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GFAP mutation and astrocyte dysfunction lead to a neurodegenerative profile with impaired synaptic plasticity and cognitive deficits in a rat model of Alexander disease

Abbreviated title: Neurodegeneration and cognitive deficits in AxD

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Abstract

Alexander disease (AxD) is a rare neurological disorder caused by dominant gain-of-function mutations in the gene for glial acidic fibrillary protein (*GFAP*). Expression of mutant protein results in astrocyte dysfunction that ultimately leads to developmental delay, failure to thrive, and intellectual and motor impairment. The disease is typically fatal, and at present there are no preventative or effective treatments. To gain a better understanding of the link between astrocyte dysfunction and behavioral deficits in AxD we recently developed a rat model that recapitulates many of the clinical features of the disease, including failure to thrive, motor impairment, and white matter deficits. In the present study, we show that both male and female AxD model rats exhibit a neurodegenerative profile with a progressive neuroinflammatory response combined with reduced expression of synaptic and mitochondrial proteins. Consistent with these results AxD rats show reduced hippocampal long-term potentiation and are cognitively impaired, as demonstrated by poor performance in the Barnes maze and novel object recognition tests. The AxD rat provides a novel model in which to investigate the impact of astrocyte pathology on central nervous system function and provides an essential platform for further development of effective treatments for AxD and potentially other neurodegenerative diseases with astrocyte pathology.

Significance Statement

Alexander disease (AxD) is a fatal neurodegenerative disorder caused by gain-of-function *GFAP* mutations. We recently developed a *Gfap*$^{+/R237H}$ rat model which demonstrates hallmark astrocyte pathology, myelin deficits, and motor impairment. Here, we show that *Gfap*$^{+/R237H}$ rats also exhibit reduced synaptic plasticity and cognitive deficits as additional clinically relevant phenotypes, further demonstrating its utility as a model. Hippocampal transcriptomic analysis in young adult animals reveals a neurodegenerative signature with an innate immune response and loss of synaptic and metabolic gene expression, features that are typically associated with chronic diseases of aging. These results reveal mechanisms by which astrocyte dysfunction leads to learning and memory deficits in AxD and perhaps contributes to other diseases such as Alzheimer's and Parkinson's.

Introduction

Alexander disease (AxD) is a progressive and generally fatal disorder of the central nervous system (CNS), with a range of clinical phenotypes including cognitive and motor impairments (Messing et al., 2012; Messing, 2019; Hagemann, 2022). In early onset cases, AxD is associated with prominent white matter deficits, especially in the frontal lobes (van der Knaap et al., 2001), leading to its original classification as a leukodystrophy, whereas later onset cases are associated with lesions of the posterior fossa (Prust et al., 2011). In all cases, the hallmark pathological feature is the presence of Rosenthal fibers, protein aggregates found within the cytoplasm of astrocytes, particularly in perivascular, subpial, and subependymal locations (Alexander, 1949). Over 90% of cases of AxD result from heterozygous missense mutations in the gene encoding the astrocyte intermediate filament, glial fibrillary acidic protein (GFAP) (Brenner et al., 2001). Since this discovery, AxD has become a model system in which to explore the consequences of primary astrocyte dysfunction in the CNS (Messing et al., 2012). Over the past few decades, the role of astrocytes in regulating synapse formation and activity in both development and disease has become an active area of research (Araque et al., 1999; Allen and Eroglu, 2017; Blanco-Suarez et al., 2017). Although deficits in the hippocampal pyramidal layer (CA1) and striatum have been demonstrated in some cases of AxD (Borrett and Becker, 1985), neuronal loss has not been widely reported (Sosunov et al., 2018).

Despite the initial description of AxD as an intellectual disability syndrome (Alexander, 1949), disturbances in cognitive function have received little attention. Generalizations about the prevalence of the cognitive phenotype in different Alexander disease sub-types are frequently stated in print, but it is not clear how often and the degree to which patients are cognitively impaired. Indeed, many questions remain unanswered, including which domains of cognitive function are affected, whether impairments localize to known sites of pathology, and whether

cognitive deficits, once characterized, might be useful as outcome measures in the testing of experimental treatments.

We recently developed a rat model of AxD which demonstrates myelin loss, clinically relevant motor phenotypes, and increased mortality, more closely recapitulating the human disease compared to earlier mouse models (Hagemann et al., 2006; Hagemann et al., 2021). Previous behavior testing in AxD model mice demonstrated deficits in learning and memory (Hagemann et al., 2013), but these deficits were subtle and strain specific. In this report, we take advantage of the rat model to investigate whether astrocytes compromised by GFAP mutation impair synaptic, neuronal, and cognitive function.

Materials and Methods

Rat model of AxD

The *Gfap*$^{+/R237H}$ rat model of AxD, hereafter referred to as R237H, was generated as previously described (Hagemann et al., 2021) and maintained as heterozygotes on a Sprague-Dawley genetic background (Charles River CD IGS rat). All animals were bred at the University of Wisconsin-Madison. For molecular, histological, and physiological analysis, animals were housed under specific pathogen free conditions in the AAALAC-accredited Waisman Center Rodent Models Core. Rats were co-housed as wild type and R237H pairs whenever possible under a 12-hour light cycle and fed ad libitum. For behavioral phenotyping, rats were bred at the University of Wisconsin-Madison (UW-Madison) and pregnant dams sent to the University of California Davis (UC Davis) M.I.N.D. Institute Intellectual and Developmental Disabilities Research Center (IDDRC). Animals housed at UC Davis were kept under the same conditions as those in the Waisman Center at UW-Madison (co-housed as wild type and R237H pairs

under a 12-hour light cycle and fed ad libitum). All animal studies were approved by the Animal Care and Use Committees at UC Davis or under the College of Letters and Sciences and Vice Chancellor Office for Research at UW-Madison.

