Age-dependent impact of early-life stress on glia and synapses
Substrates for increased risk for Alzheimer's disease
Kotah, J.M.

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CHAPTER 5
Early-life stress exposure impacts on the hippocampal synaptic proteome in a mouse model of Alzheimer’s disease: age-dependent effects on mitochondrial proteins

Janssen M. Kotah¹*, Mandy S.J. Kater²*, Lianne Hoeijmakers¹, Niek Brosens¹, Sylvie L. Lesuis¹, Roberta Tandari¹, Luca Marchetto¹, Ella Yusaf¹, August B. Smit², Paul J. Lucassen¹, Harm Krugers¹, Mark H.G. Verheijen²#, Aniko Korosi¹#

¹Brain Plasticity Group, Swammerdam Institute for Life Sciences – Center for Neuroscience, University of Amsterdam
²Department of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

*shared first authorship
#shared last authorship
Abstract

Epidemiological evidence indicates that early life stress (ES) exposure increases the risk for later-life diseases, such as Alzheimer’s disease (AD). Accordingly, we and others have shown that ES aggravates the pathological response to amyloid-beta (Aβ) in animal models. While the mechanisms behind this are unclear, ES-exposed transgenic mice were previously shown to have deficits in cognitive flexibility and synaptic function. As such, we investigated here how ES, using the limited bedding and nesting model, affects the synaptic proteome across age in both wildtype and APP/PS1 transgenic mice.

We found that hippocampal synaptosomes of APP/PS1 mice at an early pathological stage (4-months-old) expressed more mitochondrial proteins, but less proteins involved in actin dynamics. Interestingly, ES exposure in wildtype mice had similar effects on the level of mitochondrial and actin-related synaptosomal proteins at this age, whereas ES exposure had no additional effect on the synaptosomal proteome of early-stage APP/PS1 mice. Accordingly, ultrastructural analysis of the synapse using electron microscopy in a follow-up cohort showed fewer mitochondria in pre-synaptic and postsynaptic structures in both APP/PS1 and ES-exposed mice, respectively, at the age of 4 months.

The hippocampal synaptic proteome of APP/PS1 mice at a later pathological stage (10-month-old), revealed an upregulation of proteins related to Aβ processing, that was accompanied by a downregulation of proteins related to postsynaptic receptor endocytosis. ES exposure no longer had an effect on the synaptic proteome of wildtype animals by this age, whereas it dysregulated the expression of astrocytic proteins involved in lipid metabolism in APP/PS1 mice. We confirmed that the latter was dysregulated in a separate cohort of 12-month-old mice, by immunostaining for the alpha subunit of the mitochondrial trifunctional protein and fatty acid synthase in astrocytes.

In conclusion, our data suggest that ES and amyloidosis share pathogenic pathways involving synaptic mitochondrial dysfunction and astrocytic lipid metabolism. These pathways might be underlying contributors to the long-term aggravation of the APP/PS1 phenotype by ES, as well as the ES-associated risk for AD progression.

These data are publicly accessible online as a web app via https://amsterdamstudygroup.shinyapps.io/ES_Synaptosome_Proteomics_Visualizer/.
Introduction

Despite advances in understanding the role of genetics in Alzheimer’s disease (AD)\textsuperscript{1,2}, recent evidence emphasizes the important role of environmental (i.e., non-genetic) factors in its etiology\textsuperscript{3} including stress\textsuperscript{4–6}. In particular, there is emerging epidemiological evidence that early-life stress (ES) history might increase the risk to develop cognitive decline\textsuperscript{7–9}, AD and other dementias\textsuperscript{10–17}.

This association has been further substantiated and tested in animal models\textsuperscript{18,19}, e.g. in transgenic APP/PS1 mice which overexpress mutated versions of the human amyloid precursor protein and presenilin-1\textsuperscript{20,21}. These mutations result in amyloid-beta (A\textsubscript{\beta}) plaques by 6 months of age\textsuperscript{20,21}, and, eventually, impaired cognition\textsuperscript{22,23}, synaptic structure\textsuperscript{24}, and neuroinflammation\textsuperscript{23,25}, among other phenotypes\textsuperscript{26}. When such mice are exposed to ES, via e.g. the limited bedding and nesting (LBN) model\textsuperscript{27,28}, we and others have reported age-dependent alterations in plaque load\textsuperscript{29} and neuroinflammation\textsuperscript{29,30}, as well as deficits in cognitive flexibility and synaptic function\textsuperscript{31–34}.

The synapse is one of the most prominently affected substrates in AD patients, who exhibit progressive synaptic loss especially in the hippocampus\textsuperscript{35,36}. Such synaptic loss is thought to underlie the cognitive symptoms of the disease\textsuperscript{37}, leading to a view of AD as a synaptic disorder\textsuperscript{38,39}. Before actual synaptic loss, synaptic alterations are already present during early and prodromal stages of the disease\textsuperscript{40,41}. Animal model studies suggest these to occur as a function of the appearance of A\textsubscript{\beta} pathology\textsuperscript{24,42}. Given the hypothesis of ES effects on the aging process\textsuperscript{43}, and the fact that aging remains the biggest risk factor for AD\textsuperscript{44}, we hypothesized that ES exposure might age-dependently affect the alterations to the hippocampal synapse in an AD mouse model.

We therefore set out to study how the hippocampal synaptic proteome is affected in transgenic APP/PS1 mice during early, pre-plaque stages of pathology, how these changes compare to those observed during advanced pathological stages, and how prior ES exposure (via the LBN model during the first postnatal week) might further modulate these alterations. We studied this using label free mass spectrometry in APP/PS1 mice and their wildtype (WT) littermates at 4 (4M) and 10 months (10M) of age, representing stages with low and high A\textsubscript{\beta} pathology, respectively\textsuperscript{29}. The hippocampi used in this study were derived from a cohort from which we previously reported the effects of ES on the cognition, neurogenesis, and neuroimmune (i.e., microglial and astrocytic) profiles of APP/PS1 mice\textsuperscript{29,30,45}.

We found similar changes in synaptosomal proteins associated with mitochondria and actin dynamics in both APP/PS1 and ES mice at 4M, with no further alterations in ES-exposed APP/PS1 mice. At 10M, A\textsubscript{\beta}-associated alterations were pronounced in APP/PS1 brains, with previous ES-exposure also further altering (mitochondrial) lipid metabolism in APP/PS1 mice. Our data revealed changes not only in neuronal proteins but, as earlier reported\textsuperscript{24,46–48}, also in proteins that are highly expressed by cell types closely interacting with synapses. We then characterized ultrastructural mitochondrial alterations by performing electron microscopy in 4M mice, and validated our main findings via immunostaining, showing that alterations in astrocytic lipid proteins in ES-exposed APP/PS1 mice persisted until 12 months.
Chapter 5

Materials and Methods

Animal use
Male bigenic hemizygous APPswe/PS1dE9 (TG) mice on a C57Bl/6J background and their wildtype (WT) littermates were used for this study. Three cohorts of APP/PS1 mice were used for synaptosome proteomics. Cohort 1 (WT: 7, APP/PS1: 7) consisted of WT and TG mice sacrificed at 4 months of age (4M). Cohorts 2 and 3 consisted of WT and TG mice exposed to control (CTL) or early-life stress (ES) conditions during the first postnatal week and were sacrificed at 4M (WT-CTL: 5, WT-ES: 4, TG-CTL: 4, TG-ES: 4) and 10 months of age (10M, WT-CTL: 5, WT-ES: 9, TG-CTL: 5, TG-ES: 6). We have previously reported on the behavior, neurogenesis, and neuroinflammation of the mice used in Cohorts 2 and 3.

Two more cohorts were generated to follow up the proteomics data. Cohort 4 consisted of ES-exposed WT and TG littermates that were sacrificed at 4M to investigate mitochondria using electron microscopy (WT-CTL: 7, WT-ES: 5, TG-CTL: 5, TG-ES: 6). Cohort 5 consisted of CTL or ES-exposed TG mice sacrificed at 12-months, and was used to validate two proteins that were found to be differentially expressed in the 10M proteomics data (TG-CTL: 8, TG-ES: 6).

