

Supplementary Materials for
**Protein lifetimes in aged brains reveal a proteostatic adaptation linking
physiological aging to neurodegeneration**

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Tables S1 to S7

Supplementary texts

Text S1: Interpretation of concomitant lifetime and protein abundance changes (related to Fig. S8B).

Text S2: Changes in protein lifetimes with regional or synaptic specificities that could be explained by biochemical properties (related to Fig. S10).

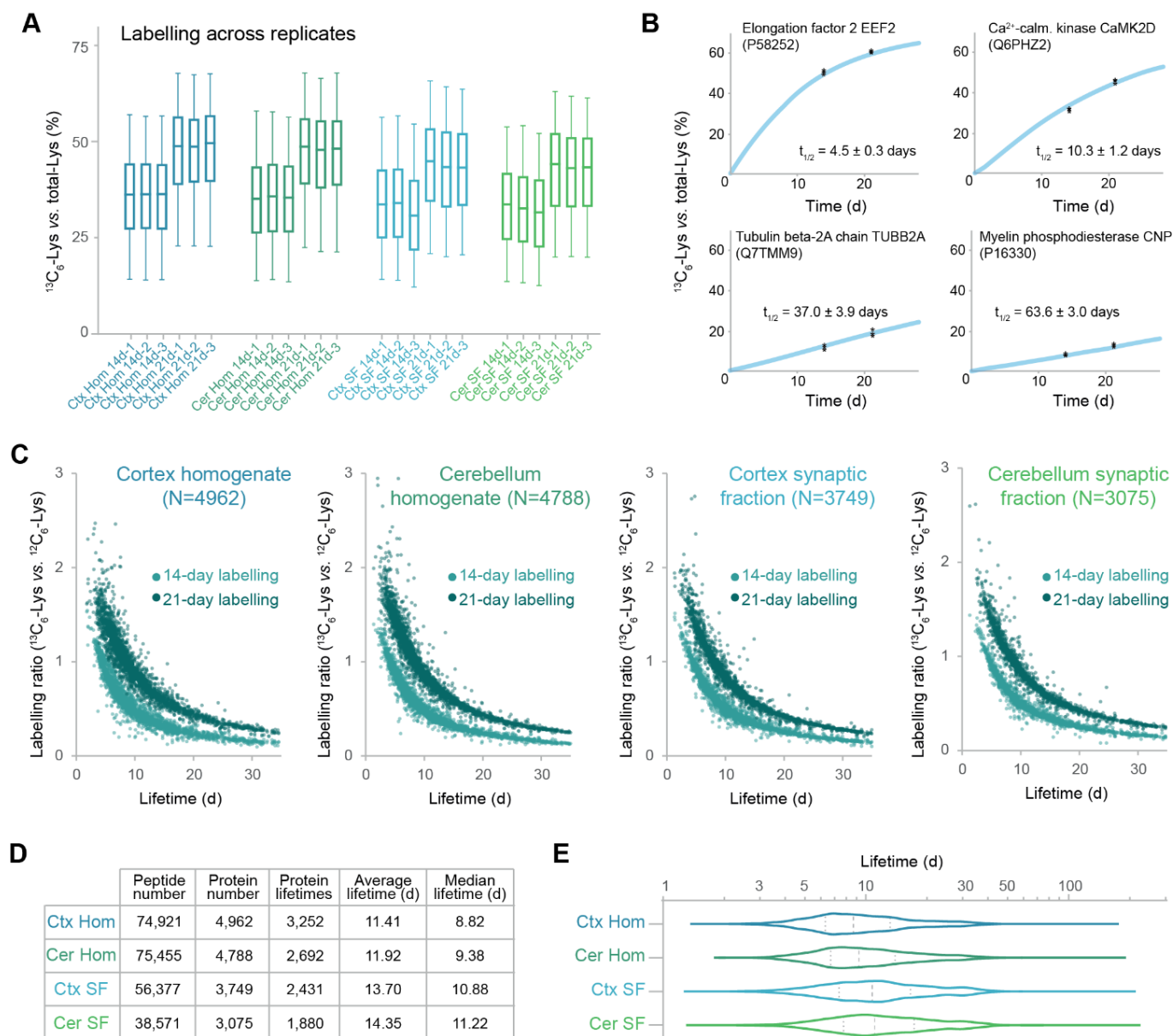


Fig. S1. Summary of labeling efficiencies, and overall data quality control for the metabolic labeling and the mass spectrometry measurements in the aged mouse brain cortex, cerebellum, and respective synaptic fractions. (A) Boxplots summarizing the percentage of $^{13}\text{C}_6$ -lysine (heavy) versus total lysine for each time point and for each single biological replicate for cerebellum total homogenate, cortex total homogenate and the respective synaptic fractions. Boxplots represent median, 25th to 75th percentile with whiskers showing 5th-95th percentile. (B) Representative fittings for four proteins of the cortex homogenate with different lifetimes, calculated as in (18). Note that for several biological replicates (black asterisks) the variation is virtually nonexistent, mirroring the reproducibility of the data. (C) Scatter plots, obtained after fitting as described in (18), summarizing the interdependence of labeling ratio and lifetimes. The different color hues indicate different labeling times. (D) Supplementary Table summarizing the number of proteins, peptides, lifetimes, and relative average lifetime and median for the four datasets. (E) Violin plots representing the distribution of lifetimes calculated in days. The thicker segmented line in the middle of each plot represents the median, while the thinner segmented lines represent lower 25th percentile and higher 75th percentile. Note that the graph has a logarithmic scale.

