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Antisense suppression of glial fibrillary acidic protein as a treatment for Alexander disease

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Abstract

Objective—Alexander disease is a fatal leukodystrophy caused by autosomal dominant gain-of-function mutations in the gene for glial fibrillary acidic protein (GFAP), an intermediate filament protein primarily expressed in astrocytes of the central nervous system. A key feature of pathogenesis is over-expression and accumulation of GFAP, with formation of characteristic cytoplasmic aggregates known as Rosenthal fibers. Here we investigate whether suppressing GFAP with antisense oligonucleotides could provide a therapeutic strategy for treating Alexander disease.

Methods—In this study we use GFAP mutant mouse models of Alexander disease to test the efficacy of antisense suppression and evaluate the effects on molecular and cellular phenotypes and non-cell autonomous toxicity. Antisense oligonucleotides were designed to target the murine *Gfap* transcript, and screened using primary mouse cortical cultures. Lead oligonucleotides were then tested for their ability to reduce GFAP transcripts and protein, first in wild-type mice with normal levels of GFAP, and then in adult mutant mice with established pathology and elevated levels of GFAP.

Results—Nearly complete and long-lasting elimination of GFAP occurred in brain and spinal cord following single bolus intracerebroventricular injections, with a striking reversal of Rosenthal fibers and downstream markers of microglial and other stress related responses. GFAP protein was also cleared from cerebrospinal fluid, demonstrating its potential utility as a biomarker in future clinical applications. Finally, treatment led to improved body condition and rescue of hippocampal neurogenesis.

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Author contributions

TLH, BP, CM, GH, ES, and AM contributed to the conception and design of the study. TLH, BP, AK and SW contributed to the acquisition and analysis of data. TLH and BP contributed to preparing the figures, and all authors contributed to drafting the text.

Potential Conflicts of Interest

BP, CM, AK, GH and ES receive salaries from and are shareholders in Ionis Pharmaceuticals. TLH, SW, and AM have no conflicts of interest.

Interpretation—These results demonstrate the efficacy of antisense suppression for an astrocyte target, and provide a compelling therapeutic approach for Alexander disease.

Alexander disease (AxD) is a rare and generally fatal disorder resulting from dominant missense mutations in GFAP, the major intermediate filament protein of astrocytes.¹ Expression of mutant GFAP initiates a cascade of effects within astrocytes that lead to cytoplasmic stress protein aggregates, known as Rosenthal fibers (RFs). Reactive gliosis and astrocyte dysfunction lead to a variety of secondary changes in neurons and other types of glia.² Indeed, because GFAP expression in the adult is almost entirely restricted to mature astrocytes, AxD has become a prototype for understanding how primary dysfunction of astrocytes impacts the other cells of the central nervous system. At present, there is no effective treatment.

Human GFAP contains 432 amino acids, and disease-causing mutations occur throughout the rod and tail domains, involving > 70 of these amino acids. Nearly all are point mutations, or short in-frame insertions or deletions, and occur in the heterozygous state (<http://waisman.wisc.edu/alexander/mutation-table.pdf>). In contrast to most mutations of other intermediate filaments, which lead to loss-of-function,³ no null mutations for GFAP have ever been found in human disease, and the *Gfap*-null mouse has a relatively mild phenotype.^{4–6} Based on mouse models and cell cultures, GFAP mutations associated with Alexander disease (AxD) appear to produce a gain-of-function,² and a key step in pathogenesis is elevation of GFAP to a threshold that causes toxicity.^{7, 8} In both mouse^{8–10} and human,^{11, 12} the levels of GFAP roughly correlate with disease severity (or in the case of humans, more precisely defined as age of onset).

Given the large number of disease-causing mutations, and the apparent minimal consequence of the null state in the mouse, we previously proposed general suppression of GFAP as an approach for therapy.¹³ We have conducted screens to identify existing drugs capable of general suppression, using primary cultures of mouse astrocytes.¹⁴ The resulting hits (such as clomipramine) were active in wild type mice but subsequently failed in mutant mice. Other known drugs under study such as ceftriaxone (for glutamate transporters) and lithium (to increase autophagy) either failed completely or were only modestly effective with unacceptable side effects.^{15, 16}

As an alternative, antisense oligonucleotides (ASO) are rapidly becoming a realistic option for manipulating gene expression in the central nervous system (CNS).^{17, 18} In mouse models of Huntington's disease, the use of ICV infusion to bypass the blood brain barrier led to long-lasting suppression by relatively short term treatments.¹⁹ ASOs for the treatment of spinal muscular atrophy (SMA) recently passed through Phase III clinical trials and have been approved by the FDA.^{20, 21} However, little is known about the accessibility of astrocytes to ASO delivery and the amenability of a highly-expressed astrocyte target to ASO suppression.

In this report, we use ASOs to suppress GFAP in mouse models of AxD with remarkable results. Although rare, AxD is one of only a few primary disorders of astrocytes, and as

such, specifically demonstrates astrocyte responsiveness to ASO treatment. More importantly, this is the first significant advance toward therapy for this devastating disease.