Mice were bred in-house, and kept under standard housing conditions (temperature 20–22 °C, 40–60% humidity level, chow/water ad libitum, 12/12 h light/dark schedule). All experiments were approved by the Animal Experiment Committee of both universities.

Early-life stress paradigm
Mice used in ES experiments were bred and randomly assigned to control or ES groups at PND2 as previously described. Briefly, CTL litters were housed in standard amounts of sawdust, along with a 5x5cm piece of nesting material (Tecnilab-BMI, Someren, The Netherlands). ES nests were housed on a fine-gauge stainless steel mesh on top of 1/3 the regular amount of CTL bedding material, along with half of a 2.5x5 cm square of nesting material. All nests were covered with filter tops. Nests were transferred to standard cage conditions at PND9 and allowed to age until used in our experiments.

Synaptosome proteomics
Tissue collection and synaptosome isolation
To study how Aβ pathology affects the hippocampal synaptosome, mice from Cohorts 1, 2, and 3 were sacrificed via rapid decapitation. The hippocampi of these mice were dissected, snap frozen on dry ice, and stored at -80°C until further use.

Synaptosomes were isolated on a discontinuous sucrose gradient as described previously. In short, homogenization buffer (0.32 M sucrose, 5 mM HEPES, in PBS pH=7.4, with protease inhibitor cocktail [Roche]) was added to hippocampi samples, after which they were mechanically homogenized by a Dounce homogenizer (12 strokes, 900 rpm). Samples were centrifuged at 1000 x g for 10 min and supernatant was collected. This was layered on a 0.85/1.2 M sucrose gradient and centrifuged at 30,000 x g for 2h. We collected synaptosomes from the interface, which were further diluted with 5 ml homogenization...
buffer and centrifuged at 20,000 x g for 30 min to obtain a synaptosomal pellet. All steps were performed on ice.

**FASP in-solution digestion of proteins**
Samples were digested by filter-aided sample preparation (FASP) as previously described\(^{48}\). Ten µg of synaptosomes from each sample was incubated with 75 µl reducing agent (2% SDS, 100 mM TRIS, 1.33 mM TCEP) at 55 °C for 1h at 900 rpm. And incubated with 500 mM MMTS for 30 min at RT. The samples were transferred to YM-30 filters (Microcon, Millipore) and a washing solution (200 µl 8 M urea in 100 mM TRIS (pH=8.8)) was added to wash five times by spinning at 14,000 x g for 15 min. Next, the samples were washed with 50 mM NH\(_4\)HCO\(_3\) for four more times. Samples were incubated overnight at 37 °C with 100 µl of Trypsin (0.6 grams in 100 µl 50 mM NH\(_4\)HCO\(_3\)). Digested peptides were eluted from the filter with 50 mM NH\(_4\)HCO\(_3\). The samples were dried using a SpeedVac and stored at -20 °C.

**Mass spectrometry-based analysis**
Samples were loaded onto an Ultimate 3000 LC system (Dionex, Thermo Scientific) as described before\(^{24,48-50}\). Spectronaut 14 (Biognosys) was used for data analysis of the raw files. The spectral library was created with crude hippocampal synaptosomes containing spiked-in peptides (Biognosys), analysed with a TripleTOF 5600 in data-dependent acquisition mode. The obtained library was searched against mouse proteome (UP000000589_10090.fasta and UP000000589_10090.addition.fasta) in MaxQuant Software (version 1.3.0.5). Data quality control and statistical analysis were performed by using the downstream analysis pipeline for quantitative proteomics (MS-DAP version 0.2.6.3, manuscript currently under review). Outliers were removed in case of large within or between sample variations observed by deviating distribution plots, or in case of disturbed protein detection observed by altered retention time plots.

**Data analysis**
Peptide abundance values were normalized and the MSqRob algorithm was used for peptide-level statistical analysis. The threshold for significance was set at FDR<0.05 and Log\(_2\) fold change cut off at -0.1/0.1. Venn diagrams were created using the ggVenndiagram package on R, v1.1.0\(^{51}\).

For contrasts with at least 5 differentially expressed proteins, we performed Gene Ontology (GO) analysis for biological processes (BP) and cellular components (CC) using gProfiler2 R package, v0.2.0\(^{52}\), analyzing up- and down-regulated proteins separately at a significance threshold of FDR<0.05. GO analysis results with >10 terms were also semantically clustered using RRVGO to reduce redundancy\(^{53}\). Synaptic GO analyses were done using the SynGO database v1.1\(^{54}\). Mitochondrial proteins and pathway analyses were done by importing annotations from the MitoCarta3.0 database\(^{55}\) into gProfiler2. To gain insight into whether specific cell-types are more altered based on the differentially expressed proteins, we performed expression weighted cell-type enrichment analysis, using a hippocampal single cell RNAseq dataset\(^{56,57}\). Data visualization and statistics were performed in RStudio v1.4.1717\(^{58}\).
Ultrastructural analysis of mitochondria

Tissue preparation
To further understand what the identified differences in mitochondrial proteins in 4M TG and ES mice mean for mitochondrial structure, we studied these at the ultrastructural level via electron microscopy analysis. 4M mice (Cohort 4) were sacrificed via transcardial perfusion with ice-cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH = 7.4) under anesthesia by 120mg/kg Euthasol. Whole brains were dissected and kept in 4% PFA for 24 h after which the solution was replaced for 30% sucrose for cryopreservation of the tissue. Brain tissue was stored at -80 °C until further processed.

Fifty µm thin coronal sections of the hippocampus were made on a sliding microtome. Contrasting of the sections was realized by a mixture of 1% osmium and 1% ruthenium. The slices were then exposed to increasing ethanol concentrations (30%, 50%, 70%, 90%, 96% and 100%) and finally propylene oxide for dehydration. This was followed by embedding the sections in epoxy resin and polymerized for 72 h at 65 °C. Ultra-thin sections of 90 nm of the dorsal CA1 hippocampus were cut on an ultra-microtome (Reichert-Jung, Ultracut E). Finally, post-contrasted was realized with uranyl acetate and lead citrate in an ultra-stainer (Leica EM AC20).

Electron microscopy imaging and analysis
The grids were examined with a JEOL JEM 1011 electron microscope at 50,000 x magnification. Pictures were taken with an Olympus Moreda 11-MP camera and iTEM software (Olympus). A total of 70 pictures of randomly selected locations within the dorsal CA1 hippocampus were collected in which at least one morphologically intact mitochondrion was identified. Mitochondria along the synapse were counted and classified as belonging to either pre-synapse, post-synapse, or astrocytes. Morphological features (Perimeter, Area, Circularity) of whole mitochondria (i.e., those not on the edge of the image) were measured using ImageJ. Outliers in the morphological measurements were removed using the 1.5*IQR method. Statistical analyses were done by creating a mixed model to assess the effects of genotype, early-life condition, and their interaction, while correcting for the nesting effect of multiple mitochondrial measures being obtained from the same mice. Post-hoc analyses were conducted correcting using Tukey’s method.

Immunofluorescence
To validate ELS-induced modulation of the hippocampal synaptic proteome in APP/PS1 mice at advanced stages of pathology, we used two parallel series of 40µm thick coronal brain slices from a previously described cohort of 12-month old APP/PS1 mice exposed to ELS31 (Cohort 5) Mice were sacrificed via rapid decapitation within the first two hours of the light phase, and brains were drop-perfused in 4% paraformaldehyde overnight, then stored in 0.1M PB with 0.01% Na-Azide until slicing, and stored in antifreeze at -20°C until use.