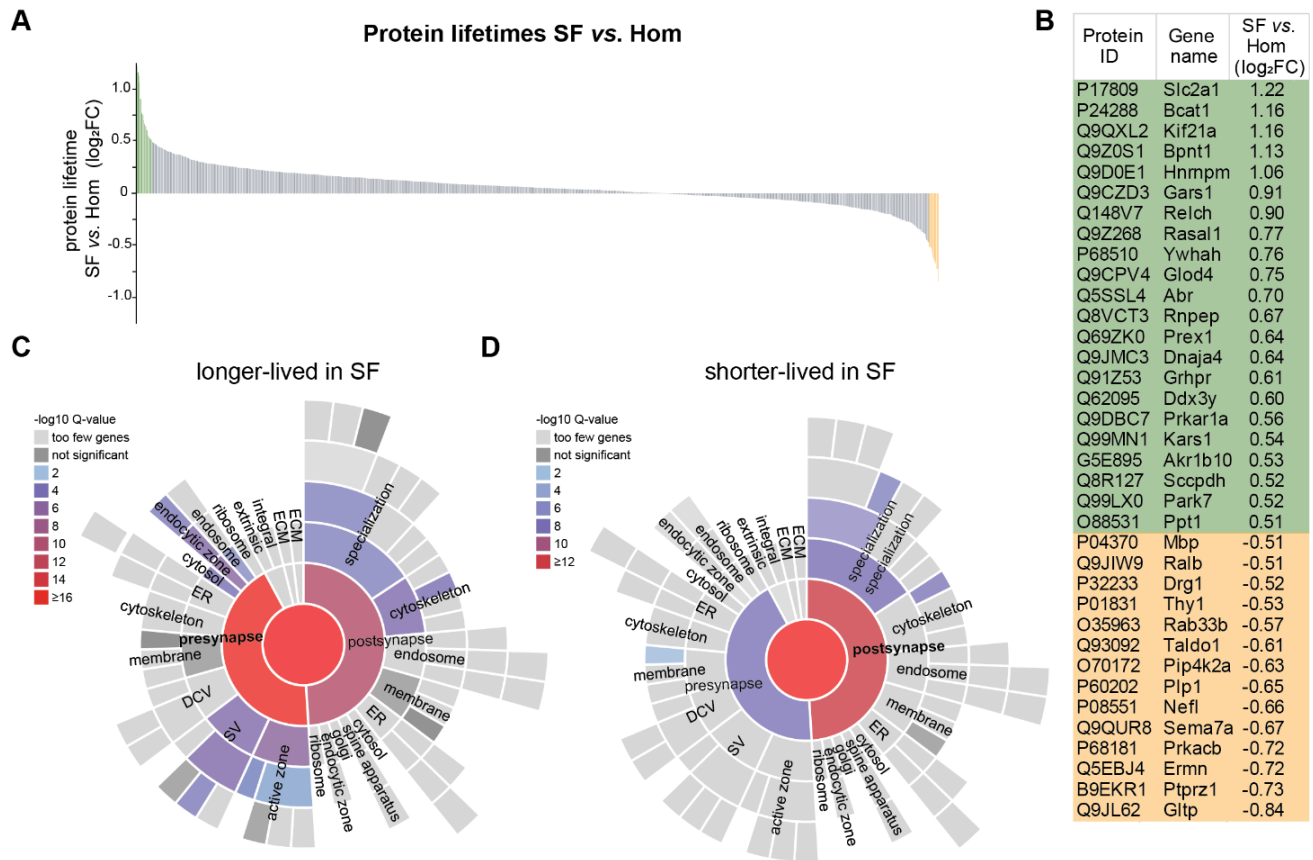


Fig. S2. Analysis of proteins longer- or shorter-lived in the synaptic fraction compared to the respective homogenates. We calculated the ratios of protein lifetimes in the synaptic fraction vs. its respective homogenate for both cortex and cerebellum. For 1143 proteins, ratios from both brain regions were obtained and averaged. Log₂FCs of these averages are shown as bars in (A). The color-coded bars represent all proteins with a log₂FC > 0.5 (green) or < -0.5 (yellow), which are also detailed in (B). All values can be inspected in **table S1**. For synaptic gene ontology enrichment analysis, SynGO (24) was used, inputting the proteins with log₂FC > 0.15 or < -0.15 for longer-lived (C) and shorter-lived (D) proteins in synaptic fraction vs. homogenate, respectively.

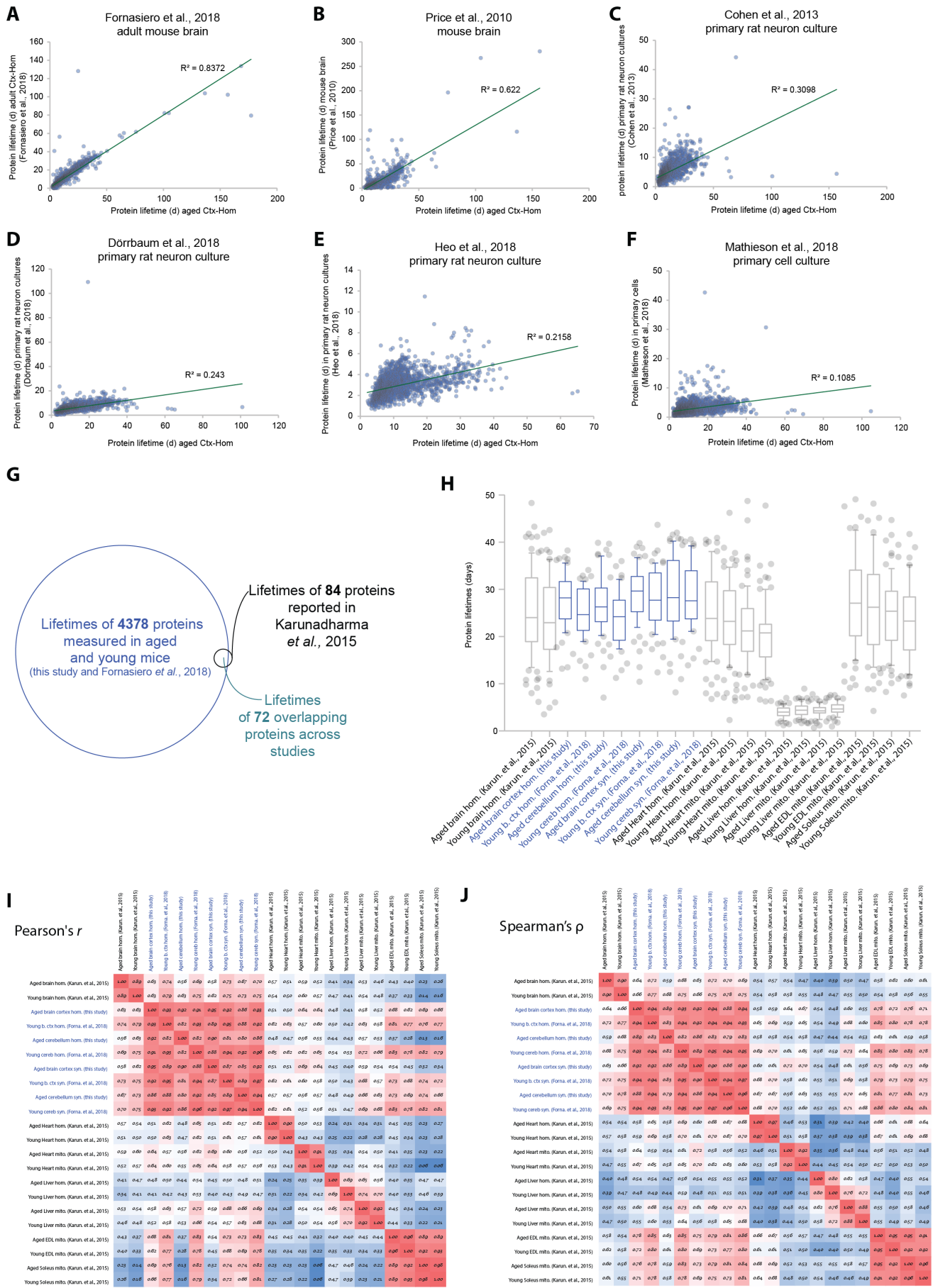


Fig. S3. Comparison of protein lifetimes between our aged cortex homogenate dataset and previously-published datasets. (A, B) Lifetimes acquired from in vivo experiments in the mouse brain; our own previously generated dataset

