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Targeting G-quadruplex DNA as cognitive function therapy for ATR-X syndrome

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38

39 **Abstract**

40 Alpha-thalassemia X-linked intellectual disability (ATR-X) syndrome is caused
41 by mutations in *ATR*X, which encodes a chromatin-remodeling protein. Genome-wide
42 analyses in mouse and human cells indicate that ATRX tends to bind G-rich sequences
43 with high potential to form G-quadruplexes. Here, we report that *Atrx* mutation induces
44 aberrant upregulation of Xlr3b expression in mouse brain, an outcome associated with
45 neuronal pathogenesis displayed by ATR-X model mice. We show that ATRX normally
46 binds to G-quadruplexes in CpG islands of the imprinted *Xlr3b* gene, regulating its
47 expression by recruiting DNA methyltransferases. Xlr3b binds dendritic mRNAs, and
48 its overexpression inhibits dendritic transport of CaMKII α mRNA, promoting synaptic
49 dysfunction. Notably, treatment with 5-ALA, which is converted into
50 G-quadruplex-binding metabolites, reduces RNA polymerase II recruitment and
51 represses *Xlr3b* transcription in ATR-X model mice. 5-ALA treatment also rescues
52 decreased synaptic plasticity and cognitive deficits seen in ATR-X model mice. Our
53 findings suggest a potential therapeutic strategy to target G-quadruplexes and decrease
54 cognitive impairment associated with ATR-X syndrome.
55

56 **Introduction**

57 ATR-X syndrome (OMIM Entry #301040) a severe intellectual disability, is
58 caused by *ATR*X mutations¹⁻³. *ATR*X encodes the switch/sucrose nonfermentable
59 (SWI/SNF)-like chromatin remodeling protein ATRX, which contains two signature
60 motifs. One is a plant homeodomain (PHD) designated the ATRX-DNMT3-DNMT3L
61 (ADD) domain, which binds histone H3 tails, specifically at H3K4me0K9me2/3⁴⁻⁶. The
62 other includes seven helicase subdomains that confer ATPase activity^{7,8}.

63 Genome-wide analysis combining chromatin immunoprecipitation with
64 next-generation sequencing (ChIP-seq) in both primary human erythroid cells and
65 mouse embryonic stem cells (ESCs) shows ATRX enrichment at G-rich variable number
66 tandem repeats (VNTRs), some of which form non-B DNA structures, including
67 G-quadruplexes⁹. ATRX functions as a part of a histone chaperone complex that
68 deposits the histone variant H3.3 onto pericentromeric heterochromatin and telomeres
69 collaborating with death domain-associated protein (DAXX) in HeLa cells¹⁰ and
70 murine ESCs^{11,12}. ATRX/H3.3 co-localization also occurs on the DNA-methylated
71 allele of many imprinted genes and is associated with differentially methylated regions
72 (DMRs) in mouse ESCs¹³. Some imprinted genes show upregulated expression in
73 forebrain of *Atrx* conditional knockout (cKO) mice^{14,15}, suggesting that ATRX silences
74 the active allele. Moreover, expression of the autism-related gene *Nlgn4* significantly
75 decreases in forebrain of *Atrx* cKO mice¹⁶. Imprinting loss in neurons leads to various
76 mental retardation syndromes, including Prader-Willi and Angelman syndromes¹⁷.
77 However, the relationship between abnormal expression and cognitive dysfunction in
78 ATR-X syndrome remains unclear.

79

Results

ATRX regulates *Xlr3b* expression in mouse brain

Atrx^{ΔE2} mice, which are engineered to lack *Atrx* exon 2, show cognitive defects, among other phenotypes¹⁸, and express a mutant protein that corresponds to a variant with an Arg37Stop (R37X) mutation in exon 2 seen in human ATR-X syndrome¹⁹⁻²¹. Moreover, *Atrx*^{ΔE2} mice show 80% reduction in ATRX protein levels^{18, 19}, similar to outcomes seen in 27 individuals with ATR-X syndrome⁸. We employed DNA microarrays to assess transcriptional profiles at post-natal day (P) 90 in hippocampus of wild-type (WT) and *Atrx*^{ΔE2} mice. To identify differentially-expressed genes, we used an algorithm combining false discovery rate (FDR) and fold-change in expression, and identified 31 genes (8 upregulated and 23 downregulated) in WT versus *Atrx*^{ΔE2} samples. Among them was *Atrx* itself, which was downregulated in *Atrx*^{ΔE2} mice (**Supplementary Table 1** for list of genes with an FDR < 0.05 and a log2 fold-change of > 0.5 or < -0.5). Among genes markedly upregulated in *Atrx*^{ΔE2} mice were a member of the lymphocyte regulated (Xlr) gene family, *Xlr3a* and the imprinted gene *Xlr3b*²²⁻²⁴ (**Fig. 1a**, FDR < 0.05 and log2 fold-change of > 0.5). *Xlr3a* and *Xlr3b* genes show 94% protein similarity, and DNA microarrays are limited in their ability to distinguish related factors. Thus, we carried out reverse transcription-PCR (RT-PCR) analysis with a common forward primer and subtype-specific reverse primers (**Supplementary Fig. 1a**). Primer efficiency was confirmed by amplifying respective cDNAs (**Supplementary Fig. 1b**), and distinct *Xlr3a* and *Xlr3b* amplicons were detected on gels (**Supplementary Fig. 1c**, lanes 1 and 2). Then, using these primers, we detected *Xlr3b*, but not *Xlr3a* mRNA, in prefrontal cortex, hippocampus, hypothalamus and cerebellum of WT and *Atrx*^{ΔE2} mice (**Supplementary Fig. 1c**). Interestingly, quantitative RT-PCR (RT-qPCR) indicated *Xlr3b* transcript upregulation in some brain areas, including hippocampus, of *Atrx*^{ΔE2} mice, but not in peripheral tissues (**Fig. 1b**).

Next, in order to investigate changes in Xlr3 protein expression, we generated an Xlr3 antibody. To confirm its specificity, we performed Xlr3 knockout (KO)-validation using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 in cultured hippocampal neurons. To do so, we transduced cultured hippocampal neurons with lentivirus harboring U6gRNA-Cas9-2A-GFP plus guide Xlr3 sgRNA (Xlr3-Cas9-GFP) or control lentivirus expressing GFP. Microscopy analysis revealed loss of Xlr3 immunoreactivity in MAP2-positive neurons infected with Xlr3-Cas9-GFP but not control GFP virus (**Supplementary Fig. 1d**). We also confirmed *Xlr3b* KO efficacy by RT-qPCR in cultured hippocampal neurons (**Supplementary Fig. 1e**).

Western blot analysis with the Xlr3 antibody also showed significantly decreased protein expression in KO relative to WT cultured hippocampal neurons (Supplementary Fig. 1f), confirming antibody specificity. Next, we used the Xlr3 antibody to examine Xlr3 protein expression in *Atrx*^{ΔE2} mouse prefrontal cortex, hippocampus, hypothalamus and cerebellum tissues by western blot, and observed significant increases in Xlr3 protein levels in brain of *Atrx*^{ΔE2} relative to WT mice (Fig. 1c).

