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### Article

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# Control of mammalian brain ageing by the unfolded protein response transcription factor XBP1

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#### Abstract:

Brain ageing is the main risk factor to develop dementia and neurodegenerative diseases, associated with a decay in the buffering capacity of the proteostasis network. We investigated the significance of the unfolded protein response (UPR), a major signaling pathway to cope with ER stress, to the functional deterioration of the brain during aging. Genetic disruption of the ER stress sensor IRE1 $\alpha$  accelerated cognitive and motor decline during ageing. Exogenous bolstering of the UPR by overexpressing an active form of the UPR transcription factor XBP1 restored synaptic and cognitive function, in addition to reducing cell senescence. Proteomic profiling of hippocampal tissue indicated that XBP1s expression attenuated age-related alterations to synaptic function and pathways linked to neurodegenerative diseases. Overall, our results demonstrate that strategies to manipulate the UPR in mammals may sustain healthy brain ageing.

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#### Main text:

Normal ageing is associated with progressive cognitive impairment, representing the most prevalent risk factor for the development of dementia and neurodegenerative disorders. Decades of research have defined the hallmarks of ageing, underscoring the biological processes that determine when and how organisms age, thus regulating healthspan and lifespan<sup>1</sup>. Subtle structural and functional alterations in synapses are the main drivers of age-related cognitive decline<sup>2</sup>, but the molecular mechanisms dictating these perturbations are still elusive. Proteostasis (homeostasis of proteins) is maintained by the dynamic integration of pathways that mediate the synthesis, folding, degradation, quality control, trafficking and targeting of proteins, and its disturbance has been posited as a pillar of the ageing process<sup>3</sup>. The complexity of synaptic architecture and its dynamic regulation highlight the need to maintain the integrity of proteostasis at the level of the secretory pathway during the organismal lifespan to sustain normal brain function<sup>4</sup>. Studies in simple model organisms demonstrated that the activity of a central node of the proteostasis network, the unfolded protein response (UPR), declines with ageing<sup>5</sup> and that enhancing the expression of the UPR transcription factor X-BOX binding

protein-1 (XBP1s) in neurons extends health and lifespan in C.  $elegans^6$ . XBP1 is a master regulator of the UPR, an adaptive pathway that mediates proteostatic recovery in cells suffering endoplasmic reticulum (ER) stress<sup>7</sup>. XBP1 is activated by the stress sensor inositol-requiring enzyme-1 alpha (IRE1), an ER-located RNase that catalyzes the unconventional splicing of the *XBP1* mRNA to eliminate a 26-nucleotide intron<sup>8</sup>. This processing event shifts the coding reading frame to generate an active and stable transcription factor termed XBP1s (for the spliced form) that controls the expression of various components of the proteostasis network<sup>7-8</sup>. In the brain, XBP1 has additional functions in the regulation of synaptic plasticity, neuronal differentiation, learning and memory<sup>9</sup> and global metabolic control<sup>7</sup> Studies in C. elegans indicate that the activity of the UPR in neurons operates as a central regulator of organismal proteostasis during aging through a cell-nonautonomous mechanism that tunes proteostasis in the periphery to extend lifespan.<sup>10-13</sup> To date, this concept has not been validated in mammals, although correlative studies indicate altered UPR signaling in aged human tissue<sup>14-16</sup>.

To determine the possible contribution of the UPR to age-associated cognitive decline in 15 humans, we analyzed RNA sequencing data from the hippocampus of elderly subjects diagnosed or not with dementia in a unique aged population-based cohort from the Adult Changes in Thought (ACT) study (http://aging.brain-map.org/). Remarkably, unbiased functional enrichment analysis of most altered transcripts in demented patients highlighted unfolded protein binding and processing as the most enriched altered biological functions (Fig. 1a). Moreover, XBP1 was predicted as a possible transcription factors driving such alterations to the proteostasis network (Fig. 1a, Table S1). In addition, chaperones of the heat shock family accounted as the most altered genes in demented patients (Fig. 1b), while some classical UPR mediators were not altered (Extended Data Fig. 1a).

To determine the capacity of the ageing mammalian brain to engage the UPR, we 25 intraperitoneally injected mice of different ages with tunicamycin, a well-established pharmacological inducer of ER stress<sup>17</sup>. Next, we evaluated UPR transcriptional responses by measuring the mRNA levels of Xbp1s, Hspa5 (Bip/Grp78), Ddit3 (Chop) and Atf3 in the hippocampus, in addition to cerebral cortex and cerebellum. Remarkably, the capacity to induce *Xbp1*s was reduced in hippocampus in middle age and aged animals following experimental ER stress although no alteration in basal levels was found (Fig. 1c-e). UPR mediators Ddit3 and Hspa5 showed the same trend in this brain region (Fig. 1c,). Similar findings were obtained in

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the brain cortex of the same animals (Extended Data Fig. 1b), whereas no differences were detected in the cerebellum (Extended Data Fig. 1c). Increased generation of reactive oxygen and nitrogen species, such as nitric oxide (NO), has been observed during ageing<sup>18</sup>. Previous reports indicated that S-nitrosylation of IRE1a, resulting from posttranslational modification of cysteine thiol (or more properly thiolate anion) groups at Cys931 and Cys951 by NO-related species, inhibits its ribonuclease activity<sup>19</sup>. Accordingly, we evaluated the levels of S-nitrosylation of IRE1a (abbreviated SNO-IRE1a) during normal brain ageing. We measured the ratio of SNO-IRE1 $\alpha$  to input IRE1 $\alpha$  by biotin-switch assay in the brains of young and old animals and found a significant increase in this ratio in aged samples (Fig. 1f), potentially accounting, at least in part, for the decrease in IRE1 $\alpha$  activity and thus compromised ability to adapt to ER stress with age. Notably, when we also performed standard immunoblots of IRE1 $\alpha$  to carefully quantify by levels by densitometry in young and aged animals, we found that total IRE1 $\alpha$  also decreased with ageing (Fig. 1g), as suggested previously based on mRNA levels<sup>20</sup>. Thus, while the absolute level of S-nitrosylated IRE1a may not be that different in young vs. aged brain, the proportion of SNO-IRE1α is significantly greater (Fig. 1f), reflecting overall inhibition of enzyme activity. These results suggest that the occurrence of specific molecular alterations in the ageing hippocampus interfere with the capacity to adapt to ER stress.

In order to evaluate and quantify cognitive and motor decline associated with normal ageing in rodents, we implemented a battery of tasks to assess the behavior of young, middle-aged, and aged wild type mice (Extended Data Fig.2a,e,h,i,j,k). Additionally, we measured compound muscular action potentials (CMAPs) in three muscles during ageing (Extended Data Fig.1). We detected spontaneous decline in the performance of animals starting at middle age and progressing thereafter using distinct cognitive and motor evaluations (Extended Data Fig.1), some of which were previously reported<sup>21</sup>. Since most of the cognitive tasks implemented here are dependent on normal hippocampal function, we also evaluated the electrophysiology of hippocampal slices derived of young or aged animals (Extended Data Fig.1m). Firing rates of CA1 neurons were recorded during spontaneous activity or following picrotoxin (PTX) treatment, an antagonist of GABAergic inhibitory interneurons that fosters excitatory activity in hippocampal circuits (Extended Data Fig. 2m). Despite no significant alterations at basal level, a decline in the firing rates of CA1 neurons of aged mice following PTX treatment was detected, indicative of functional ablation. In line with those results, density of dendritic spines in CA1

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pyramidal cells was also significantly diminished in aged animals when compared to middle aged or young mice (Extended Data Fig. 2n).

To assess the significance of the UPR to brain health span, we conditionally ablated the RNase domain of IRE1 $\alpha$  in the nervous system using CRE transgenic lines driven by the Nestin promoter (IRE1<sup>cKO</sup>) for general deletion in the brain<sup>22</sup> or by the Camk2a promoter (IRE1<sup>cKO/CaK</sup>) (Extended Data Fig. 2a), restricting the deletion to specific neuronal populations. Disruption of the IRE1 $\alpha$  pathway in the central nervous system resulted in reduced performance in agesensitive cognitive tests, including new object recognition (NOR) and contextual fear conditioning (CFC) (Fig. 2a, 2b). IRE1 $\alpha$  deletion using the Camk2a-CRE line also resulted in similar effects (Extended Data Fig.2c). Importantly, aged mice interacted with objects for the same period of time as young animals (fig. Extended Data Fig. 2b and 2f), and did not show intrinsic preferences for any of the objects or locations tested (Extended Data Fig. 2c,d,g). Remarkably, only aged IRE1<sup>cKO</sup> animals presented a significant decay in spatial memory acquisition when tested in the Barnes maze, reflected in a higher percentage of errors (Fig. 2c), although the latency to find the targets did not differ between genotypes (Extended Data Fig.3b). Notably, genetic disruption of IRE1 $\alpha$  function did not alter the cognitive performance of young animals, indicating the occurrence of age-dependent phenotypes (Fig. 2a-c, fig. Extended Data Fig. 3b-c). Since IRE1a has various signaling outputs in addition to controlling XBP1 mRNA splicing<sup>6</sup>, we further confirmed our results in XBP1 conditional knockout animals, which have normal IRE1 $\alpha$  expression, but lack XBP1 expression in the hippocampus, and observed reduced performance in the NOR assay in middle aged but not in young animals (Fig. 2d).

recombinase into the hippocampus of IRE1a floxed animals using adeno-associated viruses (AAVs) (Fig. 2e and Extended Data Fig. 3d). Middle-aged mice were tested in the NOR assay 25 prior to AAV-CRE injection and then monitored again following 4-weeks of brain surgery. Remarkably, targeting IRE1 $\alpha$  in the hippocampus of middle-aged mice impaired the capacity to discriminate novel objects when compared with empty AAV (Fig. 2f), correlating with reduced density of dendritic spines in the CA1 region (Fig. 2g). Importantly, injection of AAV-CRE into the brain of young IRE1<sup>flox/flox</sup> animals did not alter NOR performance or the distribution of 30 dendritic spines (Fig. 2f-g), confirming the occurrence of age-related impairment. We previously

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We next targeted the UPR in the brain of adult animals via local delivery of CRE

reported that XBP1 regulates the expression of brain-derived neurotrophic factor (BDNF), an important factor regulating synaptic plasticity thus facilitating learning and memory processess<sup>10</sup>. In agreement with the observed behavioral phenotypes in IRE1<sup>cKO</sup> mice, *Bdnf* expression was reduced in the aged hippocampus of these animals (Fig. 2h).

In order to determine whether IRE1 $\alpha$  ablation in the brain exacerbates age-associated motor decay, we evaluated animals at behavioral, electrophysiological and morphological levels. Aged IRE1<sup>cKO</sup> mice manifested impaired performance in the wire hanging and rotarod tests when compared to littermate control animals (Fig. 2i and 2j). However, muscle electrophysiological properties did not show significant differences between genotypes in three distinct muscles tested (Extended Data Fig. 3e-g). Similarly, analysis of neuromuscular junction (NMJ) morphology did not reveal any alterations between aged IRE1<sup>cKO</sup> and control mice (Extended Data Fig. 3h-i). We then determined if the disruption of the IRE1 $\alpha$  pathway in the brain results in altered markers of ageing. Thus, we evaluated the accumulation of senescent cells using  $\beta$ -galactosidase and  $\gamma$ -H2AX staining in hippocampal tissue, as increased senescent cells in brain tissue correlate with age-dependent cognitive decline<sup>23</sup>. Remarkably, an increase in the content of senescent cells was observed in the brain of middle-aged IRE1<sup>cKO</sup> animals but not in young animals (Fig. 2k, Extended Data Fig. 3j). Overall, our results indicate that genetic disruption of the IRE1 $\alpha$ pathway in the nervous system accelerates the natural emergence of age-associated behavioral and neuromorphological alterations in the central nervous system.