RNA isolation and hippocampal transcriptomics

Male and female littermates of each genotype were sacrificed at 3 or 8 weeks of age by CO_2 asphyxiation (N = 4 per group, 32 samples total), and hippocampus collected immediately on ice and frozen (-80°C) before subsequent processing. Hippocampus included Ammon's horn, the dentate gyrus, and the subiculum. The alveus was used as a dorsal boundary to separate the hippocampus from subcortical white matter, and parahippocampal regions were removed from the ventral/lateral boundaries. Excess white matter from the fimbria and fornix were also removed. RNA was extracted with Trizol reagent per the manufacturer's protocol (Invitrogen, ThermoFisher Scientific), treated with TURBO DNase (TURBO DNA-free Kit, Ambion, ThermoFisher Scientific), and RNA integrity determined with an Agilent 4200 TapeStation System for quality control. Libraries were prepared from 1 µg total RNA with integrity numbers (RIN) between 8.4 and 9.3 for sequencing with TruSeq Stranded mRNA Sample Preparation kit (Illumina). Libraries were quantified with PicoGreen reagent (ThermoFisher Scientific) and assayed with an Agilent TapeStation System to confirm integrity before sequencing with an Illumina NovaSeq X Plus (2x150bp, 70M reads per sample, 2 lanes). Base calling was performed using Bcl2fastq (v2.20.0.422), read trimming with Skewer (Jiang et al., 2014), read alignment with STAR (Dobin et al., 2013), expression estimation with RSEM (Li and Dewey, 2011), and differential expression estimation with EdgeR (Robinson et al., 2010). Pathway enrichment analysis for differentially expressed genes [false discovery rate (FDR) < 0.01] was performed with g:Profiler (Raudvere et al., 2019) for Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000), and the

full gene list for each comparison over all known rat genes was used as the statistical domain. Morpheus (https://software.broadinstitute.org/morpheus) was used to generate heat maps. Library preparation, sequencing, and data analysis were performed by the Gene Expression Center (Research Resource Identifier - RRID:SCR_017757), the DNA Sequencing Facility (RRID:SCR_017759), and the Bioinformatics Core Facility (RRID:SCR_017799) respectively within the University of Wisconsin – Madison Biotechnology Center. Results were deposited in the Gene Expression Omnibus repository under accession number GSE278645.

Protein isolation and cytokine analysis

Hippocampal tissues were collected from male and female rats at 8 or 12 weeks of age as described for RNA extraction. For analysis of cytokines, tissues from 8-week-old rats were homogenized with a GenoGrinder bead mill in RIPA buffer (Pierce, ThermoFisher Scientific) with 1 mM Pefabloc SC (Sigma-Aldrich), and Complete Protease Inhibitor Cocktail (Roche, Sigma-Aldrich) at 150 mg tissue per ml. Samples were centrifuged at 20,000 g for 10 min at 4°C, supernatants collected, and protein quantified using the BCA assay (Pierce, ThermoFisher Scientific). Homogenates were assayed using the Meso Scale Discovery (MSD) Proinflammatory Panel 2 (rat) V-PLEX kit to quantify IL1β, IL4, IL5, IL6, IL10, IL13, IFNγ, TNFα, CXCL1 on a MESO QUICKPLEX SQ 120 multiplex cytokine plate reader within the Small Molecule Screening Facility at UW-Madison. For analysis of chemokines, tissues from 12-week-old rats were homogenized with a GenoGrinder bead mill in ProcartaPlex Cell Lysis Buffer (Invitrogen, ThermoFisher Scientific) with 1 mM Pefabloc SC, and Complete Protease Inhibitor Cocktail at 200 mg tissue per ml. Samples were centrifuged at 16,000 g for 10 min at 4°C, supernatants collected, and protein quantified using the BCA assay. Homogenates were assayed using a ProcartaPlex Rat Chemokine 8-plex Beads panel to quantify CXCL1, CXCL2, CXCL10, CCL2, CCL3, CCL7, and CCL11 on a Luminex MAGPIX Multiplex Analyzer plate

reader within the Vision Research Core at UW-Madison. CXCL1 was below the limit of detection for the Luminex assay and values from the MSD assay are reported.

Protein isolation and western analysis

Hippocampal tissues were collected from male and female rats at 8 weeks of age as described for RNA extraction above. For analysis of post-synaptic proteins, tissues were homogenized with a GenoGrinder bead mill in 2% SDS, 50 mM Tris-HCl (pH7.5), 5 mM EDTA, 1 mM Pefabloc SC (Sigma-Aldrich), and Complete Protease Inhibitor Cocktail (Roche, Sigma-Aldrich) at 50 mg tissue per ml, and protein quantified using the BCA assay (Pierce, ThermoFisher Scientific). Protein (5 µg/lane) was separated on 10% TGX Criterion gels (BioRad) and transferred to Immobilon-IF PVDF membrane. After transfer (30V 15 hrs, Criterion transfer system, 4°C), membranes were stained with REVERT Total Protein Stain and imaged on an Odyssey imager (LI-COR Biosciences) for quantification and normalization of proteins of interest. Membranes were then placed in SEA blocking buffer (Pierce, ThermoFisher Scientific) before proceeding to incubations with primary antibodies against PSD95 (Cell Signaling 3450) or GluA1 (Cell Signaling 13185) diluted 1:1000 in TBS with 0.05% Tween20 (TTBS) at 4°C overnight. Immunoblots were washed 3 times in TTBS, incubated with secondary antibody (1:10,000 IRDye-800CW conjugated goat-anti-rabbit, LI-COR 925-32211) at room temperature for 2 hours, washed again with TTBS followed by PBS, and allowed to dry before analysis with an Odyssey imager.

For vesicular presynaptic proteins and gephyrin, hippocampi were homogenized on ice in Syn-PER synaptic protein extraction reagent (Pierce, ThermoFisher Scientific) with 1 mM Pefabloc SC, and Complete Protease Inhibitor Cocktail at 10 mg/ml using a Potter-Elvehjem tissue homogenizer (900 rpm Teflon pestle in glass tube). Protein from the synaptic fraction (P2

per manufacturer's protocol) was quantified with the BCA assay and 1 µg loaded (without heating) on 10% separating gels and transferred to nitrocellulose at 100 V for 70 min. Membranes were blocked in TTBS with 5% BSA before proceeding to protein staining (as above) and incubating with primary antibodies against SV2A (Cell Signaling 66724), VGluT1 (Cell Signaling 47181), VGAT (Synaptic Systems 131 004), or gephyrin (Proteintech 12681-1-AP) diluted 1:1000 in TTBS overnight at 4°C. Immunoblots were washed 3 times in TTBS, incubated with peroxidase conjugated goat anti-mouse, rabbit, or guinea-pig secondary antibodies (Jackson ImmunoResearch Laboratories, 115-035-003, 111-035-003, 106-035-003, diluted 1:5000) at room temperature for 2 hours, washed again with TTBS before developing with Western Lightning Plus chemiluminescent substrate (Perkin Elmer) and imaging with an Azure 500 system.

For mitochondrial proteins, hippocampi were homogenized on ice in RIPA buffer (Pierce, ThermoFisher Scientific) with 1 mM Pefabloc SC, and Complete Protease Inhibitor Cocktail at 10 mg/ml using a Potter-Elvehjem tissue homogenizer (900 rpm). Homogenates were centrifuged at 12,000 g, protein from the supernatant quantified with the BCA assay, and 8 µg loaded (without heating) on a 12% separating gel and transferred to PVDF in Bjerrum & Schafer-Nielsen transfer buffer at 100 V for 70 min. Immunolabeling was performed using the Total OXPHOS Rodent Western Blot Antibody Cocktail (Abcam ab110413) as described for synaptic proteins.