CpG island (CGI) methylation by DNA methyltransferases (DNMTs) generally represses transcription²⁵, and cKO mice lacking *Dnmt1* or both *Dnmt1* and *Dnmt3a* in forebrain neurons show increased *Xlr3b* expression^{26, 27}. Although ATRX lacks a canonical DNA methyltransferase motif, human *ATRX* mutations are associated with altered DNA methylation patterns at various genomic loci²⁸. Moreover, *Xlr3* gene CGIs display similar sequences, and a sequence potentially forming a G-quadruplex structure is specific to *Xlr3b* (Supplementary Fig. 2a). Thus, we asked whether ATRX recruits DNMTs to methylate *Xlr3b* CGIs. To do so, we employed bisulfite sequencing to compare DNA methylation levels in *Xlr3b* CGIs of whole brain samples from WT or *Atrx*^{ΔE2} mice. Relative to WT mice, *Atrx*^{ΔE2} mice showed significantly decreased DNA methylation at *Xlr3b* CGIs, while we observed no difference in DNA methylation levels in CGIs of other Xlr3 subfamily genes between genotypes (Fig. 1d, Supplementary Fig. 2b). Circular dichroism (CD) spectra of a synthetic oligodeoxynucleotide containing sequences potentially forming a G-quadruplex in *Xlr3b* CGIs (Xlr3b-ODN) showed a spectrum characteristic of parallel G-quadruplexes in 10 and 100 mM KCl, with maximum absorbance at 265 nm and minimum at 240 nm (Supplementary Fig. 3a). This conformational change in physiological conditions (100 mM KCl) was reflected by decreased DNA mobility in gel shift assays performed using native polyacrylamide gel electrophoresis (PAGE) gels (Supplementary Fig. 3b). We then undertook dimethyl sulphate (DMS) footprinting of Xlr3b-ODN to assess formation of an intramolecular parallel G-quadruplex consisting of a core of three stacked G-quartets and three loops (Fig. 1e, Supplementary Fig. 3c). This analysis confirmed that *Xlr3b* CGI sequences can form parallel G-quadruplex structures.

To assess how ATRX regulates *Xlr3b*, we performed luciferase reporter assays. To do so, we first cloned three different *Xlr3b* genomic sequences into a pGL3 luciferase reporter vector. They included: a 2.0 kb fragment (designated pGL3-2K) upstream of the *Xlr3b* ATG initiation codon; a pGL3-2K deletion mutant lacking G-quadruplex-forming sequences (pGL3-2KΔG4); and a 1 kb *Xlr3b* upstream sequence (pGL3-1KΔCGI) that lacks CGIs. Mouse neuroblastoma Neuro-2a cells transfected

with pGL3-2K showed significantly reduced luciferase activity when co-transfected with ATRX; conversely, luciferase activity relative to controls increased in cells co-transfected with ATRX shRNA (**Fig. 1f**). However, cells transfected with either pGL3-2KΔG4 or pGL3-1KΔCGI showed activity comparable to cells co-transfected with ATRX or ATRX shRNA, suggesting that ATRX regulates *Xlr3b* through the G-quadruplex structure (**Fig. 1f**). We confirmed ATRX shRNA knockdown efficacy by immunoblotting for endogenous ATRX protein in Neuro-2a cells (**Supplementary Fig 4a**). Moreover, Neuro-2a cells co-transfected with pGL3-2K plus DNMTs (DNMT1 or DNMT3A) showed significantly decreased reporter activity relative to respective controls (**Fig. 1g**). However, cells co-transfected with pGL3-2K plus DAXX or H3.3 showed comparable reporter activity. Importantly, cells co-transfected with pGL3-2KΔG4 plus DNMTs showed no change in reporter activity relative to pGL3-2KΔG4 alone. Finally, we methylated pGL3-2K, pGL3-2KΔG4 and pGL3-1KΔCGI constructs using CpG DNA methyltransferase *M.SssI* and assessed the luciferase activity. We observed decreased luciferase activity in methylated pGL3-2K and pGL3-2KΔG4, not in methylated pGL3-1KΔCGI, supporting the idea that CGI methylation inhibits *Xlr3b* expression (**Fig. 1g**).

To determine whether ATRX and interacting factors target *Xlr3b* CGIs in mouse hippocampus, we performed ChIP-qPCR with an ATRX antibody. We confirmed that hippocampal samples from *Atrx*^{ΔE2} mice contained detectable levels of ATRX protein: those lysates showed an 80% reduction in ATRX protein levels relative to lysates from WT mice, and the ATRX antibody recognized both WT and mutant ATRX protein as described in^{18, 19} (**Supplementary Fig. 4b**). Co-immunoprecipitation in hippocampal lysates revealed that ATRX interacts with DNMT1, DNMT3A, DAXX and H3.3 from both WT and *Atrx*^{ΔE2} mice, and relatively low amounts of DNMT1, DNMT3A, H3.3 and DAXX were detected in immunoprecipitated samples of *Atrx*^{ΔE2} mice (**Supplementary Fig. 4c**). We detected substantial ATRX enrichment at *Xlr3b* CGIs containing G-quadruplexes based on ChIP analysis with primers targeting *Xlr3b* region R1 in chromatin isolated from WT mouse hippocampus, while ATRX interaction with chromatin at this site was greatly decreased in *Atrx*^{ΔE2} hippocampus (**Fig. 1h**). Levels of DNMT1, DNMT3A, DAXX and H3.3 also significantly decreased in *Atrx*^{ΔE2} relative to WT chromatin in the R1 region. Also, quantitative profiling of ATRX, DNMTs, DAXX and H3.3 across an unrelated R2 region revealed small peaks, demonstrating enrichment of these proteins at *Xlr3b* CGIs (**Fig. 1h**). These observations suggest overall that ATRX binds to parallel G-quadruplexes in *Xlr3b* CGIs along with DNMTs, DAXX and H3.3, where it regulates *Xlr3b* gene expression.

188

189 Neuronal RNA granules contain Xlr3b

190 We next undertook a proteomic screen to search for Xlr3 interaction partners by
191 performing liquid chromatography-tandem mass spectrometry (LC-MS/MS) of proteins
192 pulled down from lysates of P60 WT mouse brain with an Xlr3 antibody. LC-MS/MS
193 analysis revealed an endogenous Xlr3 complex containing multiple components,
194 including actin/myosin-related proteins, dynein motor complex (DYNLL, DYNLRB,
195 DYNLT and DYNC1LI2) proteins, ribonucleoproteins (hnRNP A/B and hnRNP D),
196 40S ribosomal proteins (RPS10 and RPS25), and others (**Fig. 2a, Supplementary**
197 **Table 2**). We then confirmed association of purified GST-Xlr3b protein with hnRNP
198 A/B, hnRNP D, DYNLL and DYNC1LI2 by *in vitro* pull-down assays in mouse
199 hippocampal lysates (**Fig. 2b**). Purified Xlr3b protein did not bind F-actin, and its
200 presence did not alter actin polymerization (**Supplementary Fig. 5a, 5b**). To confirm
201 interactions *in vivo*, we performed immunoprecipitation of mouse hippocampal extracts
202 with an Xlr3 antibody followed by immunoblotting with hnRNP A/B, hnRNP D,
203 DYNLL, or DYNC1LI2 antibodies and observed co-precipitation of all with Xlr3 (**Fig.**
204 **2c**).

205 To determine Xlr3 cellular localization, we undertook immunofluorescence of
206 mature MAP2-positive cultured neurons with an Xlr3 antibody. Xlr3 was localized to
207 nuclei and perinuclear areas and seen in granules in MAP2-positive dendrites and in
208 synaptic synaptophysin-positive puncta (**Fig. 2d**). Xlr3-positive structures of this type
209 were also observed in MAP2-positive dendrites in mouse hippocampal CA1 analyzed at
210 P60 (**Fig. 2e**). In dendrites, Xlr3-positive puncta co-localized with hnRNP A/B and
211 cytotoxic granule-associated RNA binding protein 1 (TIA1), both found in RNA
212 granules²⁹, and with SYTO14-visualized RNA. Xlr3-positive granules did not
213 co-localize with DCP1a, a marker of RNA-processing bodies²⁹ (**Fig. 2f**).