Methods

Mice

Mice of various strains were used in this study. Wild-type strains included C57BL/6J (Jackson Laboratory) and FVB/N (Taconic). Mice engineered with point mutations at the *Gfap* locus included the *Gfap^{tm3Mes}* and *Gfap^{tm2Mes}* lines, carrying the R76H and R236H mutations, respectively, were maintained as heterozygotes congenic in the FVB/N or B6 genetic backgrounds.⁸ Reporter mice created with an exogenous transgene consisting of 12 kb of the murine *Gfap* promoter driving a luciferase gene²² were maintained as hemizygotes (FVB/N-Tg(*Gfap-luc*)53Xen). All experiments were carried out with mice in the FVB/N background unless noted otherwise. Mice were housed with ad libitum access to food and water under standard 12:12 hour light cycles. Both males and females were used as indicated per experiment. Mice were assigned successive ID numbers in order of date of birth and sex (males then females) after weaning and before genotyping. After genotyping, to balance treatments per genotype, sex, cage, and litter, treatment assignments were alternated (control/ASO) in order of ID number within each genotype. All studies were approved by Institutional Animal Care and Use Committees at the University of Wisconsin-Madison and Ionis Pharmaceuticals respectively, and were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Design and Screening for efficacious ASOs targeting mouse *Gfap*

Second generation 18mer 5-8-5 MOE gapmer ASOs targeting murine GFAP were designed and synthesized. ASOs consisted of 2'-O-methoxyethyl (MOE) modifications on 5 nucleotides each on the 5' and 3' ends to increase binding affinity with target RNA and resistance to nucleases, with 8 unmodified central "gap" deoxynucleotides to support RNase H1 activity.¹⁷ Four internucleoside linkages were phosphodiester at positions 2–3 and 14–15, and all other linkages were phosphorothioate to promote resistance to nucleases and uptake into tissues. For *in vitro* screening, embryos were extracted from E16 timed pregnant CD1 mice. Cortical and striatal tissue was isolated from the embryos into ice-cold Hank's Balanced Salt Solution (Life Technologies). The tissue was digested using 0.05% Trypsin/EDTA (Life Technologies) at 37°C for 8 minutes. Digestion was halted using Neurobasal media containing B27, GlutaMAX and pen/strep (Life Technologies). After trituration, cells were spun down, resuspended in complete Neurobasal media and plated on poly-D-lysine coated plates (Thermo Scientific). Cells were treated with ASOs resuspended in water at a final concentration of 8 µM and incubated at 37°C/5% CO₂ for 3 days. RNA was purified with a silica filter plate (Qiagen #74192) per manufacturer's instructions. GFAP message levels were quantified with RT-qPCR on Step One instruments (Thermo Fisher). Briefly, 20 µL RT-qPCR reactions containing 5 µL of RNA were run with Agpath-ID reagents and the primer probe sets listed in Table 1 following the manufacturer's instructions. Total RNA levels were measured with the Quant-iTTM RiboGreen[®] RNA reagent and used to normalize the GFAP data. Ten ASOs that showed superior GFAP mRNA suppression *in vitro* were selected for *in vivo* screening. They were administered individually in WT C57BL/6J mice

(Jackson Labs) as a single 500 µg dose by intracerebroventricular (ICV) injection at 8–9 weeks of age. The top 3 ASOs with greater than 90% GFAP mRNA reduction at 2 weeks post-ICV injection were selected for further characterization. These were administered as a single 30–300 µg ICV bolus injection to test for efficacy and dose responsiveness 2 weeks post-ICV. Duration of action studies were performed 1–16 weeks following a single ICV injection of 300 µg. qRT-PCR was used to assess *Gfap* mRNA reduction. These three ASOs were also used in an AxD mouse model (*Gfap*^{+R236H}) along with saline (vehicle) or a non-specific ASO control. ASO sequences were as follows: control, 5'-CCTATAGGACTATCCAGGAA-3'; ASO-4, 5'-CTTTATTTCTGCCCAGTG-3'; ASO-5, 5'-TGTCTTTATTTCTGCCCA-3'; ASO-9, 5'-GGAGAGCTGGAGCACGCC-3'. The control ASO also has 5 flanking 2'-MOE-nucleotides on each end. ASO-4 and -5 target the 3' UTR of the major *Gfap*-α isoform, while ASO-9 localizes to intron 7a and targets both *Gfap*-α and δ isoforms.

Delivery of ASOs

ASOs were dissolved at 100 mg/mL in sterile phosphate buffered saline (PBS) or 0.9% sterile saline and delivered by a single intracerebroventricular (ICV) bolus injection into the right lateral ventricle of adult mice. Anesthetized animals were positioned in a stereotaxic apparatus and scalps prepared by cleaning the clipped area with Betadine and 70% ethanol rinse. A 1–1.5 cm, slightly off-center incision was made in the scalp. A Hamilton syringe primed with 10 µL of injection solution was positioned over bregma. The needle of the syringe was moved to the appropriate anterior/posterior and medial/lateral coordinates. For ICV bolus injections the coordinates 0.3 mm anterior/posterior (anterior to bregma) and 1.0 mm medial/lateral to the right are used. The needle tip was advanced through the skull to –3.0 mm from the skull surface. The proper amount of injection solution (10 µL) was injected by hand at approximately 1 µL/second. After 3 minutes, the needle was withdrawn from the skull and the incision sutured closed.