Immunolabeling and stereology

Animals were deeply anesthetized with isoflurane and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in paraformaldehyde before cryoprotecting in 30% sucrose and collecting 40 µm coronal sections on a sliding

microtome. For stereology, every 6th section (240 µm interval) starting at the rostral extent of the hippocampus (approximately -1.4 mm from bregma) was immunolabeled for NeuN to count neuronal nuclei in the pyramidal layers and the granule cell layer of the dentate gyrus. Pyramidal cell nuclei in CA1 and CA3 and dentate granule cell nuclei were outlined separately using Stereo Investigator (MBF Bioscience) for both hippocampi for a total of 6 contours per section when all regions were present. Cell counting was performed in both hemispheres on sections anterior to the posterior commissure (approximately -4.5 bregma). For rats at 8 weeks of age, the sampling grid was 180 x 180 µm, the counting frame was 20 x 20 µm, and the disector height was 25 µm. For rats at 3 weeks, the sampling grid was 150 x 150 µm, the counting frame was 20 x 20 µm, and the disector height was 20 µm. Even numbers of males and females were analyzed in both age groups (N=3) and data combined for the final analysis after confirming there was no significant effect of sex. The Gundersen coefficient was less than 0.1 (m=1) for all samples, and the total cell population was estimated for the dorsal hippocampus using the mean section thickness.

Hippocampal long-term potentiation

Slice preparation: Coronal hippocampal slices (400 µm) were prepared from 16-week-old rats. The rats were deeply anesthetized using isoflurane and decapitated. The brain was removed from the skull within 60 seconds of decapitation and immediately placed in a slice preparation solution containing 124 mM NaCl, 1.25 mM NaH_2PO_4, 3 mM KCl, 25 mM $NaHCO_3$, 10 mM glucose, 1 mM sodium ascorbate, 3 mM kynurenic acid, 3.6 mM $MgSO_4$ and 0.8 mM $CaCl_2$. and saturated with carbogen (95% O_2/5% CO_2). Brain slices were cut with a vibratome (Model 7000 smz2, Campden Instruments, Loughborough, UK) and transferred into a submerged incubation chamber containing slice recovery solution of 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 15 mM glucose, 0.8 mM sodium ascorbate, 1.3 mM $MgSO_4$, and 2.5 mM $CaCl_2$.

Slices were recovered at 33°C for 30 minutes followed by a room temperature recovery for 60 minutes. Both the preparation and recovery solutions were buffered to pH 7.3 with an osmolality between 294–297 osmol/kgH_2O.

Long-Term Potentiation (LTP): Recordings were taken from coronal slices in a submersion recording chamber perfused with the carbogenated recovery solution at 3.0 mL/min at 30°C using fire-polished borosilicate glass recording pipettes filled with 1 M NaCl (3–5 MΩ) and evoked responses were generated using Pt/Ir concentric bipolar stimulating electrodes. WinLTP software (v2.3, Bristol University) was used for stimulation and recording. LTP was induced using three theta burst trains consisting of 40 stimuli grouped into 10 bursts of 4 stimuli each at 100 Hz with burst delivered at 5 Hz. Potentiation was defined as the mean field excitatory postsynaptic potential (fEPSP) slope during the last 10 minutes of the 60-minute LTP recording compared to the average fEPSP slope during the 10-minute period preceding the theta burst stimulus.

Novel object recognition

A total of 6 litters were used to generate 2 cohorts of R237H and wild type littermates (N=24 with 12 males and 12 females per genotype) for the novel object recognition (NOR) test. Animals were tested at 16 weeks of age. Testing took place in a black Plexiglas arena (60 x 60 x 50 cm) over two consecutive days. On day 1, rats were placed in an empty arena for 30 min to habituate them to the apparatus, and then returned to their home cage. On day 2, the familiarization phase, they were returned to the empty arena and allowed another 30 min of habituation. Two identical objects were then placed into the arena with the animals, and they were allowed 10 minutes to freely explore the two objects. Following exploration of the now familiar objects, rats were removed from the arena and isolated in Plexiglas holding cages for at least 60 minutes. One of the two objects was then replaced with a novel object. Rats were returned to the testing arena and allowed 5 minutes to freely explore the familiar and novel objects. Time spent exploring the objects was determined and used to generate a discrimination

index (DI, time exploring novel object/total time exploring both novel and familiar objects) as a measure of novel object recognition and memory for previously seen objects (Ennaceur and Delacour, 1988; Winters et al., 2008; Kinnavane et al., 2015). A glass jar and plastic cone were used as the stimulus objects. Right/left object position and designation of jar/cone as novel/familiar objects were counter-balanced across experimental groups. All trials were tracked using EthoVision software (EthoVision v.13.0, Noldus Information Technology, Netherlands) and time with the familiar and novel object was hand-scored by the investigator.

Barnes maze

A total of 13 litters were used to generate 2 cohorts of R237H (N=24; 11 males, 13 females) and wild type (N=30; 14 males, 16 females) littermates for the Barnes maze test (separate cohorts from those tested for NOR). Animals were tested at 16 weeks of age. The Barnes maze consists of a circular black Plexiglas test arena (170 cm in diameter) with 20 circular holes (12 cm in diameter) around the perimeter. One hole is designated as the escape hole and has a dark goal box underneath which the rat can enter. Rats were tested for a total of 6 days, with one habituation day and 5 training days. The maze was surrounded by black curtains and visual cues (e.g., white square, circle, triangle, and “N” shape) were attached to the curtain and present throughout training. On habituation day, the rats were placed on the arena for 10 minutes with ambient light (30 lux), with no curtain cues or goal box present. They were then returned to their home cage. The following day, rats were placed under a black Plexiglas box (approximately 20 x 20 x 15 cm) in the center of the arena for 60 seconds. Bright lights of approximately 1,000 lux and approximately 60 dB of white noise were then turned on. After 60 seconds, the rat was released from the box and was given 90 seconds to find the escape hole. If the rat did not find the escape hole, the investigator led the rat to the hole. In both cases, lights and sound were turned off when the rat entered the escape hole. The rat then remained in the escape hole for 15 seconds before starting the next trial. Each rat had 4 trials per day over

the 5-day testing period. During each trial, the investigator recorded the latency to reach the escape hole (whether or not they escaped), total distance traveled, and search strategy used on the maze (random, serial, or direct) as described previously (Fedor et al., 2010; Pitts, 2018). Briefly, spatial strategies included a direct (the rat goes directly to the escape hole with no incorrect holes chosen) or serial search around the perimeter of the maze in either the clockwise or counterclockwise direction visiting 2 or more holes. All other patterns of movement on the maze were classified as random. Latency to locate the escape hole and spatial search strategy were analyzed.