214 Xlr3b exhibits a predicted nuclear localization signal (NLS) at amino acid (AA)
215 residues 2-11; residues 74-200 also constitute a conserved Cor1/Xlr/Xmr region (Cor1
216 domain). To assess function of those sequences, we transfected Neuro-2a cells with
217 FLAG-tagged Xlr3b or similarly-tagged deletion mutants shown in Figure 2g and
218 analyzed their location by confocal microscopy (**Fig. 2g**). Based on FLAG expression,
219 full length (FL)-Xlr3b significantly co-localized with TIA1 in nuclear regions compared
220 to that of non-transfected cells. FL-Xlr3b also accumulated with hnRNP A/B in nuclei,
221 but not with DCP1a (**Fig. 2g, Supplementary Fig. 6a, 6b**). Cells transfected with
222 constructs lacking AA 2-11 (Δ NLS) or 74-123 (Δ 74-123) showed partial loss of Xlr3b
223 nuclear localization, but localization of the interacting proteins TIA1 and hnRNP A/B

224 was unchanged. These localization patterns were similar to those seen in
225 FL-Xlr3b-transfected cells (**Fig. 2g, Supplementary Fig. 6a, 6b**). Deletion of AA
226 124-200 (Δ 124-200) caused loss of nuclear Xlr3b expression and localization of tagged
227 protein in cytoplasmic ubiquitin-positive inclusion-like structures, in which DCP1a,
228 TIA1 and hnRNP A/B were also colocalized. In addition, the Δ 158-170 mutant was
229 distributed similarly to the Δ 124-200 mutant (**Fig. 2g, Supplementary Fig. 6a, 6b and**
230 **6c**). Importantly, nuclear localization of TIA1 and hnRNP A/B in cells transfected with
231 Δ 124-200 or Δ 158-170 constructs was very similar to patterns observed in
232 non-transfected cells (**Fig. 2g, Supplementary Fig. 6a**).

233 To confirm interactions of Xlr3b AA 158-170 with RNA binding proteins
234 (RBPs), we used Xlr3b inhibitory peptide (XIP), a 29-AA peptide containing Xlr3b AA
235 158-170 plus the cell-permeable antennapedia homeodomain (ANTP) peptide
236 (**Supplementary Fig. 7a**). When FL-Xlr3b-transfected cells were treated with XIP
237 (1 μ M for 4 h), TIA1 did not accumulate in the nucleus (**Supplementary Fig. 7b**). We
238 confirmed that XIP, but not the ANTP control peptide, bound to RBPs, TIA1 and
239 hnRNP A/B using pull-down assays of mouse hippocampal lysates with an ANTP
240 antibody (**Supplementary Fig. 7c**). Immunoprecipitation confirmed that FLAG-tagged
241 FL, Δ 74-123, Δ 124-200 and Δ 158-170 mutants were immunoprecipitated with a FLAG
242 antibody. Immunoblotting with TIA1 and hnRNP A/B antibodies detected
243 immunoreactive TIA1 and hnRNP A/B bands in samples transfected with FL and
244 Δ 74-123, but not in Δ 124-200 and Δ 158-170. Remarkably, interactions with TIA1 and
245 hnRNP A/B were not seen in FL-Xlr3b-transfected cells treated with 1 μ M XIP for 4 h
246 before immunoprecipitation (**Fig. 2h**), suggesting that Xlr3b residues 158-170 are
247 critical for RBP binding. Ubiquitination of Xlr3b Δ 124-200 and Δ 158-170 may prevent
248 binding to RBPs, regardless of co-localization³⁰. Thus, to assess potential Δ 124-200
249 and Δ 158-170 ubiquitination, we performed immunoprecipitation of lysates of cells
250 transfected with FLAG-tagged FL, Δ 74-123, Δ 124-200 and Δ 158-170 mutants with a
251 FLAG antibody followed by immunoblotting with a ubiquitin antibody. We confirmed
252 ubiquitination in immunoprecipitants of constructs Δ 124-200 and Δ 158-170, but
253 ubiquitination was little seen in FL or Δ 74-123 proteins (**Supplementary Fig. 6d**).

254 Phylogenetic analysis identified the FAM9 gene family as human Xlr
255 orthologues, and FAM9 proteins are predicted to have a Cor1 domain³². BLAST
256 searches indicated that Xlr3b residues 158-170 are highly conserved in Cor1 domains of
257 FAM9 family proteins, including FAM9A and FAM9B (**Supplementary Fig. 6e**). We
258 observed nuclear localization of FLAG-tagged FAM9A overexpressed in Neuro-2a cells,
259 while a similar construct lacking residues analogous to Xlr3b 158-170 was found in

260 cytoplasmic inclusion body-like structures in a pattern similar to Xlr3b Δ 158-170
261 transfected cells (**Supplementary Fig. 6e**).

262

263 **Aberrant Xlr3b expression in neurons decreases synaptic plasticity by inhibiting** 264 **transport of dendritic CaMKII α mRNA**

265 In neurons, RNA granules are rapidly transported to dendrites, a process
266 regulated by motor proteins³². Some RBPs including hnRNP A/B recognize a specific
267 *cis*-acting element termed the hnRNP A2 response element (A2RE) in mRNA 3'
268 untranslated regions (3' UTRs); these include Ca²⁺/calmodulin-dependent protein kinase
269 II α (CaMKII α), activity-regulated cytoskeleton-associated protein (Arc) and
270 brain-derived neurotrophic factor (BDNF)³³. Because Xlr3b interacts with the dynein
271 motor complex and RBPs, we hypothesized that Xlr3b regulates dendritic mRNA
272 transport via a dynein-mediated transport mechanism.

273 To test this hypothesis, we undertook RNA immunoprecipitation in mouse
274 hippocampal lysates to determine whether an endogenous Xlr3 complex associates with
275 dendritic mRNAs. After immunoprecipitation with an Xlr3 antibody, we isolated total
276 RNA from precipitates and analyzed it by qPCR with primers specific to CaMKII α , Arc
277 and BDNF mRNA 3' UTRs. Immunoprecipitates contained all three mRNAs, and XIP
278 treatment (1 μ M for 2h) of cell lysates significantly blocked these interactions (**Fig. 3a**).

279 Atrx ^{Δ E2} mice show cognitive impairment and CaMKII α dysfunction^{18,19}. Thus
280 we focused on dendritic CaMKII α mRNA. To determine whether Xlr3b protein is
281 co-transported into dendrites with CaMKII α mRNA, we used dual-color time-lapse
282 imaging with mCherry-Xlr3b and GFP-MS2-labeled CaMKII α mRNA (GFP-CaMKII α
283 3' UTR)³⁴. mCherry-Xlr3b co-localized with the GFP-CaMKII α 3' UTR, and both were
284 co-transported to dendrites of cultured neurons (**Fig. 3b**). By contrast, co-localization of
285 mCherry-Xlr3b mutants (Δ 124-200 and Δ 158-170) with GFP-CaMKII α 3' UTR
286 immunofluorescence was rarely seen (**Supplementary Fig. 8a**). To analyze dynamics
287 of dendritic CaMKII α mRNA transport, we investigated movement of the
288 GFP-CaMKII α 3' UTR in proximal dendrites (20-100 μ m away from the cell body).
289 Granules moved in anterograde (white arrowhead) and retrograde (yellow arrowhead)
290 directions (**Fig. 3c, top panel, left**). **Fig. 3c** (top panel, right) shows a representative
291 kymograph corresponding to 15-min of live imaging generated from a proximal
292 dendrite that GFP-CaMKII α 3' UTR (**also see Supplementary Video 1**). Imaging of
293 GFP-CaMKII α 3' UTR movement revealed that approximately half of the observed
294 CaMKII α mRNA granules were immobile, and the few mobile granules showed
295 bidirectional movement in cultured WT neurons. The number of bidirectionally-mobile

