1 *Research article*

2

3 **_Txn1_ mutation causes epilepsy associated with vacuolar degeneration in the**

4 **midbrain**

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Abstract

Thioredoxin (TXN), encoded by *Txn1*, acts as a critical antioxidant in the defense against oxidative stress by regulating the dithiol/disulfide balance of interacting proteins. The role of TXN in the central nervous system (CNS) is largely unknown. A phenotype-driven study of *N*-ethyl-*N*-nitrosourea-mutated rats with running seizures at around five-week of age revealed the relevance of *Txn1* mutations to CNS disorders. Genetic mapping identified *Txn1*-F54L in epileptic rats. The insulin-reducing activity of *Txn1*-F54L rats was approximately one-third that of the wild-type. Vacuolar degeneration in the midbrain, mainly in the thalamus and the inferior colliculus, was observed in the *Txn1*-F54L rats. The lesions displayed neuronal and oligodendrocyte cell death. Neurons in *Txn1*-F54L rats showed morphological changes in the mitochondria. Vacuolar degeneration began at three weeks of age, and spontaneous repair began at seven weeks; a dramatic change from cell death to repair occurred in the midbrain during a restricted period. In conclusion, *Txn1* is essential for the development of the midbrain in juvenile rats.

Keywords

Txn1, Thioredoxin, mitochondria, neuron, vacuolar degeneration, epilepsy, oxidative stress

List of abbreviations

CNS, central nervous system;

WT, wild type;

ROS, reactive oxygen species

NADPH, Nicotinamide adenine dinucleotide phosphate;

NOX, NADPH oxidases;

ENU, N-ethyl-N-nitrosourea

SOD, superoxide dismutase;

CAT, catalase;

HD, Huntington's disease;

ALS, amyotrophic lateral sclerosis;

MS, multiple sclerosis;

GSH, Glutathione;

GPx, glutathione peroxidase;

Prdx, peroxiredoxin

Txn, thioredoxin;

MRI, magnetic resonance imaging;

8-OHdG, 8-hydroxy-2'-deoxyguanosine;

4-HNE, 4-hydroxynonenal

TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling

PBS, phosphate-buffered saline;

HE, hematoxylin and eosin;

KB, Klüver-Barrera

DAB, 3,3'-diaminobenzidine;

IHC, immunohistochemistry;

GFAP, glial fibrillary acidic protein;

72   GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

73   TEM, Transmission electron microscope;

74

Introduction

The brain is more vulnerable to oxidative stress than other organs. Compared to the liver or kidney, the brain has a higher oxygen consumption, in some areas has an increased iron content that catalyzes the generation of reactive oxygen species (ROS), has elevated amounts of lipids with unsaturated fatty acids, and has lower activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [1]. Several studies have implicated oxidative stress in the development of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) [2, 3, 4].

The primary endogenous sources of ROS in mammals are the mitochondrial respiratory chain and NADPH oxidase (NOX). [5, 6, 7]. Oxidative stress modulates functions ranging from cell homeostasis to cell death [8]. Low levels of mitochondrial ROS production under physiological conditions are required for cellular signaling pathways, such as those regulating proliferation and differentiation. In contrast, excess ROS causes protein denaturation and promotes cell death. The delicate balance between the beneficial and harmful effects of ROS is a vital aspect of living cells and tissues [8].

Living organisms have a defense system that scavenges ROS produced by mitochondria. The enzymatic antioxidant system consists of SOD, CAT, GPx, and thioredoxin (Txn). Txn was first identified as a hydrogen donor for ribonucleotide reductase in *Escherichia coli* [9]. In humans, thioredoxin has been identified in the culture media of adult T-cell leukemia cell lines and Epstein-Barr virus-infected cells [10]. Subsequently, it has been reported that thioredoxin expression is upregulated in many cancer cells and that it inhibits apoptosis of cancer cells [11, 12]. Accordingly, the thioredoxin system has attracted attention as a target

100 for cancer therapy [13, 14, 15]. However, fewer studies have investigated the roles of the

101 TXN system *in vivo*. A previous study reported that transgenic mice overexpressing a

102 dominant-negative mutant of *Txn1* specifically in the heart show cardiac hypertrophy [16].

103 Homozygous *Txn1* knockout mice are embryonically lethal, whereas heterozygous mice show

104 normal development [17]. Other than these reports, little is known about the effects of

105 reduced TXN activity *in vivo*.

106

107 Here, we present a novel animal model for neurodegeneration designated the

108 *Adem* "Age-dependent mitochondrial cytopathy" rat. These animals harbor a *Txn1* missense

109 mutation (F54L). *Adem* exhibit a unique seizure phenotype that appears only at 5 weeks of

110 age. Detailed histological analyses and anatomical analyses using MRI revealed vacuolar

111 degeneration in the midbrain during the epileptic period. Our study reveals a hitherto

112 unknown function of *Txn1* in the CNS.

113

Materials and methods

115 *Animals*

116 We used *Txn1*-F54L rats (*Adem*; Age-dependent mitochondrial cytopathy rat) generated by

117 ENU-mutagenesis and a *Txn1*- T160C (p. F54L) knock-in rat generated by CRISPR-Cas 9.

118 We used the former to characterize the phenotype and the latter to confirm the reproducibility

119 of the phenotype. The *Txn1*-F54L rat was initially discovered in an ENU-induced mutant

120 archive at Kyoto University [18, 19, http://www.anim.med.kyoto-u.ac.jp/nbr] as a rare strain

121 with frequent running seizures only in its juvenile stage. All animal husbandry procedures

122 were performed according to the protocols approved by the institutional experimental animal

123 use committees Okayama University, Osaka University, and Kyoto University. Both sexes

124 were used in the present study.

125

Genetic mapping and sequencing analysis of the Adem rat generated by ENU-mutagenesis

The rats with running seizures were backcrossed for more than ten generations on the

F344/NSlc inbred background (Japan SLC, Hamamatsu, Japan). The repeated backcrosses

ensure that the frequency of potential mutations induced by ENU elsewhere in the genome is

reduced to approximately 1 in 4×10^6 bp of the genome. To identify the causative gene, we

produced 117 (*Adem×BN/SsNSlc*) × *BN/SsNSlc* backcross progeny. Genetic mapping of the

locus was determined in rats that displayed wild running or generalized tonic-clonic seizure

phenotypes. Genomic DNA was extracted from rat tail biopsies by an automatic genomic

DNA isolation system (PI-200; Kurabo, Osaka, Japan). To localize the locus to a specific

chromosomal region, genome-wide scanning on DNA samples was performed using simple

sequence length polymorphism (SSLP) markers covering all autosomal chromosomes (Chrs)

as previously reported [20].

To enrich the 1.5-Mb region (Chr 5: 75,159,000 to 76,708,000), SureSelect custom DNA

probes were designed by SureDesign under moderately stringent conditions, and generated by

Agilent Technologies (Santa Clara, CA, USA). The DNA library was prepared using

SureSelect reagents and a custom probe kit. Genomic sequence analysis was performed by

HiSeq 2500 (2×150 bp) according to the standard protocol at Takara Bio.