from young adult mice (19) and data produced by (20). (C-F) Protein lifetimes from in vitro data are generally less correlated, as previously described (19). Data were retrieved from (23, 81–83). Scatterplot representations include the squared Pearson's r . (G) Venn diagrams of lifetimes measured in this study together with the previously published data from our group (19) compared to Karunadharma *et al.*, 2015 (6), showing that 72 proteins are common to all studies. Although two completely different labelling technologies and mass spectrometry analysis workflows were used, the overlap between the mitochondrial proteins of the two studies is sufficient to carry on subsequent analyses. (H) Protein lifetime distributions across the different studies, for both aged and young tissues. Our lifetime results are perfectly in line with those previously reported by (6), since the overall average lifetimes of these proteins in the brain in both studies are in the range of ~25-30 days. This is quite remarkable, especially since we used two different labels ($^{13}\text{C}_6$ -lysine while they used $^2\text{H}_3$ -leucine), confirming the validity of both studies. Some small differences that might be due to the fact that we used different brain regions, different MS methods, different protein extraction methods, and different mouse ages. The main finding of the work of Rabinovitch and colleagues is entirely confirmed by our data. This finding can be resumed as follows: once the lifetimes are normalized to the mean of the respective tissue and treatment group, the relative lifetime differences across mitochondrial proteins are conserved. As an example, some components of Complex I, such as Ndufs 1, Ndufs 2, Ndufs 3, Ndufs 4, Ndufs 6, Ndufs 7, Ndufa 4, Ndufa 6 and Ndufa 7, tend to have relatively shorter lifetimes than the mean of the respective tissue and treatment group for these mitochondrial proteins. Likewise, most of Complex V proteins tend to have relatively longer lifetimes. For the complete set, refer to the rightmost part of the **table S2**, showing the normalized values. (I, J) The similarities across studies summarized by Pearson's r and Spearman's ρ of the data. The average Pearson's r and Spearman's ρ of our lifetimes vs. the brain fractions of both young and aged mice are 0.68 ± 0.02 and 0.70 ± 0.01 (p-values < 0.0001), indicating good agreement. Interestingly enough, the lifetimes from our study have even better correlations with the other tissues reported in Karunadharma *et al.*, 2015 than their own brain samples (our samples vs. their other tissues Pearson's r 0.59 ± 0.01 and Spearman's ρ 0.65 ± 0.01 ; while their brain samples vs. their other tissues Pearson's r 0.43 ± 0.03 and Spearman's ρ 0.53 ± 0.01). This indicates that the two studies are in very good agreement. Using the original data from Karunadharma *et al.*, 2015, also reported in **table S2**, we also compared the normal brain vs. the young brain and confirmed that also in their findings there is a significant lengthening of protein lifetimes in the aged brain (~7% higher in the aged brain for this selection of mitochondrial proteins, paired T-test $p = 0.00025$). As for the extent of this difference, we found a very similar value for this subset of proteins (averaging 7.13% when we consider all brain regions and subcellular fractions, but still significant with a paired T-test $p < 0.0001$). We also tested, using the same group- and treatment-wise normalization used in Karunadharma *et al.*, 2015, whether the lifetimes of these proteins correlate to any parameters in our bioinformatic collection. The only parameter with a significant correlation is the beta-sheet content of these proteins ($r = 0.38$, $P = 0.006$). This result is in line with the main finding of Karunadharma *et al.*, 2015, since it is likely that proteins with more compact structures (hence with higher beta-sheet content), are more stable.