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In order to test the consequences of artificially bolstering an adaptive UPR during ageing, we developed strategies to increase the levels of the spliced and active form of XBP1 in the brain. For this purpose, we initially used transgenic mice that overexpress XBP1s under the control of the PrP promoter<sup>9</sup> (referred to here as Tg<sup>XBP1s</sup>; Extended Data Fig. 4a) and evaluated their cognitive performance during ageing. Remarkably, XBP1s overexpression prevented the development of age-related deterioration in brain function, as evaluated in the NOR, NOL and Barnes maze tests; in fact, these mice performed comparably to non-transgenic young animals (Fig. 3a-c). Additionally, Tg<sup>XBP1s</sup> mice showed reduced age-dependent coordination and motor decay when compared to littermate controls in the wire hanging and rotarod tests (Fig. 3d-e). We then investigated possible molecular pathways that may explain the protective effects of XBP1s overexpression during normal brain ageing. Unexpectedly, we did not observe the upregulation

of *Bdnf* in the brain of middle age and aged Tg<sup>XBP1s</sup> mice (Extended Data Fig. 4b). To define global changes driven by the expression of XBP1s in the brain, we performed an unbiased proteomic analysis of hippocampal tissue derived from animals at different ages. Remarkably, comparison between young and aged wild-type animals indicated alterations in a cluster of cell signaling proteins involved in long-term potentiation, calcium signaling and metabolic control (Extended Data Fig. 4c, left; Table S2). Evaluation of middle aged and aged animals (Extended Data Fig.3C, right) uncovered alterations in a cluster of proteins related to synaptic vesicle recycling, endocytosis, cytoskeletal dynamics, among other processes (Extended Data Fig. 4c, Table S2). Comparison of Tg<sup>XBP1s</sup> animals with age-matched litter-mates (Extended Data Fig. 4d) indicated significant modifications in the expression of proteins involved in neurotransmission (Fig. 4f-g, Extended Data Fig. 4e-f, Table S3), consistent with the functional improvements detected in these animals at the cognitive level. Interestingly, enriched terms were associated to several age-related neurodegenerative diseases (amyotrophic lateral sclerosis, prion diseases, Alzheimer's disease), synaptic physiology (long term-potentiation, neurofilament, glutamatergic synapse and exocytosis) and myelin sheet (Fig. 3f-g, Extended Data Fig. 4e-f, Table S3).

Finally, we evaluated whether artificial activation of XBP1s-dependent responses could potentially reverse the natural decay in normal brain function observed during ageing. For this purpose, we performed bilateral injections of AAVs to express XBP1s in the hippocampi of 20 ageing mice that already manifested cognitive decline (Extended Data Fig. 5a-b). Remarkably, the administration of AAV-XBP1s to middle-aged and aged animals resulted in improved performance in the various cognitive tests compared to age-matched animals injected with control virus (Fig. 4a-d). Administration of AAV-XBP1s also had beneficial effects in electrophysiological properties of hippocampal slices (Fig. 4e-f). In fact, we observed that basal 25 firing rates and bursting activity in hippocampal CA1 neurons were decreased after treating aged animals with AAV-XBP1s. Such alterations facilitated both increased firing rates and busting activity in CA1 neurons following PTX treatment (Fig. 4e-f). Furthermore, induction of longterm potentiation (LTP), widely thought to represent an electrical correlate of learning and memory, was significantly improved in hippocampal slices derived from aged mice treated with AAV-XBP1s compared to controls (Fig. 4g; see fiber volley amplitudes in Extended Data Fig. 5c). Importantly, these findings were associated with a significant increase in dendritic spine

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density in CA1 neurons compared to age-matched control animals (Fig. 4h). Interestingly, aged mice also exhibited decreased accumulation of senescent cells in the hippocampus following XBP1s overexpression (Fig. 4i, Extended Data Fig. 5d). Lastly, we evaluated proteomic changes in the hippocampus of aged animals treated with AAV-XBP1s (Fig. 4j-k, Extended Data Fig. 5e). Gene set enrichment analysis indicated that the delivery of AAV-XBP1s into the brain modified the expression of proteins involved in synaptic function, neurofilaments and vesicle cycle (Fig. 4j-k, Extended Data Fig. 5e and Table S4), similar to the findings obtained in Tg<sup>XBP1s</sup> animals. We also detected important changes in proteins related to extracellular matrix, suggesting an impact to secretory pathway proteostasis (Table S4). Overall, our proteomic profiling suggests that the enforcement of XBP1s expression influences age-associated alterations in synaptic function, consistent with the positive effects of XBP1s administration in prolonging brain health span.

Our findings underline for the first time a protective function of the IRE1 $\alpha$ -XBP1s axis in sustaining mammalian brain healthspan, grounded on its function at synaptic physiology 15 maintenance. Surprisingly, proteomic studies could not detect evident changes in canonical XBP1s-target genes involved in proteostasis control described in other organs, but rather highlighted altered expression of a cluster of proteins related to synaptic function and neurodegenerative diseases. Many of the identified hits are cargoes of the secretory pathway, suggesting that XBP1s overexpression modulates neuronal proteostasis at some extents. Importantly, accumulating evidence suggest that UPR mediators, and more specifically XBP1, 20 have alternative functions in the nervous system by controlling synaptic plasticity<sup>24</sup> and dendritogenesis<sup>25,26</sup>. Importantly, the appearance of senescent cells in brain function was prevented by XBP1s overexpression, whereas IRE1 $\alpha$  deficiency exacerbated the accumulation of senescent cells, consistent with a role of XBP1 as an ageing modifier. Overall, our results 25 suggest that the UPR exerts global effects in sustaining proper brain function during ageing.

Prior reports have demonstrated the involvement of the IRE1a-XBP1s pathway in a variety of age-related neurodegenerative conditions, including Parkinson's disease, Alzheimer's disease, ALS and frontotemporal dementia<sup>24</sup> and retinal degeneration in diabetes<sup>27</sup>. However, whether the activity of the UPR can restore neuronal function in the context of normal mammalian ageing in the absence of disease was unknown, and the mechanisms driving brain

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ageing versus neurodegenerative diseases are predicted to be different. Unexpectedly, our proteomic profiling suggested that XBP1s overexpression modulates the expression of a variety of proteins related to several neurodegenerative diseases (Table S2, Table S3 and Table S4). These findings support the concept that targeting central components of the proteostasis network, such as the UPR, may influence the risk to develop brain diseases, where XBP1 operates as an intersection between the biology of ageing and the emergence of neurodegenerative conditions. In this line, a previous study suggested that XBP1s directly controls the expression of several Alzheimer's disease-related genes<sup>28</sup> and a polymorphism in the *XBP1* promoter operates as risk factor to develop the disease<sup>29</sup>. Studies in invertebrate models (yeast, C. elegans and D. melanogaster) have uncovered a central role of ER proteostasis and the UPR in ageing (see examples in<sup>6,10-13,30-33</sup>). Interestingly, the beneficial effects of caloric restriction, a major intervention that extend lifespan, were recently linked to modulatory effects on ER proteostasis<sup>32-33</sup>. Importantly, the positive consequences of activating neuronal and glial UPR on lifespan of worms involves the global control of organismal proteostasis through a cellnonautonomous mechanism<sup>6,12,31</sup>. It remains to be determined if an increase in XBP1s expression in the aged brain can be translated into the propagation of adaptive signals that improve the function of other organs, thus mitigating their deterioration during the course of natural ageing. Because recent studies suggested a correlation between UPR alterations in elderly human tissue<sup>14-16</sup>. strategies to improve ER proteostasis or boost the adaptive activity the UPR may extend brain healthspan, reducing the risk of developing dementia and other age-associated neurodegenerative diseases.

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#### **Figures and Figure Legends**



Fig. 1. Impaired UPR activation in the mammalian aged brain. (a) Functional enrichment analysis of altered genes in an elderly cohort of patients diagnosed with dementia versus non-demented. GO: gene ontology; Key TF: key transcription factor in TRRUST database; FDR: false discovery rate. (b) Violin plots displaying normalized FPKM values of chaperones *HSPA1a* and *HSPA1b* in RNA-sequencing of human hippocampus of demented patients and controls (n = 49,42; unpaired Kolmogorov-Smirnov test, \*\*: P < 0.01). (c-e) Young, middle-aged, and aged animals were treated with tunicamcyin (5 mg/kg) or vehicle. After 24 h, the mRNA levels of *Xbp1-s*, *Bip/Hspa5*, *Chop/Ddit3* and *Atf3* were determined in dissected hippocampus by quantitative RT-PCR. (n = 4 animals/group. One-way ANOVA followed by Tukey's post-test compared tunicamycin treated groups, \*\*: P < 0.01). (f, g) Relative levels of S-nitrosylated IRE1a (d) and total IRE1a (e) in brains from young and aged mice. Histograms show relative levels of SNO-IRE1a/input IRE1a measured by biotin-switch assay (f) and total IRE1a/GAPDH quantified by densitometry on standard immunoblots (g) for young (3 month-old) versus aged (24 month-old) mouse brains (n = 3, \*: P < 0.05 by Student's t test).

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Fig. 2. Genetic ablation of the IRE1 $\alpha$  pathway in the brain accelerates and exacerbates age-associated cognitive and motor decline in mammals. (a) Conditional knockout mice for IRE1 $\alpha$  were generated using the Nestin-CRE system (IRE1<sup>cKO</sup>) and their littermate flox/flox control animals (IRE1<sup>WT</sup>). The new object recognition test was used to evaluate the ability to discriminate novel objects after 24 h in young, middle-aged and aged mice (n = 10/ group; \*\*\*: P < 0.005 by two-way ANOVA followed by Sidak's multiple comparison test). (b) In animals of indicated genotypes, contextual fear conditioning test was performed to compare total number of freezing episodes following 24 h of aversive stimuli in young (n = 7, 10) and middle-aged (n = 9, 14) wild-type (IRE1<sup>WT</sup>) or IRE1<sup>cKO</sup> mice (\*P < 0.05 by unpaired Student's t test within each age-matched group). (c) Young (n = 9, 9), middle-aged (n = 10, 8) IRE1<sup>WT</sup> or IRE1<sup>cKO</sup> mice were evaluated in the Barnes maze to evaluate spatial memory