Statistical analyses

Data resulting from molecular, histological, and physiological analyses were analyzed using GraphPad Prism. For data sets with a single variable, an unpaired 2-tailed *t*-test was used to determine significance. For data sets with 2 variables, a 2-way ANOVA was used to determine significance. All data in graphs represent means ± standard deviation.

Behavioral data were analyzed using SPSS statistical package (IBM SPSS Statistics, Version 28.0.0.0.). For the NOR test, time spent exploring the familiar and novel objects were analyzed by a two-way repeated measures ANOVA. A discrimination index (DI) calculated as the ratio of time spent exploring the novel object divided by the total time spent exploring the novel and familiar objects (i.e., novel/novel + familiar). Using this index, a lack of preference for novel vs familiar objects yields a DI of 0.5. A one sample t-test was used to determine whether the DI differed significantly from 0.5 and was used as an index of the strength of recognition memory (Brown and Aggleton, 2001). The DI corresponds to d', one of the primary measures of recognition memory in humans (Sivakumaran et al., 2018). Latency to find the escape hole in the Barnes maze was analyzed by a mixed effects model with Šídák's multiple comparisons

post-test. Search strategy (direct, serial, and random, as described in Barnes maze methods above) used to locate the escape hole was analyzed by Chi square.

Data were examined for missing values and outliers. Outliers were identified from z-transformed data as those exceeding ± 3.29. Missing data and outliers were replaced by multiple imputation (SPSS). Levene's test for homogeneity of variance was carried out and variables violating assumptions of homogeneity were analyzed via the Mann-Whitney U non-parametric statistic. All tests were 2-tailed with the minimum probability to establish statistical significance set at $p<0.05$. All data in graphs represent means ± standard error of the mean ($\overline{X}\pm$ SE). The minimum probability for statistical significance was set at $p<0.05$. Experimenters were blinded to genotype throughout behavioral testing, although the smaller size of R237H versus WT rats was readily apparent from approximately 5 weeks of age and this difference continued through the experiments (see Figure 1F, (Hagemann et al., 2021).

Results

Neurodegenerative profile of hippocampal transcripts in R237H rat model of AxD

The R237H rat model of AxD exhibits extensive GFAP accumulation and aggregation throughout the CNS, with a particularly high pathological burden in hippocampus (Hagemann et al., 2021). Given the role of hippocampus in learning and memory, we wanted to understand the impact of reactive glia and astrocyte dysfunction on neurons and cognitive function and began with bulk RNA sequencing for differential gene expression analysis. To distinguish initiating events from chronic disease, we collected hippocampus at 3 weeks of age (P21), when CNS pathology is apparent but R237H rats are otherwise presymptomatic, and at 8 weeks of age (P56), when pathology is significantly worse and the rats are severely affected with motor impairment and increased mortality. Both male and female R237H rats were analyzed with wild

type littermate controls, and gene expression comparisons were made between genotypes within age groups. In both age groups, elevated transcripts in the R237H rat hippocampus (FDR < 0.01) were dominated by immune related genes which demonstrated the largest fold changes. Those that were decreased encoded proteins related to synaptic and metabolic function (Fig. 1A, P56 males shown; Extended Data Fig. 1-1, 1-2, 1-3, and 1-4). Given the large number of genes that were affected (Fig. 1B), those that were upregulated were analyzed for pathway enrichment separately from genes that were downregulated (2747 increased, 1907 decreased, FDR < 0.01 in P56 male R237H rats). Enrichment results for gene ontology (GO) terms were filtered using a two-stage algorithm within g:Profiler to group significant terms into sub-ontologies and identify functional drivers within these contexts (Raudvere et al., 2019). At 8 weeks of age when disease is most severe, molecular functions of genes with increased expression were related to cytokine and immune receptor binding and activity, integrin binding, and downstream effects such as protein kinase binding (Fig 1C, males shown; Extended Data Fig. 1-1 and 1-2). Enriched biological processes again included immune system components and integrin mediated signaling pathways as related drivers. Actions involved in reactive gliosis, including cytoskeletal and extracellular matrix reorganization (e.g., actin filament and supramolecular fiber organization) and pathways related to cell cycle, were also highlighted as driving processes. Enriched cellular components included lytic vacuoles (lysosomes), vesicles (including endosomes) and cell adhesion terms such as focal adhesions, integrins, and associated signaling molecules (Fig 1C; Extended Data Fig. 1-1 and 1-2).

KEGG pathway enrichment analysis demonstrated activation of NFκB and JAK-STAT signaling (Fig 1C), consistent with our previous report showing both NFκB and STAT3 are activated in R237H rat hippocampus (Hagemann et al., 2021). Transcripts related to cellular senescence and p53 signaling, including *Cdnk1a* (p21WAF), cyclins and checkpoint kinases, GADD45 genes, and Tp53 itself, were also significantly enriched, corroborating our recent work showing astrocyte senescence in the rat as well as other models of AxD (Wang et al., 2022).

Pathways associated with an innate immune response were highly enriched, and a response to danger/pathogen associated molecular patterns (DAMPs and PAMPs) through NOD-like, Toll-like, and C-type lectin receptor signaling, cytosolic DNA-sensing, and RIG-1-like receptor signaling could further contribute to astrocyte senescence and the associated secretory phenotype (SASP) (Li and Chen, 2018). It is noteworthy that lymphocyte-related pathways are enriched, and although these pathways overlap with other immune-related categories, T-cell infiltration has been documented in AxD (Olabarria et al., 2015; Boyd et al., 2021). Comparisons between age groups showed that a majority of elevated transcripts were increased at both 3- and 8-weeks, with enrichment for most of the same immune related pathways in each group (Fig. 1D,E). A heat map of significantly increased innate immune related transcripts shows a subset of genes with comparable increases at both presymptomatic (P21) and severe stages (P56) while other transcripts demonstrate a progressive increase as the animals age (Fig. 1D; Extended Data Fig. 1-1, 1-2, 1-3, and 1-4). Many of the increased transcripts with an FDR < 0.01 at P21 and not at P56 (425 transcripts, Fig. 1B) were elevated at P56 when an FDR < 0.05 was considered (97/425). Of the 425 unique transcripts elevated at P21, 101 were down regulated in R237H rats at P56 compared to R237H rats at P21; however, there were no common pathways associated with these genes.