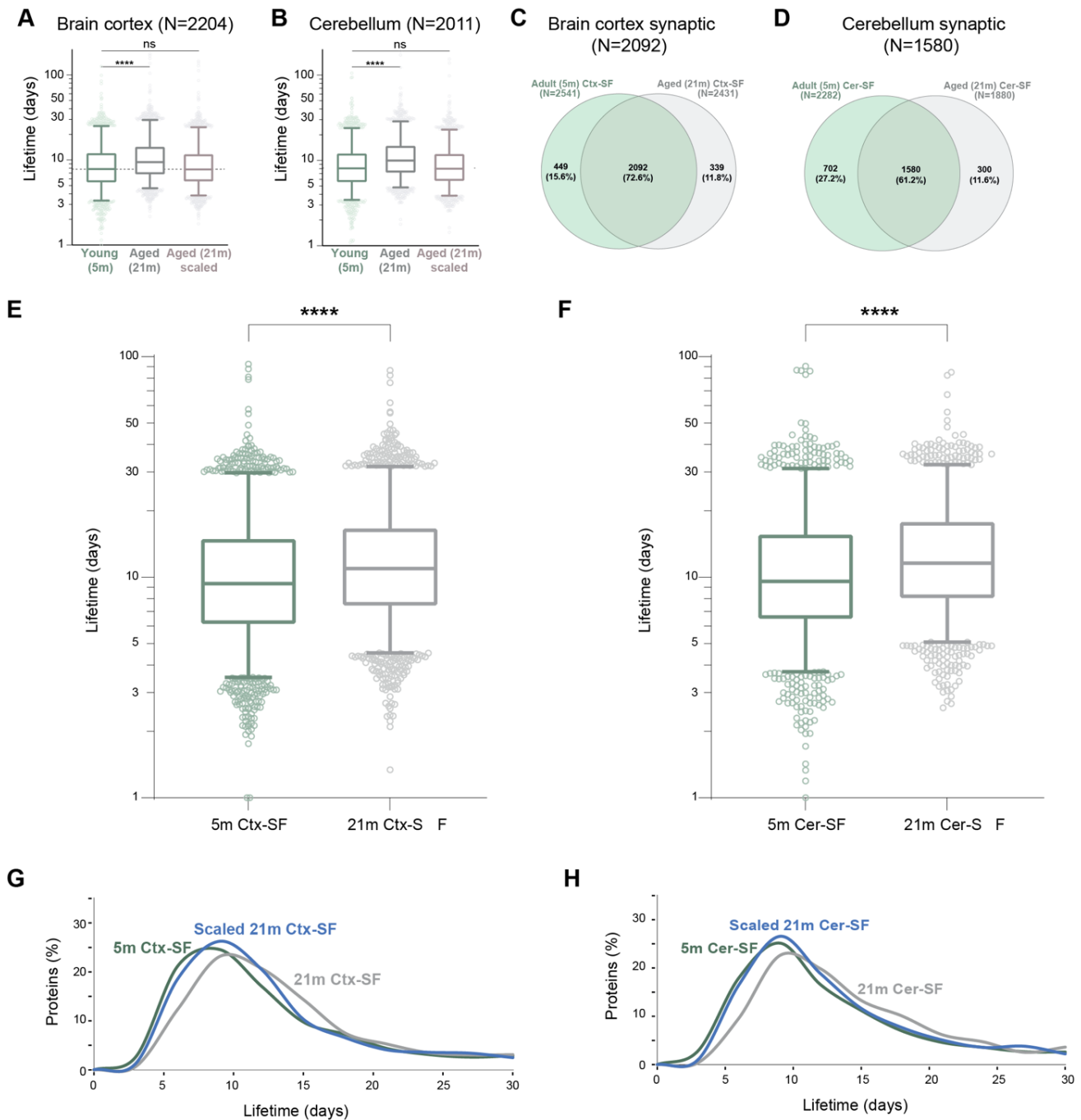


Fig. S4. Brain protein lifetime changes in synaptic fractions, before and after median rescaling (supplementing main Fig. 2). (A) Comparison of protein lifetimes in the cortex of 5m and 21m old mice. Turnover of the aged brain cortex is on average 21.7% slower compared to young adult. After median linear rescaling, accounting for this difference, lifetimes are not significantly different (nonparametric Wilcoxon matched-pairs signed rank test, N=2204, P -value: **** ≤ 0.0001 ; boxplots represent median, 25th to 75th percentile with whiskers showing 5th-95th percentile). (B) The turnover of the aged cerebellum proteome is also slower compared to the young adult cerebellum (24.3% slower at 21m vs. 5m). After median linear scaling, protein lifetimes show no significant difference (Wilcoxon matched-pairs signed rank test, N=2011, P -value: **** ≤ 0.0001). (C, D) Venn diagrams of lifetimes measured in this study ('aged mice'; 21m) and previously published data from our group (young adult mice'; 5m; (19) in synaptic fractions. For details on how synaptic fractions were prepared please refer to the methods and to our previous work (19). (E, F) Comparison of protein lifetimes in the cortex of 5m and 21m old mice. The turnover of the aged brain cortex synaptic fraction is on average slower compared to the young adult (15.14% in the SF cortex and 18.10% in the SF cerebellum). Boxplots represent median, 25th to 75th percentile with whiskers showing 5th-95th percentile. (G, H) Continuous distributions of lifetimes, showing the effect of median linear rescaling for the synaptic fractions, similar to what is represented in panels A and B.

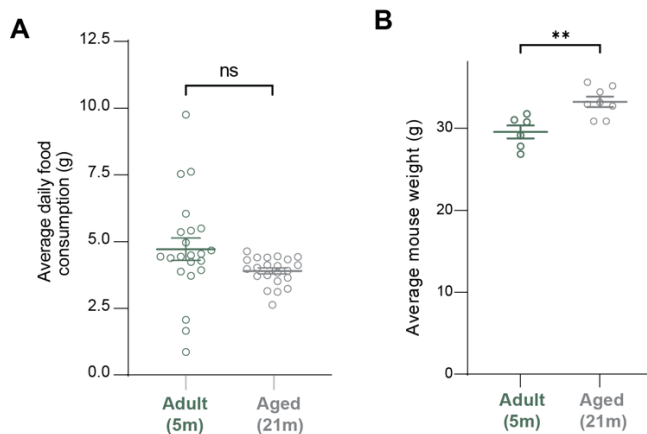


Fig. S5. Analysis of food consumption in mice at 5 and 21 months of age. (A) Average food consumption per day, measured in grams, indicating that young adult and aged animals consume on average the same amount of food (~4 grams per day). (B) average mouse weight in grams. Mean \pm SEM, two-sided t-test P -value $** \leq 0.01$. For these measurements, a total amount of 14 mice was used.



Fig. S6. STRING representation of the relatively-longer- and relatively-shorter-lived proteins in the aged brain. (A) rLL proteins in the aged brain (top 10% of consistently longer-lived proteins in all 4 datasets, resulting in 72 proteins) were analyzed with STRING v11 (28) and three representative GOs and one pathway are color coded. For the complete analysis results see **table S3**. Note that among these proteins there are several implicated in quality control, protein localization and also some key players in autophagy, such as Cathepsin C (an abundant lysosomal cysteine proteinase) and Cathepsin B (also known as APP secretase). (B) same as 'A' for rSL proteins in the aged brain (128 final number of proteins analyzed, with results reported in **table S3**). As also explained in the main text, several among these proteins are mitochondrial components implicated in mitochondrial function and metabolism, with a direct link to NDDs, as also highlighted by the very highly significant false discovery rates (FDRs) for Alzheimer's, Parkinson's, and Huntington's.