within each age-matched group). (d) Young WT (n = 8), young XBP1s<sup>cKO</sup> (n = 12), middle-aged WT (n = 18), or middle-aged XBP1<sup>cKO</sup> (n = 14) mice were evaluated in the new object recognition test to analyze the ability to discriminate between novel objects following 24 h of presentation of two identical objects (\*: P < 0.05 by unpaired 5 Student's t test comparing age-matched groups). (e) Scheme to illustrate the loss-of-function approach based on AAV2-CRE-mediated deletion of floxed IRE1 $\alpha$  in the hippocampus. IRE1<sup>flox/flox</sup> animals were evaluated using the NOR test and then received bilateral hippocampal injections with AAV2-CRE. After 4 weeks, animals were evaluated again in the NOR test and then euthanized. Brains were collected for dendritic spine and biochemical analysis. (f) Young (n = 7) and middle-aged (n = 6) IRE1<sup>flox/flox</sup> animals were evaluated in the NOR test before and 10 after AAV2-CRE intra-hippocampal injections (\*: P < 0.05 by paired Student's t test comparing each individual before and after AAV2 injection). (g) Animals of different ages were injected with adeno-associated vector (AAV-CRE or AAV2-Mock) into the CA1 region of each hippocampus. AAV constructs included a GFP cassette to express eGFP in neurons for monitoring dendritic spine density. One month after the injection, spines were imaged by confocal microscopy. Left-hand panels: Representative confocal microscopic images of dendritic spines (arrows) 15 in the CA1 region of middle-aged animals. 60x magnification; scale bar, 5 µm. Right-hand panel: Histogram of mean and SEM of spine density per  $\mu$ m (n = 24 dendrites, 4 animals; 27 dendrites, 3 animals; 31 dendrites, 4 animals; 42 dendrites, 4 animals, respectively). \*\*\*\*: P < 0.001 by unpaired Student's t test within each agematched group. (h) Quantitative PCR showing Bdnf relative mRNA levels comparing young, middle aged or aged IRE1<sup>cKO</sup> to controls (n = 3-4 animals/group. Unpaired Student's t test was performed comparing age-matched groups, \*: P < 0.05), (i) Wire hanging test performed in young, middle-aged, and aged IRE1<sup>WT</sup> or IRE1<sup>cKO</sup> to 20 evaluate motor performance. Graph indicates mean and SEM of arbitrary scores from aged (n = 18, 15), middleaged (n = 11, 15), and young (n = 10, 12) animals (\*: P < 0.05 by two-way ANOVA followed by Sidak's post-hoc test). (j) Rotarod test used to evaluate motor performance and coordination in young (n = 16, 12), middle-aged (n =8, 8), and aged (n = 18, 6) IRE1<sup>WT</sup> or IRE1<sup>cKO</sup> animals. Graph indicates mean and SEM of latencies to fall from the 25 rod (\*: P < 0.05 by unpaired Student's t test within each age group). (k) Representative photomicrographs of  $\beta$ galactosidase staining of hippocampal slices derived from young and middle-aged IRE1<sup>WT</sup> or IRE1<sup>cKO</sup> animals (n =3-4 animals/group). Graphs (at right) indicate mean and SEM of percentage of  $\beta$ -galactosidase positive cells. \*\*:  $P < \beta$ 0.01 by unpaired Student's t test within each age-matched group for each hippocampal sub-region. Magnification: 20x; scale bar: 200 µm.

acquisition. Total number of errors before finding the target hole is plotted (\*P < 0.05 by unpaired Student's t test



Fig. 3. Enforced expression of XBP1s in the brain prevents age-associated motor and cognitive decline in mammals. (a) New object recognition test was used to evaluate ability to discriminate novel objects after 24 h in Tg<sup>XBP1s</sup> or littermate non-transgenic animals (non-Tg) in young, middle-aged, and aged animals (n = 22, 6; 23, 9; 17, 12; respectively; \*: P < 0.05 by unpaired Student's t test within each age-matched group). (b) New object location test was used to evaluate the ability to discriminate changes in object location after 24 h in young, middle-aged, and aged non-Tg or Tg<sup>XBP1s</sup> (n = 20, 7; 12, 13; 19, 9 respectively; histogram shows mean and SEM of percentage of time interacting with the novel-located object (NLO). \*: P < 0.05 by unpaired Student's t test within each age-matched group). (c) Aged non-Tg and aged Tg<sup>XBP1s</sup> animals were evaluated in the Barnes maze test to compare spatial memory acquisition over a period of 5 days. Graph indicates mean and SEM of each group (n = 10 for non-

Tg [WT], 8 for Tg<sup>XBP1s</sup>). Student's t test was used to compare performances within each day (\*P < 0.05). Latencies of young/non-Tg mice also plotted for comparison (n = 10). (**d**) Young, middle-aged, and aged non-Tg or Tg<sup>XBP1s</sup> were evaluated in the wire hanging test to monitor motor performance and coordination. Mean and SEM of arbitrary scores for each group (n = 8 animals/group; \*: P < 0.05, \*\*P < 0.01 by two-way ANOVA followed by Sidak's multiple comparison test). (**e**) Young and aged non-Tg or Tg<sup>XBP1s</sup> animals were evaluated in the rotarod test to monitor motor performance and coordination. Mean and SEM for each group (n = 10 animals/group, \*\*\*: P < 0.001 by unpaired Student's t test within each matched group). (**f**) Following MS/MS analysis of the hippocampal proteome, gene set enrichment analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) library. Graphs depict most enriched terms based on combined scored computed by EnrichR platform comparing middle aged and aged Tg<sup>XBP1s</sup> with age-matched controls. Terms were divided in two graphs related to synaptic function or neurodegeneration. (**g**) Protein-protein interaction networks depicting main genes altered in middle aged and aged Tg<sup>XBP1s</sup> (PPI enrichment p-value < 1<sup>-16</sup>).



Fig. 4. XBP1s-gene delivery to aged animals reverses age-associated phenotypes at the behavioral, morphological and electrophysiological levels. (a) Middle-aged and aged WT animals were injected with AAV2-Mock or AAV2-XBP1s in both hippocampi and then evaluated in the new object location test to evaluate their ability to discriminate displacement of objects 24 h following presentation. Histogram shows mean and SEM of percentage of time interacting with the novel located object (NLO) for n = 10 animals/group, \*\*: P < 0.01 by unpaired Student's t test). Young animal performance plotted for comparison (n = 6). (b) Aged animals injected with AAV2-Mock or AAV2-XBP1s in both hippocampi were evaluated in the new object recognition test to

evaluate their ability to discriminate novel objects after 24 h. Mean and SEM of percentage of interaction time with novel objects (n = 10 animals/group, \*\*\*: P < 0.005 by unpaired Student's t test). Young animal performance plotted for comparison (n = 6). (c) Aged animals injected with AAV2-Mock or AAV2-XBP1s in both hippocampi were evaluated in the Barnes maze test to compare spatial memory acquisition. Mean and SEM of latencies to find target for each group during 4 days of testing (n = 5 for AAV2-Mock, 8 for AAV2-XBP1s; Student's t test was used to compare performances within each day (\*: P < 0.05). Young animal performance plotted for comparison (n = 10). (d) Aged animals injected with AAV2-Mock or AAV2-XBP1s evaluated by contextual fear conditioning for aversive memory acquisition 24 h after presentation of an unconditioned stimulus (n = 5 for AAV2-Mock, 7 for AAV2-XBP1s; \*\*: P < 0.01 by unpaired Student's t test). Young animal performance plotted for comparison (n =6). (e) Brain slice electrophysiological analysis assessing firing rates in hippocampal neurons. Mean and SEM of firing rates were measured during spontaneous activity or following picrotoxin treatment in brain slices from aged mice injected with AAV2-Mock or AAV2-XBP1s (n = 633-832 neurons; n = 6, 9 animals, respectively; \*\*\*\*: P < 6, 90.001 by unpaired Student's t test). (f) Basal and picrotoxin-induced burst activity measured in pyramidal and interneurons in hippocampal brain slices derived from aged mice injected with AAV2-Mock or AAV2-XBP1s (n = 832, 644 neurons from 3-4 animals, \*\*: P < 0.01 by unpaired Student's t test). (g) Mean and SEM of field excitatory postsynaptic potentials amplitudes in brain slices derived from aged animals injected with AAV2-Mock or AAV2-XBP1s 1 h after theta burst stimulus (TBS) to induce LTP in (n = 6, 9 animals, respectively; n = 17-28 slices/animal,\*\*\*\*: P < 0.001 by unpaired Student's t test). (h) Representative fluorescent images showing dendritic spines of pyramidal neurons in CA1 region of young and aged animals injected with AAV2-eGFP (mock) or aged animals injected with AAV2-XBP1s. Young animal neuron is shown for reference. Right panel: Mean and SEM of spine density per  $\mu$ m for indicated experimental groups (n = 24, 53 and 35 dendrites from 3, 6 and 5 animals, respectively; \*\*: P < 0.01 by unpaired Student's t test comparing the aged groups). 60x magnification; scale bar, 5  $\mu$ m). (i) Representative images of  $\beta$ -galactosidase staining of hippocampal slices derived from aged mice injected with AAV2 (n = 3-4 animals/group). Histograms (right) show mean and SEM of percentage of  $\beta$ -galactosidase positive cells. Magnification 20x; scale bar, 200 µm. (i) Following MS/MS analysis of the hippocampal proteome, gene set enrichment analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) library. Graphs depict most enriched terms based on combined scored computed by EnrichR platform comparing aged mice infected with AAV-Mock or AAV-XBP1s. Terms were divided in two graphs related to synaptic function or neurodegeneration. (k) Volcano plot for proteomic comparison of hippocampal tissue derived from aged animals injected with AAV-Mock or AAV-XBP1s. FDR (y-axis) and fold-change (log2) (x-axis) are indicated. Dotted lines delineate cut-off used to filter genes for functional enrichment analysis. Indicated genes associated with each enriched terms connected to synaptic function, neurodegeneration, metabolism and ECM-receptor interaction are colored.

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#### **Materials and Methods**

#### 5 Human Study Population

RNA-seq human data was extracted from The Aging, Dementia and Traumatic Brain Injury Study (https://aging.brain-map.org/overview/home) which is a detailed neuropathologic, molecular and transcriptomic characterization of brains of control and TBI exposure cases from a unique aged population-based cohort from the Adult Changes in Thought (ACT) study. Differential gene expression in RNA-seq was performed directly by the website comparing all donors clinically diagnosed as "Non demented" versus "Dementia, type unknown", "Possible Alzheimer's disease", "Probable Alzheimer's disease" irrespective to sex, years of education, TBI or pathological markers of AD pathology. Only minimum of 30M 50bp paired-end reads per sample were considered. A fold change cut-off greater than 1.2 fold was implemented in the gene list (FDR <0.5) to further functional enrichment analysis evaluation.

#### Animals

C57BL/6 mice were employed, maintained in a facility with 12 h light/dark cycle at 25°C with food and water provided ad libitum. Cohorts of aged, middle-aged, and young animals were directly obtained from the Jackson's Laboratory (USA). All animal procedures were approved by the Bioethics Committee of the Faculty of Medicine, University of Chile (protocol number 18166-MED-UCH).

#### **Stereotaxic injections**

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Young (3 month-old), middle-aged (12 month-old), and aged (18 month-old) mice received bilateral stereotaxic injections of AAV ( $1 \times 10^9$  viral genomes (VGs)/µL) in the hippocampi using the coordinates AP: -1.9, DV: +1.7, and ML: ±1.0. Male mice were deeply anesthetized using isoflurane (4%) and a stereotaxic apparatus coupled to a Hamilton microsyringe was used for the procedure. Cranium was exposed though a skin incision, and bilaterally symmetrical holes were opened using a dental drill. Injections were performed at approximately 0.5 µL/min in a total of 1 µl. Mice were returned to their home cages and kept under close monitoring until

they were awake. Mice were injected with either control AAV serotype 2 vector (Mock), AAV2-CRE (Addgene, #105545-AAV2) or AAV2-XBP1s (produced at Genzyme) under the control of the CMV promoter, in addition to an eGFP cassette to monitor transduction efficiency, as we previously reported <sup>34</sup>.

#### 5 **Behavioral tests**

Behavioral experiments were performed in a masked fashion, both for genetically-modified animals and AAV-injected mice, using groups of age-matched controls. Injected mice underwent behavioral evaluation 1 month following injections. Cognitive and motor tests were performed in the following order: New object recognition, new object location, Barnes maze, wire hanging test, rotarod, and contextual fear conditioning. Not all animals were exposed to all tests in order to avoid possible secondary effects mediated by interaction of the various tests.