Collection of CSF

CSF was collected from mice according to the method of DeMattos et al.²³ Mice were anaesthetized with a mixture of ketamine, xylazine, and acepromazine, via IP injection. A midline sagittal incision was made over the dorsal aspect of the hindbrain and three muscle layers carefully peeled back to expose the cisterna magna. The membrane covering the cisterna magna was pierced with a borosilicate glass micropipette, and CSF was collected immediately. Approximately 10 µL of CSF was collected per animal, and stored at –80°C until further processing.

RNA analysis

Animals were sacrificed by CO₂ asphyxiation and CNS regions microdissected and rapidly frozen on dry ice. For the initial dose response and time course experiments in wild-type mice (Fig 1), tissue was homogenized with sterilized porcelain beads (RayTech Industries) by vigorous shaking in a Precellys 24 tissue homogenizer (Bertin Technologies) in 5–10 volumes of guanidine isothiocyanate solution, pH 6.0 (Invitrogen, Thermo Fisher Scientific)/8% beta-mercaptoethanol (Sigma-Aldrich). RNA was isolated from 40 µL of tissue homogenate using the RNeasy 96Kit (Qiagen) according to the manufacturer's

instructions. Isolated RNA was prepared for qRT-PCR using the EXPRESS One-Step Superscript kit (Thermo Fisher) and analyzed on the StepOne Plus Real-Time PCR System (Thermo Fisher) for mRNA expression of *Gfap* and cyclophilin A (*Ppia*), a housekeeping gene used for normalization. Custom primer/probe sets (Integrated DNA Technologies) are listed in Table 1.

For additional experiments in mutant mice (Fig 2, 5), tissues were homogenized in Trizol and processed according to the manufacturer's protocol (Invitrogen, Thermo Fisher). Contra- and ipsilateral regions were homogenized separately. Complementary DNA was synthesized from 1 µg total RNA with Superscript III (Invitrogen, Thermo Fisher), and quantitative PCR performed with SYBR Select Master Mix on a ViiA7 Real Time PCR System (Applied Biosystems, Thermo Fisher). Primer sets are listed in Table 1, and values were normalized to 18S rRNA (*Rn18s*). No differences were observed between contra- and ipsilateral regions, and data represent either both regions or contralateral only.

GFAP protein analysis

To quantify GFAP protein in brain and spinal cord, tissues were collected as indicated above and lysates prepared from contralateral regions according to a modified protocol from Heaven et al.²⁴ Briefly, tissues were homogenized (1:20, w/v) in 0.5% Triton-X-100 in TEE (20 mM Tris-HCl (pH7.4), 2 mM EDTA, 1 mM EGTA, 1 mM PefablocSC) with Complete Protease Inhibitor Cocktail (Roche, Sigma), using a bead mixer mill (GenoGrinder, SPEX SamplePrep), and centrifuged at 17,000 g for 20 min at 4°C. The supernatant was collected as the Triton-X-100 soluble fraction. The insoluble pellet was resuspended in an equivalent volume of 6M urea in TEE and centrifuged at 3,000 g for 10 min at 4°C. The supernatant was collected as the Triton-X-100 insoluble fraction (urea soluble), and the RF enriched pellet was resuspended in 2% SDS in TEE and boiled for 30 min.

Protein from each fraction was quantified by BCA assay and diluted in PBS with 1 mg/ml BSA and 0.05% Tween 20 as follows: approximately 1 µg/ml Triton-X-100 soluble, 0.01 µg/ml insoluble, and 0.1 µg/ml RF enriched protein. GFAP ELISAs were performed as previously described for both protein lysates and CSF.¹⁰

Histology and immunostaining

For immunofluorescence in the initial time course study in wild-type mice (Fig 1), animals were sacrificed with CO₂ asphyxiation, brains collected and bisected at the midline. The contralateral half was immersion fixed in neutral buffered formalin, processed, embedded in paraffin and sectioned onto slides at 3 µm. Slides were deparaffinized, antigen retrieval was performed in citrate buffer at 100° C, and sections blocked in 5% normal goat serum/1% BSA in PBS for 30 min. Sections were incubated with 1:1000 primary rabbit anti-GFAP antibody (Dako, Z0334) in block for 1 hour at room temperature, rinsed in PBS, and incubated with secondary fluorescent antibody (Thermo Fisher) for 30 min at room temperature. Slides were rinsed in PBS, with one final rinse in water, dried briefly under an oscillating table fan, and images were generated using a microarray scanner (Agilent).