Transcripts which were decreased in the R237H rat consisted of genes related to synapses and metabolism and depicted a neurodegenerative profile (Fig. 1A). GO term enrichment analysis for molecular function at 8 weeks of age showed decreased expression of inorganic cation transmembrane transporters (proton, metal ion, and potassium transporters), channel regulators, and proteins involved in glutamate receptor binding. Synaptic signaling and synaptic organization drove biological processes, with all terms relating to synaptic function including G protein-coupled receptor signaling, monoatomic ion transport (potassium, calcium, metal ion transport), and aerobic respiration (mitochondrial function). Enriched cellular components also consisted of synapse related terms which included mitochondrial membranes

and the respiratory chain complex. Many of these changes were not yet apparent at 3 weeks of age in asymptomatic rats (Fig. 1D,E; Extended Data Fig. 1-3, 1-4). Enriched GO terms in rats at P21 included monoatomic ion transport and nervous system development as drivers of molecular function and biological process respectively, but only cellular component terms showed a significant enrichment of synapse related compartments (Fig. 1D,E; Extended Data Fig. 1-3, 1-4).

KEGG pathway enrichment analysis for transcripts that were decreased at 8 weeks of age in R237H rat hippocampus again highlighted a neurodegenerative profile and included "pathways of neurodegeneration – multiple diseases", as well as Alzheimer, Parkinson, and Huntington disease, and amyotrophic lateral sclerosis (ALS). Multiple synaptic profiles, including those for glutamatergic, GABAergic, cholinergic, dopaminergic, and serotonergic synapses, showed decreased expression, as predicted by GO term analysis. Transcripts related to oxidative phosphorylation and mitochondrial function were highly enriched among downregulated genes, and genes related to calcium and cyclic AMP signaling also showed decreased expression (Fig. 1C). At 3 weeks of age, pathways of neurodegeneration were not significantly enriched in R237H rats. Comparisons of expression changes for synapse related genes (GO:0045202) between age groups demonstrated expected developmental changes (within both genotypes) but a general trend for decreases in AxD model rats at both presymptomatic and severe disease stages (Fig. 1D,E). A higher number of genes related to mitochondrial function were downregulated at 8 weeks compared to 3 weeks, accounting for the shift to a neurodegenerative profile at the later age (Fig. 1D,E). Genes encoded by mitochondrial DNA (mtDNA) were not downregulated at P21, but transcripts from all 13 protein encoding genes were decreased at P56 as well as many nuclear genes related to oxidative phosphorylation and respiration, suggesting an overall reduction of mitochondrial function at this severe stage of disease (Fig. 1D).

Neuroinflammatory response to GFAP mutation in AxD model rats

We have previously demonstrated activation of NFκB and STAT3 in R237H rat hippocampus, and specifically nuclear localization of phosphorylated STAT3 (Tyr705) in astrocytes (Hagemann et al., 2021). Here, to determine whether elevation of immune related transcripts translated into a neuroinflammatory microenvironment, we quantified cytokines and small chemokines in adult rat hippocampus by ELISA (Fig. 2A). In agreement with STAT3 activation and our previous report showing a senescence phenotype in AxD astrocytes (Wang et al., 2022), IL6 was markedly elevated (24-fold, $p < 0.0001$), and TNFα was also significantly increased (2-fold, $p < 0.0001$), similar to their transcription profile (Fig. 2B). Although IL1β transcript is elevated, protein was slightly decreased, and many of the other interleukins analyzed were near the lower limits of detection and only marginally elevated, including anti-inflammatory cytokines IL10 and IL4. It is possible that the assay used may not detect pro-IL1β or oxidized/reduced forms (per the manufacturer) or that heterogeneous nuclear ribonucleoproteins may affect translation (Sirenko et al., 2002; Zhao et al., 2012). Many small chemokines were highly elevated, including CXCL1 and CCL2, suggesting potential chemotactic effects on peripheral immune cells. CXCL10, an IFNγ inducible chemokine, is also markedly elevated, although IFNγ is only marginally increased.

Regional differences in hippocampal neuron numbers

The decrease in synapse related gene expression observed in the R237H rat hippocampus (Fig. 1C) could reflect neuronal loss, and we have previously reported that granule cells in the hippocampal dentate gyrus are reduced in number (Hagemann et al., 2021). To analyze both the dentate gyrus and pyramidal cell layers, we counted pyramidal cells in CA1 and CA3 and granule cells in the dentate gyrus of the dorsal hippocampus using stereological methods at 3 and 8 weeks of age. At 3 weeks of age, there were no differences in the regions analyzed; however, a reduction in granule cell numbers was confirmed in the dentate gyrus at 8 weeks of

age (Fig. 3). This is of particular interest in view of the involvement of the dentate gyrus in the formation of precise memories for locations of objects (Lee and Jung, 2017). No change in pyramidal cell numbers were detected in CA1 or CA3 at 8 weeks (Fig. 3).

Neurodegenerative changes in synaptic and mitochondrial proteins in R237H rat hippocampus

To further assess neuronal pathology and potentially synapse function, we quantified expression of synapse related proteins in hippocampus from R237H rats at 8 weeks of age. An initial assessment by western analysis showed decreases in both pre- and post-synaptic proteins including SV2A (synaptic vesicle glycoprotein 2A) and PSD95 (postsynaptic density protein 95) respectively (Fig. 4A,B). More specific analysis of excitatory synaptic markers VGluT1 (vesicular glutamate transporter 1) and GluA1 (AMPA glutamate receptor subunit A1) also demonstrated decreases at the protein level (Fig. 4C,D). Inhibitory pre- and post-synaptic markers VGAT (vesicular GABA transporter) and gephyrin were also reduced (Fig. 4E,F). In addition, we analyzed a panel of mitochondrial proteins related to oxidative phosphorylation (Fig. 5) from complex I through V (NDUFB8, SDHB, UQCRC2, MT-CO1, ATP5A). All but complex V (ATP5A) showed a decrease in R237H rat hippocampus at 8 weeks of age, including the mitochondrially encoded cytochrome C oxidase I (complex III and IV).

Deficits in hippocampal long-term potentiation in R237H rats

To determine whether differences in expression of synaptic proteins translated into physiological deficits, we analyzed R237H rat hippocampus for long-term potentiation (LTP) in Schaffer collateral-CA1 synapses. To match the age at which we planned to test cognitive function, LTP was measured at 16 weeks, when R237H rats show improved motor performance and are past the critical survival period between 6-12 weeks of age when 15% of the animals die. Acute

coronal slices including the dorsal hippocampus were collected from both male and female rats of each genotype and subjected to a theta-burst stimulation (TBS) paradigm with three trains of 10 bursts (4x100Hz). The field excitatory postsynaptic potential (fEPSP) was measured for 10 minutes before and one hour after TBS. Potentiation for each slice was defined as the mean fEPSP slope during the final 10 minutes compared to baseline (Fig. 6A). There was significantly less potentiation ($p = 0.0008$, *t*-test) for R237H rats versus wild type rats, confirming impaired synaptic plasticity at this age in the model (Fig. 6B).