We stained against two proteins involved in lipid metabolism, Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Alpha (HADHA, rabbit polyclonal Abcam, ab54477, 1:250) and Fatty Acid Synthase (FASN, rabbit polyclonal, Abcam ab22759, 1:500). Both were co-stained with Vimentin (chicken polyclonal, EMD Millipore 5733, 1:3000 for HADHA; 1:2000 for FASN) to localize the signals to astrocytes.
HADHA immunostaining was performed on pre-mounted slices after 15 minutes of antigen retrieval at 100°C in citrate buffer (pH 6.0), while FASN was performed free floating at room temperature. Both were blocked for 1h in a 0.05M TBS mix with 5% NGS and 0.3% Triton (pH 7.6), then incubated with primary antibodies for 1h at RT, then overnight at 4°C. Sections were incubated the next day with secondary antibodies (goat anti-chicken-A488, goat-anti-rabbit-A568, and goat-anti-mouse-A647, Invitrogen, 1:800) for 2h at RT. Slices were washed with 0.05M TBS (pH7.6) between steps. We used 12 brains for HADHA staining and 14 brains for FASN staining.

Confocal microscopy and image analysis
We imaged 1µm-step Z-stacks (total Z range of 9-10µm) at 40x magnification using a Nikon A1 confocal microscope. Images were taken from six sections along the dorsoventral axis of the hippocampus to obtain an even representation. Each image was a stitched composite such that all hippocampal subregions (Stratum Oriens in the Cornu Ammonis [CA] to the Hilus in the dentate gyrus) were visible, with dorsal sections being 2 images wide and 4 images tall and ventral sections being 4 or 5 images wide and 3 images tall.

Images were analyzed with FIJI (v1.53q) by first drawing regions of interest to define the CA and dentate gyrus regions. Per slice, binary masks of Vimentin signal were generated using an automated threshold followed by particle analysis to reduce noise. The area of Vimentin signal, as well the automated thresholded area of the HADHA or FASN signal were measured, with coverage being defined as (HADHA or FASN area/Vimentin area). After testing for outliers using the 1.5*IQR method, data were analyzed using the student’s t-test.

Visualizing trajectories of proteomic alterations from 4M to 10M
To visualize the trajectory of TG-induced effects at the synapse, we adapted a ΔLog2FC approach to visualize temporal dynamics of changes in synaptosomal proteomes. We performed this analysis in differentially expressed mitochondrial proteins when comparing either WT-CTL vs TG-CTL mice or TG-CTL vs TG-ES mice. For each protein, we calculated a ratio between their Log2FC across ages (Log2FC<sub>10M</sub>/Log2FC<sub>4M</sub>, or ΔLog2FC). Using a threshold of ±0.05, we then stratified the differentially expressed proteins by whether their ΔLog2FC ratio increased (i.e., ΔLog2FC > 0.05), decreased (i.e., ΔLog2FC < -0.05), or was not affected (-0.05 < ΔLog2FC < 0.05) between 4M and 10M.

Results

Early Aβ pathology alters hippocampal synaptic proteins involved in mitochondria and actin dynamics
To identify possible synaptic alterations induced by the transgenic APP/PS1 genotype (TG) at early stages of Aβ pathology, we characterized the proteome in hippocampal synaptosomes from wildtype (WT) and TG mice at 4 months (4M) in two independent cohorts (Fig. 1A). We found a similar number of differentially expressed proteins in both cohorts (Cohort 1: 14 up, 23 down; Cohort 2: 17 up, 23 down, Fig. 1B-D, table S1, S2), of which two proteins (ABI1, WASF) were downregulated in both cohorts.
Figure 1. Upregulation of mitochondrial proteins and downregulation of actin-dynamics related proteins in synaptosomes from transgenic APP/PS1 (TG) mice at 4 months (A) Experimental design in analyzing the hippocampal synaptosomal proteome at four months (4M). (B) Table summarizing differentially expressed proteins (DEP) between two cohorts of wildtype (WT) and TG mice. Only two proteins (ABI1, WASF) were significantly downregulated in both cohorts. (C-D) Volcano plots showing differentially expressed proteins (±Log2FC > 0.1, FDR< 0.05) in cohorts 1 (C) and 2 (D). (E-F) Overview of overrepresented biological processes (BP) and cellular component (CC) terms based on gene ontology (GO) analyses of up- (light blue) and downregulated (light red) proteins in Cohorts 1 (E) and 2 (F). Italicized terms are ‘parent’ GO terms after clustering by semantic similarity using RRVGO. Size indicates number of differentially expressed proteins annotated to each GO term.

Overrepresented GO terms, based on differentially expressed proteins in both cohorts, were related to the same processes, i.e., mitochondria and actin dynamics (Fig. 1E, 1F, table S3), but notably involved different sets of proteins. TG groups in both cohorts had upregulated mitochondrial proteins that, at least in Cohort 1, were involved in fatty acid and monocarboxylic acid metabolism (ACSL6, ALDH3A2, APP, ECHS1, PNPLA8, THEM4). This
mitochondrial alteration was also evident when comparing the upregulated proteins with a curated database of mitochondrial proteins (MitoCarta3.0\(^5\), Cohort 1: ACSL6, ALDH3A2, ECHS1, NIPSNAP3B, PNPLA8, SDHD, SFXN5, THEM4, TIMM50; Cohort2: BCS1L, COA3, ECI1, ETFA, HTRA2, LACTB, NDUFA7, PRKACA).

We also found in both cohorts a downregulation of proteins (Cohort 1: ABI1, CYFIP2, WASF1; Cohort 2: ABI1, NCKAP1, WASF) involved in the SCAR complex, which plays a role in actin dynamics and cytoskeletal regulation\(^5\). The two shared downregulated proteins (ABI1, WASF) are part of this GO term. The downregulation of actin dynamics related proteins in synaptosomes from TG mice was particularly evident in Cohort 2 (Fig. 1F), wherein actin-related biological processes (e.g., polymerization/depolymerization: ABI1, ADD2, CAPZA1, CAPZA2, CAPZB, DBN1, NCKAP1, PFN1, PFN2, PPP1R9B, TMOD2) were downregulated. In terms of cellular component annotations, these proteins were localized to the cytoskeleton, as well as in the post-synapse (Fig. 1F, table S3).

To assess whether these differentially expressed proteins reflected cell-type specific TG effects, we performed expression weighted cell-type enrichment (EWCE) analysis\(^6\) using a published single-cell gene expression database from the mouse hippocampus\(^7\). TG synaptosomes from Cohort 1 exhibited astrocytic enrichment in the upregulated proteins (Fig. S1A), while downregulated proteins in both cohorts were enriched for neurons (Fig. S1A, S1B). Further analysis of the function of the downregulated proteins using a curated database of synaptic proteins (SynGO\(^5\)) similarly revealed the involvement of downregulated proteins in postsynaptic processes, specifically with respect to actin-related processes (Fig S1C-D).

**Early-life stress downregulates actin dynamics and upregulates astrocytic proteins in hippocampal synaptosomes from WT mice at 4M**

We then investigated how early-life stress (ES) exposure affected the synaptic proteome in 4-month-old WT and TG mice (Fig. 2A). First, ES exposure in WT animals resulted in 126 differentially expressed synaptosomal proteins (84 up, 42 down, Fig. 2B, table S4). Based on GO analyses, proteins enriched in WT-ES synaptosomes were involved in sodium ion homeostasis (ATP1A2, ATP1B2, GNAI2, SLC1A3, SLC8A1), and were associated with cellular component terms such as the GTPase complex, and endoplasmic reticulum of the cell (Fig. S1E, table S3). Notably, some upregulated proteins (via BCAP31, CANX, TMX2) were part of the mitochondria-associated membrane of the endoplasmic reticulum (table S3). EWCE analysis revealed enrichment of astrocytic annotations in the 126 upregulated proteins (Fig 2C). In line with this, some of these upregulated proteins (ATP1A2\(^6\), SLC1A2\(^6\), SLC1A3\(^6\), SLC6A11\(^6\)) have been described in literature to be specific for astrocytic proteins enriched around the synapse. GO analysis of downregulated proteins revealed overrepresentation of biological processes terms such as actin cytoskeletal dynamics (via ABI2, ACTN4, ADD2, BIN1, CAPZA1, CAPZA2, CAPZB, CFL1, CORO1C, DBN1, PFN1, PPM1E, PPP1R9B, TMOD2, TWF2), and cellular component terms such axonal growth cones (via CFL1, DBN1, FKBP4, HSP90AB1, PPP1R9B, TMOD2, TWF2, Fig. S1F, table S3).