Generation of Txn1- T160C (p. F54L) knock-in rat using CRISPR-Cas 9

The possibility of genomic DNA alterations around the *Txn1* gene could not be fully excluded

in *Txn1*-F54L rats generated by ENU mutagenesis. As the *Txn1* mutant rats showed a unique

phenotype, never previously reported, we generated a new *Txn1*-F54L knock-in rat to test the

reproducibility of this phenotype. A *Txn1* T160C (p. F54L) knock-in rat was generated by

CRISPR-Cas9 genome editing with long single-stranded DNA (lssDNA), as previously

reported [21]. A pair of guide RNAs (gRNAs) targeting intron 1 and intron 2 of rat *Txn1* were

151 designed. LssDNA for *Txn1* was synthesized using a long ssDNA Preparation Kit

152 (Biodynamics Laboratory Inc., Tokyo, Japan) following the manufacturer's protocol. The

153 sequences of gRNA and ssDNA are listed in Supplementary Table 1. Cas9 mRNA (400 ng/μl),

154 gRNA (200 ng/μl), and ssDNA (40 ng/μl) were delivered into 113 *F344/Jcl* embryos by

155 electroporation as previously reported [22]. The two-cell stage embryos (n = 110) were

156 transferred into the oviducts of five pseudopregnant females. Genomic DNA from founder

157 rats was extracted from the tail biopsies. The CRISPR target site was amplified using the

158 specific primer pairs listed in Supplementary Table 1.

159

160 *Genotyping*

161 The last 3 mm of the rat tail was cut at three weeks of age, and DNA was purified with a

162 DNeasy Blood &Tissue kit (Qiagen, Hilden, Germany). A DNA fragment containing a

163 mutation was amplified with a pair of primers as follows: FW 5′ - CCA CAT GGG AGA

164 GTC ACA T -3′ and RV 5′- ATA GCC TGG AAG CGG TCA GAT G -3′ (Sigma-Aldrich

165 Japan, Inc., Tokyo, Japan). The PCR products (521 bp) were then treated with the restriction

166 enzyme *Bsp*1286I (New England Biolabs, Ipswich, MA, USA), and the F54L mutant DNA

167 was digested to 260 bp and 261 bp fragments. Genotyping was determined by the size of the

168 digested fragments on a 3% agarose gel.

169

170 *Video monitoring of epileptic seizures*

171 To clarify the appearance and frequency of epileptic seizures, both the heterozygotes (n = 8)

172 and homozygotes (n = 9) were placed under a 24-hour video monitoring system (Handycam

173 HDR-CX480, SONY, Japan) from week three to nine,

174

175 *Histological analysis*

176   Brains were fixed with 10% formalin neutral buffer solution. Tissue sections were cut from

177   paraffin-embedded brain samples taken at 2, 3, 5, and 9 weeks of age and were subjected to

178   hematoxylin and eosin (HE) and Bodian and Klüver-Barrera (KB) staining using standard

179   protocols. The stained sections were examined under a BZX-700 microscope (Keyence,

180   Osaka, Japan). We evaluated brain lesions, including samples from the thalamus and the

181   inferior colliculus, in the sagittal and coronal planes.

182

183   ***Head MRI***

184   To evaluate the extent of brain lesions and their changes over time, an MRI scan was

185   performed in 1 mm slices. Three animals were examined for each genotype at 5, 7, and 9

186   weeks of age. The same animal was examined twice on days 1 and 14 as the storage period of

187   the MRI-attached breeding room was limited to no longer than two weeks. The mice were

188   anesthetized with isoflurane and laid in the prone position on a cradle. Anesthesia was

189   maintained by inhalation of 2% isoflurane in air at 1.4 L/min through a face mask.

190   Throughout the MRI measurements, respiratory rate and rectal temperature were monitored

191   using a dedicated system (Model 1025, MR-compatible Small Animal Monitoring and Gating

192   System, SA Instruments, Inc., NY, USA). The body temperature was maintained by a flow of

193   warm air using a heater system (MR-compatible Small Animal Heating System, SA

194   Instruments).

195

196   All MR images were obtained with a 4.7 Tesla preclinical MR scanner (BioSpec 47/16 USR,

197   Bruker BioSpin MRI GmbH, Ettlingen, Germany). A quadrature volume resonator (Bruker

198   BioSpin) was used for signal detection. The scanner was operated using ParaVision 6.0.1

199   (Bruker BioSpin). Two-dimensional multi-slice T2-weighted MR imaging was performed

200   using a rapid acquisition with relaxation enhancement (RARE) sequence. The whole-head

201   images were acquired in three orthogonal (coronal, sagittal, and axial) orientations. The

202   acquisition parameters were as follows: echo time (TE), 12 ms; effective TE, 36 ms; RARE

203   factor, 8; acquisition bandwidth, 62.5 kHz; in-plane spatial resolution, 0.156×0.156 mm^2;

204   slice thickness, 1 mm; slice gaps, 0 mm; the number of averages, 2. For coronal orientation,

205   repetition time (TR), 3000 ms; field of view (FOV), 25×29.3 mm^2, matrix size, 120×136

206   (zero-filled to 160×188 before image reconstruction); the number of slices, 24; acquisition

207   time, 1 min 42 s. For sagittal orientation, TR, 2500 ms; FOV, 27×27.5 mm^2, matrix size, 131

208    $\times 128$ (zero-filled to 173×176); number of slices, 18; acquisition time, 1 min 20 s. For axial

209   orientation, TR, 2500 ms; FOV, 27×30.5 mm^2, matrix size, 131×144 (zero-filled to $173 \times$

210   195); the number of slices, 16; acquisition time, 1 min 30 s.

211

212   ***Western blotting***

213   Western blotting was used to quantify the different cell types of the brain lesion and Txn1

214   expression levels in multiple organs in the rats. The protein extracts (10 µg) from rat tissues

215   were mixed with sample buffer solution (Nakalai Tesque, Kyoto, Japan), denatured at 95 °C

216   for 5 min, and loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel (Mini-protean

217   TGX Precast gels; Bio-Rad, Hercules, CA) for electrophoresis. The proteins were blotted onto

218   a polyvinylidene fluoride (PVDF) membrane using the iBlot Dry Blotting System (Thermo

219   Fisher Scientific). Membranes were incubated with anti-Thioredoxin1 rabbit antibody

220   (CST#2298; Cell Signaling Technology, Danvers, MA, USA), anti-NeuN rabbit antibody

221   (ab177487; Abcam, Cambridge, UK), anti-Olig2 rabbit antibody (ab109186; Abcam),

222   anti-glial fibrillary acidic protein (GFAP) rabbit antibody (ab7260; Abcam), anti-Iba1 rabbit

223   antibody (ab178847; Abcam), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

224   rabbit antibody (CST #2118). Mouse and rabbit primary antibodies were detected using

225   anti-mouse IgG-HRP-linked antibody (CST #7076) and anti-rabbit IgG-HRP-linked antibody

226   (CST; #7074), respectively. Chemiluminescence was detected using Western Lightning ECL

227   Pro (PerkinElmer Japan, Kanagawa, Japan) and the ChemiDoc Touch (Bio-Rad).

228

Immunohistochemistry (IHC) analyses

230 A standard IHC protocol was used to stain the brain tissue samples. In brief, 5-µm-sized

231 paraffin-embedded tissue sections were de-paraffinized with xylene. Antigen retrieval was

232 performed by autoclaving at 120 °C for 5 min. Endogenous peroxidases were quenched by

233 treatment with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. They

234 were then incubated with blocking serum for 1 h at 22-26 °C. Sections were incubated with

235 the following primary antibodies to determine the cell type in the brain lesion: anti-NeuN

236 rabbit antibody (ab177487; Abcam, Cambridge, UK) and anti-Olig2 rabbit antibody

237 (ab109186; Abcam) for 1 h at room temperature. To determine DNA oxidative injury and

238 lipid peroxidation, sections were stained with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG)

239 mouse antibody (MOG-020P, JaICA, Japan), and anti-4-hydroxynonenal (4-HNE) mouse

240 antibody (MHN-020P, JaICA, Japan) overnight at 4 °C, respectively. The sections were then

241 reacted with a biotinylated rabbit polyclonal secondary antibody (PK-6200, Vector

