Glial fibrillary acidic protein exhibits altered turnover kinetics in a mouse model of Alexander disease

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Mutations in the astrocyte-specific intermediate filament glial fibrillary acidic protein (GFAP) lead to the rare and fatal disorder, Alexander disease (AxD). A prominent feature of the disease is aberrant accumulation of GFAP. It has been proposed that this accumulation occurs because of an increase in gene transcription coupled with impaired proteasomal degradation, yet this hypothesis remains untested. We therefore sought to directly investigate GFAP turnover in a mouse model of AxD that is heterozygous for a disease-causing point mutation (Gfap<sup>R236H+</sup>) (and thus expresses both wild-type and mutant protein). Stable isotope labeling by amino acids in cell culture, using primary cortical astrocytes, indicated that the in vitro half-lives of total GFAP in astrocytes from wild-type and mutant mice were similar at ~3–4 days. Surprisingly, results obtained with stable isotope labeling of mammals revealed that, in vivo, the half-life of GFAP in mutant mice (15.4 ± 0.5 days) was much shorter than that in wild-type mice (27.5 ± 1.6 days). These unexpected in vivo data are most consistent with a model in which synthesis and degradation are both increased. Our work reveals that an AxD-causing mutation alters GFAP turnover kinetics in vivo and provides an essential foundation for future studies aimed at preventing or reducing the accumulation of GFAP. In particular, these data suggest that elimination of GFAP might be possible and occurs more quickly than previously surmised.

Alexander disease (AxD)<sup>2</sup> is a rare and often fatal human disease of the central nervous system caused by dominant mutations in the astrocyte intermediate filament glial fibrillary acidic protein (GFAP) (1). Most AxD patients have de novo mutations in GFAP (2), encoding for various missense mutations as well as small in-frame insertions and deletions. The hallmark feature of the pathology is the formation of aggregates, known as Rosenthal fibers (RFs), within the cell bodies and processes of astrocytes, along with variable degrees of white matter deficits. Although the genetic basis for AxD is clear, the mechanisms by which GFAP mutations lead to astrocyte dysfunction and the cascade of secondary effects on other cells in the central nervous system remain unresolved. Previous studies demonstrated that simple overexpression of wild-type GFAP to high levels induces the formation of RFs (3), and indeed increased levels of GFAP are consistently present in autopsy samples from patients with AxD (4–8). Mouse models engineered to express mutations equivalent to common human mutations (9) illustrate a connection between GFAP levels and severity of disease, which has led to the concept of “GFAP toxicity.”

The excessive accumulation of GFAP and the formation of RFs in AxD presumably reflect a fundamental alteration in proteostasis. Proteostasis, or protein homeostasis, involves a vast network of pathways that control protein synthesis, folding, trafficking, aggregation, and degradation. Protein aggregation disorders such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis are thought to challenge normal proteostasis, whereby aggregation-associated proteotoxicity overcomes the “normal” levels of protein degradation and clearance (10, 11). In AxD, work in cell culture models demonstrates impairment in proteasome activity (12, 13), although autophagy appears to be enhanced (6). At the same time, considerable evidence exists for a rise in levels of GFAP mRNA, which would suggest an increase in GFAP synthesis (9, 14, 15). However, astrocytes in vivo differ markedly from those in cell culture, and the net effect, as well as the relevance of these findings for disease mechanisms in vivo, remains unclear.

GFAP expression is primarily regulated at the level of gene transcription (16), and only a few studies have examined the kinetics of GFAP protein degradation, all involving wild-type protein. For instance, using radioactive tracers and primary cultures of rat astrocytes, two groups found evidence for biphasic degradation: a fast decaying pool with a half-life of ~18 h and a slow-decaying pool with a half-life of ~6 days (17, 18). Again using rat primary astrocytes, Morrison et al. (19) found only monophasic decay with a half-life of 7.5 days. In contrast to these in vitro studies, Eng and co-workers (20) used pulse labeling with radioactive tracers to examine degradation of GFAP in mouse spinal cord and found a half-life of ~9 weeks.
Protein turnover represents the balance between synthesis and degradation, the two complex processes that determine protein concentration (21, 22). Recently, new non-radioactive methods (23, 24) for quantitating turnover via mass spectrometry have been developed. Using these procedures, in the context of analyzing turnover kinetics of the whole brain proteome of the mouse, Price et al. (25) reported a half-life for wild-type GFAP of 28 days. With the goal of understanding whether alterations in turnover kinetics can explain the rise in GFAP levels in AxD, we have re-examined GFAP turnover using both cell culture and mouse models of AxD. Surprisingly, we find that GFAP turnover in vivo is increased in the mutant mice, suggesting an increase in both synthesis and degradation. These results also underscore the increasingly common observation that results with cell cultures must be interpreted with caution and, for many diseases, do not faithfully recapitulate the situation in animals.