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Figure 2. Early-life stress (ES) exposure upregulates astrocytic mitochondrial proteins and downregulates actin-dynamics related proteins
(A) Experimental design within cohort 2 and summary of differentially expressed proteins (DEP) across contrasts at 4 months of age (4M). (B) Volcano plot showing differentially expressed proteins (±Log2FC > 0.1, FDR< 0.05) in synapses from ES-exposed WT mice. (C) Expression weighted celltype enrichment (EWCE) analysis shows enrichment of astrocytic annotations in upregulated proteins and oligodendrocytic annotations in downregulated proteins. (D) Overlap of differentially expressed proteins when comparing proteomic changes in synaptosomes from TG (light blue) and ES (dark red) mice. (E) Upregulated proteins in synaptosomes from TG-CTL and WT-ES mice are mitochondrial, as determined by the MitoCarta database. (F) Volcano plot showing differentially expressed proteins (±Log2FC > 0.1, FDR< 0.05) when comparing synapses from ES-exposed WT and TG mice.

Possible shared effects between ES exposure and APP/PS1 genotype on the hippocampal synaptic proteome at 4M
Surprisingly, ES exposure did not lead to further significant changes in the synaptosomal proteome of TG mice at this age (table S5). This stands in contrast to the detected alterations to synaptomes from both TG-CTL and WT-ES mice, suggesting a degree of convergence between ES and TG effects at this age. To explore this, we investigated the 18 proteins (7 up, 11 down) that were differentially expressed in the synaptosomes of both TG-CTL or WT-ES mice (Fig. 2D, table S7). While the group of upregulated proteins did not show any overrepresentation of GO terms, the downregulated proteins were associated with actin-related biological processes (e.g., actin polymerization or depolymerization: ADD2, CAPZAY, CAPZA2, CAPZB, DBN1, PFN2, PPP1R9B, TMOD2) and were overrepresented for cellular component terms such as the postsynapse (ADD2, CAPZB, DBN1, MOB4, NSF, PFN2, PPP1R9B) and dendritic spines (CAPZB, DBN1, MOB4, PPP1R9B, table S8).
Beyond actin-related alterations, inspection of the shared upregulated proteins also suggested mitochondrial changes in both ES and TG synaptosomes. This is evidenced firstly by the reported mitochondrial expression of DPM1\textsuperscript{63}, the protein with the highest log2 fold change value in TG-CTL (Fig. 1D) and WT-ES (Fig. 2B) synaptosomes. Several shared upregulated proteins are also either expressed in mitochondria (e.g. COA3\textsuperscript{64}, CHCHD2\textsuperscript{65,66}), or have been reported to interact with mitochondria and regulate their function (e.g. RAB5C\textsuperscript{67,68}, ETL4-SKT\textsuperscript{69}, SLC4A3\textsuperscript{70}). Lastly, as found in synaptosomes from TG-CTL mice, several upregulated proteins in synaptosomes isolated from WT-ES mice also overlapped with MitoCarta (ACAA2, ACAD8, ACADM, COA3, DECR1, GLUD1, IDH2, ETFA, MPST, NDUFA3, PRDX5, Fig. 2E).

Despite these similarities, ES and TG effects were not entirely identical. When comparing ES-exposed WT and TG mice, we found 17 differentially expressed proteins (4 up, 13 down, Fig. 2F, Table S6). There were insufficient upregulated proteins to perform GO analysis, but these were notably involved in Aβ processing (APP, DOCK9, NCSTN) and GABA biosynthesis (GAD2). GO analysis of downregulated proteins revealed one overrepresented cellular component term: the hippocampal CA3 mossy fiber synapse (ADCY1, SHANK2, SYT7, Table S3). Still, these data suggest the possibility that early proteomic alterations in synapses from TG mice are similar to those induced by ES.

**Ultrastructural analyses of synaptic mitochondria at 4M reveal a distinct loss of mitochondrial numbers in the hippocampus of both APP/PS1 and ES-exposed mice**

To further explore the extent to which TG and ES effects overlap in the changes they induce at the hippocampal synapse at this age, we next generated a follow-up cohort of 4M WT and TG mice exposed to ES to study their mitochondria (Fig. 3A). We determined the number of mitochondria and assessed mitochondrial morphology at the ultrastructural level within the presynapse, postsynapse, dendrites, and astrocytes in the CA1 subregion of the hippocampus of these mice (Fig. 3B).

While there was no overall TG or ES effect on total number of mitochondria (Fig. S2A), we found interaction effects of both factors on the number of mitochondria in the pre-synapse (condition: F(1,19)=0.0218, p=0.8842; genotype: F(1,19)=2.1204, p=0.1617; interaction: F(1,19)=12.001, p=0.0026) and post-synapse (condition: F(1,16)=2.5384, p=0.1307; genotype: F(1,16)=0.0555, p=0.8168; interaction: F(1,16)=11.8658, p=0.0033, Fig. 3C). Mitochondria within astrocytes or dendritic structures were not affected. Using pairwise post-hoc tests, we found the pre-synaptic effect to be due to a decrease in mitochondria in TG-CTL compared to WT-CTL synapses (p=0.0109), while the postsynaptic interaction effect was driven by a decrease in the number of mitochondria in WT-ES compared to WT-CTL synapses (p=0.0171).
Figure 3. Investigating ultrastructural alterations to synaptic mitochondria in ES-exposed WT and TG mice. (A) Overview of cohort 4, generated for electron microscopy (EM) analysis of mitochondria. (B) Representative EM image analyzed. Mitochondria (m) at the CA1 region of the hippocampus were counted and traced across 70 images per animal to analyze their morphology. Analysis was done separately in sub-synaptic structures, as labeled and outlined in the image: pre-synapse (yellow), post-synapse (red), astrocyte (blue), dendritic compartment (green). (C) Interaction of TG and ES effects on mitochondrial counts at the pre- and post-synapse, but not in dendrites or astrocytes. (D) Alterations to pre-synaptic mitochondrial counts are not accompanied by alterations to mitochondrial size. (E) Interaction of TG and ES effects on mitochondrial area at the post-synapse. &, interaction effect, *p*<0.05; post-hoc effects: a, different from WT-CTL, *p*<0.05; b, difference between WT-CTL and TG-CTL, *p*<0.05; c, difference between WT-ES and TG-ES, *p*<0.05.

The effects on mitochondrial numbers were not accompanied by alterations in mitochondrial area at the pre-synapse (Fig. 3D), although there was an interaction between TG and ES effects on the postsynaptic mitochondrial area (Fig. 3E, condition: $t(20)=-1.7961$, *p*=0.0876; genotype: $t(20)=-2.2274$, *p*=0.0376; interaction: $t(20)=3.3099$, *p*=0.0035). Post-hoc analyses reveal that this interaction is explained by significant but opposite effects of genotype in CTL ($t(20)=2.227$, *p*=0.0376) and ES ($t(20)=-2.452$, *p*=0.0235) synapses. Mitochondrial area in other regions, as well as perimeter and circularity, were not significantly altered by either experimental variable (Fig. S2B-D).