#### 1. Novel object recognition

Novel object recognition (NOR) was performed as previously described<sup>35-36</sup>. Briefly, mice were 15 first trained and then placed in an arena facing the wall. They were presented with two identical objects (Lego blocks, 3 x 4 cm) located in a distal position towards the animal. For the training phase, mice were allowed to explore both objects for a total time of exploration of 20 s during a single trial. If exploration did not occur within 20 s, a maximum time of 5 minutes was given for each animal to explore the objects. After 24 h, mice were again placed in the arena facing the wall but at this time were introduced to a new object of different color, shape and texture. In the test phase, mice were again allowed to explore objects for a maximum of 20 s or 5 minutes if the criterion of exploration was not accomplished in the initial time allotment. Four different Lego blocks with equivalent sizes  $(3-4 \times 4 \times 4 \text{ cm})$  were presented in a randomized fashion for each animal. Trials were recorded on a digital camera coupled to a computer for subsequent 25 evaluation of time spent exploring objects by a blinded researcher. Both the objects and the arena were cleaned with 70% ethanol before each session to avoid olfactory cues. Exploration time was defined as the amount of time mice had their noses oriented toward an object with its nose within 3 cm or less. Other behaviors such as rearing near the object or resting against the object were not considered as exploration. Exploration time spent during the training phase was also



recorded. Animals that failed to interact with one or both objects, or that showed signs of stress were excluded from the analysis. All analysis was performed in a masked fashion.

#### 2. Novel object location

The novel object location task (NOL) was performed as previously described  $^{35-36}$ . Mice were placed in the same arena used for the NOR assay. The arena was divided into quadrants. For the training phase, mice were introduced to two identical objects (Lego blocks, 5 x 4 x 4 cm) placed in two randomized quadrants of the arena. Objects were placed equidistant from one other. At 3 or 24 h after the test phase, mice were again placed in the arena facing the wall but now with one of the objects placed in a distinct quadrant. Trials were recorded on a digital camera coupled to a computer for subsequent evaluation of time spent exploring objects. Both the objects and the arena were cleaned with 70% ethanol before each session to avoid olfactory cues. Exploration time was defined as detailed above by a blinded researcher. As per standard protocol, animals that failed to interact with one or both objects or showed signals of stress were excluded from the analysis.

#### 3. Barnes maze

The Barnes maze task was performed on a white circular surface (0.9 m in diameter) with 20 holes equally spaced around the perimeter, as previously described<sup>37</sup>. A dark escape box ( $10 \times 20 \times 7.8$  cm) was located under one of the holes, which was the target. A ramp was placed under the target hole so that mice could reach the escape tunnel easily. The circular open field was elevated 75 cm above the floor. Distal spatial cues (with different colors and shapes) were placed outside of the maze. The maze was rotated daily, with the spatial location of the target unchanged with respect to the distal visual room cues. A cylindrical start chamber was placed in the center of the maze and removed after 10 s. Training sessions consisted of four trials per day conducted for 4 days with a maximum time of 3 minutes each. If animals were unable to find the target after 3 minutes, they were gently placed in the right hole by the tail. A stopwatch sound was used as an aversive stimulus in order to induce exploration. Once a mouse reached the target, the noise was immediately stopped, and the mouse was left inside the box for 1 minute and then returned to its home cage. A maximal period of 15 minutes was given for the inter-trial interval. The maze and the escape box were cleaned with 70% ethanol between each trial to avoid olfactory cues. The numbers of primary pokes and the primary latency to reach the target were manually counted, as

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defined by the number of pokes and time to reach the target for the first time, as some animals did not promptly enter the escape box during training sessions. Every trial was recorded on a video camera placed in the ceiling and fed to a computer. On the fifth day, the escape box was removed, and one single trial was performed as a probe trial to evaluate spatial memory acquisition with a maximum time of 90 s. Then, following 7 days without training, mice were again exposed to a test phase to evaluate long-term memory formation.

#### 4. Contextual fear conditioning

On the first day, mice were placed in the contextual fear-conditioning chamber (Med Associates). During the first 2 min, no stimulus was applied, following which 80 db of white noise (the conditioned stimulus; CS) was generated for 30 s. Two seconds later, mice were exposed to a 0.5 mA electric shock (the unconditioned stimulus) for 2 s. After the shock, mice were let in the cage for 2 more minutes and freezing episodes, represented by the percentage of freezing response to total activity, were measured by an automated system. On the next day, mice were again placed in the same chamber and exposed to the same protocol but this time without any electroshock. Freezing events and freezing percentage time were recorded for 5 min using an automated system.

#### 5. Wire hanging test

The wire hanging test is a well-established protocol for measuring muscular strength in mice. We generated an arbitrary score to take into consideration the number of times one animal fell from the wire during the course of the test (total of 180 s). Every time an animal fell from the wire, its arbitrary score on the test was decreased by 1 (starting at 10 and decreasing to unity). If an animal fell more than 9 times, the test was stopped. Every time an animal reached one side of the wire, the stopwatch was paused, and the animal was placed in the middle of the wire before the stopwatch was started again. A soft cotton sheet was placed under the wire to avoid an harm to the animal during the falls. The test was scored as follows:

Final Score = (10 - number of falls) x time remaining on the wire (s).

#### 6. Rotarod test

Mice were placed on a rotating cylinder and trained to learn how to walk on it at constant speed. Mice that could not learn the task after successive trials were excluded. Mice that learned the

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task were then analyzed in 5 trials with the apparatus configured to accelerate from 4-40 RPM (rotations per minute) over 10 minutes during the course of 3 days. The mean time until the mouse fell off of the cylinder for each trial was computed as the score.

#### **Tissue collection and processing**

Briefly, mice were deeply anesthetized with ketamine/xylazine and perfused with ice cold saline. The brain was removed from the skull and separated into two hemispheres. The hippocampus, cerebral cortex and cerebellum of the left hemisphere were dissected out and stored frozen at -80 °C until analysis. The right hemisphere was postfixed in 4% paraformaldehyde in PBS overnight at 4 °C, followed by cryopreservation in 30% sucrose in PBS and freezing medium (OCT, TissueTek). Subsequently, 40 µm-thick sagittal sections were obtained free-floating on a Leica cryostat for immunostaining using anti-NeuN (Millipore, MAB377, 1:100) to label neurons. Five serial sections every 200 µm were stained per animal. Fluorescence images were acquired using a confocal microscope (Nikon C2+).

#### **Dendritic spine imaging**

Brain slices were cut at 40-µm thickness on a cryostat. AAV2-GFP fluorescence was previously confirmed in injected animals to validate viral transduction using an inverted epifluorescence microscope and then imaged on a confocal microscope, Nikon Eclipse T1, at 60x magnification with additional digital zoom of 3x. Similar regions were compared among animals (CA1 region, spines in primary and secondary dendrites between the *stratum radiata* and the pyramidal layer, AP: -1.9 to -2.1 from the bregma). Z-stacks were acquired in 0.5-µm slices, laser intensity at 0.5-1% and 12.5us/pixel at 1024x1024 resolution. Z-Stacks were then summed using ImageJ software for total maximum intensity to generate one single stacked 8-bit image. The number of spines was manually quantified in scaled images and divided by the length of the dendrite analyzed; 5-10 dendrites per animal were used for analysis.

#### 25 Immunofluorescence

Brain slices were incubated in citrate buffer at 96 °C for 30 min for epitope exposition and washed in PBS. After this, slices were washed in TBS and incubated in blocking solution (3% BSA and 0.05% Triton X-100) for 60 min at room temperature. Slices were then incubated overnight at 4 °C with primary antibody anti-phospho-Histone H2AX Ser139 (Sigma-Aldrich,

05-636) diluted 1:1000 in 3% BSA in TBS. Secondary antibody was Alexa Fluor 568 (Invitrogen A-11031) diluted 1:2000 in 3% BSA in TBS. The samples were washed 3 times using TBS, and in the final wash, DAPI was added and incubated for 5 minutes. Images were taken with Leica TCS SP8 confocal microscope with a 40x objective magnification. ImageJ and LAS X software were used to process the stacked images. The percentage of positive cells for p-H2AX was graphed. Representative images are shown.

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#### Senescence-associated beta-galactosidase (SA-ßgal) staining

Histochemical detection of SA-βgal activity was performed according to Debacq-Chainiaux et al.<sup>38</sup>. Briefly, slices were incubated in a 1 mg/mL of solution of 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside in 0.04 M citric acid/sodium, 0.005 M K<sub>3</sub>FeCN<sub>6</sub>, 0.005 M K<sub>4</sub>FeCN<sub>6</sub>, 0.15 M NaCl and 0.002 M MgCl<sub>2</sub>, and diluted in phosphate-buffered saline (pH 6) for 16 h. After incubation, slices were washed with TBS and mounted on superfrost microscope slides (ThermoFisher 6776214) using Fluoromount-G (ThermoFisher, 00-4958-02). Images were acquired on a Leica DM500 binocular microscope equipped with a Leica ICC50 W camera using 4x and 10x objective magnifications. ImageJ software was used to process the images. Positive area for SA-βgal activity was measured and representative images are shown.

#### **Electrophysiological measurements**

#### 1. Excitatory postsynaptic field recordings

Hippocampal slices were prepared as we previously reported<sup>39-40</sup>. Briefly, mice were deeply anesthetized with isoflurane (Forene B506 AbbVie), brains quickly removed, and hippocampi sectioned into 300-µm-thick slices in ice-cold dissection buffer (in mM: 2.6 KCl, 1.23 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 212.7 sucrose, 10 dextrose, 3 MgCl<sub>2</sub>, and 1 CaCl<sub>2</sub>, equilibrated with 95% O2 and 5% CO2) using a vibroslicer (Leica VT1200S, Leica Microsystems, Nussloch, Germany). Slices recovered for 1 h at room temperature in an artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 dextrose, 1.5 MgCl<sub>2</sub>, and 2.5 CaCl<sub>2</sub> bubbled with a mixture of 5% CO<sub>2</sub> and 95% O<sub>2</sub>) and then transferred to a submerged recording chamber superfused with ACSF (30 °C, 2 ml/min). Synaptic responses were evoked with 0.2 ms pulses delivered through theta glass micropipettes (TG200-4, Warner Instruments) filled with ACSF, and extracellularly recorded in the stratum radiatum of CA1. Baseline responses were recorded at

0.033 Hz using a stimulation intensity that evoked a half-maximal response, defined as the maximal response without a population spike (pop-spike). Slices were discarded if the postpike appeared in the initial rising phase, paired-pulse facilitation at a 50 ms interval was less than 10%, or the baseline was not stable. LTP was induced using theta burst stimulation (TBS; 10 trains of four pulses each at 100 Hz; 5 Hz inter-burst interval) delivered at 0.1 Hz. LTP magnitude was calculated as the average (normalized to baseline) of the responses recorded 50–60 min after conditioning stimulation.

#### 2. Multielectrode array (MEA) recording

The same brain hippocampal slices were used to record both multielectrode arrays (MEA, 252 electrodes, Multichannel Systems) and local field potentials. Hippocampal slices were mounted on a MEA matrix bathed in an ACSF medium (in mM: NaCl 125, KCl 2.5, glucose 25, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO4 1.25, CaCl<sub>2</sub> 2, and MgCl<sub>2</sub> 1) at 32 °C and constantly bubbled with 95% O<sub>2</sub> and 5%  $CO_2$ . Spike sorting Spyking-circus used. as described was at (http://www.yger.net/software/spyking-circus), to detect individual neuronal action potentials (Spikes) using the default parameters that consist of four main steps: filtering raw extracellular traces, whitening them (to remove correlated noise), clustering action potential waveforms, and fitting them on the whitened traces.

#### 3. Firing and burst rate recordings

Firing rate was computed as the number of spikes divided by the recording length using Neuroexplore (https://www.neuroexplorer.com/) software. Burst rate analysis was computed by an inter-spike interval routine implemented by a custom Matlab procedure<sup>41</sup>. Spontaneous Activity (SA) was recorded for 10 min, following a 30-min application of picrotoxin (PTX, 100  $\mu$ M).