Deficits in novel object recognition in R237H rats

The novel object recognition (NOR) task was used as an initial test to evaluate recognition memory in the R237H rat. This test is based on the tendency of rats to spend more time exploring novel objects than exploring familiar objects (Ennaceur and Delacour, 1988). Current evidence supports a critical role for the perirhinal cortex and its projections to the hippocampus, including the dentate gyrus, in object memory encoding, consolidation, and retrieval (Brown and Aggleton, 2001; Winters et al., 2008; Kinnavane et al., 2015). R237H rats and littermate controls were tested at 16 weeks of age. Wild type rats showed longer exploration of the novel object compared to the familiar object with a significant effect of genotype (two-way RM ANOVA, $p = 0.0001$), while R237H rats did not show a preference (Fig. 7A, data from one female R237H rat was excluded from analysis due to a lack of exploratory activity during the testing phase). A discrimination index (DI) representing the time exploring the novel object as a fraction of the total time exploring both objects was calculated as an additional measure of NOR performance. DIs for recognition memory performance in male (*$p = 0.018$) and female wild type (**$p = 0.006$) rats were significantly greater than 0.5 (i.e., chance) indicating memory for previously presented objects (Fig. 7B). In contrast, DIs were not significantly above chance for either male or female R237H rats.

Impaired Barnes maze performance in R237H rats

The Barnes maze is a behavioral test of spatial memory (Barnes, 1979). It shares similarities with the Morris water maze (Morris, 1984) in that it requires an animal to use distal environmental cues to locate a fixed escape location and has the advantage of not requiring stress associated with swimming. The goal of this task is for the rats to learn to seek shelter from bright lights by finding a dark escape hole. Male and female R237H rats and littermate controls were tested with 4 trials per day over 5 consecutive days at 16 weeks of age, and data were analyzed separately for each day as well as the average daily score across the four daily trials using a day-by-genotype-by-sex repeated measures ANOVA. Data for trial 1 on each test day was used to assess spatial reference in the Barnes maze and proved to be the most reliable as rats tended to perform poorly over repeated trials each day once they had located the escape hole in trial 1. Figure 8A shows the average latency (seconds) to find the escape hole in trial 1 on each of the 5 testing days. As expected, there was a significant overall decrease in latency to find the escape hole ($F_{4,188}$ =9.80, $p<0.001$) across test days. There was also a significant difference between genotypes ($F1,47=4.74$, $p<0.05$). Sex differences and interactions were not statistically significant. Comparisons of daily escape latencies between wild type and R237H rats showed that wild type rats had shorter escape latencies on test day 5 compared to R237H rats ($F1,52=4.22$, $p<0.05$). Escape latencies did not differ on test days 1-4. The three nominal search strategies used by the rats (random, serial, or direct) were also analyzed for Trial 1 on each test day, as well as the average percent use of each strategy over the four trials on each test day. As shown in Figure 8B, rats begin the task by using mainly a random search strategy but shift to serial, and then a direct spatial strategy over the course of training. By test days 4 and 5, wild type rats were using a search pattern that differed significantly by Chi square analysis from R237H rats ($p<0.05$ and $p<0.01$, respectively) in that fewer wild type rats were using a random search strategy, and more were using a direct spatial

strategy. The same general pattern was found when all four daily trials were included in a Chi square analysis (Extended Data Fig. 8-1).

Discussion

Cognitive delays and deficits are frequent clinical features of Alexander disease, particularly in early onset cases, but relatively few publications provide detailed information about the cognitive phenotype. Most studies are single case reports, presenting evidence for cognitive decline using a variety of tests including unspecified IQ tests, Wechsler Intelligence scales for adults and children, and tests for dementia. More recent studies have provided additional information about cognitive function, including impaired visual and verbal memory, using different assessment tools. For example, memory problems were reported in 3 subjects with adult onset AxD, including impairments in short-term memory and tests of object recall (Anis et al., 2023). In another case study, 2 of 3 adult onset AxD patients showed impaired verbal and visual memory in the Montreal Cognitive Assessment (MoCA) test (Lichtenstein et al., 2017). In a study of four children with AxD, ages 5-15, verbal short-term memory, backwards verbal short-term memory and narrative microstructure were impaired, along with word recall (Zampini et al., 2023). Additional case studies of patients with infantile onset support the conclusion that cognitive impairments, and memory deficits in particular, are common features of AxD (Draghi et al., 2019; Kirsch et al., 2021), but a better understanding of the cognitive phenotype and its underlying mechanism is necessary for the development and testing of potential therapeutics to address this aspect of the disease.

Memory processes involve interactions among widespread regions of the brain, including prefrontal cortex, amygdala, striatum, and hippocampus among others (White and McDonald, 2002; Kesner, 2009), and astrocyte pathology throughout the CNS is likely to contribute to memory impairment found in AxD (Li et al., 2025). In this report, we sought to take

advantage of the R237H rat model of AxD to determine the impact of reactive and dysfunctional astrocytes on hippocampal neurons, memory, and learning. Transcriptomic analysis of hippocampal gene expression indicated early activation of stress related pathways in AxD model rats prior to onset of overt clinical phenotypes. Pathways related to immune processes were activated at 3 weeks of age and then intensified as disease progressed, similar to our previous transcriptomic studies in an early mouse model of AxD (Hagemann et al., 2005). Genes related to metabolism, particularly those involved in lipid and cholesterol biosynthesis, were down regulated at this early stage, and a subset of synaptic and neurodevelopmental transcripts were also decreased. The decline in synapse related gene expression became more pronounced with disease progression with multiple neuronal subtypes being implicated, and pathways involved in oxidative phosphorylation and mitochondrial function were also decreased, depicting a general profile of neurodegeneration. Given the essential role of astrocytes in maintaining CNS homeostasis and that AxD is a primary disorder of astrocytes, these results suggest that astrocyte dysfunction may also be a driving factor in other neurodegenerative diseases.

We recently reported that a subpopulation of astrocytes in AxD display a senescent phenotype, as demonstrated in multiple models, including the rat, and in patients with the disease (Wang et al., 2022). In addition, we have shown that gene expression profiles from our mouse models of AxD are highly congruent with a transcriptomic portrait of human Alzheimer's disease, second only to the Alzheimer's APP/PSEN1 mouse in comparisons with large-scale gene expression data from over 500 animal models (Gammie et al., 2024). These results further suggest that chronic gliosis and astrocyte dysfunction contribute to cognitive impairment in both Alzheimer's disease and AxD, and early reports described AxD as a developmental form of Alzheimer's based on the marked oxidative stress response (Castellani et al., 1999). Here we find overlap between these two conditions in both the stress response and in the loss of

markers for synaptic and mitochondrial function. Whether metabolic changes occur in neurons, astrocytes, or multiple cell types is not clear, but studies with induced pluripotent stem cell derived astrocytes from patients with AxD also demonstrate shifts in glycolysis and oxidative phosphorylation (Jones et al., 2018). Intermediate filaments have direct and indirect interactions with mitochondria (Schwarz and Leube, 2016), and GFAP mutations have been linked to lipoxidation and oxidative stress (Viedma-Poyatos et al., 2022). Mitochondrial dysfunction may also contribute to a cell-intrinsic innate immune response (Banoth and Cassel, 2018; Newman and Shadel, 2023), and future studies on the role of lipid metabolism and mitochondrial function will be important in understanding how astrocytes contribute to the neurodegenerative profile (Cali et al., 2024).