CaMKII α mRNA granules significantly decreased in cultured neurons of transgenic mice overexpressing Xlr3b driven by the neuron-specific Thy1.2 promoter (Thy1-Xlr3b TG mice; **Supplementary Fig. 9a-9d**), relative to effects seen in WT neurons. Moreover, in cultured neurons of Atrx ^{$\Delta E2$} mice, the number of bidirectionally-mobile CaMKII α mRNA granules significantly decreased. Xlr3b-shRNA transfection or XIP treatment antagonized decreases in dendritic CaMKII α mRNA transport seen in Atrx ^{$\Delta E2$} neurons, suggesting that Xlr3b acting through RBPs inhibits CaMKII α mRNA transport (**Fig. 3c, bottom**). We confirmed efficacy of Xlr3b knockdown by immunoblotting of endogenous Xlr3 protein in cultured neurons (**Supplementary Fig 9e**). In addition, dynamics of CaMKII α mRNA transport in distal dendrites (100-200 μ m from the cell body) was similar to that seen in proximal dendrites: bidirectionally-mobile CaMKII α mRNA granules significantly decreased in Thy1-Xlr3b TG and Atrx ^{$\Delta E2$} neurons relative to effects seen in WT neurons. Furthermore, Xlr3b-shRNA transfection or XIP treatment rescued decreases in dendritic CaMKII α mRNA transport seen in Atrx ^{$\Delta E2$} neurons (**Supplementary Fig. 8b, Supplementary Video 2**).

RNA granules localize beneath postsynaptic sites in dendrites, and CaMKII α mRNA is located in dendrites and active postsynaptic regions³³. Indeed, the number of double-positive protein puncta containing the GFP-CaMKII α 3' UTR and the postsynaptic marker PSD95 significantly decreased in cultured neurons of Thy1-Xlr3b TG and Atrx ^{$\Delta E2$} mice relative to the number seen in WT mice, and cultured neurons from Atrx ^{$\Delta E2$} mice transfected with Xlr3b shRNA or treated with XIP showed a significant increase in the number of double-positive puncta (**Fig. 3d**). Next, we performed western blot analysis for CaMKII α protein in synaptosomal membrane fractions, containing PSD95 isolated from cultured hippocampal neurons (**Supplementary Fig. 9f**). CaMKII α protein was decreased in the synaptosomal membrane fractions from cultured neurons of Thy1-Xlr3b TG and Atrx ^{$\Delta E2$} mice relative to that seen in WT mice. XIP treatment in Atrx ^{$\Delta E2$} neurons significantly increased in the synaptosomal CaMKII α protein levels without changes in both CaMKII α and PSD95 levels in total cell lysate fractions between groups (**Supplementary Fig. 9f**).

Dendritic CaMKII α mRNA translation is regulated by synaptic activity, and postsynaptic CaMKII α protein regulates hippocampal long-term potentiation (LTP), which is critical for learning and memory³⁵⁻³⁸. To assess a potential effect on LTP, we undertook electrophysiological analysis of brain slices from hippocampal CA1. Consistent with our previous study¹⁹, we observed markedly reduced high frequency stimulation (HFS)-induced LTP in Atrx ^{$\Delta E2$} mice, which was significantly rescued by a 2 h bath application of XIP to Atrx ^{$\Delta E2$} mouse samples, but not following application of an

332 ANTP control peptide, suggesting that impaired LTP seen in *Atrx*^{ΔE2} mice is in part
333 Xlr3b-dependent (**Fig. 3e**). Notably, Thy1-Xlr3b TG mice also showed impaired
334 hippocampal LTP (**Supplementary Fig. 9g**). We observed no significant difference in
335 basic electrophysiological properties, among them input-output relations and
336 paired-pulse ratio, between groups (**Supplementary Fig. 9h**). LTP induction, which
337 strengthens synapses, results in CaMKII phosphorylation throughout the dendritic area,
338 suggesting widespread CaMKII activation including in spines^{39, 40}. In WT mice, levels
339 of CaMKIIα phosphorylation significantly increased following hippocampal LTP
340 induction. Consistent with LTP impairment, CaMKIIα phosphorylation did not increase
341 following LTP induction in hippocampus of *Atrx*^{ΔE2} and Thy1-Xlr3b TG mice. Notably,
342 bath application of XIP, but not ANTP, significantly restored LTP-induced CaMKIIα
343 phosphorylation in the *Atrx*^{ΔE2} hippocampus (**Supplementary Fig. 9i**).

344 To assess whether Xlr3b overexpression affects cognitive function, we employed
345 a novel object recognition task. In training trials, we observed no differences in the
346 discrimination index using the same object between WT and Thy1-Xlr3b TG mice (data
347 not shown). After a 24 h retention interval between trial and test sessions, Thy1-Xlr3b
348 TG mice showed a significantly lower discrimination index for a novel object than did
349 WT mice (**Supplementary Fig. 9j**). To assess contextual memory, we undertook a
350 fear-conditioned passive avoidance task and observed no significant differences in
351 latency to enter a dark room in the absence of foot shock between WT and Thy1-Xlr3b
352 TG mice (data not shown). However, latency to enter the dark compartment was
353 markedly decreased 1-week after foot shock in Thy1-Xlr3b relative to WT mice
354 (**Supplementary Fig. 9k**). We then used a Y-maze to test spatial working memory and
355 observed that Thy1-Xlr3b mice showed impairment based on the percentage of
356 alternation behaviors relative to WT mice without a change in the total number of arm
357 entries (**Supplementary Fig. 9l**).

359 **5-ALA treatment reduces RNA polymerase II recruitment and represses *Xlr3b*** 360 **transcription by modifying G-quadruplex structure.**

361 G-quadruplex-binding small molecules such as porphyrin function as
362 transcriptional repressors⁴¹. Thus, we asked whether *Xlr3b* repression via porphyrin
363 binding its G-quadruplex structure would antagonize synaptic dysfunction and cognitive
364 deficits seen in *Atrx*^{ΔE2} mice. To do so, we first assessed intracellular generation of
365 porphyrins following administration of 5-aminolevulinic acid (5-ALA), which can be
366 metabolized to porphyrins, protoporphyrin IX (PpIX) and hemin⁴² (**Fig. 4a**). We
367 confirmed PpIX and hemin binding to Xlr3b-ODN G-quadruplexes using UV melting

analysis. The ΔT_m for PpIX and hemin was 2.0 and 2.3°C, respectively, indicating that PpIX and hemin both moderately bind *Xlr3b* G-quadruplexes structure. The ΔT_m for TMPyP4, a well-known G-quadruplex binding ligand, was higher than 4°C (Supplementary Fig. 10a). To determine whether 5-ALA treatment alters *Xlr3b* gene expression, we transfected Neuro-2a cells with the pGL3-2K reporter, treated cells with varying concentrations of 5-ALA or TMPyP4, and assessed effects of *Xlr3b* expression based on luciferase assays. As expected, treatment with 5-ALA or TMPyP4 reduced luciferase activity dose-dependently but had no effect on cells transfected with pGL3-2KΔG4 (Fig. 4b, Supplementary Fig. 10b). Decreased luciferase activity in 5-ALA treated cells was efficiently rescued by co-treatment with the 5-ALA dehydratase inhibitor succinylacetone (Fig. 4c). Next, we asked whether treatment with G-quadruplex ligands would inhibit aberrant *Xlr3b* gene expression seen in *Atrx*^{ΔE2} mouse brain. To do so, we administered 5-ALA (p.o. daily from P30 to P90) or TMPyP4 (i.p. twice weekly from P30 to P90). Either treatment significantly blocked increases in hippocampal *Xlr3b* mRNA and *Xlr3b* protein seen in vehicle-treated *Atrx*^{ΔE2} mice (Fig. 4d and 4e, Supplementary Fig. 10c and 10d).

