in A–T-derived cerebral astroglia that significantly impacts neuronal survival in coculture experiments. We have identified decreases in expression of enzymes that are critical for maintaining proper GSH homeostasis as a component of the A–T cerebellar phenotype. Our *in vitro* models, together with the specific defects, we uncovered, might offer a new *in vitro* screening platform for potential therapeutic approaches.

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FIGURE 1.

Effect of A-T and WT astroglial on cerebellar neuron survival and neurite extension. (A, B) Quantification of the total number of A-T PNs (A) or A-T GCs (B) cocultured on astroglia monolayers derived from either WT (white bar) or A-T (dark bar) cerebellum and grown for 3 days. Cells were labeled with DAPI to determine the number of live cells/well as defined by the absence of pyknotic nuclei. Shown are the average numbers of cells from independent experiments. A total of approximately 200 cells/well for PNs and 2000 cells/well for CNS were plated on day 0. Error bars represent SEM. Quantification was conducted using Student's *t*-test. * $P = 0.014$ with $n = 10$ for PNs and $P = 0.02$ with $n = 6$ for CGNs. (C, D) The left panels show representative images of A-T CGNs (labeled with anti- β -III tubulin in red), grown on WT (C) or A-T (D) astrocyte monolayers (astrocytes are labeled with anti-GFAP in green shown on the right panel). All cultures were also labeled with DAPI to

visualize nuclei. (Note: the right panel represents the triple-labeled composite. To clearly visualize neurons grown on astrocytes, the green astrocyte labeling is omitted in the left panel). (E, F) Quantification of the average length of neurites extended from (E) A–T and (F) WT CGNs cultured on WT or A–T astroglia. An average of 500 neurons from three different dissections were analyzed. Error bars represent standard error of the mean (SEM) *** $P < 0.005$ with $n = 4$ by Student's t -test.

FIGURE 2.

Effect of A-T astroglia conditioned medium on neuronal survival and outgrowth. (A, B) For these experiments, we cultured A-T (A) and WT (B) CGNs on poly-L-lysine (PLL)-coated wells in the presence of ACM and determined the number of surviving cells after 3 days in culture. ACM collected from WT astroglia served as positive control. Minimal media devoid of growth factors serves as negative control. $**P < 0.01$ by One-way ANOVA followed by Bonferroni posttest. $n = 3$ for WT CGNs and $n = 4$ for A-T CGNs. (C) Images show CGNs grown in culture and labeled with anti- β III tubulin in WT ACM and A-T ACM and represent color inversions to enhance contrast. (D, E) Quantification of average neurite length of A-T CGNs (D) and WT CGNs (E) cultured in ACM from A-T or WT astroglia. $*P < 0.05$ with $n = 3$ by one-way ANOVA followed by Bonferroni post-test.

FIGURE 3.

Decreased GSH content of A-T astroglia reduces neuronal survival. (A, B) Cerebellar A-T astroglia show a 40% decrease in (A) cellular and 85% decrease in (B) extracellular GSH relative to WT controls as measured by LC-MS/MS. ** $P < 0.01$, *** $P < 0.001$ with $n = 6$ for cell lysates and $n = 9$ for ACM by Student's t -test. *In vitro* survival of (C) A-T and (D) WT CGNs was increased by the addition of exogenous 250 μ M GSH to A-T ACM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with $n = 3$ by two-way ANOVA followed by Bonferroni post-test.

FIGURE 4.

GSH pathway proteins and mRNA expression levels are altered in A-T cerebellar astroglia and human A-T cerebellar tissue. (A) Representative immunoblots and (B) expression levels of key GSH pathway proteins in A-T cerebellar astroglia relative to WT controls. A-T cerebellar astroglia show reductions in xCT, GST-μ, and GR. (C) qPCR analysis shows decreased GR and markedly decreased xCT mRNA expression in A-T cerebellar astroglia. (D) Representative immunoblots of human cerebellar tissue show a reduction in GPx1, GR, GST-μ, and xCT in the A-T cerebellar sample relative to an age matched control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Mann-Whitney test. For immunoblot analysis, $n = 3$ (xCT, GSS, MRP1), $n = 6$ (GPx1), $n = 8$ (GST-μ), $n = 9$ (GR), and $n = 10$ (γGCS). For RNA expression analysis, $n = 4$.

FIGURE 5.

A-T cerebellar astroglia exhibit decreased L-cystine incorporation into GSH. (A) Labeled ^{13}C -L-cystine is transported into astroglia via xCT-mediated uptake and incorporated into GSH. The resulting stable isotope-labeled GSH (^{13}C GSH) can be detected within the cell or the ACM using LC-MS/MS. (B) A-T cerebellar astroglia showed a significant decrease in ^{13}C GSH in cell lysates after 18 h compared to WT controls. ** $P < 0.01$ with $n = 8$ by Student's t -test. (C) LC-MS/MS measurements of ^{13}C GSH in ACM from A-T astroglia showed significantly less ^{13}C GSH compared to control following an 18-h treatment with $^{13}\text{C}_6^{15}\text{N}_2$ L-cystine. *** $P < 0.001$ with $n = 8$ by Student's t -test.

FIGURE 6.

NAC increases GSH production in A–T cerebellar astroglia. (A, B) Addition of the alternative cysteine source, NAC, to A–T cerebellar astroglia leads to a statistically significant increase in intracellular 12C GSH (A) and trend toward increased 13C GSH (B) content as measured by LC-MS/MS $*P < 0.05$, with $n = 8$ for control samples, $n = 4$ for NAC- and Trolox-treated astroglia by one-way ANOVA followed by Bonferroni posttest. (C, D) Addition of NAC or Trolox to mutant A–T astroglia led to a slight increase in total GSH in the ACM at this time point but did not reach statistical significance. Addition of Trolox has no effect on the total GSH levels in the ACM ($P > 0.05$) with $n = 8$ for control samples, $n = 4$ for NAC- and Trolox-treated astroglia by one-way ANOVA followed by Bonferroni post-test.