242 Laboratories, Burlingame, CA, USA). To reveal the staining, we used an avidin-biotinylated

243 peroxidase complex (PK-6200, Vector Laboratories). After washing, the slides were

244 incubated with 3,3'-diaminobenzidine (DAB) (SK-4100, Vector Laboratories) and

245 immediately washed with tap water after color development. The slides were then

246 counterstained with hematoxylin, mounted with dibutyl phthalate xylene (DPX) and observed

247 under a BZX-700 multifunctional microscope (Keyence, Osaka, Japan). Quantification of

248 IHC was performed by assessing five randomly selected fields in the inferior colliculus and

249 thalamus. The percentages of positive cells or positive areas were calculated using BZX

250 Analyzer software (Keyence).

251

Transmission electron microscopy (TEM)

253   Samples were immersed in 2% glutaraldehyde and 2% paraformaldehyde in 100 mM

254   phosphate buffer (PB) for 24 h at 4 °C. They were then postfixed with 2% osmium tetroxide

255   in 100 mM PB for 1.5 h at 4 °C, after which they were rinsed with 100 mM PB, and

256   dehydrated through a graded series of ethanol treatments. They were embedded in Spurr resin

257   (Polysciences Inc., Warrington, PA, USA), cut into ultrathin sections, and stained with uranyl

258   acetate and lead citrate. The ultrathin sections were observed using a Hitachi H-7650 TEM

259   (Hitachi High-Tech Corp., Tokyo, Japan).

260

261   ***Plasmids and purification of recombinant proteins***

262   For recombinant Thiredoxin1 proteins, rat *Txn1* cDNAs were amplified by PCR using

263   LA-Taq polymerase (Takara Bio Inc, Shiga, Japan) with FW-primer: 5′-GGA TCC ATG

264   GTG AAG CTG ATC GAG AG-3′; and RV-primer, 5′- GTC GAC TTA GCT GTC CAT

265   GTG CTG GCG TTC GAA TTT AGC GGT TTC TTT GAA TTC GGC AAA CTC CGT

266   AAT AGT GG-3′ (Sigma-Aldrich Japan, Inc., Tokyo, Japan). The PCR products were cloned

267   into a pCMV-Tag2 plasmid (Stratagene, San Diego, CA, USA) digested with *Bam*HI and *Sal*I

268   containing FLAG-tag and S-tag sequences. The DNA sequence of the plasmids was

269   confirmed by DNA sequencing using a BigDye Terminator FS Ready-Reaction Kit (Applied

270   Biosciences, Little Chalfont, Buckinghamshire, UK) and an ABI 3130x Genetic Analyzer

271   (Applied Biosciences). The plasmids were transfected into HEK293 human embryonic kidney

272   cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). The recombinant proteins

273   were purified with anti-FLAG agarose affinity gel (A2220; Sigma-Aldrich Japan), eluted with

274   FLAG peptide (A3290; Sigma-Aldrich Japan), and filtered using an ultrafiltration membrane

275   (Amicon Ultra 3 K; Millipore, Burlington, MA, USA).

276

277   ***Insulin-reducing activity assay***

278 Thioredoxin activity was measured using a thioredoxin activity assay kit (Redoxica, Little

279 Rock, AR, USA), according to the manufacturer's instructions. In brief, the purified

280 recombinant thiredoxin1 proteins or protein extracts from rat thalamus and cortex were

281 reacted with insulin and NADPH in assay buffer, and thioredoxin activity was determined as

282 a change in the amount of oxidized NADPH by measuring the decrease in absorbance at 340

283 nm per minute using a spectrophotometer DeNovix DS-11 (DeNovix, Tokyo, Japan).

284

285 ***Primary fibroblast and neuron culture***

286 Newborn rats were sacrificed under deep anesthesia with isoflurane on the day of birth. The

287 animals were dissected in the surgical suite and disinfected with 70% alcohol. For primary

288 fibroblast culture, the abdominal skin tissues were cut into small pieces in DMEM (Fujifilm

289 Wako Pure Chemical Corporation, Osaka, Japan) and disrupted by pipetting. The cell

290 suspension was transferred and maintained in DMEM supplemented with 10% fetal bovine

291 serum (Thermo Fisher Scientific, Waltham, MA, USA), 500U/ml penicillin, and 500 ug/ml

292 streptomycin (Sigma-Aldrich Japan, Inc., Tokyo, Japan) in a fully humidified atmosphere of

293 5% CO_2. After about two weeks, fibroblast cells were cultured in DMEM supplemented with

294 10% fetal bovine serum, 100U/ml penicillin, and 100 ug/ml streptomycin.

295

296   For primary neuronal culture, we referred to a protocol using a postnatal mouse [23] and

297 modified it. We used the cerebral cortex of rats on the first postnatal day. Tissues were treated

298 with 0.125% trypsin (Thermo Fisher Scientific) and 0.004% DNase-I (Sigma-Aldrich) at

299 37 °C for 15 min and dissociated mechanically. Cells were plated on poly-l-lysine and

300 laminin-coated glass-bottomed 35-mm culture dishes. Cells were maintained in a culture

301 medium with DMEM, 100 µg/ml penicillin-streptomycin (Thermo Fisher Scientific) and 10%

302 fetal calf serum (Thermo Fisher Scientific) at 37 °C in a humidified incubator with 95% air

303 and 5% CO_2. On the second day, the culture medium was replaced with a medium containing

304   DMEM, 2% B-27 supplement (Thermo Fisher Scientific), and 5% fetal calf serum. The

305   culture medium was refed with a medium containing DMEM, α-MEM, F-12 nutrient mixture,

306   2% B-27 supplement, 0.34% glucose, 25 μM 5-fluoro-deoxyuridine, 25 μM uridine, one mM

307   kynurenic acid (Sigma-Aldrich), and 1% fetal calf serum on the fourth day. Cultures were

308   used for the experiments on days 9–15. Mitochondria and nuclei were stained using

309   MitoBright LT Red (Dojindo, Kumamoto, Japan) and Cellstain-Hoechst33258 solution

310   (Dojindo, Kumamoto, Japan), respectively.

311

312   ***Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay***

313   For determining apoptosis, paraffin-embedded brain tissues at three weeks of age and primary

314   fibroblasts were assessed using the In Situ Cell Death Detection Kit, TMR red

315   (Sigma-Aldrich Japan, Inc., Tokyo, Japan), following the manufacturer's instructions. We

316   compared cell death during incubation of primary fibroblasts with and without the addition of

317   300 μM hydrogen peroxide for 3h. In brief, the cells were cultured on an 8-chamber culture

318   slide (BD Falcon, Franklin Lakes, NJ, USA), washed with phosphate-buffered saline (PBS),

319   fixed with 10% formalin neutral buffer solution (Fujifilm Wako Pure Chemical Corporation,

320   Osaka, Japan) for one h at room temperature, and treated with permeabilization solution for 2

321   min on ice. TMR red-labeled dUTP was incorporated into 3′-OH DNA ends by terminal

322   deoxynucleotidyl transferase, and the fluorescence signal was detected using a BZX-700

323   microscope (Keyence, Osaka, Japan) or a fluorescence microscope (Olympus IX71; Olympus

324   Corporation, Tokyo, Japan).

325

326   ***Statistical analysis***

327   Results are expressed as mean ± SEM. The sample size for each experiment is indicated in the

328   figure and figure legends. One-way analysis of variance (ANOVA) was used to compare

329   between three groups. Statistical significance was set at $p < 0.05$. Bonferroni adjustment was

330   used to determine the statistical differences between the groups. All significant statistical

331   results are indicated within the figures using the following conventions: $*$ $p < 0.05$, $**$ $p <$

332   0.01, $***$ $p < 0.001$.