To investigate mechanisms underlying these effects, we undertook bisulfite sequencing to compare DNA methylation levels. *Xlr3b* CGIs in hippocampus and prefrontal cortex of *Atrx*^{ΔE2} mice showed significantly decreased DNA methylation relative to WT mice, an effect unchanged by 5-ALA treatment (Fig. 4f, Supplementary Fig. 10e). Next, we performed ChIP-qPCR of ATRX and RNA polymerase II (Pol II) within the *Xlr3b* gene, as G-quadruplex structures hinder Pol II passage^{43, 44}. The prediction of quadruplex structures within the *Xlr3b* gene was supported by the G-quadruplex analysis tool QGRS Mapper⁴⁵ (Fig. 4g, top). In WT mouse hippocampus, ATRX and Pol II accumulated at *Xlr3b* CGIs containing G-quadruplexes, based on analysis using primers targeting amplicon 1, but little signal was seen at amplicons 2, 3 and 4 (Fig. 4g, bottom). However, in *Atrx*^{ΔE2} mouse, we detected an elevated Pol II signals across all amplicons in the *Xlr3b* gene body as well as CGIs, indicating enhanced *Xlr3b* transcription. Furthermore, elevated Pol II signals were significantly attenuated following 5-ALA treatment (Fig. 4g, bottom). These results suggest that the porphyrin metabolites PpIX and hemin, which are derived from 5-ALA, bind *Xlr3b* G-quadruplex structures, partially reducing Pol II recruitment.

Treatment with G-quadruplex ligands counteracts cognitive deficits seen in *Atrx*^{ΔE2} mice.

403 Next, we asked whether 5-ALA treatment rescued decreases in dendritic
404 CaMKII α mRNA transport seen in Atrx ^{Δ E2} neurons. Imaging of GFP-CaMKII α 3' UTR
405 movement revealed that the decreased number of bidirectionally-mobile CaMKII α
406 mRNA granules was significantly rescued in 5-ALA treated Atrx ^{Δ E2} neurons (7 days,
407 1 μ M) at proximal and distal dendrites (**Fig. 5a, Supplementary Fig. 11a**). The
408 decreased number of double-positive protein puncta containing GFP-CaMKII α 3' UTR
409 and PSD95 in Atrx ^{Δ E2} neurons was also significantly rescued (**Fig. 5b**). Moreover,
410 reduced HFS-induced LTP in hippocampus of vehicle-treated Atrx ^{Δ E2} mice was
411 significantly restored in 5-ALA-treated Atrx ^{Δ E2} mice (p.o. daily from P30 to P90)
412 without a change in basic electrophysiological properties (**Fig. 5c, Supplementary Fig.**
413 **11b**). Finally, 5-ALA treatment significantly blocked decreased CaMKII α protein level
414 at synaptosomal membrane fractions in cultured hippocampal neurons from Atrx ^{Δ E2}
415 mice, and restored LTP-induced CaMKII α phosphorylation in hippocampus of Atrx ^{Δ E2}
416 mice (**Supplementary Fig. 11c**).

417 We next administered 5-ALA (p.o. daily from P30 to P90) or TMPyP4 (i.p.
418 twice weekly from P30 to P90) to Atrx ^{Δ E2} mice and subsequently assessed
419 memory-related behaviors. We observed no differences in a novel object recognition
420 task in the discrimination index using the same object in all groups in training trials
421 (data not shown). After a 24h retention interval between trial and test sessions, Atrx ^{Δ E2}
422 mice showed a significantly lower discrimination index for a novel object than did WT
423 mice. The discrimination index for the novel object in Atrx ^{Δ E2} mice treated with 5-ALA
424 or TMPyP4 was significantly higher than that seen in vehicle-treated groups (**Fig. 5d,**
425 **Supplementary Fig. 11d**). In a fear-conditioned passive avoidance task, we observed
426 no significant differences in latency to enter a dark room in the absence of foot shock in
427 all groups (data not shown). However, latency to enter a dark compartment was
428 markedly decreased 1-week after foot shock in Atrx ^{Δ E2} relative to WT mice. 5-ALA or
429 TMPyP4 administration also significantly rescued reduced latency time (**Fig. 5e,**
430 **Supplementary Fig. 11e**). In a Y-maze test, Atrx ^{Δ E2} mice showed impairment based on
431 the percentage of alternation behaviors relative to WT mice without a change in the total
432 number of arm entries. 5-ALA or TMPyP4 administration significantly increased the
433 percentage of spontaneous alternation behaviors in Atrx ^{Δ E2} mice (**Fig. 5f,**
434 **Supplementary Fig. 11f**). Many patients with ATR-X syndrome exhibit abnormal
435 social behaviors, especially shyness and social withdrawal⁴⁶. Interestingly, in
436 interactions with WT mice, Atrx ^{Δ E2} mice showed enhanced passivity, greater escape
437 duration and decreased active social behaviors, such as following and sniffing. These
438 changes were dramatically ameliorated in Atrx ^{Δ E2} mice (**Supplementary Fig. 11g**).

439 Acute administration of a single 5-ALA dose (10mg/kg, p.o.) did not ameliorate
440 impaired learning and memory-related behaviors in $Atrx^{\Delta E2}$ mice (data not shown).

441 To confirm that TMPyP4 was present in brain, we measured TMPyP4
442 fluorescence levels, as described in ⁴⁷. Chronic intraperitoneal injection of TMPyP4 (i.p.
443 twice weekly from P30 to P90) in mice increased fluorescence levels in some tissues,
444 including brain, suggesting that treatment allows intracerebral transferability and
445 enables TMPyP4 accumulation in brain tissue (**Supplementary Fig. 11h**). However, we
446 stopped chronic TMPyP4 administration on day 60 due to toxicity, as evidenced by
447 reduced body weight (**Supplementary Fig. 11i**). Unlike the case with TMPyP4, 5-ALA
448 pharmacokinetics and biodistribution, including in brain, has been reported in humans ⁴⁸
449 and rodents ⁴⁹. We confirmed that 5-ALA crosses the blood brain barrier after oral
450 administration. In mouse brain, 5-ALA (3mg/kg, p.o.) levels peaked at 30min and then
451 decreased to basal levels by 24h after administration (**Supplementary Fig. 11j**).

452 To investigate expression of other genes underlying these effects, we performed
453 DNA microarray analysis of RNA extracted from hippocampus of P90 WT, $Atrx^{\Delta E2}$, or
454 5-ALA treated $Atrx^{\Delta E2}$ mice, and focused on 31 genes differentially expressed (8
455 upregulated and 23 downregulated) in WT versus $Atrx^{\Delta E2}$ samples using a threshold of
456 FDR <0.05 and log2 fold-change of > 0.5 or < -0.5 (**Supplementary Table 1**). Rescued
457 or not rescued genes were assessed using the difference between the average value in
458 log2 global normalization. 5-ALA treatment of $Atrx^{\Delta E2}$ mice significantly blocked
459 differential expression of 71.0% of genes including *Xlr3b* (**Fig. 5g, Supplementary**
460 **Table 3**). Following analysis of mice treated with TMPyP4, we selected 138 genes
461 differentially expressed (38 upregulated and 100 downregulated) in WT versus $Atrx^{\Delta E2}$
462 hippocampal samples using a threshold of log2 fold-change of > 1.0 or < -1.0. TMPyP4
463 treatment of $Atrx^{\Delta E2}$ mice blocked differential expression of 92.8% of genes including
464 *Xlr3b*, leaving only 10 of 138 genes remained differentially expressed (**Supplementary**
465 **Table 4**).