For histology in mutant mice (Fig 4B–G), brains were collected immediately after CSF collection and decapitation. Tissues were immersion fixed in methacarn and processed for

paraffin sections and H&E. For immunofluorescence in mutant mice (Fig 7C–D), animals were anesthetized with isoflurane and transcardially perfused with phosphate buffered saline. Brains were collected and bisected at the midline and the ipsilateral half immersion fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected in sucrose, and 30 µm sections collected on a sliding microtome. Floating sections were blocked in 5% normal goat or donkey serum with 0.3% Triton-X-100 in PBS for 2 hours and incubated with primary antibodies, including rabbit anti-Iba1 (Wako, 019–19741) or goat anti-doublecortin (Santa Cruz Biotech, sc-8066), diluted 1:500 in 1% BSA/0.3% TX-100 for 72 hrs at 4°C. After rinsing in PBS with 0.05% TX-100 (3 times), sections were incubated with secondary antibodies overnight (Alexa Fluor 488 conjugated goat anti-rabbit or donkey anti-goat at 1:500; Invitrogen, Thermo Fisher), rinsed and mounted on slides with ProLong Gold with DAPI (Invitrogen, Thermo Fisher). Fluorescent images were taken with a Nikon A1R-Si confocal microscope at equivalent gain and laser power within each experiment.

Luciferase assay

Tissues from contralateral regions were homogenized (1:20, w/v) in Glo Lysis Buffer (Promega), and protein quantified by BCA assay. Luciferase activity was measured with a Turner Glo Runner Luminometer using 40 µl of lysate and 40 µl One Glo Plus substrate in microtiter plate. Relative luminescent units were normalized to total mg protein. To measure total GFAP protein, SDS was added for a final concentration of 2% and lysates boiled for 20 min. Protein was quantified and diluted to 0.5 – 2 µg/ml for GFAP ELISA as described above.

Western analysis

For ubiquitin westerns, 3 µg of RF enriched protein (described above for ELISA) was analyzed per sample. For pSTAT3 westerns, total protein lysates were prepared by homogenizing tissues (ipsilateral) in 2% SDS, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM PefablocSC with Complete Protease Inhibitor Cocktail, and boiling for 20 min. Protein was quantified by the BCA assay and 30 µg loaded per lane on a 10% Criterion precast TGX gel (BioRad). Protein was transferred to Immobilon-FL PVDF membrane (EMD Millipore), stained with REVERT Total Protein Stain Kit, and imaged on an Odyssey infrared imaging system (LI-COR). Immunoblots were blocked with SeaBlock (Pierce, Thermo Fisher), and incubated with mouse anti-ubiquitin (1:500, Chemicon MAB1510) or rabbit anti-phospho-STAT3-Tyr705 antibody (1:2,000, Cell Signaling 9145) in TBS with 0.05% Tween 20 (Pierce, Thermo Fisher 28360). Immunoblots were rinsed 3 times in TBS/Tween20 and probed with DyLight 800 conjugated goat anti-mouse and -rabbit (Thermo Fisher SA5-10176, 35571) for 2 hrs at room temperature. After washing 3 times with TBS/Tween20 followed by PBS, blots were dried and imaged with an Odyssey infrared imager.

Statistics

Statistical analysis was performed with GraphPad Prism 6 software. For experiments analyzing RNA and protein, an unpaired 2-tailed t-test was used to compare treated and untreated mice. A two-way ANOVA was used for comparisons of body weights between treatments and genotypes. Tests for each experiment are indicated in the figure legends. P values less than 0.05 were considered significant.

Results

Identifying active ASOs targeting mouse *Gfap*

To test astrocyte responsiveness to ASO treatment and whether GFAP suppression could be an effective strategy for treating AxD, ASOs were designed against mouse *Gfap*, and screened in primary mouse cortical cultures for efficacy. Three of the most promising candidates demonstrated over 50% suppression of *Gfap* transcript *in vitro* (Fig 1A–B), and were tested further in wild-type (WT) mice via stereotaxic injection into the lateral ventricle. The lead ASOs (depicted in Fig 1A) elicited dose responsive and significant reduction of cortical *Gfap* mRNA (up to 77% reduction compared to vehicle-treated controls) 2 weeks following a single ICV bolus injection (Fig 1C), with ASO-4 and –5 being slightly more effective than ASO-9. For further comparison, ASO-5 and –9 were subjected to a more detailed time course study, testing animals from 1–16 weeks following a single injection. Reduction of cortical *Gfap* mRNA was maximal at 4 weeks post-injection (93%) and persisted to a lesser extent (60%) through the duration of the experiment. Reduction of *Gfap* mRNA in spinal cord reached >95%, which was sustained through the entire 16 week period (Fig 1D). GFAP protein was assessed in mice at 16-weeks post-injection by immunofluorescent staining of sagittal sections using an anti-GFAP antibody, which demonstrated clear reduction at the protein level across all areas examined (Fig 1E).

Gfap targeted ASOs reduce elevated GFAP transcript and protein in mouse models of AxD

To determine whether *Gfap* targeted ASOs could suppress the markedly higher levels of transcript and protein observed in mouse models of AxD, we next tested the effects of treating adult mice after pathology and gliosis are well established.⁸ In the *Gfap*^{+/R236H} mouse model, Rosenthal fibers (RFs) are prominent by p21 in hippocampus, olfactory bulb and corpus callosum, as well as subpial, perivascular and periventricular regions. Based on the results from wild-type mice, AxD mice and littermate controls were treated with the more effective ASO-5 or saline at 12 weeks of age, and brain and spinal cord were collected 2 weeks post-injection. ASO treatment again suppressed *Gfap* transcript in *Gfap*^{+/R236H} mice to nearly undetectable levels, and below those of WT littermates receiving saline, in all CNS regions tested (Fig 2A). Comparable results were observed after treating *Gfap*^{+/R236H} mice and a second mutant line, *Gfap*^{+/R76H}, with ASO-4 (Fig 2B). We tested for specificity by analyzing the mRNA for another type III intermediate filament, peripherin, which exhibits 67–70% identity at the RNA level within the GFAP rod domain, and found no evidence of suppression (Fig 2C). In addition, treatment with a nonspecific control ASO did not alter *Gfap* transcript levels in either WT or mutant mice when compared with saline treated mice (Fig 2D).