Results

GFAP peptides

We used SILAC and SILAM in combination with mass spectrometry to assess the turnover dynamics of GFAP in primary cultures of cortical astrocytes and in a mouse model of AxD, both of which were either wild-type or heterozygous for the R236H mutation in the central rod domain. The peptides used for turnover calculations are shown in Fig. 1. Although SILAC and SILAM use different techniques to address protein turnover (heavy labeling of arginine alone versus all amino acids), our examination showed nine peptides that were informative in both in vitro and in vivo analysis, suggesting concordance of the kinetic scrutiny between the two methods. The peptides used for turnover analysis spanned almost the entire length of the protein, including the head and tail domains, and were of variable length. None of the peptides contained the R236H associated R236H mutation in the central rod domain is in bold red type.

In vitro analysis reveals no difference between GFAP+/+ and GFAPR236H/+ turnover kinetics

To investigate half-life differences between GFAP+/+ and GFAPR236H/+ primary cortical astrocytes, we employed SILAC and collected cellular lysates following various periods of 15N-arginine labeling (0 h, 6 h, 18 h, 1 day, 3 days, 5 days, 7 days, and 9 days). After trypsin digestion of cell lysates, relative isotope abundance (RIA) ratios of 15N-arginine-labeled peptides at all time points were quantitated following mass spectrometric analysis. Representative isotopic envelope spectral data show the mass shift from 14N-arginine to 15N-arginine over time (Fig. 2). The RIA ratios were used for non-linear regression curve fitting (one-phase association) to generate turnover rate constants and half-life calculations for each 15N-arginine-labeled GFAP peptide identified. Peptides selected for turnover calculations were required to be identified in at least seven of eight time points and have a R² of ≈ 0.9 after non-linear regression curve fitting. The kinetics of the individual peptide populations used for GFAP+/+ and GFAPR236H/+ half-life calculations, as well as R1A, turnover rate constants (k0), lag times ( τL), R² values, and half-lives, are shown in Fig. 3 and Table 1, respectively. Peptide turnover rate constants and half-lives were averaged to obtain data for the total protein. GFAP from GFAP+/+ primary cortical astrocytes had a k0 of 0.18 ± 0.03 day⁻¹, corresponding to a half-life of 3.85 ± 0.73 days. In GFAPR236H/+ primary cortical astrocytes, GFAP had a k0 of 0.20 ± 0.04 day⁻¹, corresponding to a half-life of 3.57 ± 0.70 days. An apparent lag time in 15N-arginine incorporation (0–24 h) was observed in all peptides and may be attributed to a temporary halt in gene expression as the cells recover after plating, a phenomenon observed in C6 and U251 glioma cells (26, 27).

GFAPR236H/+ half-life is shorter than GFAP+/+ half-life in vivo

In vitro culture systems lack the cellular heterogeneity, structural organization, and complex cell-cell interactions that are present in vivo. To isotypically label animals, 9-week-old male GFAPR236H/+ mice and GFAP+/+ littermate controls were fed a nitrogen-free diet supplemented with 15N-labeled Spirulina for various time points (0, 2, 8, 32, and 64 days) before sacrifice and brain collection. Total brain homogenates were analyzed by LC/MS/MS following SDS/PAGE fractionation and in-gel trypsin digestion.

Computational data analysis was performed using a series of previously described data processing modules (25, 28). Following identification and assignment by the online Protein Prospector search engine (University of California, San Francisco), only GFAP peptides that were found in both GFAP+/+ and GFAPR236H/+ samples were included in subsequent analysis. Representative spectral data show the incorporation of 15N
isotopic envelope patterns when comparing GFAP from our samples. Negligible differences were observed in isotopic envelopes from other abundant proteins identified experimentally. We performed a manual inspection of the isotopic envelope patterns and found that their tissues contain a mixture of both mutant and wild-type protein in the case of the R416W mutation. Alexander disease is a gain of function disorder that in part results from the accumulation of GFAP (a combination of mutant and wild-type protein) above a putative toxic threshold. Our goal in the current study was to examine the turnover kinetics of GFAP, both in vitro and in vivo, as a foundation for understanding the basis for this rise in protein levels, and as a guide for future studies that are aimed at reversing this trend. Although previous studies clearly support an increase in GFAP synthesis as one contributing factor, there is conflicting evidence on whether GFAP degradation is impacted as well. We found that the turnover rate GFAP in primary cultures of astrocytes at 0, 3, 5, and 9 days. Isotopic envelopes from GFAPR236H/+ have been shifted by +0.1 m/z for clarity.

Figure 2. Isotopic envelopes from representative GFAP+/+ and GFAPR236H/+  peptide FADLTDAASR over the time course of in vitro SILAC. A–D, overlays of observed isotopic envelopes from GFAP+/+ (black) and GFAPR236H/+ (red) primary astrocytes at 0 (A), 3 (B), 5 (C), and 9 days (D). Isotopic envelopes from GFAPR236H/+ have been shifted by +0.1 m/z for clarity.