466

Discussion

Here, we make several critical observations relevant to activities of ATRX (Supplementary Fig. 12). (1) ATRX binds to parallel G-quadruplexes in *Xlr3b* CGIs together with DNMTs, DAXX and H3.3, where it regulates *Xlr3b* gene expression. (2) Cognitive deficits seen in *Atrx*^{ΔE2} mice are accompanied by *Xlr3b* upregulation, which inhibits CaMKIIα mRNA transport in neuronal dendrites. (3) Abnormal neuronal phenotypes exhibited by *Atrx*^{ΔE2} mice are rescued by treatment with 5-ALA, a G-quadruplex ligand that inhibits *Xlr3b* expression.

Among genes altered in *Atrx*^{ΔE2} mouse brain, the imprinted gene *Xlr3b* is significantly overexpressed. ATRX is enriched with DNMTs at G-quadruplexes in *Xlr3b* CGIs. We observed that reduced ATRX levels at a given site were accompanied by reduced DNMT levels and substantial DNA demethylation, suggesting that ATRX regulates DNA methylation by DNMT recruitment to G-quadruplexes. Notably, forebrain-specific cKO of either DNMT1 or both DNMT1 and DNMT3A reportedly promotes increased *Xlr3b* expression in excitatory neurons^{26, 27}. Moreover, recent genome-wide analysis revealed preferential ATRX localization at the DNA-methylated allele of many imprinted DMRs in mouse ESCs and that ATRX-bound CGI sequences were methylated to a greater extent than that seen in other parts of the genome¹³. ATRX deficiency also correlates with reduced H3.3 incorporation and with Pol II stalling at G-rich intragenic sites, indicating that ATRX influences Pol II-mediated transcription¹⁶. Accordingly, we detected Pol II accumulation at *Xlr3b* CGIs harboring G-quadruplexes in WT hippocampus. We also found that cells in the *Atrx*^{ΔE2} hippocampus showed enriched Pol II signals across the *Xlr3b* gene relative to cells from WT brain, and elevated Pol II signals in *Atrx*^{ΔE2} hippocampus were significantly attenuated following 5-ALA treatment without changing DNA methylation levels. These results suggest that the porphyrin metabolites PpIX and hemin, which are derived from 5-ALA, bind G-quadruplex structures, partially inhibiting Pol II recruitment, a mechanism supported by biophysical analysis of G-quadruplexes in the *HIF1A*, *KRAS*, *CMYB* and *CMYC* genes⁴¹. The G-quadruplex structure formed behind advancing Pol II may be recognized by other factors, including positive transcription elongation factor b⁵⁰. In this context, further studies are required to define dynamic transcriptional structures interacting with the region bearing G-quadruplex motifs and how they regulate ATRX-mediated DNA methylation and Pol II recruitment.

We also report a novel role for *Xlr3b* as a component of RNA granules containing RBPs, which inhibit CaMKIIα mRNA transport into neuronal dendrites. *Xlr3b* interacted with the dendritic mRNAs CaMKIIα, Arc and BDNF via a 13 AA Cor1

domain, which binds RBPs. This sequence is required for CaMKII α mRNA transport, suggesting that RBP binding is also necessary for transport. Interestingly, that Xlr3b sequence is highly conserved in human FAM9 proteins, which also exhibit a Cor1 domain. Thus, binding ability may be common to proteins containing Cor1/Xlr/Xmr motifs. In addition, our fluorescence imaging and electrophysiological analysis supports the idea that Xlr3b negatively regulates synaptic plasticity by inhibiting dendritic CaMKII α mRNA transport in Atrx ^{Δ E2} mice. Mice engineered to lack the CaMKII α mRNA 3' UTR showed reduced levels of CaMKII α transcripts at postsynaptic densities, reduced LTP, and impaired spatial memory³⁸. Dendritic transport and local translation of BDNF and Arc mRNA in synapses also enhances neuronal activity³³, strongly suggesting that Xlr3b-mediated dendritic mRNA transport is related to learning and memory functions. 39X^mO mice, a murine model of the female developmental disorder Turner syndrome, show aberrantly high *Xlr3b* expression in brain, an outcome associated with difficulties in performing a reversal learning test, relative to 40XX and 39X^pO mice²⁴, suggesting that Xlr3b governs mouse behaviors. However, mechanisms underlying these activities remain unknown. We also demonstrated that Thy1-Xlr3b TG mice show impaired LTP and memory deficits, indicating that aberrant neuronal Xlr3b expression partially affects dendritic mRNA transport and learning behaviors in Atrx ^{Δ E2} mice.

We also show that administration of the G-quadruplex ligands 5-ALA or TMPyP4 significantly rescues neuronal phenotypes seen in Atrx ^{Δ E2} mice by inhibiting Xlr3b transcription. However, recovery of cognitive deficits in Atrx ^{Δ E2} mice following 5-ALA administration may be due in part to the binding of ligand to G-quadruplexes of other ATRX targets, although this activity remains unknown. G-quadruplex motifs broadly influence transcription and translation⁴¹. In addition, ATRX can either repress or activate gene expression through G-quadruplexes^{9, 51, 52}. Although potential off-target effects remain to be investigated, our findings suggest a novel therapeutic strategy aimed at blocking Xlr3b expression through small molecule binding to G-quadruplex DNA in patients with ATR-X syndrome. Among candidate ligands, 5-ALA has been applied clinically with minimal risk and approved for use following intracranial tumor resection in Europe, Canada, and Japan⁵³, where it has been used as a photosensitizer in photodynamic diagnostics applied in neurosurgery⁵⁴. 5-ALA is also safe when administered chronically up to 200 mg per day in humans⁵⁵. Thus, its approval time would be short and the cost of clinical trials would be reduced, as pre-existing absorption, distribution, metabolism, excretion (ADME) and toxicity data is available. Finally, the risk of failure is reduced as data relevant to 5-ALA safety and

539 pharmacology is available.

540 In summary, our study indicates that G-quadruplex ligands regulate gene
541 expression through a transcriptional mechanism involving Xlr3b and could be used to
542 treat symptoms associated with ATR-X syndrome.

543

544 **Accession codes availability:** Microarray raw data are available at Gene Expression
545 Omnibus (GEO); accession numbers, GSE103031 and GSE103032.