During advanced pathological stages, APP/PS1 synaptosomes are depleted in proteins involved in presynaptic vesicle release and postsynaptic receptor endocytosis. We then characterized the effects of TG genotype and prior ES exposure on the hippocampal synaptosomal proteome in 10-month-old (10M) mice (Fig. 4A).
Figure 4. Downregulation of proteins involved synaptic APP/PS1 genotype downregulates synaptic processes in hippocampal synaptosomes at 10 months
(A) Experimental design within cohort 2 and summary of differentially expressed proteins across contrasts at 10 months (10M). (B) Volcano plot showing differentially expressed proteins (±Log2FC > 0.1, FDR < 0.05) in synaptosomes from TG-CTL mice at 10M. (C) Protein alterations in synaptosomes from 10M TG mice are distinct from changes in TG mice at 4M. (D) Expression weighted celltype enrichment (EWCE) analysis shows enrichment of astrocytic and microglial annotations in upregulated proteins in synaptosomes from TG-CTL mice. Downregulated proteins are enriched for annotations of excitatory neurons. (E) Further analysis of downregulated proteins using SynGO reveal disruptions to synapse organization, presynaptic function, and postsynaptic processes. (F) Volcano plot showing differentially expressed proteins (±Log2FC > 0.1, FDR < 0.05) between ES-exposed WT and TG mice at 10M.
Synaptosomes isolated from TG-CTL mice were massively affected at this age, resulting in 170 dysregulated proteins (11 up, 159 down, Fig. 4B, table S9), which were largely distinct from differentially expressed proteins in TG-CTL synaptosomes at 4 months (Fig. 4C). Upregulated proteins in 10M TG-CTL mice were overrepresented for 52 clusters of biological processes and 13 clusters of cellular components GO terms, mostly driven by APP-associated pathways (e.g., Aβ processing: APP, APOE, CLU, NCSTN; cholesterol metabolism: APP, APOE, ARL8B, PSAP), and neuroinflammation (e.g., glial response: APP, CLU, GFAP, NCSTN; immune response: APP, APOE, ARL8B, CLU, NCSTN, PSAP). Downregulated proteins were associated with 23 clusters of biological processes and 17 clusters of cellular components, including cell communication, neuronal projection development, transport vesicle membranes, and DNA damage (Fig. S3A, table S10).

Cell type enrichment analysis using EWCE revealed upregulated proteins to be microglial and astrocytic, whereas downregulated proteins were mostly neuronal (Fig. 4D), consistent with the well-documented neuroinflammatory activation and synaptic dysfunction that results from Aβ pathology. To further investigate the synaptic pathways associated with the downregulated proteins, we performed an overrepresentation analysis using SynGO (Fig. 4E, S3B, table S11). This analysis revealed a depletion in the synapses of 10M TG mice of proteins involved in both presynaptic vesicle exocytosis (via GIT1, PPFIA3, RAB3A, RIMBP2, RPH3A, STX1A, STXBP1, SV2B, SYT7, VAMP2), as well as postsynaptic organization (via ACTB, DLG1, DLG3, GIT1, MPP2, RIMBP2) and receptor endocytosis (via AKAP5, AP2B1, AP2M1, AP2S1, HPCA, SYNJ1).

These TG-induced effects were also evident when comparing ES-exposed WT and TG mice (Fig. 4F), where 44 proteins were differentially expressed (35 up, 9 down, table S13). While there were no overrepresented GO terms in the downregulated proteins, the 35 upregulated proteins were overrepresented for biological processes terms mostly driven by APP, APOE, and CLU (table S10), as well as cellular component terms involving high-density lipoproteins, lysosomes, and mitochondria. Notably, 11 out of 44 differentially expressed proteins (upregulated: APOE, APP, CLU, DOCK9, GFAP, NCTSN; downregulated: LGI1, L1CAM, MPP3, SLC30A3, SYT7) were similarly differentially expressed between synaptosomes from TG-CTL and WT-CTL mice, suggesting these to be ‘core’ alterations that Aβ pathology induces on hippocampal synaptosomes at this age.

**ES exposure strongly alters the hippocampal synaptosome in 10M APP/PS1 but not WT mice**

We previously hypothesized that ES-associated effects in later ages would be minimal at baseline, but would be more pronounced upon exposure to challenges later in life. In line with this, and in contrast to the data at 4M, only 19 proteins (13 up, 6 down) were differentially expressed in 10M ES-exposed WT synapses (Fig. 5A, table S12). The one GO term associated with upregulated proteins did not seem synapse related, involving regulation of blood pressure (Fig. S3C), whereas downregulated proteins were associated with 19 clusters of GO terms (13BP, 6 CC) involving endoplasmic reticulum calcium transport (Fig. S3C, via ATP2A2, RYR2, table S10). Also, in contrast to the data at 4 months, where we found 18 shared differentially expressed proteins between ES and TG effects, only one protein (KCNQ2) was significantly altered in both contrasts at this age.
Figure 5. ES exposure further alters (astrocytic) mitochondrial lipid metabolism in TG synaptosomes with advanced pathology
(A) Volcano plot showing differentially expressed proteins (±Log2FC > 0.1, FDR< 0.05) in ES-exposed WT at 10M.
(B) Volcano plot showing further differential expression in synapses from ES-exposed TG mice at this age. (C) Expression weighted celltype enrichment (EWCE) analysis shows enrichment for interneuronal annotations in upregulated proteins in ES-exposed TG mice. Downregulated proteins are enriched for astrocytic annotations. (D-E) Differentially expressed proteins in this contrast overlap with MitoCarta (D) and are functionally annotated to detoxification and reactive oxygen species metabolism (E). (F) Overview of cohort 5 (F), used to confirm lasting alterations to astrocytic expression of HADHA (G) and FASN (H) within ES-exposed TG hippocampus at 12 months (I). *, condition effect, p<0.05.
On the other hand, ES history led to further alterations in the proteomic profile of hippocampal synapses from TG mice at 10M, with 561 differentially expressed proteins (549 up, 12 down, Fig. 5B, table S14). GO analysis of the upregulated proteins (Fig. 5D, table S10) revealed terms related to the metabolism of different molecules such as carbohydrates, aldehydes, NAD, organonitrogens, organophosphates, hydroxy-compounds, as well as GO terms related to DNA metabolic processes and chromosome structure. Cellular component analysis indicated 8 clusters of GO terms that suggest alterations at the nucleus, cytosol, (microtubule) cytoskeleton, proteasome complex, and V-type proton ATPases. Also, the GO terms that were associated with downregulated proteins in this contrast (Fig. 5E, table S10) were involved in fatty acid metabolism and beta-oxidation in the mitochondria, driven by four proteins (BCKDHA, DECR1, HADHA, HADHB).

EWCE analysis of the differentially expressed proteins revealed the upregulated proteins to be interneuronal, and downregulated proteins to be astrocytic (Fig. 5C). Upregulated proteins were not enriched for any SynGO annotated pathways (not shown). Additionally, ES exposure strongly affected mitochondrial proteins in TG synaptosomes at this age, with 47 out of 565 differentially expressed proteins (41 up, 6 down) overlapping with MitoCarta (Fig. 5D, in contrast with 2 mitochondrial proteins, HMCL and MPST, altered between WT-CTL and TG-CTL synaptosomes). GO analysis on these proteins using MitoCarta-annotated pathways revealed an overrepresentation of proteins associated with type II fatty acid synthesis, detoxification, ROS/glutathione metabolism, and sulfur metabolism (Fig. 5E). Taken together, these data suggest ES exposure to result in further, later alterations to lipid metabolism TG hippocampal synapses, in part through mitochondrial alterations.

To validate the results from the proteomic analysis, as well as investigate the temporal persistence of the presumed ES effects on lipid metabolism in the hippocampi of TG mice, we stained for HADHA and FASN (down- and upregulated in the proteomics data, respectively) in a cohort of 12-month-old (12M) TG mice exposed to ES (Cohort 5, Fig. 5F). Because of the strong expression of these two proteins in astrocytes and the EWCE data suggesting further astrocytic dysfunction in ES-exposed TG mice, we quantified the expression of these proteins together with Vimentin as an astrocyte-specific marker (Fig. 5G, 5H). We found a decrease in astrocytic HADHA (t(9.98)=2.2486, p=0.0483, Fig. 5I) and trend for increased astrocytic FASN (t(6.422)=-2.2454, p=0.0630, Fig. 5I), specifically within the dentate gyrus (DG) of the hippocampus. Beyond corroborating the protein data, these results also suggest that alterations to hippocampal astrocytic lipid metabolism in ES-exposed TG mice occur and persist beyond just the synapse.