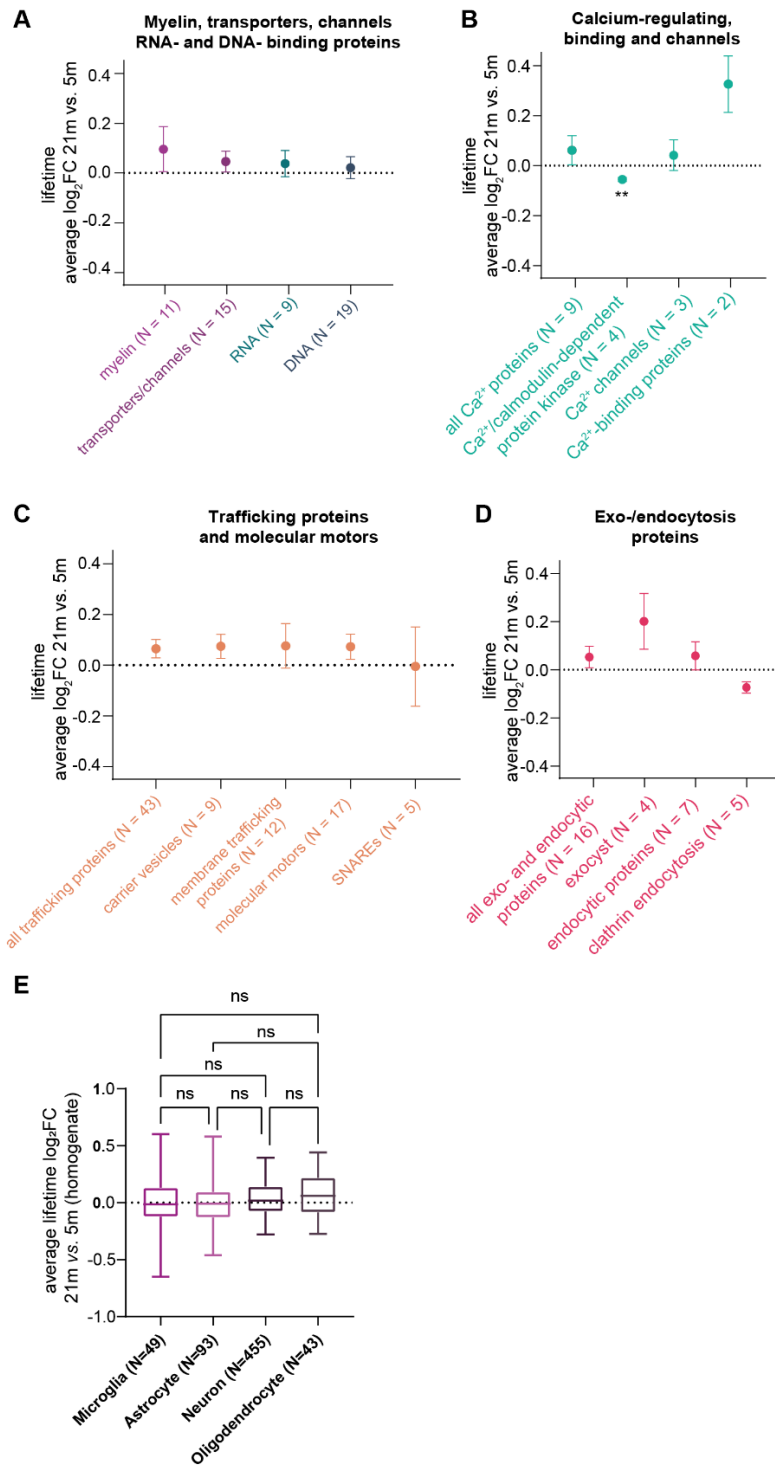


Fig. S7. Remaining categories for protein lifetimes changes supplementing Fig. 21. (A-D) Proteins either rLL or rSL in aged mice according to their manually-curated organelle and/or functional affiliation (see (19) and table S5). The mean \pm SEM of the log₂FC (21m vs. 5m) is represented. Numbers of proteins per category are indicated in parentheses. Significance against the list of consistently changing lifetimes was calculated using Brown-Forsythe and Welch analysis of variance (ANOVA) followed by Dunnett multiple comparison correction (*P*-values: ** ≤ 0.01 and not significant if nothing is indicated). (E) There are no significant differences in changes in lifetimes between microglia, astrocytes, neurons, and oligodendrocytes (assigned according to (48), see Methods for details). The average log₂FC 21m vs. 5m of cortex and cerebellum homogenate was calculated and proteins were sorted according to their dominant cell type. Boxplots represent median, 25th to 75th percentile with whiskers showing 5th-95th percentile; $p > 0.05$ for all comparisons, one-way ANOVA with Tukey's multiple comparison correction.