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#### 4. Compound muscle action potential (CMAP) recordings

CMAPs were measured in isoflurane-anesthetized mice using an electromyographic apparatus (Keypoint, Dantec, Les Ulis, Francer AD Instruments, Oxford, UK). Briefly, the sciatic nerve was stimulated by two electrodes placed over the lumbar vertebral column. CMAP was recorded by two electrodes placed in the belly and in the tendon of the right gastrocnemius, tibialis anterior, and triceps muscles. A reference earth electrode was placed in the left gastrocnemius and connected to the electromyography apparatus<sup>42</sup>.

#### **Biochemical and molecular analysis**

Total tissue RNA was extracted using Trizol<sup>™</sup> Reagent (Invitrogen) according to the manufacturer's instruction. cDNA was synthesized with random primers using a High Capacity cDNA Reverse Transcription KIT (Applied Biosystems) and subsequently subjected to quantitative PCR analysis using HOT FIREPol® EvaGreen® qPCR Mix plus (ROX) (Solis Bio Dyne) on a Stratagene Mx3000P machine (Agilent Technologies). Actin mRNA expression was used to normalize all samples. The sequences of primers used were as follows:

Gene	Forward primer	Reverse primer
Xbp1s	TGC TGA GTC GGC AGC AGG TG	GAC TAG CAG ACT CTG GGG AAG
Hspa5	TCA TCG GAC GCA CTT GGA A	CAA CCA CCT TGA ATG GCA AGA
Ddit3	TGG AGA GCG AGG GCT TTG	GTC CCT AGC TTG GCT GAC AGA
Ernl	CCG AGC CAT GAG AAA CAA GAA	GGG AAG CGG GAA GTG AAG TAG
Actin	TAC CAC CAT GTA CCC AGG CA	CTC AGG AGG AGC AAT GAT CTT
Bdnf	CCT GCA TCT GTT GGG GAG AC	GCC TTG TCC GTG GAC GTT TA
exon1		

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#### Detection of S-nitrosylated (SNO-)IRE1a and total IRE1a levels

We performed biotin-switch assays using whole-brain tissue samples as previously described with minor modifications <sup>43</sup>. Briefly, brain tissue extracts were prepared in 400  $\mu$ l HEN-RIPA buffer (100 mM Hepes pH 7.5, 1 mM EDTA, 0.1 mM neocuproine, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a thiol blocking reagent (20 mM methyl methanethiosulfonate [MMTS]) in a 3 ml homogenizer. After centrifuging to remove tissue debris, SDS was added to each sample to a final concentration of 1%. Samples were then incubated for 30 min at 42 °C to block free thiol groups. After removing excess MMTS by acetone precipitation, S-nitrosothiols were reduced to thiols with 10 mM ascorbate. The newly formed thiols were then linked with the sulfhydryl-specific biotinylating reagent N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (Biotin-HPDP). The biotinylated proteins were pulled down with Streptavidin-agarose beads, and western blot analysis performed to detect SNO-IRE1a (1:1000, Cell Signaling 3294S). To monitor the amount of "input" protein

by immunoblot, a 20  $\mu$ l aliquot of the sample was saved prior to the step of Streptavidin-agarose bead addition in the biotin-switch assay. Total IRE1 $\alpha$  was monitored by quantitative densitometry of standard immunoblot assays. GAPDH was monitored as a control to ensure equal loading (1:1000, Millipore MAB374).

#### 5 **Quantitative proteomic analysis**

The hippocampi of mice transgenic, non transgenic or transduced with AAV2 (Mock or XBP1s) were homogenized in TEN buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% NP-40) and protein inhibitor cocktail (Roche), and then sonicated for 15 s at 30% amplitude (QSonica). For each sample lysate, 20 µg were precipitated with chloroform/methanol. Samples for mass spectrometry analysis were prepared as described <sup>44</sup>. Air-dried pellets were resuspended in 1% RapiGest SF (Waters) and diluted to final volume in 100 mM HEPES (pH 8.0). Proteins were reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (Thermo Fisher) for 30 min and alkylated with 10 mM iodoacetamide (Sigma Aldrich) for 30 min at room temperature in the dark. Proteins were then digested for 18 h at 37 °C with 0.5 µg trypsin (Promega). After digestion, the peptides from each sample were reacted for 1 h with the appropriate tandem mass tag (TMT) isobaric reagent (Thermo Fisher) in 40% (v/v) anhydrous acetonitrile and quenched with 0.4% ammonium bicarbonate for 1 h. Samples with different TMT labels were pooled and acidified with 5% formic acid. Acetonitrile was evaporated on a SpeedVac and debris removed by centrifugation for 30 min at 18,000g. MudPIT microcolumns were prepared as described<sup>45</sup>. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a Q-Exactive HF mass spectrometer equipped with an Ultimate 3000 nLC 1000 (Thermo Fisher). MudPIT experiments were performed by 10 µl sequential injections of 0, 10, 20, 30, ..., 100% buffer C (500 mM ammonium acetate in buffer A) and a final step of 90% buffer C/10% buffer B (100% acetonitrile, 0.1% formic acid, v/v/v), with each step followed by a gradient from buffer A (95% water, 5% acetonitrile, 0.1% formic acid) to buffer B. Electrospray was performed directly from the analytical column by applying a voltage of 2.2 kV with an inlet capillary temperature of 275 °C. Data-dependent acquisition of MS/MS spectra was performed with the following settings: Eluted peptides were scanned from 300 to 1800 m/z with a resolution of 120,000. The top 15 peaks for each full scan were fragmented by higher energy collisional dissociation (HCD) using a normalized collision energy of 38%, isolation window of 0.7 m/z, a resolution of 45,000, AGC target 1e5, maximum IT 60 ms, and scanned from 100 to

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1800 m/z. Dynamic exclusion was set to 10 s. Peptide identification and protein quantification was performed using Proteome Discoverer 2.4 (ThermoFisher). Spectra were searched using SEQUEST against a UniProt mouse proteome database. The database was curated to remove redundant protein and splice-isoforms, and common contaminants were added. Searches were carried out using a decoy database of reversed peptide sequences using Percolator node for filtering and the following settings: 10 ppm peptide precursor tolerance, 6 amino acid minimum peptide length, trypsin cleavage (maximum 2 missed cleavage events), static Cys modification of 57.021517 (carbamidomethylation), and static N-terminal and Lys modification of 229.1629 (TMT-sixplex), FDR 0.01, 2 peptide IDs per protein. Normalization of TMT reporter ion intensities was carried out based on total peptide abundance in each channel, and subsequently, TMT ratios for each identified protein were calculated in reference to a common pooled sample. Finally, the scaled, reference-normalized TMT intensities were compared between young WT and XBP1s Tg (n=4,4), middle aged WT and XBP1s Tg (n=3,4) and aged WT and XBP1s Tg (n=3,4) transduced samples and significance was assessed using a background based t-test with multiple testing correction in the Reporter Ions Quantifier node. Significantly altered genes were statistically determined (FDR <0.1) irrespective to fold change between groups. Gene set enrichment analysis was performed in EnrichR or STRING platforms using Kyoto Encyclopedia for Genes and Genomes (KEGG) and Gene Ontology annotations<sup>46-48</sup>. Heatmaps and hierarchical clustering were generated using Morpheus (https://software.broadinstitute.org/morpheus).

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#### Neuromuscular junction (NMJ) staining and analyses

The levator auris longus (LAL) muscles were dissected and whole-mount fixed in 0.5% formaldehyde (FA) for 90 min. Samples were blocked with 4% bovine serum albumin (BSA) dissolved in PBST 12-16 h at 4 °C. Muscles were incubated with mouse monoclonal antibodies against neurofilament (2H3, 1:300) and synaptic vesicles (SV2, 1:50; both from the Developmental Studies Hybridoma Bank, DSHB, of the University of Iowa, USA) in 4% BSA-PBST for 30 min at RT and then 12-16 h at 4 °C. Tissues were then incubated with the respective secondary antibodies (1:300, Jackson Immuno Research, West Grove, PA, USA) in 4% BSA-PBST containing Alexa488-conjugated α-bungarotoxin (BTX, 1:500, Invitrogen, Carlsbad, CA, USA) and DAPI (1:1000, Thermo Fisher, Waltham, MA, USA) for 12-16 h at 4 °C. Samples

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were post-fixed with 1% FA in 1X PBS for 10 min at 22 °C, flat mounted and imaged. Z-stack images were collected at 1-µm intervals on a Zeiss LSM 700 confocal microscope. Maximal intensity projection images were reconstructed in 3D using ImageJ software. The morphology of >50 NMJs per mouse was manually determined and expressed as a percentage of the total. The area of >50 acetylcholine receptor (AChR) densities per mouse was determined for each postsynaptic structure using ImageJ software.

#### Statistical analysis

Statistical significance respective to age was queried for single comparisons with a Student's t test and for multiple comparisons with a one-way ANOVA followed by Tukey's post-hoc test. When analyzing both age and genotype in the same comparison, a two-way ANOVA followed by Tukey's post-hoc test was implemented. P values were considered significant when they were < 0.05. The number of animals in each group varied from 4-15 depending on the experiment based on a Power Analysis of prior data.

#### Data availability

15 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>49</sup> partner repository with the dataset identifier PXD020539.

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 CH, SPS, BK, JCC, AP, LP, JPH, SAL: Funding Acquisition, Resources, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing.

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**Competing interests:** CH and FCM declare a conflict of interest for a submitted patent application. Inventors: Claudio Hetz, Felipe Cabral Miranda. Title: Treatment of aging or age-related disorders using XBP1. Provisional application for patent at USPTO, application number 62800229. Submitted 01/02/2019. Status: patent pending.

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20 All data generated or analyzed during this study are included in this published article (and its supplementary information files).

#### Supplementary Information is available for this paper.

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#### **Extended data:**



**Extended Data Figure 1:(a)** Violin plots depicting normalized FPKM values fin RNA-seq readings of UPR mediators Xbp1, Hspa5 and Ddit3 in the hippocampus of demented patients versus controls (n = 49,42; unpaired Kolmogorov-Smirnov test, \*\*: P <0.01) (b-c) Young, middle-aged, and aged animals were treated with tunicamcyin (5 mg/kg) or vehicle. After 24 h, the mRNA levels of Xbp1-s, Bip/Hspa5, Chop/Ddit3 and Atf3 were determined in dissected cortex (b) and cerebellum (c) by quantitative RT-PCR. (n = 4 animals/group. One-way ANOVA followed by Tukey's post-test compared tunicamycin treated groups, \*: P < 0.05