We next tested the degree to which GFAP protein levels in the mutants were reduced, given that the starting point is much higher than WT and the normal half-life of GFAP *in vivo* is on the order of 1–2 months.^{25–27} Using the biochemical criteria of Heaven et al.,²⁴ we analyzed soluble, insoluble and RF-enriched fractions (see Methods) after single ASO injections into adult mice. *Gfap*^{+/R236H} mice were treated with ASO-5 at 8 weeks of age, and brain and spinal cord collected 8 weeks post-injection. To our surprise, GFAP was dramatically reduced in all 3 protein fractions in all regions analyzed, including

hippocampus, olfactory bulb, cerebral cortex and cervical spinal cord (Fig 3). GFAP levels for animals treated with ASO were either below that of saline treated *Gfap*^{+/+} mice or at the lower limit of detection for the assay. Western analysis of the RF enriched fraction confirmed that elevation of ubiquitinated proteins in mutant mice is normalized by GFAP suppression (Fig 4A). In addition, histopathology demonstrated clearance of RFs (Fig 4B–I).

Elevation of GFAP in AxD brain parenchyma is also reflected in cerebrospinal fluid (CSF), suggesting that GFAP itself could serve as a useful biomarker to monitor therapeutic response to ASOs.^{10, 12} We therefore measured GFAP levels in CSF from adult *Gfap*^{+/R236H} mice 8 weeks after treatment with ASO-5. GFAP remained elevated in all mutant mice treated with saline, whereas those receiving ASO had no detectable GFAP in CSF (Fig 4J).

Suppression of GFAP in AxD mice mitigates molecular, cellular, and clinical phenotypes

To test whether reduced levels of GFAP transcript and protein alleviate additional phenotypes associated with the R236H mutation, we first measured stress and immune-related transcripts known to be elevated in AxD mice or otherwise associated with gliosis. Elevation of lipocalin-2 (*Lcn2*) is a consistent feature of astrogliosis,²⁸ and *Lcn2* is also elevated in *Gfap*^{+/R236H} mice. Similarly, small chemokines *Ccl2* and *Cxcl1*, microglial *Itgb2* (Mac1), the phase II antioxidant response target *Nqo1*, and the acute-phase protein ceruloplasmin (*Cp*) are also elevated (Fig 5). At two weeks post-treatment with ASO-5, a time point when *Gfap* mRNA is clearly suppressed, all of these transcripts were reduced in most regions analyzed. *Itgb2* expression was only significantly reduced in olfactory bulb, suggesting that the secondary response by microglia may take longer to normalize. Taken together the transcription profile indicates astrocytes are beginning to recover as early as 2 weeks post-treatment.

To further assess the extent of astrocyte and CNS recovery, we next analyzed AxD phenotypes at a time-point when we know GFAP protein is reduced. Transactivation of the *Gfap* promoter is another feature of the astrocyte stress response, and contributes to the toxic elevation of GFAP expression.^{10, 22} We therefore crossed *Gfap*^{+/R236H} mice with a *Gfap*-luciferase reporter and analyzed mutant and WT littermates 8 weeks after treatment with ASO-4. Analysis of hippocampus, olfactory bulb, and cortex in ASO versus saline-treated mutant mice indicated a significant reduction in *Gfap* promoter activity in all regions (Fig 6A). Total GFAP protein levels were also measured and those from ASO-4 treated mice were at or below levels from saline treated WT mice (Fig 6B) similar to results shown for ASO-5 (Fig 3). Furthermore, a simple assessment of body weights at 8 weeks post treatment shows normalization for ASO-treated mutant mice (Fig 7A).

We recently demonstrated that the signal transducer and activator of transcription, STAT3, is activated in *Gfap*^{+/R236H} mice,¹⁶ and thus could serve as a transcriptional activator of many downstream pathways. AxD mutant mice treated with ASO-5 show reduced STAT3 activation in hippocampus at 8 weeks post-injection, as indicated by western analysis of phospho-Tyr705 (Fig 7B). There was no change in pSTAT3 in WT mice treated with ASO versus saline. To determine whether the reduction in proteotoxic stress in astrocytes eventually leads to a decrease in secondary effects on other cell types, we evaluated the microglial response by immunostaining for Iba1 at 8 weeks post-treatment. ASO treated

mutant mice showed a clear decrease in activated microglia compared with those treated with saline, with no apparent increase in ASO treated WT mice (Fig 7C).