546

547 **Data Availability Statement:** Summary of all statistical data was shown in
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550

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564

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789 **Figure legends (for main text only)**

790

791 **Figure 1. ATRX regulates *Xlr3b* gene expression.** **a**, Heat map summarizing
792 expression data, and a list of genes exhibiting differential expression between WT and
793 *Atrx*^{ΔE2} mouse hippocampus at post-natal day (P) 90 (an FDR < 0.05 and a log2
794 fold-change of > 0.5). See also **Supplementary Table 1** (a list of genes with an FDR <
795 0.05 and a log2 fold-change of > 0.5 or < -0.5). n = 7 mice each. **b**, Quantitative
796 real-time RT-PCR showing *Xlr3b* mRNA expression in mouse lysates. ***P* < 0.01 by
797 two-way ANOVA with Bonferroni's post hoc test; n = 8-12 mice at P90. PC, (n=8 each);
798 HP, (n=10 each); HT, (WT, n=10; *Atrx*^{ΔE2} n=8); CE, (WT, n=9; *Atrx*^{ΔE2}, n=10); lung,
799 liver, heart, and kidney, (n=12 each). PC, medial prefrontal cortex; HP, hippocampus;
800 HT, hypothalamus; CE, cerebellum. **c**, (top) Representative immunoblot of P90 mouse
801 brain lysates probed with indicated antibodies. (bottom) Densitometric analysis of *Xlr3*
802 normalized to β-tubulin (arbitrary units, A.U.). ***P* < 0.01, **P* < 0.05 by two-way
803 ANOVA with Bonferroni's post hoc test; n = 4 mice each. **d**, Percentage of
804 5-methylcytosine (5-mC) as determined by bisulfite sequencing analysis in whole brain
805 from male P90 WT or *Atrx*^{ΔE2} mice. ***P* < 0.01 by two-sided unpaired t test. n = 12
806 clones each. (n = 3 mice each, 4 clones were sequenced per mouse). See also
807 **Supplementary Fig. 2. e**, *Xlr3b*-ODN showing intramolecular parallel G-quadruplexes,
808 which formed three separate G-quartets stacked 5' to 3' with three loops. **f** and **g**,
809 Luciferase activity of Neuro-2a cells co-transfected with plasmids identified in left
810 margin plus other indicated plasmids. Luciferase activity is presented relative to its
811 activity in mock cells. ***P* < 0.01 by one-way ANOVA with Bonferroni's post hoc test;
812 n = 6 biologically independent samples. **h**, (top) Schematic diagram of *Xlr3b*:
813 G-quadruplex-forming sequence (yellow), CGI (gray) and R1 and R2 regions. (bottom)
814 Analysis of R1 and R2 regions in chromatin from hippocampus of *Atrx*^{ΔE2} and WT mice
815 by chromatin immunoprecipitation with indicated antibodies. Results are expressed as
816 percent input. ***P* < 0.01, **P* < 0.05 by two-sided unpaired t test. n = 6 mice at P90.
817 Full-size scans of western blots shown in **Supplementary Fig. 13**.

818

819 **Figure 2. *Xlr3b* is found in neuronal RNA granules.** **a**, Pull-down assay and
820 LC-MS/MS analysis of *Xlr3*-interacting proteins from P60 mouse brain lysates using an
821 *Xlr3* antibody. (left) Silver-stained SDS-PAGE gel of protein A sepharose column
822 eluates with rabbit IgG (lane 1) or an *Xlr3* antibody (lane 2) in mouse brain lysates.
823 (right) Distribution of identified proteins in a shotgun proteomics experiment. **b**,
824 Immunoblot analysis of GST affinity-column eluates using P60 mouse hippocampal

lysates. Aliquots of extracts (1% input) and eluates were subjected to SDS-PAGE and immunoblotted with indicated antibodies. The experiments were repeated three times with similar results. **c**, Immunoprecipitation (IP) assay with an Xlr3 antibody from P60 mouse hippocampal lysates. Precipitated proteins and inputs were immunoblotted with indicated antibodies. Aliquots of extracts (1% input) and eluates were subjected to SDS-PAGE. The experiments were repeated three times with similar results. **d**, Confocal images showing co-localization of Xlr3 (green) with synaptophysin (red) and MAP2 (blue) in primary mouse cultured neurons at day 21 *in vitro*. Arrowheads indicate synaptophysin-positive puncta. Scale bars: (top) 10μm, (bottom) 5μm. **e**, Confocal images show co-localization of Xlr3 (green) with MAP2 (magenta) in mouse hippocampal CA1. Images at bottom are high-magnification. Scale bars, 10μm. **f**, Confocal images showing co-localization of Xlr3 (magenta) with hnRNP A/B, TIA1, SYTO14 and DCP1a (green) in primary mouse cultured neurons at day 21 *in vitro*. Images at left are enlarged from corresponding boxed areas. Scale bars, 10μm. **g**, (top) Schematic representation of Xlr3b construct. Xlr3b contains a predicted typical nuclear localization signal (NLS) from residues 2-11 and a Cor1/Xlr/Xmr conserved region (Cor1) domain at residues 74-200. (bottom), Confocal images show co-localization of FLAG-tagged Xlr3b constructs (green) with TIA1 (red) in Neuro-2a cells. Nuclear DNA is labeled with DAPI (blue). Scale bars, 10μm. **h**, Neuro-2a cells were transfected with FLAG-tagged Xlr3b constructs, cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, and western blots (WB) were probed with indicated antibodies. To validate an inhibitory effect of XIP, cells were treated 4 h with XIP peptides (1μM). In **d-h**, the experiments were repeated three times with similar results. Full-size scans of western blots shown in **Supplementary Fig. 13**.

Figure 3. Aberrant Xlr3b expression perturbs synaptic plasticity by inhibiting dendritic CaMKIIα mRNA transport. **a**, Xlr3 interacts with CaMKIIα, Arc and BDNF mRNAs in an RNA immunoprecipitation assay. Total mRNA was precipitated from P90 mouse hippocampal lysates with an Xlr3 antibody. cDNA was analyzed with primers amplifying CaMKIIα, Arc and BDNF mRNAs. $^{**}P < 0.01$ by one-way ANOVA with Bonferroni's post hoc test; n = 5 mice each. **b**, Time-lapse images of mCherry-Xlr3b (red) and GFP-CaMKIIα 3' UTR (green) co-transported in a dendrite of cultured neuron at day 21 *in vitro*. Granules moved in both anterograde (white arrowhead) and retrograde (yellow arrowhead) directions. Scale bars, 3μm. The experiments were repeated three times with similar results. **c**, (top, right) Representative

861 kymograph of movement of GFP-CaMKII α 3' UTR in a proximal dendrite. Scale bars,
862 5 μ m (x-axis) and 5min (y-axis). (top, left) Granules moved in anterograde (white
863 arrowhead) and retrograde (yellow arrowhead) directions. Scale bars, 2 μ m. See also
864 **Supplementary Video 1**. (bottom) Relative frequency of movement of GFP-CaMKII α
865 3' UTR granules. $**P < 0.01$ by two-way ANOVA with Bonferroni's post hoc test; $n = 5$
866 neurons each, a proximal dendrite (20-100 μ m away from the cell body) per neuron to
867 measure relative frequencies. Cells were treated with XIP (1 μ M) 4 h before imaging.
868 Imm., immobile; Bidirect., bidirectional movement; Antero., anterograde movement;
869 and Retro., retrograde movement. **d**, The number of GFP-CaMKII α 3' UTR (green) and
870 PSD95 (magenta) double-positive puncta. $**P < 0.01$ by one-way ANOVA with
871 Bonferroni's post hoc test; $n = 20$ neurons each, a proximal dendrite (20-100 μ m away
872 from the cell body) per neuron. Whiskers represent minima and maxima. **e**, (left)
873 Representative field excitatory post-synaptic potentials (fEPSPs) were recorded from
874 the hippocampal CA1 region of mice. (middle) Changes in fEPSP slope following high
875 frequency stimulation (HFS) recorded in hippocampal CA1. (right) Changes in fEPSP
876 slope following HFS at 1 or 60 min based on analysis shown at left. $**P < 0.01$, $*P <$
877 0.05 by two-way ANOVA with Bonferroni's post hoc test; WT, Atrx $^{\Delta E2}$, Atrx $^{\Delta E2}$ + XIP, n
878 $= 8$ mice; Atrx $^{\Delta E2}$ + ANTP, $n=6$ mice.