Extended Data Figure 2. Functional tests for age-associated cognitive and motor decline. (a) Animals were evaluated on the new object recognition test, comparing the new/total interaction time with novel objects following 24 h of presentation of two identical objects. Histogram shows mean and SEM of percentages of interaction time with novel objects (n = 14, 17, 18 for the various ages, respectively). \*\*: P < 0.01, \*\*\*P < 0.005 by one-way ANOVA followed by Tukey's post-hoc test. (b) Animals were evaluated on the first day of the new object recognition (NOR) test to compare the total time of interaction with any of the objects presented. Maximum time of interaction was set at 5 minutes. Histogram show mean and SEM of interaction time with any object (n = 40, 29, 21 for the various ages, respectively). (c) Animals were introduced to the four different types of objects implemented in the NOR test. Histogram shows mean and SEM of total time of interaction with each type of object (n = 9, 8, 9, 10 for each object). (d) Animals were allowed to interact with identical objects in the two different quadrants of the arena used in the NOR test (left or right). Histogram shows mean and SEM of time spent on objects placed in different guadrants in the cage (n = 14 animals/group). (e) Animals were evaluated in the new object location test comparing the percentage of interaction time with novelly-located object (NLO) following 24 h of presentation to one object placed in a different position. Histogram shows mean and SEM of percentages of interaction time with the novelly-located object (n = 18, 13, 17 for the various ages, respectively). \*P < 0.05, \*\*\*P < 0.005 by oneway ANOVA followed by Tukey's post-hoc test. (f) Animals were evaluated on the first day of the NOL test to compare the total time of interaction with any of the objects presented. Maximum time of interaction was set at 5 minutes. Histogram shows mean and SEM of the sum of interaction time with any object (n = 19, 20, 19 for the various ages, respectively). (g) Animals were allowed to interact with identical objects in the 4 different quadrants of the arena used in the NOL test (quadrant 1, 2, 3 or 4). Histogram shows mean and SEM of time spent with objects placed in different guadrants in the cage used in the test (n = 17-23 animals/group). (h) Barnes maze test measure spatial memory ability. Graph shows mean and SEM of primary latency to reach the target hole on each day of the test (n = 10/group). \*P < 0.5 by Student's t test with Bonferroni correction performed on each day. (i) Animals were evaluated in the contextual fear conditioning test 24 h after presentation of an unconditioned stimulus. Histogram shows mean and SEM of percentage of freezing time (n = 6/group). \*P < 0.05, \*\*: P < 0.001 by one-way ANOVA followed by Tukey's post-hoc test). (j)

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Animals were evaluated in the wire hanging test with a score based on total time of hanging and falls off of the wire in a 3 min period. Histogram shows mean and SEM of score in arbitrary units (au; n = 9, 11, 15 for each age group, respectively). \*\*P < 0.01 by one-way ANOVA followed by Tukey's post-hoc test. (k) Animals were evaluated in the rotarod test to analyze muscular function and coordination. Histogram shows mean and SEM of latencies to fall from the rod under constant acceleration in 4 trials split over 4 5 consecutive days (n = 16, 8, 17 for each age group, respectively). \*\*: P < 0.01 by one-way ANOVA followed by Tukey's post-hoc test. (I) Animals were deeply anesthetized and had their compound muscle amplitude potentials (CMAPs) measured in the gastrocnemius, tibialis anterior, and triceps muscles. Histogram shows mean and SEM of differences between maximum and minimum amplitudes (n = 8, 8, 8 for each age group, respectively). \*\*: P < 0.01, \*\*\*P < 0.001 by one-way ANOVA followed by Tukey's post-hoc test. (m) Brain slice electrophysiological analysis assessing firing rates in CA1 pyramidal neurons (n = 4 10 animals/group; n = 832-1584 neurons/animal). Mean and SEM of firing rates were measured during spontaneous activity or following picrotoxin treatment. Unpaired Student t test compared picrotoxin-treated groups. \*\*\*\*: P < 0.001)... (**n**) Animals were injected with adeno-associated vector (AAV) into the CA1 region of the hippocampi bilaterally to express eGFP in neurons. Dendritic spines (indicated by arrows) were imaged by confocal microscopy. 60x magnification; scale bar, 5 µm. Left panel: representative fluorescent images showing dendritic spines (labeled AAV-Mock). Right panel: Mean and SEM of spine density per µm (n = 3 animals/group, n = 6-16 dendrites/animal). One-way ANOVA followed by Tukey's post-test was performed. \*\*\*\*: P < 0.001. 15



**Extended data Figure 3.** (a) Relative mRNA levels of *Ern1* sequence (exon 20-21) by qPCR from hippocampi of young, middle-aged, and aged IRE1<sup>flox/flox</sup> or IRE1<sup>cKO</sup> animals (n = 4 per genotype at each age). \*\*: < 0.01 by unpaired Student's t test for each age group). (b) IRE1<sup>cKO</sup> were evaluated on the Barnes maze. Graphs shows mean and SEM of primary latency to reach the target hole on a given day (n = 10, 10, 8 for each age group, respectively). (c) New object recognition test was performed comparing IRE1<sup>WT</sup> and IRE1<sup>cCamk2a</sup> animals at young (n = 20,8) middle-aged (n = 18,11). Histogram shows mean and SEM of interaction time with the novel object (NO). \*: P < 0.05, comparing age-matched groups by Student's t test. (d) qPCR of relative mRNA levels of *Ern1* sequence exon 20-21 in hippocampi of middle-aged IRE1<sup>flox/flox</sup>, IRE1<sup>cKO</sup>, and IRE1<sup>flox/flox</sup> mice injected with AAV2-CRE (n = 4 animals/group). \*P < 0.05, \*\*\*P < 0.05 by one-way ANOVA followed by Tukey's post-hoc test. (e-g) Compound muscle amplitudes potentials (CMAPs) of various muscles from young, middle-aged, and aged IRE1<sup>WT</sup> or IRE1<sup>cKO</sup> mice. Histograms show mean and

SEM for difference between the minimum and maximum amplitudes (n = 10-11 animals/group). There was no statistical difference by unpaired Student's t test within each age-matched group. (**h**-**i**) Morphological analysis of neuromuscular junctions (NMJs) of levator auris longus muscle in elderly mice from aged IRE1<sup>WT</sup> or IRE1<sup>cKO</sup> animals. After immunolabeling presynaptic neurofilaments and synaptic vesicles (NF+SV) and postsynaptic acetylcholine receptors (AChR), were imaged by confocal microscopy (40x magnification; scale bar, 25 µm). Representative images for aged WT and aged IRE1<sup>cKO</sup> are shown. Histograms show mean and SEM of percentage of fragmentation, synaptic contact and overlap of NMJs (n = 4 animals/group). (**j**) Immunofluorescence for  $\gamma$ -H2AX comparing young and middle aged IRE1<sup>cKO</sup> with IRE1<sup>WT</sup> animals. Magnification, 40x; scale bar, 50 µm. (n = 3-4animals/group). Mean and SEM of percentage of  $\gamma$ -H2AX-positive cells. \*: P < 0.05, \*\*: P < 0.01 by unpaired Student's t test within each age-matched group for each hippocampal sub-region.



**Extended data Figure 4. (a)** Relative mRNA levels of *Xbp1s* by qPCR in middle-aged and aged non-Tg or Tg<sup>XBP1s</sup> hippocampi (n=3-6 animals/group). \*: P < 0.05, \*\*: P < 0.01 within age-matched groups by unpaired Student's t test). (b) Quantitative PCR showing *Bdnf* relative mRNA levels comparing middle aged or aged Tg<sup>XBP1s</sup> to controls (n = 3-4 animals/group, p values > 0.05 following unpaired Student's t test). (c) Following MS/MS analysis of the hippocampal proteome, gene set enrichment analysis was

performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) library. Graphs depict most enriched terms based on combined scored computed by EnrichR platform comparing young and aged Non Tg animals (left) and middle aged and aged Non Tg animals (right). **(d)** Scheme depicting the rational in proteomic analysis for  $Tg^{XBP1s}$  animals. Proteomic alterations in the hippocampus of  $Tg^{XBP1s}$  mice were compared to age-matched Non Tg littermates followed by gene set enrichment analysis using KEGG libraries trough EnrichR platform. Enriched terms were compared to the ones found during ageing in Non Tg mice. Protein-protein interaction networks were generated using genes that accounted to enriched terms both in ageing and within genotype comparisons using STRING v11. **(e)** Heatmaps depicting significant proteomic alterations (FDR <0.1) related to enriched terms comparing Non Tg with  $Tg^{XBP1s}$  in various ages. **(f)** Volcano plots for proteomic comparisons of hippocampal tissue derived from young, middle-aged and aged non-Tg and  $Tg^{XBP1s}$  animals indicating *P* values (y-axis) and fold-change (log2) (x-axis). Dotted lines delineate cut-off used to filter genes for functional enrichment analysis. Indicated genes associated with each enriched terms connected to synaptic function, neurodegeneration, spliceosome and ECM-receptor interaction are colored, as indicated.



**Extended data Figure 5. (a)** qPCR of relative mRNA levels of *Xbp1s* in hippocampi of aged mice injected with AAV2-XBP1s or AAV2-Mock control.(b) Representative confocal photomicrograph of immunolabel for NeuN (red) and GFP (green) from CA1 region of hippocampus of aged mouse brain 4 weeks after injection with AAV-XBP1s. (c) Mean and SEM of fiber volley amplitudes in brain slices derived from aged animals injected with AAV2-Mock or AAV2-XBP1s 1 h after theta burst stimulus (TBS) to induce LTP in (*n* =

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6, 9 animals, respectively; n = 17-28 slices/animal). (d) Immunofluorescence of  $\gamma$ -H2aX (red) counterstained with nuclei (blue) comparing aged mice injected with AAV-Mock or AAV-XBP1s. Magnification: 40x ;Scale bar: 50µm. Histograms show mean and SEM of percentage of  $\gamma$ -H2A- positive cells. Magnification: 40x; scale bar, 50 µm. \**P* < 0.05, by unpaired Student's t test comparing the aged groups. (e) Heat map indicating relative expression of significant genes related to enriched terms in aged mice injected with AAV-Mock or AAV-XBP1s.

**Table S1:** Significantly altered genes in RNA-seq comparing the hippocampus of demented patients with controls (fold change > 1.2) in the *Aging, Dementia and Traumatic injury Study* database. Most altered genes compose a functional network of interactions related to unfolded protein binding and processing. Table indicates gene symbols, fold changes, p Values and enriched terms in gene ontology and Kyoto Encyclopedia of Genes and Genomes databases.

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Gene	Fold Change	p-Value	Gene Ontology	KEGG
HSPA1A	2.019	4.16E-01	response to unfolded protein	Protein processing in ER
DNAJB1	1.986	4.08E-01	response to unfolded protein	Protein processing in ER
HSPA6	1.764	4.07E-01	response to unfolded protein	Protein processing in ER
HSPA1B	1.74	4.31E-01	response to unfolded protein	
SERPINH1	1.543	4.11E-01	response to unfolded protein	
BAG3	1.303	4.44E-01	protein folding	
HSPB1	1.33	4.51E-01	protein folding, refolding	

15 Table S2: Table depicts most enriched terms in KEGG database comparing Non Tg mice in different ages. Table indicates terms, associated genes and combined score computed in EnrichR platform.