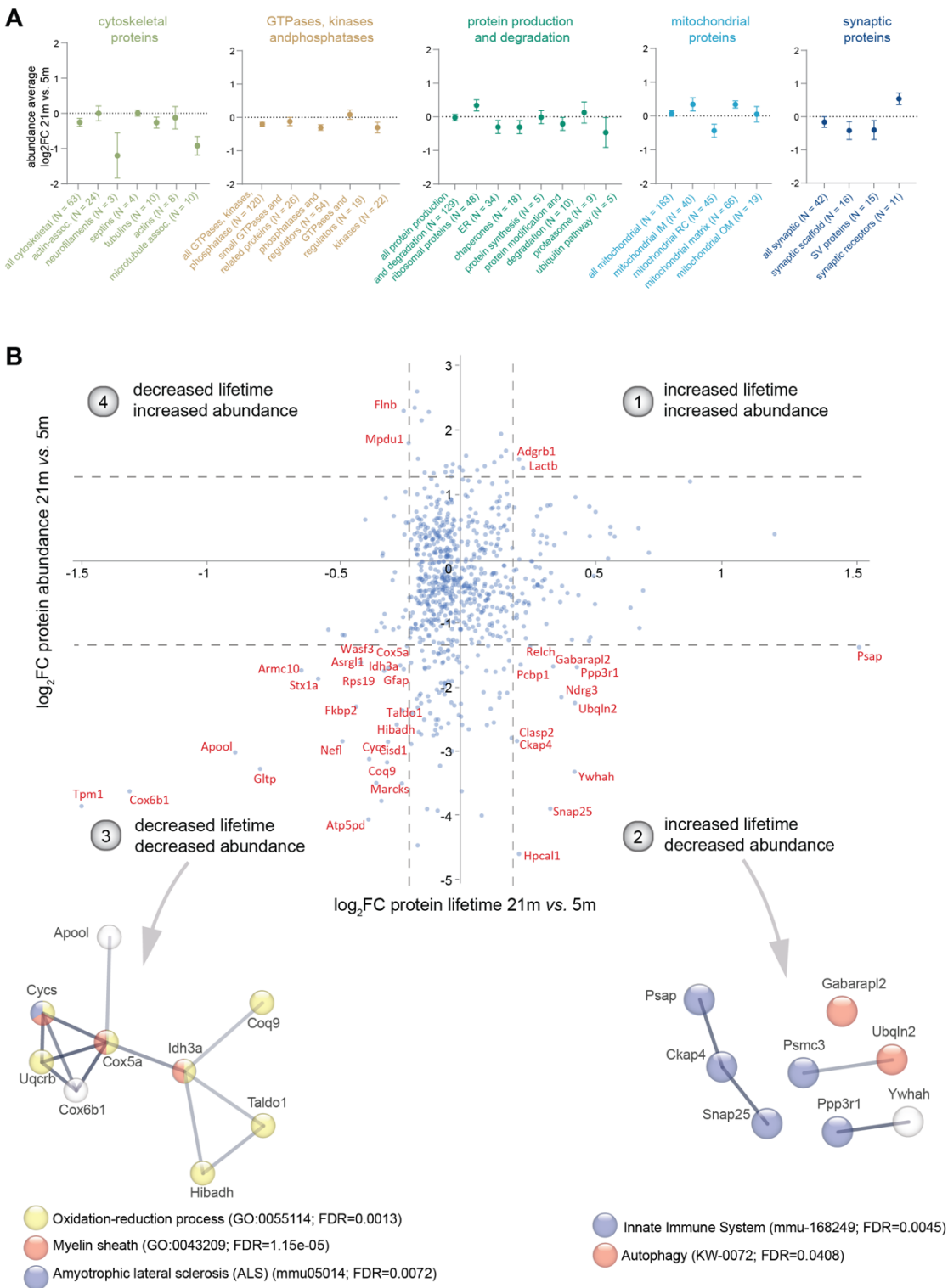


Fig. S8. Correlation between protein lifetime changes and protein abundance changes. (A) Protein abundance changes corresponding to the classes of protein lifetimes presented in main Fig. 2I. Protein abundances, calculated as LFQ and detailed in tables S1 and S5, are summarized for the respective affiliations, following the categorization used in main Fig. 2I. The mean \pm SEM of the \log_2FC LFQ (21m vs. 5m) is represented. Numbers of proteins per category are indicated in parentheses. Significance against the list of consistently changing abundances across tissue groups was calculated using Brown-Forsythe and Welch analysis of variance (ANOVA) followed by Dunnett multiple comparison correction. No significant differences were found. (B) \log_2 fold changes of protein abundance and lifetimes of aged 21m mice and young adult 5m mice are plotted (751 proteins consistently changed (>3) in both lifetime and abundance change, see table S5) and the top 10% in each quadrant are highlighted in red. Insets below show the STRING analysis of quadrants 2 and 3. See Supplementary Text 1 for interpretation

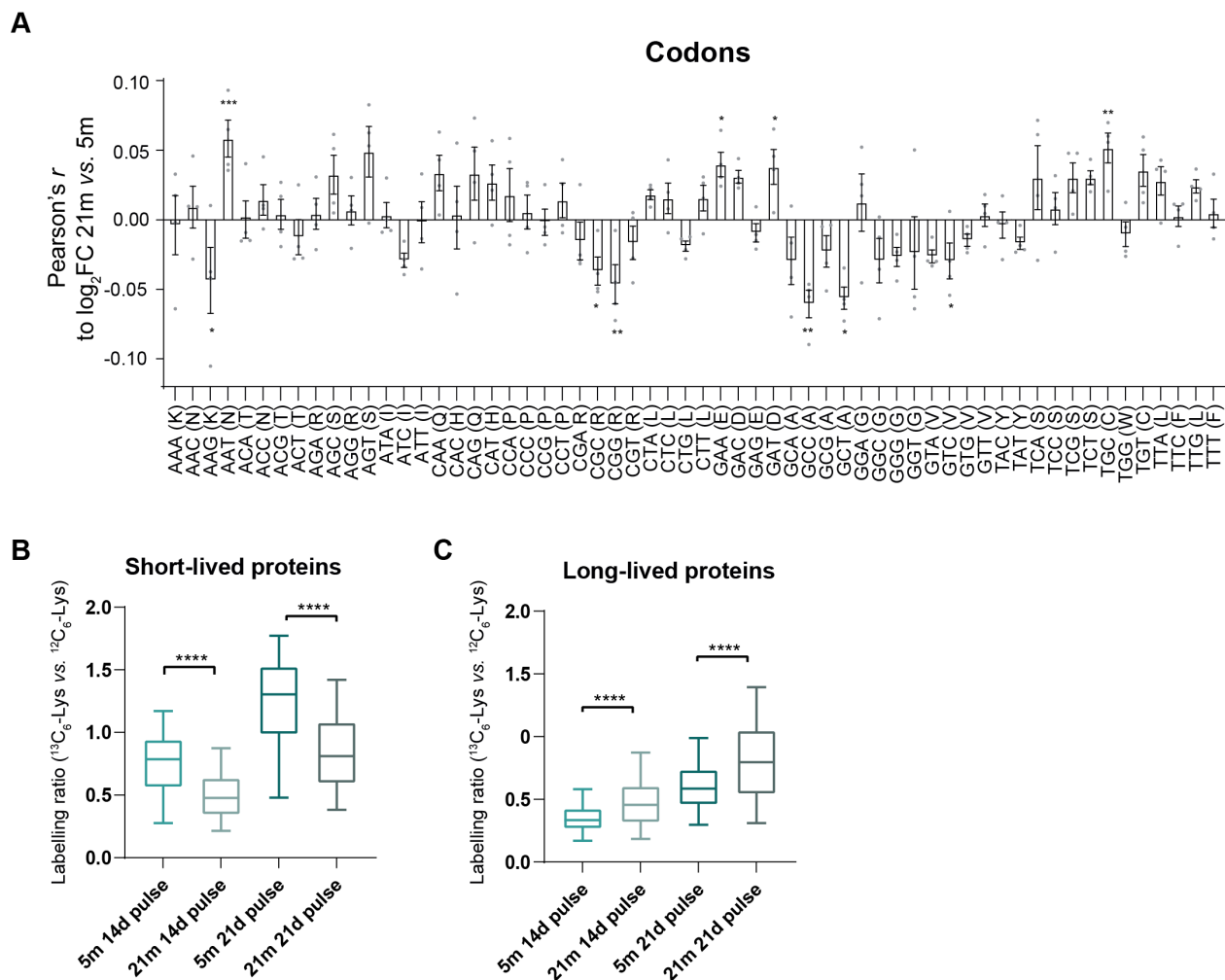


Fig. S9. Correlation to protein lifetime change during brain aging to amino acid composition, codon composition and relative labeling in short-lived and long-lived proteins. (A) Pearson's coefficients show correlations of lifetime \log_2 fold changes to codon usage. Bars represent mean \pm SEM and points are changes in the individual datasets (Pearson's P -values: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001). (B, C) Compression of aged proteome lifetimes as seen in Fig. 3F verified with the original data of heavy to light lysine ratios (ratio H/L) to exclude observations due to our workflow and lifetime fitting (see table S7). In B, short-lived proteins ($<25^{\text{th}}$ percentile of all lifetimes measured in this study, averaged between 5m and 21m mice) are depicted and significantly lower H/L ratios are observed in the 21m aged mice (grey shaded colors), meaning slower turnover of these proteins. This is true for both the 14d as well as the 21d pulse duration. (C) same as in B, but for generally long-lived proteins ($>75^{\text{th}}$ percentile). Paired two-sided t-tests between 5m and 21m, P -values **** ≤ 0.0001 ; boxplots represent median, 25^{th} to 75^{th} percentile with whiskers showing 5^{th} - 95^{th} percentile.