333

Results

334

335   ***Discovery of a novel epileptic rat with Txn1 missense mutation***

336   A strain exhibiting unique running seizures was identified in an archive of ENU-mutated rats.

337   Thus, we decided to identify the responsible gene. By analyzing simple sequence length

338   polymorphism (SSLP) markers on mutant (n = 21) and sibling (n = 25) DNA pools,

339   the *Adem* locus was roughly mapped on chromosomes 2 and 5. The locus was subsequently

340   refined by 26 and 33 SSLP markers for each chromosome, and mapped to a 1.5-Mb genomic

341   region, between markers D5Mit17 (Chr 5: 75,159,412–75,159,546) and D5Rat113 (Chr

342   5:76,707,365–76,707,563) (RGSC_v3.4) (Fig. 1A). Ten known or predicted genes within

343   the *Adem* region

344   (*Ptpn3*, *Palm2*, *AC134204.1*, *Akap2*, *LOC685849*, *Txn1*, *Txndc8*, *Svep1*, *Musk*, *Lpar1*) were

345   identified from the Ensembl database (http://www.ensembl.org).

346

347   We performed next-generation sequencing analysis of genomic DNA from 75,159,000 to

348   76,708,000 on Chr5 using the SureSelect target enrichment approach (Agilent). In mutant

349   DNA, we found only one heterozygous missense mutation in the exon region, c. T160C, in

350   exon 3 of the *Txn1* gene (NM_053800, ENSRNOG00000012081.6) by comparing the

351   parental strain F344/NSlc. This variant caused a substitution of phenylalanine for leucine at

352   residue 54 (Fig. 1B).

353

354 Txn1 is widespread in prokaryotes and eukaryotes, and its amino acid sequence is well

355 conserved in mammals (Fig. 1D). Amino acid sequences are 90% identical in humans and rats.

356 Since the structure of rat thioredoxin has not been reported, we plotted the position of F54 on

357 the 3D structure of human thioredoxin (UniProt, https://www.uniprot.org/). The positions of

358 amino acids with important functions in Txn1 were plotted on the structure. Txn1 has an

359 active center sequence Cys32-Gly-Pro-Cys35 motif and carries out redox reactions by

360 dithiol/disulfide exchange reactions between Cys32 and Cys35 [24, 25, 26]. Both Cys69 and

361 Cys73 are nitrosylated in response to nitric oxide (NO) [26, 27]. Cys-73 can serve as a donor

362 for the nitrosylation of target proteins. The location of the F54L mutation is remote from these

363 active sites (Fig. 1C and Fig. 1D), suggesting that it is not directly involved in the

364 dithiol/disulfide exchange reaction or S-nitrosylation. However, the PolyPhen-2 prediction

365 (http://genetics.bwh.harvard.edu/pph2/index.shtml) found that the F54L mutation is possibly

366 damaging with a score of 0.785, and the Sorting Intolerant From Tolerant (SIFT,

367 http://www.ngrl.org.uk/Manchester/page/sift-sorting-intolerant-tolerant.html) sequence is

368 deleterious.

369

370 *Vacuolar degeneration appears during an epileptic period*

371 Video monitoring showed that the frequency of seizures peaks at 5-week of age in both

372 heterozygotes (Fig. 2A) and homozygotes (Fig. 2B). There were no differences in seizure

373 frequencies between heterozygotes and homozygotes.

374

375 Next, a histological examination was performed at five weeks to determine whether a

376 structural change was the cause of epilepsy. Surprisingly, vacuolar degeneration was found in

377 the inferior colliculus and thalamus of the mutants. (Fig. 2C). No changes were observed in

378 the hippocampus, which is prone to epilepsy. Bodian staining, which is used to observe nerve

379 fibers, and Klüver-Barrera (KB) staining, which is used to observe the myelin sheath,

380   exhibited decreased staining in the mutants. These histological tests showed that homozygotes

381   had more severe lesions than heterozygotes. We examined when these lesions had started

382   developing (Fig. 2D). There were no remarkable changes in the inferior colliculus at two

383   weeks of age. However, vacuoles became evident in the inferior colliculus and thalamus at

384   three weeks of age and were then widespread at five weeks of age. They almost disappeared

385   completely after nine weeks.

386

387   ***MRI showed transient high signals of T2-weighted images in the midbrain***

388   MRI detected hyperintense regions of T2-weighted images (T2WI) spread around the

389   thalamus in mutant rats at five weeks of age (Fig. 3A). The hyperintense regions of the

390   heterozygous and homozygous T2WI were symmetrical. The lesions were localized in the

391   thalamus, inferior colliculus, superior colliculus, and hypothalamus in homozygotes (Fig. 3B).

392   The hyperintensity region of T2WI in homozygous rats was wider than that in heterozygous

393   rats (see also Supplementary Fig. S1, S2, and S3). Temporal lesions were also examined using

394   MRI in the same individual. Serial MRI examination revealed that the lesions at three weeks

395   spread or were similar in size at five weeks. However, the lesions shrank at seven weeks and

396   disappeared at nine weeks (Fig. 3C). The weekly changes observed on MRI were similar to

397   those seen in the histological examination.

398

399   ***Neuronal and oligodendrocyte cell loss occurs in the midbrain***

400   Western blotting of the midbrain using NeuN, Olig2, GFAP, and anti-IbaI antibodies showed

401   a decrease in NeuN and Olig2 and an increase in GFAP and IbaI expression in heterozygotes

402   and homozygotes (Fig. 4A). Immunohistochemical studies showed significantly decreased

403   numbers of NeuN-positive neurons (Fig. 4B and 4C) and Olig2-positive oligodendrocytes

404   (Fig. 4D and 4E). NeuN-positive neurons in heterozygotes and homozygotes had larger cell

405   bodies than wild-type (WT) rats. Electron microscopy of the lesions in the mutant rats

406 revealed that microtubules in the vacuoles and myelin sheath were wrapped around the

407 vacuoles (Fig. 4H and 4I). These findings indicate that the vacuoles were dilated axons of the

408 neurons. Examination of the mitochondrial morphology showed sparse cristae in the mutant,

409 even though the neurons retained their structures (Fig. 4J and 4 K).

410

411 ***Txn1-F54L shows decreased insulin-reducing activity***

412 Fig. 5A shows the thioredoxin/peroxiredoxin system. Four central enzyme systems defend

413 against oxidative stress by scavenging ROS generated in the body. These include SOD, which

414 scavenges superoxide, CAT, GPx, and thioredoxin-dependent peroxiredoxin (Prdx).

415 Thioredoxin forms a system with nicotinamide adenine dinucleotide phosphate (NADPH) and

416 thioredoxin reductase (Txnrd) to reduce disulfide bonds in target proteins. Thioredoxin is

417 responsible for converting the target protein from oxidized to the reduced form and

418 scavenging H_2O_2 via Prdx. To confirm whether the reduced expression of Txn1 underlies the

419 changes in the brain during 3–5 weeks of age, we examined the expression level of Txn1

420 protein in the brain, kidney, heart, and liver by western blotting at four weeks of age when

421 vacuoles appeared and at 16 weeks of age when repair occurred. We found that Txn1 was

422 equally expressed in all organs, and there was no change between 4 and 16 weeks of age (Fig.

423 5B).

424

425 Next, we measured thioredoxin activity by using recombinant protein (Fig. 5C), the thalamus

426 at five weeks of age, where vacuolar degeneration was observed (Fig. 5D), and the frontal

427 cortex (Fig. 5E), where vacuolar degeneration did not occur. Our results showed that the

428 insulin-reducing activity of *Txn1*-F54L was decreased to approximately one-third of that of

429 the WT. Similarly, insulin-reduction activity in the thalamus was reduced in heterozygotes

430 and reduced much further in homozygotes compared to that in WT mice (Fig. 5D).