over time (Fig. 4). We observed faster 15N incorporation in GFAPR236H/+ brain samples, indicating increased GFAP synthesis, which is best illustrated at day 32 (Fig. 4C). The previously described data processing pipeline (28) was used to extract GFAP turnover rate constants from raw LC/MS/MS data files. The kinetics of the total protein populations, including 95% confidence intervals, as well as RIA values, turnover rate constants (k0), lag times (t0), R2 values, and half-lives for total protein are shown in Fig. 5 and Table 2, respectively. Individual peptides used in total protein calculations and their RIA data, k0, t0, R2, and half-lives are shown in Tables 3–6. GFAP from GFAP+/+ mice had a k0 of 0.025 ± 0.001 day−1, corresponding to a half-life of 27.46 ± 1.56 days. In GFAPR236H/+ mice, GFAP had a k0 of 0.045 ± 0.002 day−1, corresponding to a half-life of 15.41 ± 0.53 days. To confirm that the difference seen in GFAP half-life is protein-specific and not a nutritional artifact, we performed a manual inspection of the isotopic envelope patterns from other abundant proteins identified from our samples. Negligible differences were observed in isotopic envelope patterns when comparing GFAP+/+ and GFAPR236H/+ in vivo samples for four additional proteins (representative peptides shown in Fig. 6), indicating that the change in GFAP half-life in AXD mutant mice is specific to this protein and not reflecting a generalized change in protein turnover.

Total GFAP levels remain unchanged from 9–18 weeks of age in GFAP+/+ and GFAPR236H/+ mice

To determine whether total GFAP levels were changing during the period of our analysis and in turn artificially skewing our turnover data, we performed semiquantitative Western blotting and quantitative ELISA analysis of the same brain homogenates used for mass spectrometry. Both the Western blot (Fig. 7A) and ELISA (Fig. 7B) show no change in total GFAP in either GFAP+/+ or GFAPR236H/+ brain tissue during the 9-week time course of the experiment.

Discussion

Alexander disease is a gain of function disorder that in part results from the accumulation of GFAP (a combination of mutant and wild-type protein) above a putative toxic threshold. Our goal in the current study was to examine the turnover kinetics of GFAP, both in vitro and in vivo, as a foundation for understanding the basis for this rise in protein levels, and as a guide for future studies that are aimed at reversing this trend. Although previous studies clearly support an increase in GFAP synthesis as one contributing factor, there is conflicting evidence on whether GFAP degradation is impacted as well. We found that the turnover rate of GFAP in primary cultures of astrocytes is unaffected by the presence of mutant GFAP. However, in vivo, using mice that are exact genetic mimics of the human disease, we found that the turnover rate of GFAP is markedly increased by the presence of mutant protein. The simplest interpretation of these results is that both synthesis and degradation of GFAP are increased in the setting of disease, a finding that will have significant implications for the design and testing of therapeutic strategies aimed at correcting GFAP toxicity (29).

GFAP, like other intermediate filament proteins, undergoes a process of synthesis and assembly into multimeric structures, in the case of GFAP also potentially including other intermediate filaments such as vimentin and nestin (30), along with binding partners such as αB-crystallin (31, 32) and plectin (5). The IF components first form dimers, then tetramers, and then octamers or oligomers before finally assuming the structures visualized by electron microscopy as 10-nm filaments (33). The processes of filament disassembly and degradation are relatively less well studied, although evidence exists for regulation by post-translational modifications such as phosphorylation (34, 35), and roles for several proteases including calpain (36–38), caspase 3 (39), and caspase 6 (40).

The mutations associated with Alexander disease are genetically dominant, and all patients identified so far have been heterozygous (an updated list of all published mutations is maintained by the Waisman Center), which leads to the prediction that their tissues contain a mixture of both mutant and wild-type protein. Der Perng et al. (41) proved co-assembly of mutant and wild-type protein in the case of the R416W muta-
tion, the only one for which mutant specific antibodies are available. The exact stoichiometric ratios of mutant:wild-type GFAP is not yet known, although preliminary studies suggest that the mutant may actually be less than the wild type.3 We note that in our studies we have measured total GFAP, and the peptides selected for analysis did not contain the Arg-236 site of the murine mutation. We also do not distinguish between the GFAP-H9251 and GFAP-H9254 isoforms, although H9251 likely represents more than 90% of the total in the CNS (42, 43). Differences in solubility exist between the GFAP isoforms and between mutant and wild-type protein (39, 41, 44, 45), although not under the conditions used for extraction in the experiments reported here.

Our data on the turnover rate of GFAP in primary cultures of astrocytes showed a rate that was much faster than the in vivo rate, and no difference between wild-type and mutant cells, but should be interpreted with caution. First, these cells were grown under high oxygen conditions, which might induce oxidative stress that masks differences that would otherwise exist between the two cell types (46). Second, transcription profiling has revealed numerous differences between cultured astrocytes and their in vivo counterparts (47), and even newer 3D culture

3 M. Brenner, personal communication.

Figure 3. Kinetics of GFAPR236H/+/ and GFAPR236H+/− peptides following in vitro SILAC. Representative 15N-labeled GFAP+/+ (solid, circle) and GFAPR236H+/− (dashed, square) peptides fit with a delayed exponential function used for turnover kinetic calculations. A, KVESLIEEIQFLR; B, LADVYQAELR; C, ALAELNQLR; D, LEAENLAAAYR.