#### Young vs Aged

20	Enriched Term	Genes	Score
	Long-term potentiation	PPP3R1;ADCY1;CALM2	321.4
	Glycolysis / Gluconeogenesis	ENO1;ENO2	130.4
	RNA degradation	ENO1;ENO2	97.5
25	Insulin secretion	ADCY1;STX1A	92.9
	Calcium signaling pathway	PPP3R1;ADCY1;CALM2	76.8
	Glucagon signaling pathway	PPP3R1;CALM2	73.3
	HIF-1 signaling pathway	ENO1;ENO2	71.3
	Prion diseases	C1QA	71
30	Glutamatergic synapse	PPP3R1;ADCY1	62.73
	Amyotrophic lateral sclerosis	PPP3R1	40.3
	Alzheimer disease	PPP3R1;CALM2	33.7
	Cellular senescence	PPP3R1;CALM2	31
	cAMP signaling pathway	ADCY1;CALM2	25.4
35	Synaptic vesicle cycle	STX1A	23.4
	GABAergic synapse	ADCY1	18.8
	Longevity regulating pathway	ADCY1	15.7
	Neurotrophin signaling		
	pathway	CALM2	12.2
40	Dopaminergic synapse	CALM2	10.3
	Middle Aged vs Aged		
	Enriched Term	Genes	Score
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	ECM-receptor interaction	COL1A1;COL1A2;HSPG2	48.8
	Necropiosis		00.04
	Chuachusia / Chuachachachacha		29.01
50	Giycolysis / Giuconeogenesis		20.2
50	Endocytosis		25.1
	Currentia vegiale avala		20.1
			21.21 15.90
	SINARE INTERACTIONS		15.82
55	Alzhoimer diagogo		10.14
55	AIZHEIMEI UISEase	CONSA, CALIVIZ, UQURH	14.0

5	HIF-1 signaling pathway	ENO1;ENO2	13.13
	Oxidative phosphorylation	COX5A;UQCRH	8.57
	Insulin signaling pathway	PRKAR2B;CALM2	8.04
	Parkinson disease	COX5A;UQCRH	7.56
	Ribosome	RPL23;RPLP2	5.62
	Long-term potentiation	CALM2	5.39
	Calcium signaling pathway	ATP2B4;CALM2	4.61
	Huntington disease	COX5A;UQCRH	4.48
	Huntington disease	COX5A;UQCRH	4.48
	cAMP signaling pathway	ATP2B4;CALM2	3.74

Table S3. Table depicts most enriched terms in KEGG database comparing Tg<sup>XBP1s</sup> with age-matched non Tg mice. Table indicates enriched terms and their associated genes. Symbols highlight when those terms are enriched in Tg<sup>XBP1s</sup> animals within each age-comparison.

15	within each age-companson.	within each age-comparison.		
10	Enriched Terms	Genes		
20	Synaptic function Synaptic vesicle cycle *	CLTB;CLTA;SLC6A11;SLC17A7; SLC1A3		
20	Long-term potentiation* Glutamatergic synapse * Calcium signaling pathway # Dopaminergic synapse *	GRIA1;PPP3CA;GRIN2A;RAP1B;CAMK2D;PPP3R1;ADCY1 GRIA1;PPP3CA;GRIN2A;SLC17A7;PPP3R1; SLC1A3;ADCY1 PPP3CA;GRIN2A;PTK2B;SLC8A1;CAMK2D; PPP3R1;ADCY1; ATP2B4 GRIA1;PPP3CA;GRIN2A; CAMK2D		
25	Long-term depression & cAMP signaling pathway # GABAergic synapse * Neuroactive ligand-receptor interactior	GRIA1 GRIA1;GRIN2A;RAP1B;CAMK2D;ADCY1; ATP2B4 SLC6A11;ADCY1 % GRIA1;GRIN2A		
30	MAPK signaling pathway # Axon guidance #	PPP3CA;CACNG8;TAOK3;STMN1;RAP1B;PPP3R1;TAOK3;HSPA1A PPP3CA;CAMK2D;PPP3R1;PTPN11		
	<b>Neurodegeneration</b> Amyotrophic lateral sclerosis (ALS) *	GRIA1;PPP3CA;GRIN2A;PPP3R1;NEFL;NEFM;NEFH		
35	Alzheimer diseases * Apoptosis ** Necroptosis # Cellular senescence *	PPP3CA;GRIN2A;PPP3R1 LMNA;LMNB2;LMNA;LMNB2 HIST3H2A;H2AFY;CAMK2D;HIST3H2A;H2AFX PPP3CA;PPP3R1		
40	Metabolism/Bioenergetics Glucagon signaling pathway * Glycolysis / Gluconeogenesis & GnRH signaling pathway *	PPP3CA;LDHB;CAMK2D;PPP3R1 LDHB PTK2B;CAMK2D;ADCY1		
45	Other Terms Spliceosome **	HNRNPM;DDX5;HNRNPA3;HNRNPK;SRSF1; SRSF2:HNRNPU;SRSF4:HNRNPC:PRPF19:HSPA1A		
	Ribosome *	RPLP2;RPS13;RPL18		
50	* Term enriched in Young and Middle Aged TgXBP1s			
	& Term enriched only Young TgXBP1s			
55	# Term enriched in Young, Middle Aged and Aged Tg <sup>XBP1s</sup>			
	** Term enriched in Young and Aged 1	Гg <sup>ХBP1s</sup>		

Table S4. Table depicts most enriched terms in KEGG database comparing aged mice injected with AAV-Mock or AAV-XBP1s. Table indicates enriched terms and their associated genes. Symbols highlight whether enriched terms correlate with terms found during the natural course of ageing in Non Tg animals.

5	Enriched Terms	Genes
U	Synaptic function Endocrine and other factor-regulated calcium reabsorption & SNARE interactions in vesicular transport #	CALB1;CLTB;CLTA STX7;VAMP1
10	Long-term potentiation * Synaptic vesicle cycle * Endocytosis # Calcium signaling pathway * Glutamatergic synapse *	PPP3R1;CALM2 CLTB;CLTA CLTB;CLTA;ASAP2;RAB11FIP2 PPP3R1;CALM2;SLC8A2 PPP3R1;GNG2
15	Neurodegeneration	
20	Prion diseases * Amyotrophic lateral sclerosis * Alzheimer disease * Necroptosis # Huntington disease &	C1QB;C1QA;PRNP;LAMC1 PPP3R1;NEFL;NEFM APP;PPP3R1;NDUFV2;CALM2 HIST3H2A;H2AFY2;H2AFY;H2AFV CLTB:TFAM:CLTA:NDUFV2
_ •	Cellular senescence * Parkinson disease #	PPP3R1;CALM2 NDUFV2
25	Metabolism / Bioenergetics Glycolysis / Gluconeogenesis * Glucagon signaling pathway * Oxidative phosphorylation #	ENO1;ENO2 PPP3R1;CALM2 NDUFV2 CALM2
30	HIF-1 signaling pathway *	TRF;ENO1;ENO2
	Other terms ECM-receptor interaction #	COL1A1;LAMA5;COL1A2;COL4A2; LAMA2;LAMB2;LAMB1;LAMC1;AGRN; HSPG2
35	RNA degradation * Spliceosome	ENO1;ENO2 RBMXL1;SNRPD2;SNRPD1;TRA2B; SRSF1;PLRG1;
	Ribosome	SNRPB RPL4;MRPS9;RPL31;RPL12;RPLP2; RPL19
40	<ul> <li>* Term associated to Young WT x Aged WT</li> <li>&amp; Term associated to Young WT x Middle Aged WT</li> <li># Term associated to Middle Aged WT x Aged WT</li> </ul>	
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55		

#### **Supplementary information:** Detailed analysis of proteomic data and additional findings in Tg<sup>XBP1s</sup> animals

We performed gene set enrichment analysis using only protein hits with a false discovery rate values (q) < 0.1 as a filter for significantly altered expressed genes between groups<sup>1</sup>. Next, we eliminated keratins, a common contaminant, from sample analysis<sup>2</sup>. First, to gain insight into age-associated alterations in the hippocampus, we compared samples of wild type animals among different ages looking for age-associated proteomic hallmarks (Extended Data Fig.3b-c). We found 26 hits significantly altered in this time window (n = 4 animals/group; q < 0.1). When comparing middle aged with aged animals, we found 80 significant alterations (n = 3, 4/group; q<0.1). Interestingly, many hits in our analyses matched transcriptomic alterations previously reported in Gene Expression Omnibus comparing young with middle aged (46.7%) or young and aged (16.7%) animals (GSE29075, GSE29075, GSE49699, GSE49699) in the same age.

Next, we evaluated proteins significantly altered (q<0.1) within age-matched Tg<sup>XBP1s</sup> animals and wild types, and again, performed gene-set enrichment analysis for each comparison 15 (Extended Data Fig.3D). Then, we looked for enriched terms that superposed with ageassociated alterations in wild type mice (Extended Data fig.3C; Table S2) and performed proteinprotein interaction networks analysis (Extended Data Fig.3D). Notably, many enriched terms found in this comparison were the same comparing wild type animals during ageing (Fig. 3G, left and Extended Data Fig.3C, left; also refer to Table S2 and Table S3). Most clusters of 20 proteins were directly associated with synaptic functioning (long-term potentiation, synaptic vesicle cycle, calcium signaling pathway); enriched by the same genes: calcineurin subunit Ppp3r1, Calmodulin, Calbindin and Camk2d; neurofilament proteins (Nefl and Nefm) and neurotransmitter vesicle cycle (clathrin light chains a and b). Interestingly, young Tg<sup>XBP1s</sup> also 25 presented altered levels of glutamate receptors Gria1 and Grin2a although those alterations are not recapitulated in middle or advanced age (Extended Data Fig.3e-f). Neurodegenerative diseases (ALS, Prion diseases, Alzheimer's) also showed up as enriched terms when comparing wild type animals and Tg<sup>XBP1s</sup> mice (Table S3). Genes associated were: Camk2d; calmodulin; protein phosphatases PPP3ca and Ppp3r1; and complement C1q subunit A and B. A strong cluster of proteins related to necroptosis and cellular senescence was also altered in Tg<sup>XBP1s</sup> mice 30 (H2afx, H2afv, H1f0, Hist1h1e and Hist1h1a) in addition to enolases isoforms ENO1 and ENO2. Finally, gene ontology also highlighted terms associated to myelin sheet (GO:0043209) comparing middle aged wild types with Tg<sup>XBP1s</sup> (Fig. 3g).

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#### Proteomics findings in aged animals injected with AAV-XBP1s

The most enriched KEGG term in this analysis was "Extracellular matrix interaction receptor" (Table 4) related to altered expression of collagens, laminin subunits and heparan 40 sulfate proteoglycan 2; in addition to "glycolysis/ gluconeogenesis", related to enolases 1 and 2. Those findings coincide with important age-associated alterations in wild type animals (Extended Data Fig.3C). Just as observed in Tg<sup>XBP1s</sup> animals, enriched terms in the AAV approach covered many neurodegenerative diseases such as Prion diseases (prion protein, C1QA and B; and laminin subunit gamma-1) and ALS (Nefl, Nefm) in addition to necroptosis (Hist3h2A; H2afy2; H2afy; H2afy).

#### Potential relation between XBP1s, calcium signaling and ageing

XBP1s was reported to modulate intracellular calcium dynamics in neurons, correlating with increased neurotoxicity in an AD model<sup>3</sup> and calcium homeostasis unbalance has long been proposed to be a central player in neuronal ageing<sup>4</sup>. Our findings indicate that many proteins associated with calcium signaling in neurons; namely calcineurin, calmodulin, calcium transporter ATP2B4 and other signaling components such as adenylyl cyclase, are altered following XBP1s overexpression in aged mice (Tables S3 and S4). Likely, increased long-term potentiation, dendritic spike stabilization and increments in memory formation in aged mice reported here are also dependent on calcium homeostasis.

#### Extension to human ageing

One central query in our hypothesis is whether our findings can be extended to human ageing, as we point that unfolded protein machinery account as the main changes observed in the hippocampus of demented patients (Fig. 1A-B; Table S1) . Interestingly, a recent large-scale proteomic human study has pinpointed promising protein targets that sustain cognitive stability during ageing<sup>5</sup> showing that myelin-associated proteins NEFL, NEFM and MBP are associated with cognitive resilience. Those findings overlap with ours, both in the Tg<sup>XBP1s</sup> and AAV-XBP1s approaches, as those proteins were altered following XBP1s overexpression (Table S3, Table S4). Also, altered expression of extracellular matrix component laminin-A and glial fibrillar associated protein (GFAP), two proteomic hits highlighted in our analysis, were also linked to cognitive resilience in two human cohorts studies<sup>5</sup>.