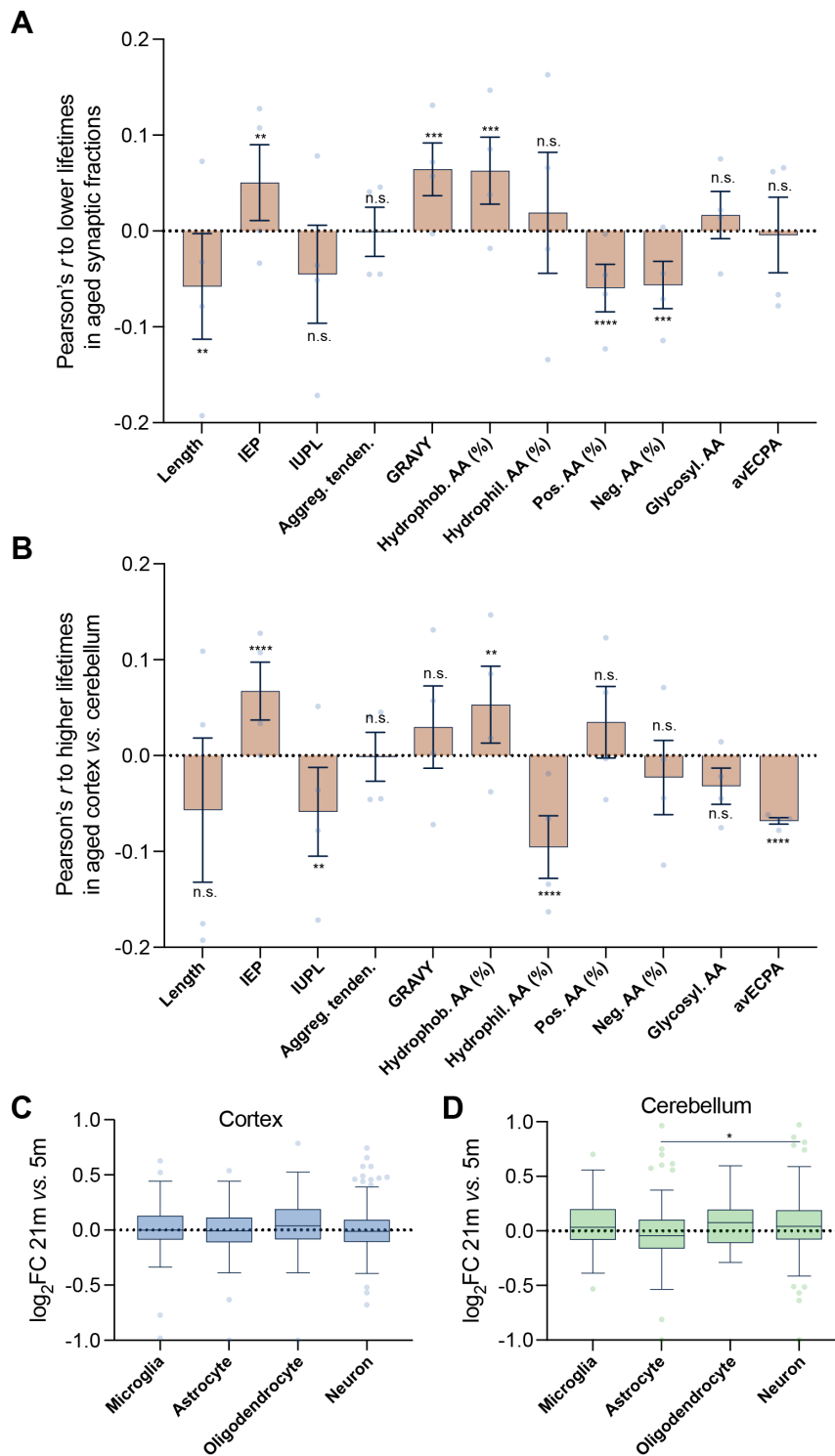


Fig. S10. Correlation between biochemical parameters in synaptic fractions, brain cortex and cerebellum. Lifetime shifts in different cell types. (A, B) Pearson's r correlation analysis between the biochemical parameters also used in Fig. 4 and the relative lifetime changes observed in 21m mice vs. the 5m in synaptic fractions or in cortex and cerebellum. Correlations are shown either to lower lifetimes in synaptic fractions (A) or higher lifetimes in the brain cortex (B). Linear correlation significances are tested including all lifetime changes found at least three times in all fractions (table S6). (C, D) Distributions of \log_2 FC indicating the relative change in protein lifetimes in 21m vs. 5m mice across different cellular populations as previously defined (40) and summarized in table S6. All significances were tested with ANOVA followed by Tukey post-test normalized by multiple comparison.

Supplementary Text 1 (related to Fig. S8B)

In general, we observed a significant positive correlation between changes in protein levels and changes in protein turnover during aging (Pearson's $r = 0.0536$, $P = 0.0042$). Nevertheless, since turnover measurements capture the instantaneous velocity of protein replacement, it is not surprising that this correlation is small. Ideally, the more appropriate comparison between turnover and protein levels should involve one protein turnover measurement and a set of two protein level measurements, the first performed shortly before and the second performed shortly after the turnover measurement. When measured in this fashion, the relative protein level changes and the respective protein turnover measurement would correlate well. As an example, a protein whose lifetime increases will tend to be accumulated in a short temporal scale. In the particular case of our work, in which we compare two turnover measurements (aged adult and young adult), we would need to involve four protein level measurements (before and after the turnover measurements in both age groups).

In reality, protein abundance measurements are known to suffer from reproducibility issues (84), and such comparisons may hold small correlations across time spans, since protein levels can change with complex trajectories over time. This implies that protein turnover measurements and protein levels should be considered as two inherently different measurements, similarly to mRNA turnover and mRNA level measurements (85). Thus, the two measurements should be considered complementary rather than consequential, which explains why it has always been difficult to interpret the comparison between protein lifetime changes and protein level changes (85).

Nonetheless, we were interested in identifying the proteins whose lifetimes consistently change in these long time-scales, either in the same or in the opposite direction with respect to protein levels, which enables us, in some instances, to infer changes in protein production and degradation trajectories. For this purpose, we compared the lifetime changes (\log_2 FC protein lifetime 21m vs. 5m) with their change in protein abundance in aged animals vs. adult animals (\log_2 FC protein abundance 21m vs. 5m). We only considered proteins with up- or downregulation in at least three out of the four dataset (including brain cortex, brain cerebellum, synaptic cortical fraction and synaptic cerebellar fraction) comparisons for both abundance and lifetime, which resulted in 751 proteins (**table S5** and **fig. S8B**). These proteins can be subdivided following four scenarios: 1) Proteins with increased lifetime and increased abundance (which are less efficiently degraded and thus tend to get accumulated); 2) Proteins with increased lifetimes but decreased abundance (which have slow translation rates and tend to become less abundant); 3) Proteins with decreased lifetimes and decreased abundance (which are more efficiently degraded and, as a consequence, have decreased levels); 4) Proteins with decreased lifetimes but increased abundance (which have high translation rates and tend to get accumulated). This analysis revealed that among these four classes, the most populated is the third scenario (39.15% of all 751 proteins considered), where proteins have decreased levels due to increased degradation (**fig. S8B**, lower left corner).