Previously we reported that *Gfap*^{+R236H} mice have reduced numbers of proliferating progenitors in the hippocampal subgranular zone (SGZ), and virtually no new neurons as indicated by fate analysis and doublecortin (DCX) staining.²⁹ To test for rescue of this phenotype, we analyzed ASO-treated mice for hippocampal DCX expression at 8 weeks post-injection. For these experiments, to avoid potential confounding effects of skewing the ratio of Gfap isoforms, we used ASO-9 to target both the major α isoform and Gfap δ (Fig 1A) which is prominent in progenitor cells.³⁰ Immunostaining showed a clear increase of DCX in the granule cell layer of the hippocampus in ASO-treated mutant mice compared to those treated with saline (Fig 7D). Both mutant and WT mice treated with ASO show similar staining of DCX positive neurons compared to WT mice treated with saline.

Discussion

AxD is caused by dominant gain of function mutations in the gene for GFAP that lead to GFAP accumulation, protein aggregation, gliosis, and non-cell autonomous toxicity in the CNS. We propose that GFAP knockdown using antisense technology is a viable approach for treating AxD. In this report, we have shown that bolus delivery of ASOs to the ventricular system of adult mice results in rapid and long-lasting suppression of GFAP mRNA and protein. The same treatment administered to AxD-mice with elevated levels of GFAP and established astrocyte pathology, reduces both mRNA and protein below wildtype levels, reverses pathology, and rescues downstream markers of disease.

Mutant GFAP activates stress and immune related responses that lead to astrogliosis and the associated transactivation of *Gfap* transcription.^{8, 10, 31} Previous work using cell culture models of AxD suggested that proteasome function is also impaired, likely by GFAP oligomers in the soluble fraction.³² Studies with brain lysates from mouse models and patients with AxD showed a marked increase in high-molecular weight ubiquitinated proteins, again implying proteasomal dysfunction.¹¹ We have hypothesized that elevated transcription and reduced degradation are both culprits in the accumulation of GFAP. Since even normal GFAP is a long-lived protein, with a half-life *in vivo* estimated at 1–2 months^{25–27}, a key question is whether antisense suppression of *Gfap* transcript can be sustained for a long enough period to allow for protein degradation. Our results show that ASO targeted transcript suppression leads to nearly complete elimination of GFAP protein, including insoluble aggregate forms, within a relatively short period of time. These results are consistent with our recent finding that the turnover rate of GFAP in mutant mice is actually faster than in wild-type.²⁵ The rapid clearance of GFAP and other ubiquitinated proteins suggests that the proteasome system can recover once new mutant protein is no longer being produced. Experiments are underway to determine a more specific timeline of when GFAP mRNA and protein decrease and return after ASO treatment. Although we do not understand the mechanism behind the weight differences in these mice, it will be interesting to see if weight loss occurs upon the return of mutant GFAP.

The AxD model mice used in the current study, designed with knock-in mutations in the endogenous *Gfap* gene, display many features in common with the human disease, at both the pathological (Rosenthal fibers, mislocalization of TDP-43) and biochemical (activation of stress pathways, inhibition of the proteasome, changes in expression of cytokine and transporter genes) levels. Nevertheless, they have no evident changes in white matter, and thus do not have a leukodystrophy. Some have suggested that these models more closely resemble the adult or Type II forms of the disease,^{33, 34} and it is an open question whether GFAP suppression can effectively correct myelin deficits such as those that are prominent in Type I patients.

Gfap transcripts include several different isoforms with α and δ being the most prominent in the CNS.³⁰ Two of the ASOs in this study (ASO-4, -5) target the major GFAP isoform α , whereas the third (ASO-9) targets both α and δ (Fig 1A). Although *in vitro* analysis has shown that shifting the ratios between α and δ can lead to GFAP aggregation,³⁵ our experiments suggest this is not the case *in vivo*. Both ASO-4 and -5 cleared protein aggregates, and led to similar phenotypic outcomes as ASO-9 (Fig 7A). Given that the role of GFAP δ is still unknown, these ASOs provide the tools for manipulating *Gfap* isoforms *in vivo* and for additional studies to confirm whether both isoforms should be targeted for therapy in humans.

Elimination of GFAP, whether by genetic knockout or ASO targeting, has no obvious detrimental effects in mice under normal circumstances. Here we show improved body condition in AxD mutant mice with two different ASOs. However, there are studies of genetic knockouts showing increased susceptibility to infection, injury, kainate induced seizures and experimental autoimmune encephalomyelitis.^{6, 36–40} While the current work demonstrates proof of concept, one goal of our continued pre-clinical studies is to titrate the dose of ASO and determine the level of suppression necessary to achieve clinical improvement. Similar experiments have been performed in wild-type animals, but given the differences in protein level and turnover rate, dose response studies in mutant animals will be essential. In addition, it will be important to assess improvements in behavioral phenotypes such as the cognitive deficits observed in models of AxD.²⁹

Another key finding in this study was the concomitant reduction of GFAP in CSF, indicating that GFAP itself could be used as biomarker to assess ASO efficacy and duration of suppression.^{10, 12} How closely linked the temporal changes in CSF are to those in brain remains to be determined, but these results suggest progress could be monitored longitudinally. We have also reported that GFAP is elevated in blood of some patients with AxD¹², and improved methods for astrocyte exosome purification^{41–43} may increase the utility of this more accessible body fluid for analysis.