431   Interestingly, reduced activity was also observed in the cortex, where there was no vacuolar

432   degeneration (Fig. 5E).

433

434   ***Oxidative damage in the midbrain and susceptibility to cell death under oxidative stress***

435   Next, we investigated the pathogenesis underlying the vacuolar degeneration. Staining with

436   8-OHdG, which indicates oxidative stress-induced DNA damage, and 4-HNE, which

437   indicates lipid oxidation, showed brain staining in the mutant lesions (Fig. 6A). The area of

438   8-OHdG (Fig. 6B) and 4-HNE (Fig. 6C) positive cells were significantly increased in

439   heterozygous and homozygous rats compared to WT cells. TUNEL assay of

440   paraffin-embedded sections, including the thalamus, showed slight staining in heterozygous

441   and homozygous rats at three weeks of age (Fig. 6D). Next, we examined cellular

442   vulnerability to H_2O_2 using primary fibroblasts and cortical neurons derived from WT and

443   homozygous rats. The TUNEL assay showed that homozygous fibroblasts were slightly

444   stained even under standard culture medium, and the staining became significantly stronger

445   when H_2O_2 was added. In contrast, TUNEL-positive cells in WT fibroblasts did not increase

446   with the addition of 0.3 mM H_2O_2 (Fig. 6E). We also found that WT primary neurons showed

447   no significant changes, whereas homozygous neurons showed cell death and nuclear rupture

448   on treatment with 0.3 mM H_2O_2 (Fig. 6F).

449

450   ***Txn1-F54L rat generated by genome editing replicates vacuolar degeneration in the***

451   ***midbrain***

452   Finally, we conducted reproducibility experiments to confirm whether the *Txn1*-F54L

453   mutation is the sole cause of the F344/NSlc phenotype. Genome editing using CRISPR-Cas9

454   was used to generate F344/Jcl rats with the *Txn1*- F54L mutation. We placed 5-week-old

455   F344/Jcl *Txn1*-F54L heterozygous rats under 24-hour video monitoring and recorded running

456   seizures eight times/24 h (a total of N = 5) (Supplementary Video 2). Running seizures and

457 transition from running to tonic seizures were observed. These seizure symptoms were similar

458 to those observed in *Adem* rats harboring *Txn1*-F54L. The lesions were localized in the

459 thalamus, hypothalamus, and superior and inferior colliculi and were more extensive in

460 homozygous than in heterozygous rats (Fig. 7A and 7 B). The vacuoles in the midbrain

461 started developing at two weeks of age, became apparent at three weeks, and recovered at

462 nine weeks (Supplementary figure 4). Although the genetic background of F344/NSlc

463 generated by ENU-mutagenesis and F344/Jcl is somewhat different, transient vacuolar

464 degeneration localized in the midbrain was reproduced in F344/Jcl rats carrying the *Txn1*-

465 F54L mutation.

466

467 **Discussion**

468 The function of TXN in vivo remains unclear. Txn1 is located in the cytoplasm, nucleus, and

469 extracellular region [25, 28], whereas Txn2 is located in the mitochondria [29]. A

470 homozygous *TXN2* mutation has been linked to an infantile-onset neurodegenerative disorder

471 with severe cerebellar and optic atrophy and peripheral neuropathy [30]. To the best of our

472 knowledge, there are no reports of *TXN1* mutations that cause human diseases. Herein, we

473 discovered a link between *Txn1* mutations and CNS disorders in mammals for the first time.

474 The loss of Txn1 function leads to neuronal death with abnormal mitochondrial structure in

475 the midbrain.

476 Txnrd catalyzes the reduction of oxidized Txn in an NADPH-dependent manner. Genetically

477 modified mice with inactivation of Txnrd1 or Txnrd2 show embryonic lethality [31, 32], so

478 the Txn/ Txnrd systems have not been elucidated in vivo beyond the fact that they are

479 essential for fetal development. Other important interacting proteins include the Prdx family,

480 which scavenges H_2O_2. Knockout mice for *Prdx1*, *Prdx2*, *Prdx3*, *Prdx4*, or *Prdx5* exhibited

481 various kinds of disorders such as hemolytic anemia, metabolic abnormalities, inflammation,

482 cancer, and age-related phenotypes [33]. The discovery of *Adem* rats may shed light on new

483 roles of the Txn/ Txnrd systems and Txn/ Prdx systems.

484

485 *Adem* rats exhibited running seizures and a transition from running to tonic seizures. The

486 focus on this unique epileptic seizure has led to a significant discovery. Running seizures are

487 rarely reported as symptoms of epileptic seizures. According to reports from the 1980s and

488 the 1990s, electrical stimulation of the inferior colliculus and midbrain reticular formation [34,

489 35, 36] or intense acoustic stimulation [37] induced running seizures. In addition, some

490 evoked running seizures are followed by tonic seizures [35, 36, 37]. The seizure symptoms in

491 the *Adem* rats share similarities with these symptoms. The time of spontaneous appearance

492 and suppression of epileptic seizures and the time of onset and repair of the midbrain lesion

493 were almost the same. This suggests that the cause of epilepsy is a midbrain lesion involving

494 the inferior colliculus and thalamus.

495

496 Upon observing the unique vacuolar degeneration in the midbrain of *Adem* rats, we had

497 several questions. First, is there a reduction in cell number in the midbrain? Second, is there

498 an alteration in the reducing activity of *Txn1*-F54L? Third, are the cells derived

499 from *Adem* rats more prone to cell death in response to excess ROS? Fourth, why are

500 vacuoles confined to the midbrain at 3–5 weeks of age? We have discussed our findings based

501 on these questions below.

502

503 Western blotting and immunohistochemistry showed a reduction in both neurons and

504 oligodendrocytes in the *Adem* rats. The cell bodies of NeuN-positive cells were more

505 significant than in the WT, and pronounced axon swelling was identified by TEM. These

506 findings suggest that vacuoles are involved in neuronal and oligodendrocyte cell death.

507 Neuronal activity controls oligodendrocyte development and myelination [38, 39, 40], and

508 neuronal cell death inhibits myelination. Further, as neurons and oligodendrocytes are closely

509 related during development, cell death can occur.

510

511 Our recombinant protein assay showed that the insulin-reducing activity in the mutant rats

512 decreased to approximately one-third that of the WT. In addition, reduced enzymatic activity

513 was observed in the thalamus, where there was a lesion, and in the cortex, where no lesion

514 was present. F54L is located far from the CGPC motif, which plays a central role in TXN

515 activity. However, PolyPhen-2 and SIFT predicted that the F54L mutation was a

516 loss-of-function mutation. The missense mutation may cause a structural change in the region

517 where the dithiol/disulfide exchange reaction occurs.

518

519 In primary cultured neurons and fibroblasts derived from *Adem* rats, cell death was induced

520 more readily than in the WT in the culture medium with H_2O_2. IHC with 8-OHdG and 4-HNE,

521 and TUNEL assays stained more intensely in the brain lesions in *Adem* rats than in the WT.

522 These results suggest that the cells in *the Adem* rats are vulnerable to excessive ROS. Txn1

523 has anti-apoptotic functions in various cells [41, 42, 43]. Loss of function of Txn1 can cause

524 cell death [44, 45, 46]. Proteins that are associated with apoptosis through interaction with

525 Txn1 include apoptosis signal-regulating kinase 1 (ASK1) [47, 48] and Caspase-3 (CASP3)

526 [49]. In addition to apoptosis, other mechanisms of neuronal death could be involved, such as

527 phagocytosis, pyroptosis, and ferroptosis [50]. Western blotting of 5-week-old brains showed

528 a dense band reacting to anti-Iba1 antibody, suggesting an increase in microglia. Microglial

529 activation is a common feature of neurodegenerative diseases. Activated microglia have been

530 reported to kill neurons by releasing TNF-α, glutamate, reactive oxygen, and nitrogen species,

531 which can cause apoptosis, excitotoxicity, and necrotic death of surrounding neurons [51, 52].