Table 1
Measured kinetic parameters from individual peptides following in vitro SILAC

| Sequence         | 0 days | 0.25 day | 0.75 day | 1 day | 3 days | 5 days | 7 days | 9 days | $k_0$  | $t_0$  |
|------------------|--------|----------|----------|-------|--------|--------|--------|--------|-------|--------|
| GFAP+/+ ALAELNQLR | 0.0014 | 0.0066   | 0.0097   | 0.0100 | 0.1593 | 0.6864 | 0.7959 | 0.8491 | 0.1900 | 0.4922 |
| PADLTDASR        | 0.0057 | 0.0028   | 0.0101   | 0.0058 | 0.1500 | 0.7024 | 0.8229 | 0.9212 | 0.2026 | 0.5024 |
| LADVYQAELR       | 0.0199 | 0.0159   | 0.0152   | 0.0257 | 0.1667 | 0.6834 | 0.7731 | 0.8214 | 0.1820 | 0.4212 |
| KVESLIEEIQFLR    | 0.0069 | 0.0716   | 0.1092   | 0.0671 | 0.2047 | 0.6479 | 0.8016 | 0.8149 | 0.1794 | 0.1858 |
| LEAENLAAAYR      | 0.0048 | 0.0127   | 0.0189   | 0.0143 | 0.1803 | 0.8585 | 0.8562 | 0.1853 | 0.4734 |
| LEEEOQSLKEMAR    | 0.0116 | 0.0116   | 0.0123   | 0.0185 | 0.1558 | 0.5377 | 0.6270 | 0.6415 | 0.1289 | 0.4167 |
| LRLDQLTANSAR     | 0.0045 | 0.0295   | 0.0243   | 0.0564 | 0.2070 | 0.7713 | 0.8918 | 0.9199 | 0.2257 | 0.3866 |
| GFAPR236H+/−     |        |          |          |       |        |        |        |        |       |        |
| ALAELNQLR        | 0.0068 | 0.0034   | 0.0075   | 0.0053 | 0.1987 | 0.6849 | 0.8441 | 0.8863 | 0.2038 | 0.4834 |
| PADLTDASR        | 0.0041 | 0.0047   | 0.0131   | 0.0058 | 0.2382 | 0.6961 | 0.9038 | 0.9152 | 0.2208 | 0.4659 |
| LADVYQAELR       | 0.0322 | 0.0227   | 0.0345   | 0.0204 | 0.2392 | 0.7149 | 0.8501 | 0.8856 | 0.2090 | 0.3643 |
| ESASYQEALAR      | 0.0128 | 0.0066   | 0.0134   | 0.0087 | 0.1898 | 0.7063 | 0.8504 | 0.9024 | 0.2076 | 0.4644 |
| GTNESLIR         | 0.0921 | 0.0174   | 0.0483   | 0.0363 | 0.8143 | 0.9104 | 0.9085 | 0.2745 | 0.2158 |
| ITIPVQTPSNLQIR   | 0.0059 | 0.0071   | 0.0085   | 0.0051 | 0.1712 | 0.06839 | 0.8695 | 0.8976 | 0.2053 | 0.4914 |
| KVESLIEEIQFLR    | 0.0295 | 0.0129   | 0.0303   | 0.0235 | 0.2585 | 0.7933 | 0.7971 | 0.1726 | 0.3609 |
| LEAENLAAAYR      | 0.0143 | 0.0114   | 0.0127   | 0.0119 | 0.2037 | 0.6781 | 0.8311 | 0.8719 | 0.1988 | 0.4479 |
| LEEEOQSLKEMAR    | 0.0162 | 0.0082   | 0.0148   | 0.0104 | 0.1661 | 0.5568 | 0.6446 | 0.6397 | 0.1328 | 0.4145 |
| LRLDQLTANSAR     | 0.0243 | 0.0113   | 0.0169   | 0.0126 | 0.1381 | 0.6124 | 0.8031 | 0.8684 | 0.1803 | 0.4629 |

$Y = 1 - \exp (-k_0(x - t_0));$ $x = time$
systems (48) fail to fully replicate the profile of astrocytes in vivo where they reside in a normal anatomic and physiological context and engage in complex interplay with neurons and other glia. Third, as noted by Price et al. (25), many cell types accelerate their turnover rate of proteins when grown under standard culture conditions.

Turnover, as defined by the shift from $^{14}$N- to $^{15}$N-containing peptides, represents the net effect of both synthesis and degradation. Because the GFAP mutant mice exhibited more rapid turnover than their wild-type littermates, one or both of these processes must have changed. Previously we have shown that Alexander disease tissues contain increased levels of GFAP mRNA (4, 9) and that, using mouse models, there is increased activity of the $Gfap$ promoter itself (15), providing strong evidence for an increase in synthesis. However, here we also show that GFAP levels of adult mutant mice remained stable, albeit elevated, throughout the postnatal period of 63–127 days under investigation.

The simplest interpretation of these results is that, during this particular postnatal period, both synthesis and degradation have increased. Whether these changes occur in similar fashion in the different biochemically defined pools of GFAP, regions of the CNS, or types of astrocytes, is not yet known. We also cannot be certain that all disease-causing mutations in GFAP will have the same effect. Nevertheless, our finding that GFAP turnover is accelerated in AxD mutant mice has significant implications for the design and testing of therapeutic strategies aimed at reversing its toxic accumulation.