Immune-related responses are prominent at an early age in rodent models of AxD (Hagemann et al., 2005; Olabarria et al., 2015), and although neuroinflammation precedes severe cognitive deficits in neurodegenerative disorders of aging such as Alzheimer's disease, effects on mild cognitive impairment in early stages of disease are not clear (Heneka et al., 2015). Microglia play a pivotal role in recognizing amyloid β as a DAMP and triggering innate immune signaling, and although reactive astrocytes contribute to the inflammatory milieu, genetic risk factors such as TREM2 and CD33 variants implicate microglia as key players in Alzheimer's disease. In AxD, the primary insult is in astrocytes, but many of the same genes and pathways are activated in both diseases. Astrocyte specific transcription profiling in AxD model mice at postnatal day 14, an early stage in GFAP accumulation and aggregation, shows a similar elevation in innate immune related transcripts (Gammie et al., 2024), and our recent study analyzing the role of STAT3 in GFAP elevation and the astrocyte stress response further indicates that the immune response is initiated in astrocytes (Hagemann et al., 2023). Nevertheless, microglia are reactive and in close association with astrocytes in AxD (Olabarria et al., 2015; Hagemann et al., 2021), and innate immune pathways are likely activated in both

591 cell types. Future studies will include single cell analysis to follow the trajectory of astrocyte
592 pathology within different subtypes and non-cell-autonomous effects on microglia, neurons, and
593 oligodendrocytes over time (Srinivasan et al., 2016; Mathys et al., 2019; Habib et al., 2020;
594 Liddelow et al., 2020; Allen et al., 2022; Sadick et al., 2022; Mathys et al., 2024; O'Dea and
595 Hasel, 2024).

596 The importance of the hippocampus for memory is well established (Scoville and Milner,
597 1957; Squire and Zola-Morgan, 1991), but the specific contributions of hippocampal subregions
598 (e.g., dentate gyrus, CA1, CA3) and adjacent structures (e.g., perirhinal, entorhinal cortex) to
599 explicit aspects of memory are still not well understood (Hainmueller and Bartos, 2020). To
600 assess whether reduced expression of synaptic markers reflect neuronal loss in R237H rats, we
601 quantified granule cells in the dentate gyrus and pyramidal cells in CA1 and CA3 and found no
602 change at 3 weeks but reduced numbers of granule cells at 8 weeks of age. We have previously
603 shown that AxD model mice and rats exhibit deficits in adult neurogenesis, with virtually no
604 detectable doublecortin expressing neurons (Hagemann et al., 2013; Hagemann et al., 2021),
605 which may contribute to the differences in cell number observed in the dentate gyrus. Further
606 analysis of synaptic proteins demonstrated a decrease in pre- and post-synaptic markers for
607 both excitatory and inhibitory synapses, suggesting that neuronal deficits are not selective and
608 supporting our transcriptomic analysis showing downregulation of several neurotransmitter
609 signaling pathways including those for glutamatergic and GABAergic synapses. Importantly, we
610 demonstrate that neuronal deficits translate into functional deficits, both physiologically with
611 reduced hippocampal long-term potentiation and synaptic plasticity, and behaviorally with
612 impaired performance in tests of learning and memory. These findings contrast with earlier work
613 showing increased hippocampal long-term potentiation and plasticity in GFAP-null mice (McCall
614 et al., 1996), and further demonstrate the toxic gain-of-function caused by GFAP mutation.

Given the many differences we observed in immune factors, metabolic pathways, and synaptic proteins, and their potential effects on neurodevelopment, synaptic plasticity, and cognitive functions, we cannot attribute a single cause for impaired LTP and cognitive deficits in the R237H rat (Li et al., 2025). Astrocytes are intricately associated with synapses and respond to many of the same neurotransmitters via G-protein coupled receptor activation and intracellular calcium mobilization (Durkee and Araque, 2019), and previous studies have shown that Ca^{2+} dependent astrocyte release of D-serine is necessary for NMDA receptor activation and LTP induction (Henneberger et al., 2010; Adamsky et al., 2018; Ahrens et al., 2024). A recent report further demonstrates activation of c-Fos and increased Ca^{2+} activity in hippocampal astrocytes associated with engram neurons during contextual fear conditioning, and astrocyte-specific knockout of c-Fos reduces Ca^{2+} activity, impairs learning, and diminishes hippocampal LTP (Williamson et al., 2025). Astrocytes in mouse models of AxD (Saito et al., 2018) and induced pluripotent stem cell derived astrocytes from patients with the disease (Jones et al., 2018) demonstrate aberrant Ca^{2+} waves, and the transcriptomic analysis reported here shows reduced expression of genes related to calcium signaling. The marked reduction in LTP in the R237H rat model provides an opportunity for future studies to determine the degree to which loss of normal astrocyte function contributes to reduced synaptic plasticity in AxD.

Several overlapping memory processes have been proposed for the dentate gyrus, including novelty detection (Hunsaker et al., 2008), recognition memory (Jiang et al., 2023), pattern separation (Gilbert et al., 2001), pattern completion (Nakashiba et al., 2012), binding of information to spatial contexts (Lee and Jung, 2017), and working memory (Sasaki et al., 2018). Given the crucial role of the hippocampus in the formation and recall of memories for objects, places, and events (Lisman et al., 2017), abnormalities in the dentate gyrus likely contribute to deficits in both novel object recognition and the Barnes maze, as observed in AxD model rats. Computational modeling suggests that dentate granule cells perform pattern-separation on

spatial representations arriving from the entorhinal cortex (Yassa and Stark, 2011; Kesner and Rolls, 2015), and recent optogenetic studies show that the perforant pathway, and more specifically projections from the medial entorhinal cortex layer II to the dentate, are an essential memory circuit for visually guided navigation tasks like the Barnes maze (Qin et al., 2018). Given the role of the perforant pathway in memory impairment in Alzheimer's disease (Hyman et al., 1987; Harris et al., 2010), astrocyte pathology and neuronal deficits in the entorhinal cortex may also be worth future investigation in AxD.