532

533   Our results could not clearly determine why the midbrain vacuoles were confined to 3–5

534   weeks of age. The *Txn1* gene is ubiquitously expressed throughout the body (*TXN* thioredoxin

535   [Homo sapiens (human)] - Gene - NCBI https://www.ncbi.nlm.nih.gov/gene/7295). Western

536   blotting of *Adem* rats also showed that thioredoxin was expressed in several organs other than

537   the brain. The insulin-reducing activity of thioredoxin was reduced to one-third of that of the

538   WT, both in the thalamus, where vacuolar degeneration appeared, and in the cerebral cortex,

539   where vacuolar degeneration did not appear. Furthermore, *Adem* rat-derived primary cultured

540   fibroblasts and neurons showed a similar level of cell death induced by excessive ROS. These

541   results of *in vitro* experiments indicate that cell death can be caused by excessive ROS,

542   regardless of the cell type, under the same cell culture conditions. The curious phenomenon of

543   limited onset of neuronal death in the midbrain may be due to the location- and age-specific

544   microenvironment of the midbrain. For example, the neurodevelopmental stage at 3–5 weeks

545   of age might trigger an increased mitochondrial ROS leakage due to high glucose metabolism

546   in the midbrain. The F54L mutation-specific inability of *Txn1* to reduce essential proteins for

547   maintaining ROS homeostasis in the midbrain or the dependence of the midbrain region on

548   the thioredoxin/peroxiredoxin antioxidant system might be high. The

549   thioredoxin/peroxiredoxin system, and not the glutathione system, was reported to be a

550   significant contributor to mitochondrial H_2O_2 removal in the brain [53].

551

552   Another surprising phenomenon in *Adem* rats is that repair of the vacuolar lesions can already

553   be seen at seven weeks on MRI, and they are almost entirely repaired at nine weeks. Both

554   astrocytes and microglia have been reported to be involved in cytotoxicity and tissue repair.

555   Astrocytes have long been thought to inhibit neuronal repair, but it has been noted that glial

556   activation induces neurogenesis and acts in repair [54, 55]. Microglia phagocytose and

557   remove debris and dead neurons, and release BDNF and IGF to repair damage to neurons [56,

558   57, 58]. In vacuolar lesions, glial cells may play roles in repair and impart neuropathic effects,

559   or their roles may change from damage to repair. Although this is an exciting point, it is far

560   from the purpose of this study, which was to identify a causative gene of epilepsy and clarify

561   the underlying pathology. We will study this in the future.

562

563   We have not yet identified the precise functions disturbed by Txn1-F54L because Txn1

564   interacts with a wide variety of proteins [59, 60, 61]. This is a limitation of this study.

565   Oxidative stress is responsible for many neurodegenerative diseases [2, 3, 4] and cerebral

566   ischemic-reperfusion injury [62, 63]. The vacuolar degeneration in *Adem* rats may share a

567   common pathological pathway with these neurological disorders. If a common pathway is

568   identified, *Adem* rats would be an attractive animal model. New therapeutic agents for

569   oxidative stress-related diseases can be evaluated more easily because neuronal death occurs

570   spontaneously in a short time and does not require surgical treatment.

571

Conclusions

572

573   We have shown that the *Txn1*-F54L mutation confers epilepsy. The underlying pathology

574   of *Adem* rats with *Txn1*-F54L is the loss of neurons and oligodendrocytes in the midbrain.

575   *Txn1*-F54L had a reduced insulin-reducing activity compared to that of the WT. Primary

576   cultured cells, both fibroblasts and neurons, derived from *Adem* rats exhibited cell death under

577   excess H_2O_2. The mutation might be affecting the midbrain microenvironment in a specific

578   manner leading to the death of neurons and oligodendrocytes in that region. Further studies

579   are required to determine this midbrain-specific function of Txn1.

580

Author contributions

T.M discovered the *Adem* rat; I.O. and M.O. performed the phenotypic characterization of this strain; T.M. and S.I. created the genome-edited mutant rats; H.I. supervised the MRI examination and analyzed the data; S.T. supervised the histopathological experiments; Conceptualization, I.O., M.O., and T. M.; Investigation, I.O., M.O., H.I., S.I., S.T., and T.M.; Writing – Original Draft, I.O.; Writing – Review & Editing, M.O. H.I., S.I., S.T., and T. M.; Project Administration, I.O.; Funding Acquisition, M.O., I.O., and T.M. All authors approved the final manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

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779

780 **Figure legends**

781 **Fig. 1.** Discovery of a novel epileptic rat with a *Txn1* missense mutation.

782 (A) *Adem* locus mapped to a 1.5-Mb genomic region, between markers D5Mit17 and

783 D5Rat113.

784 (B) DNA sequencing exhibiting c. T160C in exon 3 of the *Txn1* gene, causing a substitution

785 of phenylalanine for leucine at residue 54.

786 (C) Functional sites and F54 were plotted in the 3D structure of human thioredoxin.

787 (D) Alignments of the sequence of Txn1 gene in mammals.

788

789 **Fig. 2.** Vacuolar degeneration appears during an epileptic period.

790 (A) Total seizure frequency of heterozygotes at each week (N = 8).

791 (B) Total seizure frequency of homozygotes at each week (N = 9).

792 (C) Histology at five weeks of age. At least three animals of each genotype were examined.

793 (D) The representative changes of inferior colliculus lesions from 2 to 9 weeks of age for each

794 genotype.

795

796 **Fig. 3.** MRI demonstrates transient high signals of T2-weighted images in the midbrain.

797 (A) The representative MRI findings at five weeks for each genotype (sagittal slices).

798 (B) The coronal slices of homozygotes at five weeks.

799 (C) The temporal changes of the brain lesion for each genotype from three to nine weeks.

800

801 **Fig. 4.** Neuronal and oligodendrocyte cell loss occurs in the midbrain.

802 (A) Protein levels of each cell in the midbrain at five weeks. Anti-NeuN, anti-Olig2,

803 anti-GFAP, and Iba1 bodies are markers of neurons, oligodendrocytes, astrocytes, and

804 microglia.

805 (B) IHC of the anti-NeuN-positive cells in the inferior colliculus for each genotype.

806   (C) Quantification of NeuN-positive cells. Quantification was performed by assessing five

807   randomly selected fields in the inferior colliculus and thalamus for each genotype.

808   (D) IHC of Olig2-positive cells in the inferior colliculus for each genotype.

809   (E) Quantification of Olig2-positive cells. Quantification was performed by assessing five

810   randomly selected fields in the inferior colliculus and thalamus for each genotype.

811   (F) Representative TEM image in the inferior colliculus of WT rat at four weeks.

812   (G) Magnification of the axon in (F). The yellow arrowhead indicates myelin sheath.

813   (H) Representative TEM image of the synapse in the inferior colliculus of WT rat at four

814   weeks. The yellow arrowhead indicates mitochondria with normal morphology, and the red

815   arrowhead indicates postsynaptic density, corresponding with the synaptic terminal.

816   (I) Magnification of the vacuole in (H). The yellow arrowhead indicates the myelin sheath,

817   and the red arrowhead indicates a microtubule.

818   (J) Representative TEM image of the axon in the midbrain of the heterozygous rat at four

819   weeks. The yellow arrowhead indicates mitochondria with sparse cristae.