ES modulates the temporal pattern of synaptic mitochondrial protein alterations seen in TG mice
Lastly, we aimed to contextualize these further alterations to (mitochondrial) lipid metabolism proteins in ES-exposed TG mice, by juxtaposing them against how the TG-associated differential expression of these proteins progress over time. To do so, we selected MitoCarta annotated proteins that were significantly altered (±Log2FC > 0.1, FDR <0.05) in the WT-CTL vs TG-CTL and WT-CTL vs WT-ES contrasts at either age (Fig. 6A). Doing so revealed 55 unique MitoCarta proteins, of which 48 were present in all datasets.
Figure 6. Exploring how ES exposure influences the temporal pattern of mitochondrial protein expression in synaptosomes from TG mice. (A) Workflow to visualize trajectory of mitochondrial protein alterations in TG synapses. We identified proteins significantly altered ($\log_{2}FC > 0.1$, FDR < 0.05) in synaptosomes from TG-CTL and ES-exposed TG mice from cohorts 2 and 3. A Log2FC ratio between both ages was calculated (Log2FC$_{10M}$ – Log2FC$_{4M}$) to visualize the ‘standard’ progression of these changes in TG-CTL mice. (B) Based on a threshold of $\Delta$Log2FC > ±0.05, proteins were stratified based on whether their alterations in TG mice (light blue lines) get stronger ($\Delta$Log2FC >0.05), weaker ($\Delta$Log2FC <-0.05), or are consistent ($\Delta$Log2FC < ±0.05) with age. The $\Delta$Log2FC of the same proteins in ES-exposed TG mice are then visualized (dark blue lines). (C) Functional annotations using MitoCarta reveal that mitochondrial proteins lastingly altered in the synaptosomes of TG mice have various metabolic roles, which are presumably further impacted by ES exposure.
These proteins are affected in different ways: for instance, of the proteins upregulated (Log2FC > 0.1) in TG mice at 4M, some are downregulated (Log2FC < -0.1) by 10M (PRKACA), while some are upregulated to a similar extent (HADHA, HADHB, SFNX5, table S15). On the other hand, certain proteins downregulated in synaptosomes from 4M TG mice no longer altered at 10M (AKR1B10, ETFA, LAP3, MSRB2, PRDX5). We thus wanted to quantify the extent to which these 48 proteins are differentially expressed in synapses from TG mice with age.

To visualize this, we calculated a ratio of the Log2 fold change in the expression of each protein when comparing synaptosomes from WT-CTL versus TG-CTL mice at 4M and 10M ($\Delta\text{Log2FC} = \text{Log2FC}_{10\text{M}} - \text{Log2FC}_{4\text{M}}$, adapted from 24). This proxy measure, which has the limitation of not being able to account for the absolute magnitude of change in each protein across ages, is nonetheless able to give an impression of the ‘persistence’ of TG effects on synaptic protein expression (i.e., whether upregulation/downregulation in synapses from 4M TG mice remain or are further upregulated/downregulated at 10M). As such, proteins were stratified (using a $\Delta\text{Log2FC}$ threshold of ±0.05) by whether the ratio of their relative expression increases ($\Delta\text{Log2FC} > 0.05$) decreases ($\Delta\text{Log2FC} < -0.05$), or does not change ($\pm \Delta\text{Log2FC} < 0.05$) between both ages in TG mice (Fig. 6B, light blue lines).

We then visualized the $\Delta\text{Log2FC}$ ratio of these same proteins in the hippocampal synaptosomes based on their Log2FC in ES-exposed TG mice (Fig. 6B, dark blue lines). Analyzing the functional annotations of these proteins based on MitoCarta confirm reveal that mitochondrial aberrations in the synapses of TG mice are mostly to do with mitochondrial metabolism (Fig. 6C). Notably, ES exposure seems to lead to a different temporal pattern in the expression of these proteins. This is particularly striking for the proteins with a negative $\Delta\text{Log2FC}$, as well as for those where the Log2FC ratio is not changed in TG-CTL mice. These proteins seem to be in part involved in lipid metabolism and (ROS) detoxification, suggesting that further ES alterations to TG synapses might act through these pathways.

Discussion

We here studied the hippocampal synaptosomal proteome in transgenic amyloid-beta (Aβ) overexpressing mice after postnatal early-life stress (ES) exposure, induced via the limited bedding and nesting model. Interactions were studied at 4 and 10 months of age, representing early and advanced stages of Aβ pathology, respectively. At 4M, synaptosomes isolated from both control (CTL)-APP/PS1 and ES-exposed wildtype (WT) mice exhibited a similar upregulation of mitochondrial proteins and downregulation of actin-dynamics related proteins, while ES-exposure did not further alter synaptosomes from APP/PS1 mice. At 10M, proteins involved in Aβ processing were found to be upregulated, while neuronal proteins involved in pre- and post-synaptic processes were downregulated in synaptosomes from APP/PS1 mice. At this age, while there were minimal ES effects on the synaptosomes in WT mice, ES-exposed APP/PS1 mice exhibited further alterations in the expression of synaptosomal proteins involved in lipid metabolism. Our temporal analysis on the effect of ES on the synaptic proteome indicates that mitochondrial metabolism is lastingly altered in
synaptosomes of APP/PS1, which ES exposure seems to both mimic during early pathological stages and exacerbate during advanced pathological stages.

**Synaptic mitochondria are altered both early pathological changes induced by transgenic overexpression of Aβ as well as ES history**

We found both mitochondrial proteins and morphology to be altered in APP/PS1 mice at an age representing an early stage of Aβ pathology. Namely, synaptosomes from APP/PS1 mice across both cohorts displayed an upregulation of mitochondrial proteins, which was accompanied by a decrease in the number of pre-synaptic mitochondria, as well as smaller post-synaptic mitochondria.

Our findings support reports that mitochondrial dysfunction contributes to early Alzheimer’s disease (AD) pathogenesis. This is thought to be in part caused by the interactions between Aβ species and mitochondria, as Aβ has been shown to alter mitochondrial morphology, fusion/fission balance, and proteostasis. In fact, AD patients exhibit region-specific mitochondrial loss in the central nervous system, leading to the notion that mitochondrial alterations drive AD pathogenesis.

The loss of mitochondria has major implications for synapses, which have high energy demands. The consequent disrupted energy balance is particularly evident in the hippocampi of AD patients. This might be due to deficient oxidative phosphorylation and electron transport, as found in studies using whole hippocampi of 3- and 6-month-old APP/PS1 mice. Our data suggest that these alterations occur also specifically in the synapses of APP/PS1 mice, in line with a study that found reduced number of mitochondria in the same model at 6 months of age. Importantly, the fewer pre-synaptic mitochondria we found were not larger in area, suggesting that the reduction in number is not due to increased fusion events, but likely due to the loss of mitochondria. As such, the increased expression of mitochondrial proteins we observed in synapses from APP/PS1 mice, especially at 4M, might reflect compensatory mechanisms to overcome these putative deficits. The functional implications of these changes for synaptic energy homeostasis (e.g., ATP production) remain to be determined.

Our data also highlight how ES leads to mitochondrial alterations at the synapse. As in APP/PS1 mice, synaptosomes from 4M WT mice exposed to ES were also enriched for mitochondrial proteins. These changes were also accompanied by a decrease in mitochondrial counts at the ultrastructural level (with no increase in mitochondrial area), which altogether imply the loss of mitochondria. Notably, this decrease in mitochondrial count was found in the post-synapse, in contrast to the pre-synaptic mitochondrial deficits in APP/PS1 synapses. The upregulation in mitochondrial proteins might then, analogously, be compensatory against the effects of such deficits. This would explain a previous finding of increased fatty acid synthase (FASN) mRNA expression in the whole lysates from the contralateral hippocampus of the same WT-ES mice used in this study. This link between stress and mitochondrial function is in line with a study showing the impairment of respiratory processes in synaptosomal mitochondria of mice subjected to chronic stress in adulthood. Additionally, we had previously reported an ES-mediated disruption of the electron transport chain activity in the hypothalamus and muscle tissues in postnatal pups, which persisted until at
least 10 months of age\textsuperscript{84}. That same study reported an age-associated change in expression of hippocampal mitochondrial fission proteins after ES, with Fis1 being less expressed in ES-exposed pups yet more highly expressed in 10M WT-ES mice.