Experimental procedures

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. The animals were housed with ad libitum access to water and diets as described below, on a standard light-dark cycle of 14:10.

Primary astrocyte culture

Enriched primary cortical astrocyte cultures were generated from GFAP$^{+/+}$ and GFAP$^{R236H/+}$ AxD mutant mice as previously described (49). Briefly, primary cortical astrocytes were obtained from postnatal days 0–2 FVB/129S6 F1 hybrid GFAP$^{+/+}$ and GFAP$^{R236H/+}$ littermates ($n = 24$/genotype). After being freed of meninges, cortices from individual pups were mechanically dissociated into single cells in DMEM (Gibco) and suspended in medium containing DMEM supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cell suspensions from individual pups were seeded into T-25 flasks and maintained at 37 °C in a humidified incubation chamber containing 5% CO$_2$. Serum was reduced to 1% after 48 h. Astrocytes from both genotypes were grown for 3 weeks or until 95% confluent. Flasks were shaken overnight (200 rpm, 37 °C) to remove oligodendrocytes and microglia, leaving adherent astrocytes, which were detached by brief incubation with 0.25% trypsin-EDTA (Gibco). Following washing, astrocytes (purity ≥ 95%; data not shown) were pooled by genotype and replated at $4 \times 10^5$ cells/ml before being subjected to SILAC.

SILAC

Following replating, astrocytes were fed with DMEM deficient in natural L-arginine hydrochloride (custom medium;
Gibco) and supplemented with 84 µg/ml [15N]l-arginine (Cambridge Isotope Labs), 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (further referred to as “heavy medium”). Astrocytes from both genotypes were grown in heavy medium for a defined period (0 h, 6 h, 18 h, 1, days, 3 days, 5 days, 7 days, and 9 days). Following the switch to heavy medium, adherent cells were trypsinized (0.25%) and pelleted before lysis with 1% SDS, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1× Complete protease inhibitor mixture (Roche Diagnostics) and 1 mM Pefabloc (Sigma-Aldrich). Lysates were cleared by boiling for 15 min and total protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific).

**Table 2**
Measured kinetic parameters from total protein following in vivo SILAM

| Sample | 15N-Labeled fraction of protein population at time (RIA) | k | t | R² | ln(2)/k₀ (half-life) |
|--------|--------------------------------------------------------|---|---|----|---------------------|
| GFAP⁺⁺/⁻ | 0.0000 0.0482 0.0905 0.4874 0.8292 | 0.0243 | 1.8690 | 0.991 | 28.573 |
| GFAP⁺⁺/⁻ replicate 2 | 0.0000 0.0327 0.2624 0.5812 0.7939 | 0.0263 | −0.9323 | 0.995 | 26.366 |

**Table 3**
Measured kinetic parameters from individual peptides following in vivo SILAM from GFAP⁺⁺/⁻ replicate 1

| Sequence | 15N-Labeled fraction of peptide population at time (RIA) | Y = 1 − exp(−k₀(x − t₀)); x = time | k₀ | t₀ | R² | ln(2)/k₀ (half-life) |
|----------|--------------------------------------------------------|--------------------------------------|---|---|----|---------------------|
| ESASYQERALR | 0.0000 0.0000 0.0414 0.5195 0.8622 | 0.0258 | 2.3419 | 0.981 | 26.871 |
| FADLTDAAAR | 0.0000 0.0169 0.1202 0.5464 0.8277 | 0.0258 | 1.2220 | 0.997 | 26.366 |
| PLQQKYN | 0.0000 0.0113 0.0600 0.3959 0.7703 | 0.0253 | 1.7266 | 0.985 | 27.346 |
| HQLQYDNLNVK | 0.0000 0.0380 0.1053 0.4220 0.7891 | 0.0210 | 0.9221 | 0.988 | 32.940 |
| HPIQYQFENLQIR | 0.0000 0.3554 0.0967 0.4327 0.9487 | 0.0230 | −3.4021 | 0.888 | 30.174 |
| IYEEVVR | 0.0000 0.0000 0.0180 0.4900 0.9078 | 0.0261 | 2.6831 | 0.966 | 26.539 |
| LDLVYQELAR | 0.0000 0.2566 0.1007 0.5277 0.9480 | 0.0269 | −1.3996 | 0.948 | 25.803 |
| LQDETNLR | 0.0000 0.0313 0.1190 0.4227 0.7789 | 0.0205 | 0.8614 | 0.992 | 32.828 |
| LEAEELXVLR | 0.0000 0.0000 0.0878 0.4184 0.9006 | 0.0213 | 1.8885 | 0.987 | 32.617 |
| LLQDETNLR | 0.0000 0.0182 0.1080 0.5224 0.8195 | 0.0247 | 1.3041 | 0.996 | 28.053 |
| QRIQDLTANSAR | 0.0000 0.0332 0.2059 0.7789 0.9380 | 0.0253 | 1.7174 | 0.995 | 27.368 |
| QLEAEENLR | 0.0000 0.0000 0.0180 0.5224 0.9006 | 0.0213 | 1.8885 | 0.987 | 32.617 |
| SYASETVVR | 0.0000 0.0000 0.0180 0.4900 0.9078 | 0.0261 | 2.6831 | 0.966 | 26.539 |
| SYASETVVR | 0.0000 0.0000 0.0180 0.4900 0.9078 | 0.0261 | 2.6831 | 0.966 | 26.539 |