In this report, we show that in addition to motor and myelin deficits (Hagemann et al., 2021), the rat model of AxD demonstrates neuronal and cognitive impairment as clinically relevant phenotypes. Future studies can take advantage of the model to better understand astrocyte dysfunction in AxD and neurodegenerative disease more generally.

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Figure legends

Figure 1. Neurodegenerative profile of hippocampal transcripts in R237H rat model of AxD. (**A**) Volcano plot showing fold change (log_2FC) values versus the false discovery rate (log_{10}FDR) for differentially expressed genes (DEG) in 8-week-old male R237H rats compared to wild type (FDR < 0.01.) Genes related to the immune system process (GO:0002376) are highlighted in red, and those related to synapses (GO:0045202) are in blue. (**B**) Venn diagrams showing overlap in the number of DEG (increased and decreased) at P21 and P56. (**C**) Enrichment analysis for gene ontology (GO) terms and KEGG pathways for DEG (FDR < 0.01) in male rats at 8 weeks of age (N=4). For pathways with a -log_{10} adjusted P value greater than 20, values are indicated within the graph. (**D**) Venn diagrams showing overlap in the number of DEG related to different GO terms: Immune system process (GO:0002376), Innate immune response (GO:0045087), Synapse (GO:0045202), and Mitochondrion (GO:0005739) at P21 and P56. (**E**) Heat maps for DEG between wild type (WT) versus R237H (RH) rats within male (M) or female (F) groups, at P21 or P56 matched to different GO term gene lists as indicated. Average TPM (transcripts per million) values were transformed to the color-coded minimum and maximum scale for each gene and K-means clustering used to highlight different patterns in developmental expression versus genotype related changes. For each group, N=4, except for WT females at P21, N=3; and R237H females at P21, N=5. Extended Data Files 1-1, 1-2, 1-3, and 1-4 support this figure.

Figure 2. Neuroinflammatory response to GFAP mutation in AxD model rats. (**A**) Cytokines and chemokines were quantified by ELISA in adult R237H rats and WT littermate controls. Interleukins, IFNγ, TNFα, and CXCL1 were analyzed at 8 weeks, and the remaining chemokines were analyzed at 12 weeks of age. Data were analyzed with a 2-tailed unpaired *t*-test with Welch's correction, *p < 0.05, **P < 0.01, ****p < 0.0001, N = 5 males and 5 females for each

894 group. Error bars are standard deviation. (**B**) Comparison of R237H versus WT fold change for
895 transcript and protein expression for the same cytokines and chemokines shown in A (nd
896 indicates transcript not detected).

897

898 **Figure 3**. Regional differences in hippocampal neuron numbers in R237H rats compared to wild
899 type. Stereological counting of hippocampal neurons labeled with NeuN at P21 (**A**) and P56 (**B**).
900 NeuN labeled nuclei were counted in the dentate gyrus granule cell layer (DG) and CA1 and
901 CA3 pyramidal cell layers throughout the dorsal hippocampus (anterior boundary to posterior
902 commissure). Data were analyzed by 2-way ANOVA and Sidak's multiple comparisons test (**p
903 = 0.0073, N = 6 per group consisting of 3 males and 3 females). There was no significant effect
904 of sex when sex was included as a variable in a 3-way ANOVA.

905

906 **Figure 4**. Changes in synaptic protein expression in R237H rats. (**A-B**) Western analysis of pre-
907 and post-synaptic proteins SV2A and PSD95 respectively in hippocampus. Representative
908 images are shown for immunoblots and corresponding protein stain from the same region of the
909 gel with molecular weight marker positions indicated on the right. Values for each protein are
910 normalized to total protein and expressed as a fraction of the wild type group. (**C-D**) Western
911 analysis of excitatory pre- and post-synaptic markers VGluT1 and GluA1 (**E-F**) and inhibitory
912 markers VGAT and gephyrin. Data were analyzed with a 2-tailed unpaired *t*-test, *p < 0.05, **P
913 < 0.01, ****p < 0.0001, N = 5 males and 5 females at 8 weeks of age for each group.

914

915 **Figure 5**. Changes in mitochondrial proteins in R237H rats. (**A-B**) Western analysis of subunits
916 of the oxidative phosphorylation complexes I through V in rat hippocampus. (**A**) Representative
917 images are shown for immunoblots and corresponding protein stain from the same region of the

gel with molecular weight marker positions indicated on the right. (**B**) Values for each subunit are normalized to total protein and expressed as a fraction of the wild type group. Data were analyzed with a 2-tailed unpaired *t*-test, *p < 0.05, **P < 0.01, ****p < 0.0001, N = 3 males and 3 females at 8 weeks of age for each group.

Figure 6. Deficits in hippocampal long-term potentiation in R237H rats. (**A**) Mean fEPSP slope for wild type (WT) animals before and after a theta-burst stimulation (TBS) paradigm with three trains of 10 bursts (4x100Hz). (**B**) Comparison of potentiation, defined as the average mean fEPSP slope during the last 10 minutes compared to baseline. Data were analyzed with a 2-tailed unpaired *t*-test, ***p = 0.0008, N = 5 WT (2 male, 3 female), and 6 R237H (3 male, 3 female) rats. Error bars = standard deviation.

Figure 7. Impaired novel object recognition (NOR) performance in R237H rats compared to wild type. (**A**) Time (seconds) exploring novel and familiar objects for wild type (WT) and R237H rats in the NOR test. Both novelty and genotype were significant sources of variation (novelty = 9.5% of variation, p = 0.0033; genotype = 12.4% of variation, p = 0.0001; 2-way repeated measures ANOVA with uncorrected Fisher's LSD post-tests, p = 0.0104). (**B**) Discrimination index (DI) as a measure of NOR performance for WT and R237H rats. A DI significantly greater than 0.5 (chance) indicates a preference for exploration of the novel object, whereas a DI that is not significantly different from 0.5 indicates impairment in recognition memory (one sample t-test, ***p = 0.0002). N = 24 WT (12 male, 12 female) and 24 R237H (12 male, 12 female) rats at 16 weeks of age for A and B.

Figure 8. Barnes maze performance for wild type and R237H rats. (**A**) Latency to escape during the first trials over 5 consecutive days of training, ($p = 0.018$ for effect of genotype, mixed-effects model; $^{**}p < 0.01$, Sidak's post-test, error bars = SEM). (**B**) Search strategy patterns (random, serial, direct; see Methods section) used by rats to learn the location of the escape hole in the Barnes maze. The first trials for each of the 5 training days are shown for comparison between wild type (WT) and R237H rats (Chi Square; $^{*}p<0.05$ and $^{**}p<0.01$). N = 30 WT (14 male, 16 female), 24 R237H (11 male, 13 female), at 16 weeks of age for A and B. Average latency to escape and search strategies for all 4 trials are shown in Extended Data Fig. 8-1.