Crucially, we also found evidence for an association between the effects of ES exposure and APP/PS1 genotype on hippocampal synaptic mitochondria. As discussed above, the effects of ES on synaptic mitochondria seem to mirror the changes occurring in APP/PS1 synapses. Importantly, these effects do not completely overlap, as evident in the difference in postsynaptic mitochondrial area when comparing ES-exposed WT and APP/PS1 mice. The interaction between the two experimental factors is particularly pronounced at 10M, where 39 mitochondrial proteins were upregulated in ES-exposed APP/PS1 mice (compared to only 2 when comparing WT and APP/PS1 mice at this age). These mitochondrial disruptions might possibly be caused by the increased Aβ plaque load observed in ES mice at this age\textsuperscript{29}, as mitochondrial numbers have been shown to decrease as a function of amyloid plaques proximity\textsuperscript{95}.

**Actin dynamics as a substrate for synaptic alterations in both APP/PS1 and ES-exposed WT mice**

We also found evidence for a downregulation of actin-dynamics-related processes in two cohorts of APP/PS1 mice at 4M. This seemed to be mediated by the SCAR Complex, an evolutionarily conserved family of proteins involved in de novo assembly of actin branches\textsuperscript{59,96}. Further, downregulated proteins in APP/PS1 synapses are also involved in the regulation of actin polymerization and depolymerization.

The actin cytoskeleton is important for synaptic structure, being involved e.g., in the formation, maintenance, or elimination of dendritic spines, as it dynamically reorganizes in response to synaptic signals\textsuperscript{97}. Importantly, there is evidence for dysfunctional actin in the brains of AD patients and mouse models, seen as neuropathological ‘rods’ formed by actin-depolymerizing factor (ADF) and Cofilin “rod” aggregates\textsuperscript{98}. These aggregates can be induced via cellular exposure to stressors such as reactive oxygen species (ROS) and Aβ, and form along mitochondria-deficient parts of the neurite\textsuperscript{99,100}. This process, consisting of Cofilin-mediated formation of “rods,” accompanied by arrests to actin (de)polymerization, is thought to be part of the cellular response to stress\textsuperscript{101}, reducing the rate of ATP-loss\textsuperscript{102}, which is sensible given the energetic costs associated with actin disassembly\textsuperscript{103}.

Mitochondrial trafficking also occurs via actin networks\textsuperscript{104}, and mitochondrial function (e.g., fusion/fission) are influenced by actin remodeling\textsuperscript{105}. As such, it is tempting to speculate that the alterations to actin dynamics at the synapse is a result of a diversion of energy expenditure to meet energetic demands brought about by, e.g., loss of mitochondria at this age. Although this might have energetic benefits on the short term, it is important to consider that it might lead to long term disruptions. While Aβ-induced deficits in actin dynamics are most prominent in synaptosomes from 4M APP/PS1 mice, these terms are still present by 10M (table S3 and S10). Actin dynamics are also important for the trafficking of receptors to the synapse\textsuperscript{106,107}, as well as synaptic vesicle release and recycling\textsuperscript{108,109}, and their prolonged disruption might explain the downregulation of presynaptic release and postsynaptic receptor endocytosis in synapses from 10M APP/PS1 mice. Additionally, the
disruption of actin dynamics could also have downstream consequences, e.g., in the motility of perisynaptic astrocytes, which need cytoskeletal remodeling to respond to neuronal activity.\textsuperscript{46,48}

It is also intriguing to consider how ES might impact this, given that it was also affected in the synaptosomes isolated from ES-exposed WT mice at 4M. These actin dynamics alterations are in line with studies on early life social isolation in rats has been shown to decrease actin turnover by ADF/cofilin inactivation, leading to impaired trafficking of AMPA receptors.\textsuperscript{110,111} These mechanisms might explain the altered distribution of receptors that have been described in the synapses of mice exposed to ES using the same model.\textsuperscript{32} Interestingly, while the downregulated actin-related proteins in ES mice overlapped considerable with those from APP/PS1 mice, the downregulated proteins in ES mice were flagged by EWCE to be enriched in oligodendrocytic annotations, cells that have also been detected in other synaptosomal preparations.\textsuperscript{24,46–48} Actin dynamics via formation of growth cones is important for maturation and migration of cells of an oligodendrocyte lineage, which have been reported to form synaptic contacts with both excitatory and inhibitory neurons in the hippocampus. Cells from this lineage have also been reported to be affected by ES exposure, and potentially represent another substrate through which actin dynamics are altered in ES mice. That said, our data altogether suggest a contribution of cytoskeletal alterations in APP/PS1 and ES phenotypes. The extent to which these changes relate to those found in mitochondria, overlap between these experimental treatments, and are viable targets for preventive/intervention strategies, remains to be investigated in the future.

ES effects on the trajectory of Aβ-induced synapse pathology: impacts on (astrocytic) mitochondrial metabolism

While ES exposure did not further alter protein expression in synapses from APP/PS1 mice at 4M, it did lead to vast differences in protein expression at 10M. Because we studied mice from these experimental groups at both 4M and 10M, we also explored how ES impacted the trajectory of Aβ-associated changes in hippocampal synaptosomes. We focused our current analysis on mitochondria, given its prominence in the proteomic data, aiming to provide evidence that ES exposure modulates the TG effects on these proteins.

The idea that ES could modulate AD- and other aging-associated trajectories is not new.\textsuperscript{43} We have previously shown that ES exposure has age-dependent effects on Aβ pathology in AD mice, decreasing cell associated amyloid at 4M while increasing Aβ plaque load in the hippocampus of stressed TG mice at 10M.\textsuperscript{29} Similarly, ES exposure increases the expression of amyloidogenic proteins such as BACE1 in the hippocampus of 6- and 12-month-old TG mice.\textsuperscript{34} This has also been studied in the context of microglia and astrocytes, where we found e.g., that ES leads to age-dependent alterations in microglial morphology and gene expression in APP/PS1 mice.\textsuperscript{29,30} The synaptic alterations we detected, especially in ES-exposed APP/PS1 mice, likely reflect the age-associated increase in Aβ pathology.

We hypothesized that the lack of further ES effects in 4M APP/PS1 mice to be indicative of a convergence between ES effects with genotype effects at this age. The lack of differentially expressed proteins is consistent with the reported effects of stress exposure during different life stages on AD-like pathology.\textsuperscript{117} For instance, maternally separated WT
rats show upregulated protein expression of BACE1 and Aβ, and mice exposed to chronic psychosocial stress similarly increased expression of AD-associated proteins. We partly explored this hypothesis by EM analysis of mitochondria in both ES-exposed WT and APP/PS1 mice, where we found decreased numbers of presynaptic mitochondria in APP/PS1 hippocampi, and fewer mitochondria in the post-synapse of WT-ES mice. The shared mitochondrial dysfunction at these respective synaptic compartments might result in energetic consequences that are not further impaired in ES-exposed APP/PS1 mice. This will need to be functionally demonstrated in further studies.

In addition, ES exposure strongly affected synaptosomes from APP/PS1 mice at 10M, when Aβ is more severe and widespread. This is in contrast to minimal changes in protein expression in synaptosomes from 10M WT mice, supporting the hypothesis that ES effects at later ages would necessitate secondary challenges (as Aβ would provide) to be unmasked. These effects were prominent in the dysregulation of mitochondrial proteins, which were involved in ROS as well as fatty acid metabolism. Strikingly, these processes seem to be particularly affected in astrocytes, in line with the prominent role of these cell types in energy production in the brain. Astrocytes were also indicated to be affected in our EWCE analysis of differentially expressed proteins across several contrasts, in line with their detection in other synaptosomal studies.