820   (K) Representative TEM image of the synapse in the inferior colliculus of WT rat at four

821   weeks. The yellow arrowhead indicates mitochondria with sparse cristae, and the red

822   arrowhead indicates postsynaptic density.

823

824   **Fig.5.** *Txn1*-F54L shows decreased insulin-reducing activity.

825   (A) Thioredoxin/peroxiredoxin system.

826   (B) Protein level of thioredoxin in the brain, kidney, heart, and liver.

827   (C) Insulin-reducing activity determined using recombinant proteins.

828   (D) Insulin-reducing activity of the thalamus for each genotype.

829   (E) Insulin-reducing activity of the cortex for each genotype.

830

831   **Fig. 6.** Oxidative damage in the midbrain and susceptibility to cell death under oxidative

832   stress.

833   (A) Oxidative damage in the midbrain was assessed by IHC using anti-8-OHdG antibody and

834   4-HNE.

835   (B) Quantification of 8-OHdG-positive cells for each genotype.

836   (C) Quantification of 4-HNE-positive cells for each genotype.

837   (D) TUNEL assay of the paraffin-embedded sections including thalamus for each genotype.

838   (E) TUNEL assay of the primary fibroblasts derived from the WT and homozygous rats.

839   TUNEL staining was performed under the standard medium with or without 0.3 mM H_2O_2.

840   (F) Mitochondria and nucleus of primary neurons derived from WT and homozygous rats

841   treated with standard medium with or without 0.3 mM H_2O_2.

842

843   **Fig. 7.** *Txn1*-F54L rat generated by genome editing replicates the vacuolar degeneration in the

844   midbrain. The yellow arrowheads indicate vacuolar degeneration.

845   (A) Representative HE staining of the sagittal brain section at three weeks for each genotype.

846   (B) The coronal slices of the brain for each 500 mm section in the heterozygote.

847

848   **Supplementary Fig. 1.** Representative MRI for WT at five weeks.

849   (A) Sagittal slices.

850   (B) Coronal slices.

851   (C) Horizontal slices.

852

853   **Supplementary Fig. 2.** Representative MRI for heterozygote at five weeks.

854   (A) Sagittal slices.

855   (B) Coronal slices.

856   (C) Horizontal slices.

857

858 **Supplementary Fig. 3.** Representative MRI for homozygote at five weeks.
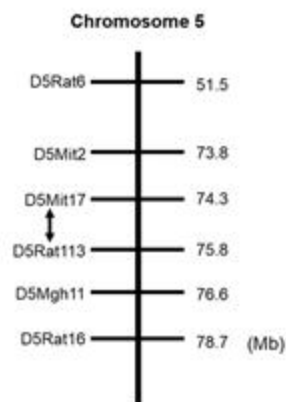
859 (A) Sagittal slices.
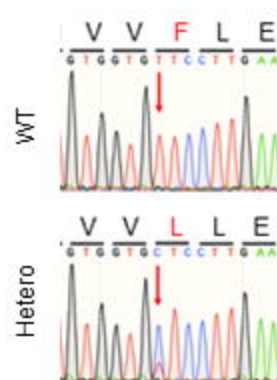
860 (B) Horizontal slices.

861

862 **Supplementary Fig. 4.** *Txn1*-F54L rat generated by genome editing replicates the transient

863 vacuolar degeneration in the midbrain from two to nine weeks of age.
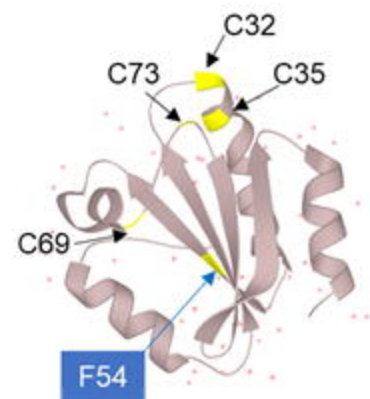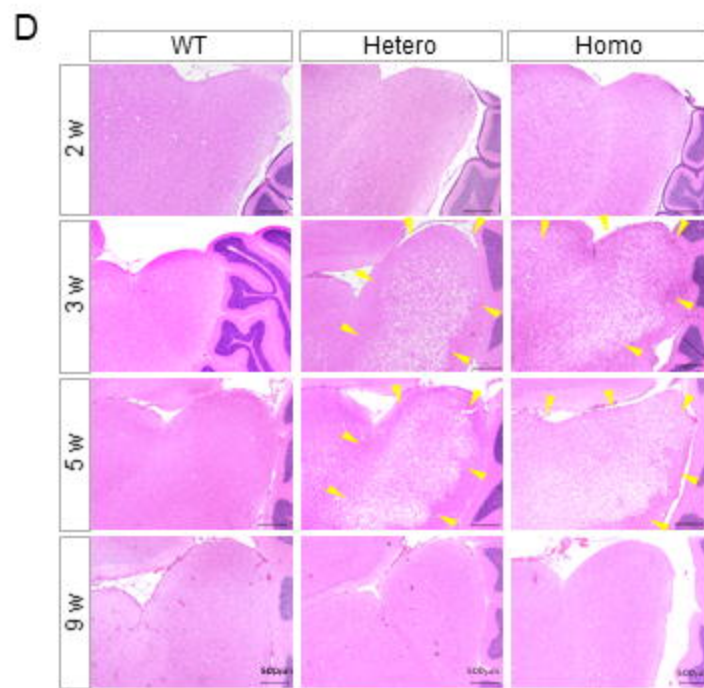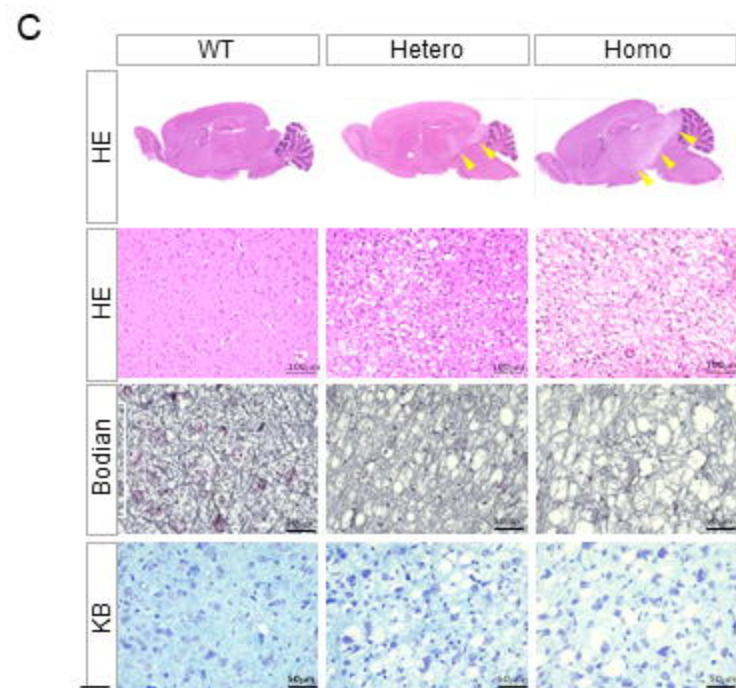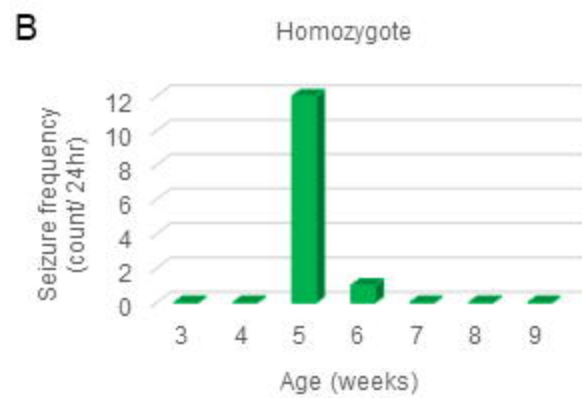
A