**Table 4**
Measured kinetic parameters from individual peptides following in vivo SILAM from GFAP⁺⁺/⁻ replicate 2

| Sequence | 15N-Labeled fraction of peptide population at time (RIA) | Y = 1 − exp(−k₀(x − t₀)); x = time | k₀ | t₀ | R² | ln(2)/k₀ (half-life) |
|----------|--------------------------------------------------------|--------------------------------------|---|---|----|---------------------|
| IYEEVVR | 0.0000 0.0188 0.1531 0.5419 0.8650 | 0.0202 | −0.0053 | 0.989 | 34.251 |
| LLEGEENR | 0.0000 0.1135 0.1170 0.5194 0.6653 | 0.0186 | −1.5191 | 0.987 | 37.250 |
| LQDETNLR | 0.0000 0.0000 0.3719 0.5464 0.9018 | 0.0286 | −0.6287 | 0.960 | 24.246 |
| LRLQYDNLNVK | 0.0000 0.0173 0.5864 0.9863 0.9170 | 0.1052 | 0.6553 | 0.988 | 6.589 |
| NAELLRL | 0.0000 0.0000 0.0262 0.4604 0.7425 | 0.0205 | 2.4279 | 0.990 | 33.819 |
| QAQDEALTAR | 0.0000 0.0645 0.0670 0.5015 0.7346 | 0.0208 | 0.6812 | 0.990 | 33.357 |
| SYASETVVR | 0.0000 0.0148 0.1587 0.5654 0.9311 | 0.0363 | −1.2256 | 0.941 | 19.075 |

**Mass spectrometry and data analysis of SILAC samples**

Total cell lysates were subjected to TCA/acetone protein precipitation before trypsin digestion and analysis with LC/MS/MS using an Agilent G2226A NanoPump HPLC coupled to a Thermo Fisher Scientific Orbitrap XL mass spectrometer. The Trans-Proteomic Pipeline (Institute for Systems Biology) was used for data file conversion before Mascot database searching (Matrix Science, version 2.2), using the Mus musculus NCBI RefSeq organism database. A sequence-reversed decoy copy of each protein, as well as common contaminant sequences such as keratins and trypsin, was appended to the search, with and without a modification to account for 15N-
GFAP turnover in alexander disease

Table 5

| Sequence | 15N-Labeled fraction of peptide population at time (RIA) | Y = 1 − exp(−k0(x − t0)) | ln(2)/k0 (half-life) |
|----------|-----------------------------------------------------|-------------------------|---------------------|
|          | 0 days | 2 days | 8 days | 32 days | 64 days | k0 | t0 | R² | days |
| ALAASNQQLR | 0.0000 | 0.1636 | 0.1962 | 0.7511 | 0.9283 | 0.0390 | −0.4292 | 0.990 | 17.777 |
| EASAYQDAALR | 0.0000 | 0.1068 | 0.2112 | 0.8872 | 0.9322 | 0.0506 | 0.4717 | 0.987 | 13.712 |
| PADLDAASAR | 0.0000 | 0.0237 | 0.3397 | 0.7497 | 0.9436 | 0.0463 | 0.3953 | 0.997 | 14.971 |
| FLEQNK | 0.0000 | 0.0339 | 0.6013 | 0.8730 | 1.0000 | 0.1000 | 0.3158 | 0.988 | 16.883 |
| HLQYQDLNVK | 0.0000 | 0.2380 | 0.2157 | 0.9124 | 0.9387 | 0.0517 | −0.4563 | 0.975 | 13.409 |
| ITIPQTVSNLQR | 0.0000 | 0.1489 | 0.2493 | 0.8576 | 0.9536 | 0.0503 | −0.0426 | 0.992 | 13.793 |
| TYEEVR | 0.0000 | 0.3122 | 0.4255 | 0.8941 | 0.9462 | 0.0672 | −0.1239 | 0.989 | 10.314 |
| KVEELEELQFLR | 0.0000 | 0.1551 | 0.1960 | 1.0000 | 0.9548 | 0.0591 | 0.3935 | 0.962 | 11.729 |
| LADVQAEELR | 0.0000 | 0.0129 | 0.2480 | 0.7936 | 0.9080 | 0.0448 | 0.8985 | 0.996 | 15.479 |
| LDQLTANSAR | 0.0000 | 0.5317 | 0.4591 | 0.8603 | 0.9340 | 0.0637 | −3.1979 | 0.952 | 10.885 |
| LEAAANLALYR | 0.0000 | 0.1052 | 0.2717 | 0.6643 | 0.9076 | 0.0351 | −0.6009 | 0.999 | 19.760 |
| LEVERDNFADQLQTLRA | 0.0000 | 0.0372 | 0.2969 | 0.8155 | 0.9623 | 0.0506 | 0.6324 | 0.998 | 13.685 |
| LEEGSNER | 0.0000 | 0.0951 | 0.3532 | 0.7211 | 0.8595 | 0.0392 | −0.8024 | 0.994 | 17.687 |
| LRLDQTLANSAR | 0.0000 | 0.0877 | 0.1716 | 0.4225 | 0.6694 | 0.0167 | −1.9786 | 0.997 | 41.472 |
| QREADETLAR | 0.0000 | 0.0753 | 0.3596 | 0.7318 | 0.9305 | 0.0443 | −0.2639 | 0.997 | 15.663 |
| QLQALTC-DLGSLR | 0.0000 | 0.0074 | 0.2893 | 0.9471 | 0.9639 | 0.0623 | 1.0635 | 0.989 | 11.133 |
| SYASETQVVR | 0.0000 | 0.4340 | 0.2054 | 0.6722 | 0.9411 | 0.0316 | −4.6271 | 0.932 | 21.924 |