Finally, our data highlight the important role of astrocytes as a substrate for both APP/PS1 and ES effects. We have previously speculated that astrocytes in ES-exposed APP/PS1 mice, while similarly reactive to control APP-PS1 as quantified by GFAP immunostaining, might exhibit alterations also in other functional domains (e.g. lipid metabolism, BBB maintenance), due to the increased plaque load in their surroundings. Here, we show evidence that astrocytic lipid metabolism is further disrupted in ES-exposed APP/PS1 mice. For instance, we find evidence for decreased astrocytic expression of HADHA, a key enzyme in the beta-oxidation of fatty acids, as well as increased astrocytic of FASN, an enzyme involved in de novo synthesis of fatty acids. These changes seemed to occur both at the synapse at 10M and in the rest of the dentate gyrus at 12M, possibly indicating a shift in lipid homeostasis. Beyond indicating a compensatory response to energy deficits, altered lipid metabolism in the hippocampi of ES-exposed APP/PS1 mice could have consequences on neuronal circuits, given the recent evidence that the contents of saturated lipids from reactive astrocytes can have neurotoxic properties. At the same time, an imbalance in the synthesis of fatty acids may mean increased rates of oxidative stress, given their propensity to be peroxidized by free radicals. These seemed to predominantly occur in astrocytes, in line with the important role of their mitochondria in energy production.

In this context, our observation that the synapses of ES-exposed APP/PS1 mice are upregulated in mitochondrial proteins associated with ROS metabolism might be a compensatory adaptation brought about by this shift in lipid synthesis. A similar effect was found in work using the 5xFAD TG model. Importantly, our lab has shown age-dependent consequences of ES on fatty acid profiles across brain regions in WT mice, where e.g. hippocampal polyunsaturated fatty acids (PUFA) are decreased in ES-exposed pups and increased in ES-exposed adult mice at 6 months. Notably, the alteration of PUFA species, via dietary intervention, has been shown to rescue both lipid levels and cognitive deficits.
in ES-exposed adults. The consequences of these altered lipid profiles in the ES-exposed brain (e.g., regarding ROS metabolism), as well as the functional consequences of ES on these processes in the APP/PS1 brain, will require further investigation.

Conclusion

Our work reveals that ES modulates Aβ pathology-induced alterations to the synaptosomal proteome. We show that the effects of ES alone on synaptic proteome are very similar as those of early Aβ pathology, and accordingly, ES exposure does not further alter the proteomic profile in APP/PS1 mice at this age. We also report that ES leads to additional effects on the proteomic alterations caused by Aβ at advanced pathological stages, which was minimal in the WT condition. These data highlight the importance of the synapse as a long-term substrate for APP/PS1 pathology, and show how ES influences the trajectory of these alterations with advancing age. These interactions between the two factors also suggest that Aβ pathology, especially early on, leads to a synaptic signature similar to that after early stress exposure. Our data are publicly available and can be accessed online using a web app, via


Supplementary information and data availability

Our proteomics data can be accessed via the linked Shiny web app. Supplementary tables can be accessed via https://bit.ly/ES_synaptosome_supplemental. All other data will be made available upon request.
References


Early-life stress effects on wildtype and APP/PS1 hippocampal synaptic proteomes


Early-life stress effects on wildtype and APP/PS1 hippocampal synaptic proteomes


Figure S1. Overview of (top) GO pathways altered by APP/PS1 genotype and ES exposure at 4M (related to figs. 1, 2) (A-B) Top overrepresented gene ontology (GO) terms in Cohort 1 (A) and Cohort 2 (B) when comparing wildtype (WT) vs transgenic APP/PS1 (TG) synaptosomes. (A) Expression weighted celltype enrichment (EWCE) analysis in Cohort 1 shows upregulation of astrocytic proteins and downregulation of neuronal proteins TG vs WT synaptosomes. (B) EWCE analysis in WT-CTL vs TG-CTL synaptosomes from Cohort 2 reveal downregulation of neuronal proteins. (E-F) Top GO terms overrepresented in significantly upregulated (E) and downregulated (F) proteins in 4M WT-CTL vs WT-ES synaptosomes. All GO analyses performed based on biological processes (BP) and cellular components (CC). Italicized terms are ‘parent’ terms after clustering by semantic similarity using RRVGO. Size indicates number of differentially expressed proteins annotated to each GO term.
Figure S2. Other measures of mitochondrial morphology are unaffected in ES or TG synapses at 4M (related to fig. 3)
(A) Total number of mitochondria is not affected in the hippocampi of ES-exposed WT and TG mice. (B) Mitochondrial area in the dendritic (DEND), and astrocytic (ASTRO) compartments are not affected at 4M. (C-D) Mitochondrial perimeter (C) and circularity (D) in the PRE, post-synaptic (POST), DEND, and ASTRO compartments are not at 4M.
Figure S3. Overview of (top) overrepresented pathways altered by APP/PS1 genotype and ES exposure at 10M (related to figs. 4, 5).

(A) Top overrepresented gene ontology (GO) terms from downregulated proteins (light red) when comparing synaptosomes from 10M WT-CTL and TG-CTL mice. (B) SynGO analysis of downregulated proteins (light red) in 10M TG-CTL mice reveals alterations to pre-synaptic vesicle cycling and post-synaptic receptor endocytosis. (C) Top overrepresented gene ontology (GO) terms from up- (dark red) and downregulated (light red) synaptosomal proteins in ES-exposed WT mice. (D-E) Top overrepresented GO terms from upregulated (D, dark blue) and downregulated (E, light blue) proteins in synaptosomes from ES-exposed TG mice. All GO analyses performed based on biological processes (BP) and cellular components (CC). Italized terms are 'parent' terms after clustering by semantic similarity using RRVGO. Size indicates number of differentially expressed proteins annotated to each GO term.
Supplementary Tables

Supplementary tables can be accessed at: https://bit.ly/ES_synaptosome_supplemental

- Table S1 – Table of differentially expressed proteins between WT vs TG synaptosomes at 4M (Cohort 1)
- Table S2 – Table of differentially expressed proteins between WT-CTL vs TG-CTL synaptosomes at 4M (Cohort 2)
- Table S3 – Table of overrepresented gene ontology (GO) terms and reduced parent terms from differentially expressed proteins at 4M (Cohorts 1 and 2)
- Table S4 – Table of differentially expressed proteins between WT-CTL vs WT-ES synaptosomes at 4M (Cohort 2)
- Table S5 – Table of differentially expressed proteins between TG-CTL vs TG-ES synaptosomes at 4M (Cohort 2)
- Table S6 – Table of differentially expressed proteins between WT-ES vs TG-ES synaptosomes at 4M (Cohort 2)
- Table S7 – Table of shared differentially expressed proteins between the WT-CTL vs TG-CTL and WT-CTL vs WT-ES contrasts at 4M (Cohort 2)
- Table S8 – Table of overrepresented gene ontology (GO) terms and reduced parent terms from shared differentially expressed proteins between the WT-CTL vs TG-CTL and WT-CTL vs WT-ES contrasts at 4M (Cohort 2)
- Table S9 – Table of differentially expressed proteins between WT-CTL vs TG-CTL synaptosomes at 10M (Cohort 3)
- Table S10 – Table of overrepresented gene ontology (GO) terms and reduced parent terms from differentially expressed proteins at 10M (Cohort 3)
- Table S11 – Table of overrepresented SynGO-annotated ontology terms from downregulated proteins in WT-CTL vs TG-CTL synaptosomes at 10M (Cohort 3)
- Table S12 – Table of differentially expressed proteins between WT-CTL vs WT-ES synaptosomes at 10M (Cohort 3)
- Table S13 – Table of differentially expressed proteins between WT-ES vs TG-ES synaptosomes at 10M (Cohort 3)
- Table S14 – Table of differentially expressed proteins between TG-CTL vs TG-ES synaptosomes at 10M (Cohort 3)
- Table S15 – Table of Log2FC values and functional annotations of mitochondrial proteins in the WT-CTL vs TG-CTL and TG-CTL and TG-ES contrasts at 4M and 10M