Chromosome 5

Marker	Position
D5Rat6	51.5
D5Mit2	73.8
D5Mit17	74.3
D5Rat113	75.8
D5Mgh11	76.6
D5Rat16	78.7 (Mb)

B

V V F L E
G T G G T G T T C C T T G A A
WT

V V L L E
G T G G T G C T C C T T G A A
Hetero

C

C32
C73
C35
C69
F54

D

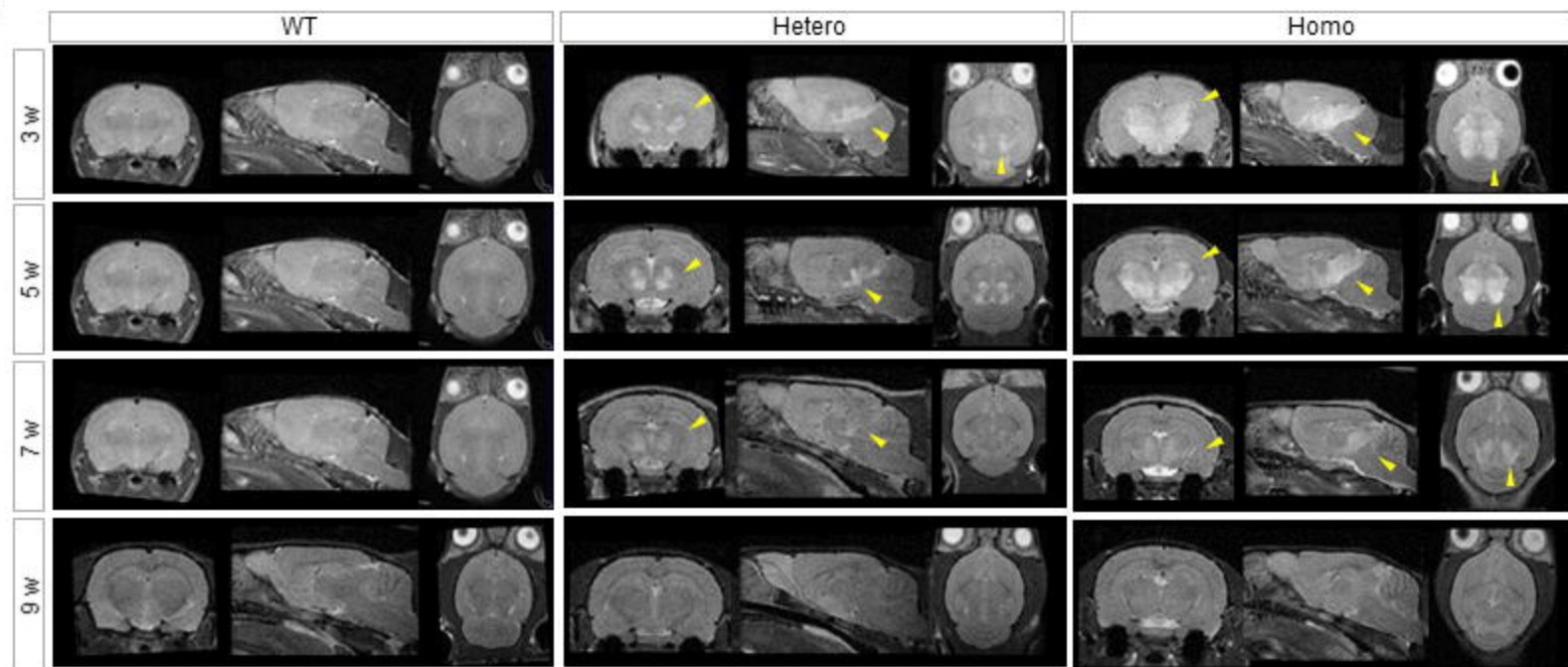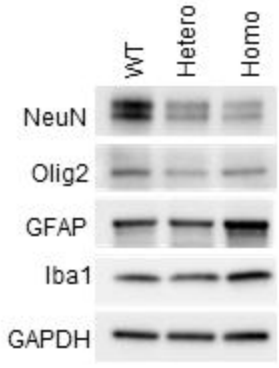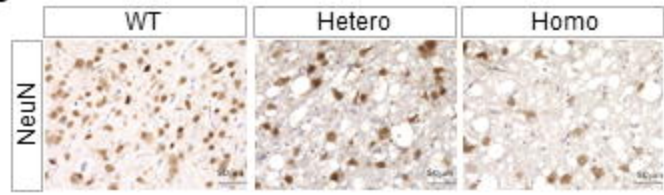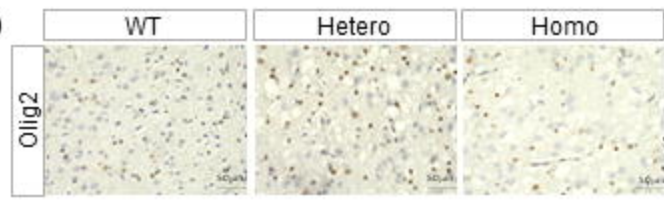|	1	32 35	54	69 73	105
Human	MVKQIESKTAFQEALDAAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVI F	LEVDVDDCQDVASE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATINELV
Rat	MVKLIESKEAFQEALAAAGDKLVVVDFSATW	CGPC	KMIKPFFHSLCDKYSNVV F	LEVDVDDCQDVAAD C EVK C	MPTFQFYKKGQKVGEFSGANKEKLEASITEFA
Mouse	MVKLIESKEAFQEALAAAGDKLVVVDFSATW	CGPC	KMIKPFFHSLCDKYSNVV F	LEVDVDDCQDVAAD C EVK C	MPTFQFYKKGQKVGEFSGANKEKLEASITEYA
Marmoset	MVKQIDSKDAFQEALDAAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVASE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATINEFV
Sheep	MVKQIESKYAFQEALNSAGEKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVAAE C EVK C	MPTFQFFKKGQKVSEFSGANKEKLEATINELI
Horse	MVKQIESKSAFQEALNSAGEKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVAAE C EVK C	MPTFQFFKKGQKVDEFSGANKEKLEATIKGLI
Cattle	MVKQIESKYAFQEALNSAGEKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVAAE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATIKELI
Pig	MVKQIESKYAFQEALNSAGEKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVASE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATINELI
Alpaca	MVKQIESKYAFQEALNSAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVAPE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATINELI
Ferret	MVKQIESKIAFQEALVGAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVASE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATINELI
Bat	MVKQIDSKDAFEEALTSAGSKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVASE C EVK C	MPTFQFFKNGKKVGEFSGANKEKLESTINEFI
Marmot	MVKQIESKEAFQEALNSAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVAAE C EVK C	MPTFQFFKKGQKVGEFSGANKEKLEATINEFV
Guinea pig	MVQQIESKEAFQEALNDAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVV F	LEVDVDDCQDVAAE C EVK C	MPTFQFFKKGKKVSEFSGANKEKLEATINELV
Chinchilla	MVQQIETKEAFQQALAAAGDKLVVVDFSATW	CGPC	KMIKPFFHSLSEKYSNVL F	LEVDVDDCQDVAAE C EVK C	MPTFQFFKKGEKVSEFSGANKEKLEATINELI

Secondary structure

β α β β α β α β β α

β strand α helix

A

Heterozygote

B

Homozygote

C

	WT	Hetero	Homo
HE			
HE			
Bodian			
KB			

D

	WT	Hetero	Homo
2 w			
3 w			
5 w			
9 w			

A

3w

WT

Hetero

Homo

B