Antisense therapy has become a realistic approach for treating neurodegenerative disease, and transcript targeting strategies range from RNase H mediated degradation, as demonstrated in this and other reports, to other applications that alter splicing and restore functional protein as in SMA.¹⁸ The mechanism of cellular uptake of ASOs is also varied. Although the process commonly involves clathrin or caveolin dependent mechanisms, ASOs may also be taken in by nonconventional endocytic pathways, and the route of entry can

affect efficacy.⁴⁴ Although ASO uptake by astrocytes has been shown previously,⁴⁵ here we use a primary disorder of astrocytes to demonstrate the efficacy of ASOs in both normal and dysfunctional reactive astrocytes. With the increasing interest in targeting astrocytes in other disorders of the CNS,^{46, 47} these results demonstrate that antisense therapy can alleviate the non-cell autonomous toxicity mediated by dysfunctional astrocytes.

In summary, these studies demonstrate ASO efficacy in astrocytes, open up new approaches for investigating the biology of intermediate filaments *in vivo*, and most importantly provide the foundation for developing GFAP therapeutics for patients with AxD.

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FIGURE 1.

GFAP targeted ASOs produce effective and long-lasting reduction of GFAP in WT mice. (A–C) Three lead GFAP targeted ASOs were identified in an *in vitro* screen, and further evaluated for dose responsiveness *in vivo*. (A) A diagram of the murine *Gfap* gene shows the location of ASOs 4 and 5 in the 3'UTR of *Gfap* α and ASO-9 in the 3'UTR of *Gfap* δ located within intron 7 of the α isoform. (B) Quantitation of *Gfap* transcript in ASO treated primary mouse cortical cultures shows more than 50% reduction for each of the lead ASOs compared to untreated controls (C, n = 11; ASOs, n = 2). (C) Administration of these ASOs to WT mice conferred dose-responsive and potent reduction of target mRNA as shown in cortex 2 weeks following a single ICV bolus injection of 30–300 μ g (n = 2 animals per dose). (D) A time course study demonstrates potent and prolonged GFAP mRNA suppression in the spinal cord (open circles and dotted lines) and cortex (closed circles and solid lines) following a single ICV bolus injection of 300 μ g (n=4 animals, error bars = standard deviation). (E) Scans of sagittal brain sections immunofluorescently labeled for GFAP demonstrate prolonged and widespread protein reduction 16 weeks post-ICV. All mice were treated between 8 and 9 weeks of age. *Gfap* transcript values (B–D) represent a percent of the average transcript measured in untreated cells (B, n = 11) or vehicle treated mice (C, D, n = 4).

FIGURE 2.

GFAP targeted ASOs reduce *Gfap* transcript in mouse models of AxD. **(A)** To assess transcript suppression, *Gfap*^{+/R236H} (squares) and *Gfap*^{+/+} (circles) littermates were treated with 500 µg ASO-5 or saline at 12 weeks of age, and spinal cord and brain regions analyzed 2 weeks post-injection (n = 2–3, males and females). **(B)** Treatment of two different GFAP point mutant lines with ASO-4 shows similar suppression in hippocampus. **(C)** Peripherin transcript levels demonstrate specificity of *Gfap* knockdown compared to another type III intermediate filament. (Asterisks represent 2-tailed unpaired t-test comparing control versus ASO treated *Gfap*^{+/R236H} mice **p<0.01, ***p<0.001, ****p<0.0001). **(D)** Comparison of *Gfap* transcript suppression by targeted ASOs 4, 5, and 9 versus a non-specific control ASO in hippocampus from WT and R236H mice (p < 0.0001, one-way ANOVA; asterisks indicate significant differences by Bonferroni post-tests comparing ASO to saline treated animals).

FIGURE 3.

GFAP targeted ASOs reduce GFAP protein in mouse models of AxD. To assess GFAP protein, mice were treated with 500 μg ASO-5 or saline at 8 weeks of age, and CNS regions collected at 8 weeks post-treatment. Tissue lysates were fractionated into Triton-X-100 soluble, Triton insoluble (urea soluble), and RF-enriched (SDS soluble) fractions, and GFAP protein measured by ELISA. Asterisks represent 2-tailed unpaired t-test comparing saline versus ASO treated *Gfap*^{+/R236H} mice (*p < 0.05, **p < 0.01, ****p < 0.0001, n = 3–4 for comparison).

FIGURE 4.

GFAP targeted ASOs reverse Rosenthal fiber formation and reduce CSF-GFAP. (A) Western analysis of RF-enriched fraction shows a decrease in ubiquitinated protein in hippocampus from ASO-5 treated *Gfap*^{+/R236H} mice compared with those treated with saline (**p = 0.0007, 2-tailed unpaired t-test comparing saline and ASO treated *Gfap*^{+/R236H} males, n = 3–4). (B–I) H&E staining in hippocampus (B, D, F, H) and olfactory bulb (C, E, G, I) demonstrates the presence of RFs in naïve *Gfap*^{+/R236H} mice prior to treatment (arrows, D, E) compared with WT *Gfap*^{+/+} mice at 8 weeks of age (B, C). (F–I) At 8 weeks after treatment, virtually no RFs are apparent in mice injected with ASO (H, I) compared to those treated with vehicle (arrows, F, G). N = 3 male *Gfap*^{+/R236H} mice injected with 300 µg ASO-5 or saline at 8 weeks of age, collected 8 weeks post-injection, scale bar = 50 µm. (J) GFAP protein in CSF collected from *Gfap*^{+/R236H} mice as measured by ELISA (n = 5–7 males treated with saline or 300 µg ASO-5 at 8 weeks of age, collected 8 weeks post-injection. **p = 0.0043, 2-tailed unpaired t-test).