To restrict our analysis to the most relevant variations among these 751 proteins, we considered proteins whose changes were higher than defined thresholds (see **fig. S8B** for details). We thus considered more in detail 39 proteins (in red in **fig. S8B**). Also among these selected proteins, the most populated scenario is scenario 3 (decreased lifetimes and decreased abundance). These include specifically some mitochondrial proteins, such as the Cytochrome C protein CYCS and the Cytochrome C Oxidase Subunit 5A, in line with increased degradation of mitochondrial proteins in the aged brain (86). Interestingly, some of these encompass myelin components, probably reflecting unproductive myelination, as observed in the optic nerve of aged animals (87). Among proteins with a putative slower translation efficiency (second scenario), we have observed some proteins with a role in autophagy such

as Ubiquilin 2, which regulates protein degradation mechanisms and pathways including the ubiquitin-proteasome system (**fig. S8B**, lower right corner).

Other degradative pathways, including the lysosomal pathway, show paradoxical increased protein expression, but actual decline in function in the aged brain and other organs (35, 55, 88). We also followed up on chaperone-mediated autophagy, which was recently connected to neuronal proteostasis in aging and NDD (7). In this context, Hsc70 recognizes the 'KFERQ' motifs of proteins, facilitating their lysosomal degradation. While overall lifetime changes are significantly positively correlated to the presence of canonical KFERQ motifs (Pearson's $r = 0.045$, $P = 0.0164$), this correlation is not significant once corrected for protein length, which in itself is positively correlated to KFERQ motif presence (Pearson's $r = -0.007$, $P = 0.6872$) (89). In other words, it seems that there is no preferential role of lysosomal degradation, but rather it suggests that longer proteins in general are more sensitive to aging-mediated derangement. For the moment, until more direct measures of *in vivo* protein production and degradation become available, this type of considerations require careful examination. As discussed above, the whole trajectory, the 'history' of protein production and degradation might lead to misleading interpretations of the data. As an additional consideration, we have analyzed here the measurements that are globally changed in all our four datasets (cortex, cerebellum and two respective synaptic fractions), but we cannot exclude that tissue- or fraction differences exist, as also summarized in the main text.

Supplementary Text 2 (related to Fig. S10)

To test whether changes in protein lifetimes that have regional or synaptic specificities could be explained by biochemical properties, we correlated the proteins lifetime changes to the parameters shown in Fig. 4. Significant correlations were found when considering synaptic fractions vs. the total brain homogenate and also when comparing two different brain regions (**Fig. S10A-B**).

For synapses, the hydrophobicity and the GRAVY hydropathic score are significantly correlated to lower lifetimes in aged animals (**Fig. S10A**), which is also corroborated by a negative correlation with negatively- and positively-charged amino acids. The most likely explanation for these findings is that many membrane proteins (enriched in hydrophobic residues) may be affected in ageing, possibly through a specific effect on synaptic transmission (90) that might prelude neurodegenerative alterations, in line with changes in synaptic vesicle protein lifetimes in APP mice (71).

For the differences between cortex and cerebellum, we noticed two main trends. First, there is a negative correlation between preferential lifetime changes in the cortex of aged mice with hydrophilic amino acid percentages, which might indicate that there is a different protein solubility tolerance between these two brain regions (4). Second, we observed a negative correlation to the content of expensive amino acids, also suggestive of differences in the metabolism of these two brain regions in aged animals.

We also addressed if in brain cortex and in cerebellum of aged mice there are cell-specific shifts in protein lifetimes (**Fig. S10C-D**). This analysis was based on proteins that can be affiliated specifically to different cell lineages in the brain, based on previous measurements (40), and also summarized in **table S6**. While in the cortex we found no statistical difference between cell types (**Fig. S10C**), in the cerebellum we found that there is a significant difference in the lifetime shift between neurons and astrocytes (**Fig. S10D**; ANOVA multiple comparisons, followed by Tukey post-test adjusted for multiple comparisons). The finding is intriguing but remains for the moment correlative until more direct forms of validations of lifetime changes in these cells will be available.

Supplementary Table captions (note that these files are available as separate files).

Table S1: In *vivo* pulsing of 21-month aged mice with $^{13}\text{C}_6$ Lysine and LC-MS protein measurements of brain cortex, cerebellum, and their respective synaptic fractions. Subsequent protein lifetime determination using the previously established method described in (18, 19). In this table we summarize identified peptides, protein groups, LFQ values, H/L ratios ($^{13}\text{C}_6$ -Lys vs. $^{12}\text{C}_6$), lifetimes in days and their confidence intervals, as well as the categorization used for **Fig. 1** and **fig. S1**. Lifetime differences between synaptic fraction and homogenate are also detailed here.

Table S2: Comparison to a previous work (6) reporting lifetimes from brain mitochondrial proteins in young and aged animals.

Table S3: Comparison of protein lifetimes in 21m aged and 5m young adult mice. Here we summarize the lifetimes in young and aged mice, the linear scaling and the consistently changed lifetimes. It also contains the full heatmap and GO analysis for **Fig. 2** and **fig. S4, S6**.

Table S4: rLL proteins in the aged mouse brain are connected to neurodegenerative disease (NDD). Details and references for proteins linked to NDDs that are marked with a red box in **Fig. 2E** are listed here.

Table S5: \log_2 fold changes of protein lifetimes (and LFQ abundance) in 21m aged vs. 5m young adult mice, categorized as in Fig. 1 and in Fornasiero et al, 2018. Used in **Fig. 2I** and **fig. S7, S8**.

Table S6: Differences in relative lifetime changes ($\log_2\text{FC}$ 21m vs. 5m) between datasets (cortex - cerebellum and homogenate - synaptic fraction). Here we summarized row z-score normalized lifetime changes for the heatmaps and for the full GO analysis represented in **Fig. 3**. Additionally, the cell type assignments are reported in accordance to previously-published results (40), used in **fig. S7**.

Table S7: Correlations between biochemical protein parameters and turnover. In this table we summarize the annotated protein features and their Pearson correlation to lifetime changes in the aged brain. We also list the relatively shorter-, middle- and longer-lived proteins with their features as well as the original H/L data used in **Fig. 4** and **fig. S9**.

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