arginine incorporation. Protein identification was performed in conjunction with in-house false discovery rate algorithms set at 5%. Quantitative analysis was performed using Census (50), producing area under the curve data for light (L) and heavy (H) tryptic peptides. RIA ratios for 15N-labeled peptides were calculated for GFAP peptides at all time points as RIA = H/(H + L) (51). Half-life was calculated for individual peptides as the incorporation of heavy label over time using non-linear regression curve fitting (least squares fit) with the delayed exponential function,

Y = 1 − exp(−k0(x − t0)),

(Eq. 1)

with Y = RIA, k0 = turnover rate constant, x = time point, and t0 = lag time (25). Half-life calculations were performed with the following equation,

\[ t_{1/2} = \ln(2)/k_0 \]

(Eq. 2)
in GraphPad Prism version 5.0 (San Diego, CA). GFAP peptides were chosen for inclusion in total GFAP half-life calculations by averaging individual peptide half-lives (mean ± standard deviation; n = 7 GFAP+/−, n = 10 GFAPR236H+/+) if RIA values were available for ≥7 of 8 time points and coefficient of determination (R²) values following non-linear regression curve fitting were ≥0.9, a method previously employed (25). GFAP turnover was comparatively analyzed from GFAP+/− and GFAPR236H+/+ astrocytes to assess differences in half-life.

SILAM

Prior to the initiation of the experiment, GFAP+/− and GFAPR236H+/+ mutant mice (males, FVB/129S6 background) from a pilot study were fed a nitrogen-free custom diet (TD.110902, Harlan Teklad) supplemented with 14N-Spirulina algae (Cambridge Isotope Labs) for 2 weeks and examined daily.
for general health and weight fluctuations. Mice maintained or gained weight during the pilot study (data not shown).

At 8 weeks of age, GFAP+/− and GFAPR236H+/− mutant mice (males, FVB/129S6 background) were fed a nitrogen-free custom diet (TD.110902, Harlan Teklad) supplemented with 14N-Spirulina algae (Cambridge Isotope Labs). Following a 1-week acclimation to the diet, 15N-labeled Spirulina algae (>98%, Cambridge Isotope Labs) was substituted for 14N-Spirulina in the aforementioned custom diet (TD.110903, Harlan Teklad) and was fed ad libitum for up to 64 days. The diet was composed of 25% (w/w) dry Spirulina algae (Cambridge Isotope Labs) and 75% custom protein-free diet (Harlan Teklad) as dry food pellets. Mice (n = 2/time point/genotype) were sacrificed at five time points (0, 2, 8, 32, and 64 days) following the introduction of the 15N-Spirulina diet. After sacrifice, the whole brain (including olfactory bulb, cerebral hemispheres, cerebellum, and brain stem) was collected and homogenized with lysis buffer (2% SDS, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1× Complete protease inhibitor mixture (Roche Diagnostics) and 1 mM Pefabloc (Sigma-Aldrich)) before total protein concentration measurement using the Pierce BCA protein assay kit (Thermo Fisher Scientific).

Mass spectrometry and data analysis of SILAM samples
Brain homogenates of equal protein concentration were separated by SDS-PAGE, and proteins were visualized using GelCode Blue Safe Protein stain (Thermo-Pierce). The 45–48-kDa region (determined in a pilot experiment to contain GFAP; data not shown) was excised independently from each of the gel lanes and subjected to in-gel digestion. To each sample, trypsin/ProteaseMax solution (10 ng/μl) was added before desalting and concentration by solid phase extraction and elution with 75% acetonitrile containing 0.1% formic acid. Eluted peptides were dried to minimal volume and diluted with 0.1% formic acid in water prior to analysis by HPLC/MS/MS.