FIGURE 5.

Suppression of *Gfap* transcript leads to early reduction in stress response. Fold-change for stress and immune-related transcripts in brain and spinal cord from ASO and saline-treated *Gfap*^{+/R236H} mice compared to saline-treated *Gfap*^{+/+} mice (log 2 scale, n = 3 mice treated at 12 weeks, 500 µg ASO-5, 2 weeks post-treatment). Asterisks 2-tailed unpaired t-test comparing saline versus ASO-treated *Gfap*^{+/R236H} mice, *p<0.05, **p<0.01, and ***p<0.001. Error bars = standard deviation.

FIGURE 6.

Diminished *Gfap* promoter activity coincides with reduction of total GFAP protein. (A) Luciferase activity in brain regions from *Gfap*^{+/+} and *Gfap*^{+/R236H} mice crossed with a *Gfap*-reporter line treated with 300 µg ASO-4 at 8 weeks of age (8 weeks post-treatment, n = 5–6 males). (B) Total GFAP protein levels (log 2 scale) as determined by ELISA for same animals shown in A. Asterisks in A and B represent 2-tailed unpaired t-test comparing control versus ASO treated *Gfap*^{+/R236H} mice, ****p<0.0001.

FIGURE 7.

Suppression of GFAP in AxD mice mitigates molecular, cellular, and clinical phenotypes. (A) Weekly body weights for *Gfap*^{+/+} and *Gfap*^{+/R236H} mice treated at 8 weeks of age with saline, ASO-4 or ASO-9 (300 µg ASO, n = 4–6 males). P values represent 2-way RM ANOVA comparison between saline and ASO treated *Gfap*^{+/R236H} mice. Asterisks indicate significant differences as indicated by Sidak's multiple comparisons, error bars = standard deviation. (B) Western analysis of phosphorylated STAT3-Y705 shows decreased activation in *Gfap*^{+/R236H} mice treated with 500 µg ASO-5 versus saline at 8 weeks post treatment (hippocampus shown, n = 3–4 C57BL/6J mice, males and females treated at 8 weeks of age, asterisks represent 2-tailed unpaired t-test comparing saline versus ASO-treated *Gfap*^{+/R236H} mice, *****p* < 0.0001). (C–D) Immunostaining in hippocampus at 8 weeks post-treatment shows reduced microglia activation (C, Iba1) and an increase in DCX-positive neurons (D) in ASO-treated *Gfap*^{+/R236H} mice compared with saline-treated mutants (C = 500 µg ASO-5, D = 300 µg ASO-9, n = 4–5 males treated at 8 weeks of age, scale bar = 100 µm, maximum projection of 12 optical slices at 1 µm intervals, equivalent exposure levels).

Table 1

Primer/probe sets for quantitative PCR

Gene	Forward primer	Reverse primer	Probe (5'FAM; 3'TAMRA)
<i>Gfap</i>	GAA ACC AGC CTG GAC ACC AA	TCC ACA GTC TTT ACC ACG ATG TTC	TC CGT GTC AGA AGG CCA CCT CAA GA
<i>Ppia</i>	TCG CCG CTT GCT GCA	ATC GGC CGT GAT GTC GA	CC ATG GTC AAC CCC ACC GTG TTC
<i>Gfap</i>	CAA CGT TAA GCT AGC CCT GGA CAT	CTC ACC ATC CCG CAT CTC CAC AGT	SYBR Green
<i>Prph</i>	CTT AAC GTC AAG ATG GCC CTG	GCT ATC CTG GAG AGG CTC CA	SYBR Green
<i>Lcn2</i>	AGA CTT CCG GAG CGA TCA GT	TCT GAT CCA GTA GCG ACA GC	SYBR Green
<i>Ccl2</i>	CTG GAG CAT CCA CGT GTT GG	CAT TCC TTC TTG GGG TCA GC	SYBR Green
<i>Cxcl1</i>	GCC ACA CTC AAG AAT GGT CG	ACC AGA CAG GTG CCA TCA GA	SYBR Green
<i>Itgb2</i>	TGC GGT GAC AAA GAA GAT GGT GAA	GTG CCC GGA TGA CAA AGG ACT G	SYBR Green
<i>Nqo1</i>	CGG TAT TAC GAT CCT CCC TCA ACA	AGC CTC TAC AGC AGC CTC CTT CAT	SYBR Green
<i>Cp</i>	CGA GCC GAA GAA GAC GAG CAC TT	TCA CCC CAT GGG CAT GTA TTG AAT	SYBR Green
<i>Rn18s</i>	CGC CGC TAG AGG TGA AAT TCT	CGA ACC TCC GAC TTT CGT TCT	SYBR Green