#### 25 Supplementary References

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# Figures



# Figure 1

Impaired UPR activation in the mammalian aged brain. (a) Functional enrichment analysis of altered genes in an elderly cohort of patients diagnosed with dementia versus non-demented. GO: gene ontology; Key TF: key transcription factor in TRRUST database; FDR: false discovery rate. (b) Violin plots displaying

normalized FPKM values of chaperones HSPA1a and HSPA1b in RNA-sequencing of human hippocampus of demented patients and controls (n = 49,42; unpaired Kolmogorov-Smirnov test, \*\*: P <0.01). (c-e) Young, middle-aged, and aged animals were treated with tunicamcyin (5 mg/kg) or vehicle. After 24 h, the mRNA levels of Xbp1-s, Bip/Hspa5, Chop/Ddit3 and Atf3 were determined in dissected hippocampus by quantitative RT-PCR. (n = 4 animals/group. One-way ANOVA followed by Tukey's posttest compared tunicamycin treated groups, \*\*: P < 0.01). (f, g) Relative levels of S-nitrosylated IRE1a (d) and total IRE1a (e) in brains from young and aged mice. Histograms show relative levels of SNO-IRE1a/input IRE1a measured by biotin-switch assay (f) and total IRE1a/GAPDH quantified by densitometry on standard immunoblots (g) for young (3 month-old) versus aged (24 month-old) mouse brains (n = 3, \*: P < 0.05 by Student's t test).



## Figure 2

Genetic ablation of the IRE1a pathway in the brain accelerates and exacerbates age-associated cognitive and motor decline in mammals. (a) Conditional knockout mice for IRE1a were generated using the Nestin-CRE system (IRE1cKO) and their littermate flox/flox control animals (IRE1WT). The new object recognition test was used to evaluate the ability to discriminate novel objects after 24 h in young, middleaged and aged mice (n = 10/ group; \*\*\*: P < 0.005 by two-way ANOVA followed by Sidak's multiple comparison test). (b) In animals of indicated genotypes, contextual fear conditioning test was performed

to compare total number of freezing episodes following 24 h of aversive stimuli in young (n = 7, 10) and middle-aged (n = 9, 14) wild-type (IRE1WT) or IRE1cKO mice (\*P < 0.05 by unpaired Student's t test within each age-matched group). (c) Young (n = 9, 9), middle-aged (n = 9, 9), and aged (n = 10, 8) IRE1WT or IRE1cKO mice were evaluated in the Barnes maze to evaluate spatial memory acquisition. Total number of errors before finding the target hole is plotted (\*P < 0.05 by unpaired Student's t test within each agematched group). (d) Young WT (n = 8), young XBP1scKO (n = 12), middle-aged WT (n = 18), or middleaged XBP1cKO (n = 14) mice were evaluated in the new object recognition test to analyze the ability to discriminate between novel objects following 24 h of presentation of two identical objects (\*: P < 0.05 by unpaired Student's t test comparing age-matched groups). (e) Scheme to illustrate the loss-of-function approach based on AAV2-CRE-mediated deletion of floxed IRE1a in the hippocampus. IRE1flox/flox animals were evaluated using the NOR test and then received bilateral hippocampal injections with AAV2-CRE. After 4 weeks, animals were evaluated again in the NOR test and then euthanized. Brains were collected for dendritic spine and biochemical analysis. (f) Young (n = 7) and middle-aged (n = 6) IRE1flox/flox animals were evaluated in the NOR test before and after AAV2-CRE intra-hippocampal injections (\*: P < 0.05 by paired Student's t test comparing each individual before and after AAV2 injection). (g) Animals of different ages were injected with adeno-associated vector (AAV-CRE or AAV2-Mock) into the CA1 region of each hippocampus. AAV constructs included a GFP cassette to express eGFP in neurons for monitoring dendritic spine density. One month after the injection, spines were imaged by confocal microscopy. Left-hand panels: Representative confocal microscopic images of dendritic spines (arrows) in the CA1 region of middle-aged animals. 60x magnification; scale bar, 5 µm. Right-hand panel: Histogram of mean and SEM of spine density per  $\mu$ m (n = 24 dendrites, 4 animals; 27 dendrites, 3 animals; 31 dendrites, 4 animals; 42 dendrites, 4 animals, respectively). \*\*\*\*: P < 0.001 by unpaired Student's t test within each age-matched group. (h) Quantitative PCR showing Bdnf relative mRNA levels comparing young, middle aged or aged IRE1cKO to controls (n = 3-4 animals/group. Unpaired Student's t test was performed comparing age-matched groups, \*: P < 0.05). (i) Wire hanging test performed in young, middle-aged, and aged IRE1WT or IRE1cKO to evaluate motor performance. Graph indicates mean and SEM of arbitrary scores from aged (n = 18, 15), middle-aged (n = 11, 15), and young (n = 10, 12) animals (\*: P < 0.05 by two-way ANOVA followed by Sidak's post-hoc test). (j) Rotarod test used to evaluate motor performance and coordination in young (n = 16, 12), middle-aged (n = 8, 8), and aged (n = 18, 6) IRE1WT or IRE1cKO animals. Graph indicates mean and SEM of latencies to fall from the rod (\*: P < 0.05 by unpaired Student's t test within each age group). (k) Representative photomicrographs of  $\beta$ galactosidase staining of hippocampal slices derived from young and middle-aged IRE1WT or IRE1cKO animals (n = 3-4 animals/group). Graphs (at right) indicate mean and SEM of percentage of  $\beta$ galactosidase positive cells. \*\*: P < 0.01 by unpaired Student's t test within each age-matched group for each hippocampal sub-region. Magnification: 20x; scale bar: 200 µm.



## Figure 3

Enforced expression of XBP1s in the brain prevents age-associated motor and cognitive decline in mammals. (a) New object recognition test was used to evaluate ability to discriminate novel objects after 24 h in TgXBP1s or littermate non-transgenic animals (non-Tg) in young, middle-aged, and aged animals (n = 22, 6; 23, 9; 17, 12; respectively; \*: P < 0.05 by unpaired Student's t test within each age-matched group). (b) New object location test was used to evaluate the ability to discriminate changes in object location after 24 h in young, middle-aged, and aged non-Tg or TgXBP1s (n = 20, 7; 12, 13; 19, 9)

respectively; histogram shows mean and SEM of percentage of time interacting with the novel-located object (NLO). \*: P < 0.05 by unpaired Student's t test within each age-matched group). (c) Aged non-Tg and aged TgXBP1s animals were evaluated in the Barnes maze test to compare spatial memory acquisition over a period of 5 days. Graph indicates mean and SEM of each group (n = 10 for non-Tg [WT], 8 for TqXBP1s). Student's t test was used to compare performances within each day (\*P < 0.05). Latencies of young/non-Tg mice also plotted for comparison (n = 10). (d) Young, middle-aged, and aged non-Tg or TgXBP1s were evaluated in the wire hanging test to monitor motor performance and coordination. Mean and SEM of arbitrary scores for each group (n = 8 animals/group; \*: P < 0.05, \*\*P < 0.01 by two-way ANOVA followed by Sidak's multiple comparison test). (e) Young and aged non-Tg or TgXBP1s animals were evaluated in the rotarod test to monitor motor performance and coordination. Mean and SEM for each group (n = 10 animals/group, \*\*\*: P < 0.001 by unpaired Student's t test within each matched group). (f) Following MS/MS analysis of the hippocampal proteome, gene set enrichment analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) library. Graphs depict most enriched terms based on combined scored computed by EnrichR platform comparing middle aged and aged TgXBP1s with age-matched controls. Terms were divided in two graphs related to synaptic function or neurodegeneration. (g) Protein-protein interaction networks depicting main genes altered in middle aged and aged TgXBP1s (PPI enrichment p-value < 1-16).



# Figure 4

XBP1s-gene delivery to aged animals reverses age-associated phenotypes at the behavioral, morphological and electrophysiological levels. (a) Middle-aged and aged WT animals were injected with AAV2- Mock or AAV2-XBP1s in both hippocampi and then evaluated in the new object location test to evaluate their ability to discriminate displacement of objects 24 h following presentation. Histogram shows mean and SEM of percentage of time interacting with the novel located object (NLO) for n = 10

animals/group, \*\*: P < 0.01 by unpaired Student's t test). Young animal performance plotted for comparison (n = 6). (b) Aged animals injected with AAV2-Mock or AAV2-XBP1s in both hippocampi were evaluated in the new object recognition test to evaluate their ability to discriminate novel objects after 24 h. Mean and SEM of percentage of interaction time with novel objects (n = 10 animals/group. \*\*\*: P < 0.005 by unpaired Student's t test). Young animal performance plotted for comparison (n = 6). (c) Aged animals injected with AAV2-Mock or AAV2-XBP1s in both hippocampi were evaluated in the Barnes maze test to compare spatial memory acquisition. Mean and SEM of latencies to find target for each group during 4 days of testing (n = 5 for AAV2-Mock, 8 for AAV2-XBP1s; Student's t test was used to compare performances within each day (\*: P < 0.05). Young animal performance plotted for comparison (n = 10). (d) Aged animals injected with AAV2-Mock or AAV2-XBP1s evaluated by contextual fear conditioning for aversive memory acquisition 24 h after presentation of an unconditioned stimulus (n = 5 for AAV2-Mock, 7 for AAV2-XBP1s; \*\*: P < 0.01 by unpaired Student's t test). Young animal performance plotted for comparison (n = 6). (e) Brain slice electrophysiological analysis assessing firing rates in hippocampal neurons. Mean and SEM of firing rates were measured during spontaneous activity or following picrotoxin treatment in brain slices from aged mice injected with AAV2-Mock or AAV2-XBP1s (n = 633-832 neurons; n = 6, 9 animals, respectively; \*\*\*\*: P < 0.001 by unpaired Student's t test). (f) Basal and picrotoxin-induced burst activity measured in pyramidal and interneurons in hippocampal brain slices derived from aged mice injected with AAV2-Mock or AAV2-XBP1s (n = 832, 644 neurons from 3-4 animals, \*\*: P < 0.01 by unpaired Student's t test). (g) Mean and SEM of field excitatory postsynaptic potentials amplitudes in brain slices derived from aged animals injected with AAV2-Mock or AAV2-XBP1s 1 h after theta burst stimulus (TBS) to induce LTP in (n = 6, 9 animals, respectively; n = 17-28 slices/animal, \*\*\*\*: P < 0.001 by unpaired Student's t test). (h) Representative fluorescent images showing dendritic spines of pyramidal neurons in CA1 region of young and aged animals injected with AAV2-eGFP (mock) or aged animals injected with AAV2-XBP1s. Young animal neuron is shown for reference. Right panel: Mean and SEM of spine density per µm for indicated experimental groups (n = 24, 53 and 35 dendrites from 3, 6 and 5 animals, respectively; \*\*: P < 0.01 by unpaired Student's t test comparing the aged groups). 60x magnification; scale bar, 5  $\mu$ m). (i) Representative images of  $\beta$ -galactosidase staining of hippocampal slices derived from aged mice injected with AAV2 (n = 3-4 animals/group). Histograms (right) show mean and SEM of percentage of  $\beta$ -galactosidase positive cells. Magnification 20x; scale bar, 200  $\mu$ m. (j) Following MS/MS analysis of the hippocampal proteome, gene set enrichment analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) library. Graphs depict most enriched terms based on combined scored computed by EnrichR platform comparing aged mice infected with AAV-Mock or AAV-XBP1s. Terms were divided in two graphs related to synaptic function or neurodegeneration. (k) Volcano plot for proteomic comparison of hippocampal tissue derived from aged animals injected with AAV-Mock or AAV-XBP1s. FDR (y-axis) and fold-change (log2) (x-axis) are indicated. Dotted lines delineate cut-off used to filter genes for functional enrichment analysis. Indicated genes associated with each enriched terms connected to synaptic function, neurodegeneration, metabolism and ECM-receptor interaction are colored.