LC/MS/MS was performed using an Agilent G2226A Nano-Pump HPLC coupled to a Thermo Fisher Scientific Orbitrap XL mass spectrometer. The mass spectrometer was fitted with an EasySpray 75-μm × 15-cm column packed with PepMap 3 μm, 100 Å C18, and maintained at 40 °C during all the analyses to maximize retention time stability across runs. Dynamic exclusion was employed using a repeat count of 1 and exclusion duration of 30 s. The Trans-Proteomic Pipeline (Institute for...
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Systems Biology) was used to convert raw acquisition data files before database searching by Mascot (Matrix Science, version 2.2). The *M. musculus* database was used for Mascot searching, to which was appended a sequence-reversed decoy copy of each protein, as well as contaminant sequences. Only the time 0 samples were subjected to database searching, because these samples would have no $^{15}$N incorporation. GFAP was found in each of the time 0 samples at a peptide false discovery rate of 0.2% with sequence coverage of 68–79% for the four time 0-in-gel digests. Further data analysis was performed using Protein Prospector (hosted at the University of California, San Francisco Mass Spectrometry Facility) to generate lists of detected GFAP peptides from the time 0 LC/MS/MS data, generated using the Batch-Tag Web tool to identify peptides from the user-entered sequence for GFAP (NCBI reference sequence NP_034407.2). The parameters for peptide identification were trypsin cleavage specificity with 1 missed cleavage allowed, 0 non-tryptic termini, monoisotopic precursors, 20-ppm precursor maximum mass error, 0.6-Da fragment ion maximum mass error, +2 or +3 precursor charge state, fixed carbamidomethylation of Cys residues, variable deamidation of Asn and Gln residues, variable oxidation of Met residues, and variable conversion of peptide N-terminal Gln residues to pyroglutamic acid. Following Batch-Tag, a list of identified GFAP peptides was generated using the Search Compare module, and subsequent data analysis was performed using a data processing pipeline for mammalian proteome dynamics studies created by S. Guan and coworkers (25, 28).

For the data processing pipeline analysis (28), the following criteria were set: +/− m/z tolerance for extracted ion chromatogram (XIC) at $t_0 = 0 = 5e-6$; start delta retention time (ΔRT) for XIC extraction at $t_0 = 0 = 1.5$; end ΔRT for XIC extraction at $t_0 = 1.5$; +/− m/z tolerance for $^{15}$N peaks (all) = 8e-6; +/− ΔRT for $^{15}$N peak averaging (all) = 0.25. GFAP peptides were included in turnover calculations if they were present in all samples and contributed to total protein $R^2$ values of $\geq 0.99$ following the data processing pipeline. Some peptides were excluded within the Pep2Prot module of the analysis pipeline based on dissimilarity of relative abundance curves to the aggregated total protein curve. The output of the data processing pipeline yielded protein turnover rate constants ($k_0$) extracted from raw LC/MS/MS data files in a series of algorithms culminating in a delayed exponential function used to fit the experimental $^{15}$N association curves (Equation 1). Non-linear regression curve fitting of the RIA values from each time point, genotype, and replicate was confirmed in GraphPad Prism version 5.0 (San Diego, CA), and 95% confidence intervals were generated to assess the preciseness of the best fit curve fitting parameter values for each genotype. Half-life was then calculated with Equation 2, and the means ± standard deviations between replicates were reported. GFAP turnover was comparatively analyzed from GFAP$^{+/+}$ and GFAP$^{R236H/+}$ mouse brain homogenates to assess differences in GFAP turnover kinetics.

**Western blot**

To determine whether total GFAP levels were changing during the 9-week experimental time course, aliquots of brain homogenates used for mass spectrometry were subjected to Western analysis. 15 μg of total protein/lane was loaded onto a 10% Criterion T GX gel (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were blocked with SEA BLOCK blocking buffer (Thermo) before probing with mouse monoclonal anti-GFAP (UC-Davis, N206A/A, catalog no. 75–240) and monoclonal anti-GAPDH (Fitzgerald, catalog no. 10R-G109A) antibodies, diluted 1:2,500 and 1:5,000, respectively, in TBS with 0.5% Tween 20 for 2 h at room temperature. Following three washes with TBS/Tween 20, the membranes were probed with goat anti-mouse AlexaFluor 680 (Invitrogen, catalog no. A21057) secondary antibodies, diluted 1:10,000 in TBS/Tween 20 for 2 h at room temperature. After washing, the blots were scanned with the Odyssey Infrared Imaging System (Li-Cor).

**ELISA**

To quantify GFAP levels during the experimental period, aliquots of brain homogenates used for mass spectrometry were subjected to ELISA analysis as previously described (15). Briefly, microtiter plates were coated with a monoclonal mixture of anti-GFAP (1:1,000; SMI-26R, Covance/BioLegend, catalog no. 837602) antibody and blocked with 5% nonfat dry milk before incubation with samples and GFAP standards of known concentration, diluted in PBS with 0.05% Tween 20 and 1% BSA. Polyclonal rabbit anti-GFAP (1:5,000; DAKO, catalog no. Z0334) was used for detection, followed by HRP-conjugated goat anti-rabbit IgG (1:10,000; Sigma, catalog no. A6154) and SuperSignal ELISA Femto maximum sensitivity substrate (Thermo). Chemiluminescence was quantitated with a Glo-Runner microplate luminometer (Turner Biosystems). Differences in GFAP levels were assessed with GraphPad Prism version 5.0 (San Diego, CA), one-way analysis of variance, Tukey’s multiple comparisons post-test.

**Author contributions**—L. R. M. conducted most of the experiments, analyzed the results, and wrote most of the paper. G. A. B.-W. and M. R. S. contributed to the experimental design, conducted experiments, and analyzed the mass spectrometry data. A. M. and M. R. S. conceived the idea for the project, and A. M. wrote the paper with L. R. M.

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