880 **Figure 4. 5-ALA represses *Xlr3b* transcription with RNA polymerase II**
881 **recruitment by modifying G-quadruplex structure.** **a**, 5-ALA metabolism to
882 indicated porphyrins. Succinylacetone inhibits 5-ALA dehydratase (ALAD). PBG,
883 porphobilinogen; PpIX, protoporphyrin IX; FECH, ferrochelatase. **b** and **c**, Luciferase
884 activity in Neuro-2a cells transfected with pGL3-2K or pGL3-2K Δ G4 and treated with
885 5-ALA (0.1, 0.3, 1, 3 or 10 μ M) (**b**), or co-administered 10 μ M 5-ALA and
886 succinylacetone at 1, 10, 100 or 1000 μ M (**c**) for 48h. Luciferase activity is shown
887 relative to that in vehicle-treated cells. $**P < 0.01$ by one-way ANOVA with
888 Bonferroni's post hoc test; $n = 3$ biologically independent samples. **d**, Quantitative
889 real-time RT-PCR showing *Xlr3b* mRNA expression in P90 mouse hippocampal lysates.
890 $**P < 0.01$ vs. vehicle-treated WT mice, $###P < 0.01$ vs. vehicle-treated Atrx $^{\Delta E2}$ mice by
891 one-way ANOVA with Bonferroni's post hoc test; $n=6$ mice each. **e**, (top)
892 Representative immunoblot of P90 mouse brain lysates probed with indicated
893 antibodies. (bottom) Densitometric analysis of Xlr3 normalized to β -tubulin (arbitrary
894 units, A.U.). $**P < 0.01$ vs. vehicle-treated WT mice, $###P < 0.01$ vs. vehicle-treated
895 Atrx $^{\Delta E2}$ mice by one-way ANOVA with Bonferroni's post hoc test; $n=4$ mice each. **f**,
896 Percentage of 5-methylcytosine (5-mC) as determined by bisulfite sequencing analysis

897 in medial prefrontal cortex (PC) and hippocampus (HP) from male P90 WT or Atrx^{ΔE2}
898 mice. $**P < 0.01$ by two-way ANOVA with Bonferroni's post hoc test; $n = 9$ clones.
899 ($n = 3$ mice. 3 clones were sequenced per mouse.) See also **Supplementary Fig. 10e, g**,
900 (top) The G-quadruplex prediction tool QGRS mapper was used to identify potential
901 G-quadruplex forming sequences in the entire Xlr3b genomic DNA sequence, which is
902 shown with exons highlighted in black and introns highlighted in gray. QGRS mapper
903 provides a G-score (plotted in blue) which indicates the likelihood of G-quadruplex
904 formation. Image is adapted from QGRS mapper
905 (<http://bioinformatics.ramapo.edu/QGRS/index.php>). (bottom) Analysis of indicated
906 amplicons in chromatin from hippocampus of P90 mice by chromatin
907 immunoprecipitation with indicated antibodies. Results are expressed as percent input.
908 $**P < 0.01$, $*P < 0.05$ vs. vehicle-treated WT mice, $##P < 0.01$, $\#P < 0.05$ vs.
909 vehicle-treated Atrx^{ΔE2} mice by one-way ANOVA with Bonferroni's post hoc test; $n = 6$
910 mice each. Full-size scans of western blots shown in **Supplementary Fig. 13**.

911

912

913 **Figure 5. Treatment with 5-ALA counteracts cognitive deficits seen in Atrx^{ΔE2} mice.**

914 **a**, (top) Representative kymograph of movement of GFP-CaMKIIα 3' UTR in a
915 proximal dendrite. Scale bars, 5μm (x-axis) and 5min (y-axis). (bottom) Relative
916 frequency of movement of GFP-CaMKIIα 3' UTR granules. Cells were treated with
917 5-ALA (1μM) for 7 days before imaging. $*P < 0.05$ vs. vehicle-treated WT neurons,
918 $##P < 0.01$ vs. vehicle-treated Atrx^{ΔE2} neurons by two-way ANOVA with Bonferroni's
919 post hoc test; $n = 5$ neurons each, a proximal dendrite (20-100μm away from the cell
920 body) per neuron to measure relative frequencies. Imm., immobile; Bidirect.,
921 bidirectional movement; Antero., anterograde movement; and Retro., retrograde
922 movement. **b**, The number of GFP-CaMKIIα 3' UTR (green) and PSD95 (magenta)
923 double-positive puncta in a proximal dendrite. $**P < 0.01$ vs. vehicle-treated WT
924 neurons, $##P < 0.01$ vs. vehicle-treated Atrx^{ΔE2} neurons by one-way ANOVA with
925 Bonferroni's post hoc test; $n = 20$ neurons each, a proximal dendrite per neuron.
926 Whiskers represent minima and maxima. **c**, (left) Representative field excitatory
927 post-synaptic potentials (fEPSPs) were recorded from the hippocampal CA1 region of
928 mice. (middle) Changes in fEPSP slope recorded following high frequency stimulation
929 (HFS) were attenuated in Atrx^{ΔE2} mice, and 5-ALA treatment significantly rescued
930 impaired LTP in hippocampal CA1. (right) fEPSP slope changes following HFS at 1 or
931 60 min, as shown at left. $**P < 0.01$, $*P < 0.05$ vs. vehicle-treated WT mice, $##P < 0.01$,
932 $\#P < 0.05$ vs. vehicle-treated Atrx^{ΔE2} mice by two-way ANOVA with post hoc

933 Bonferroni's multiple comparison test; WT + vehicle, Atrx^{ΔE2} + vehicle, and Atrx^{ΔE2} +
934 5-ALA, n = 8 mice; WT + 5-ALA, n=6 mice. **d-f**, 5-ALA treatment decreased cognitive
935 deficits in Atrx^{ΔE2} mice based on memory-related tests. Novel-object recognition (NOR)
936 test (**d**), latency time in retention trials in a passive avoidance (PA) test (**e**), and
937 alternations in a Y-maze test (**f**). ***P* < 0.01 vs. vehicle-treated WT mice, ###*P* < 0.01,
938 #*P* < 0.05 vs. vehicle-treated Atrx^{ΔE2} mice by one-way ANOVA with Bonferroni's post
939 hoc test; Respective sample sizes are indicated. **g**, Heat map summarizing expression
940 data (left), and a list of rescued or not-rescued genes (right) between WT, Atrx^{ΔE2}, and
941 5-ALA treated Atrx^{ΔE2} mouse hippocampus at post-natal day (P) 90. The list was
942 focused on 31 genes differentially expressed (8 up-regulated and 23 down-regulated) in
943 WT versus Atrx^{ΔE2} samples in **Supplementary Table 1** (an FDR < 0.05 and a log2
944 fold-change of > 0.5 or < 0.5). See also **Supplementary Table 3**, which includes
945 5-ALA treated Atrx^{ΔE2} group. Rescued or not rescued genes were assessed using the
946 difference between the average value in log2 global normalization. WT, n=7 mice;
947 Atrx^{ΔE2}, n=7 mice; Atrx^{ΔE2} + 5-ALA, n=4 mice. Full-size scans of western blots shown
948 in **Supplementary Fig. 13**.
949

950 **On-line Methods**

951

952 **Animals**

953 Male mice (C57BL/6J) were used for all experiments. Mice were housed under
954 climate-controlled conditions with a 12-h light/dark cycle and provided standard food
955 and water ad libitum. Animal studies were conducted in accordance with the Tohoku
956 University institutional guidelines. Ethical approval has been obtained from the
957 Institutional Animal Care and Use Committee of the Tohoku University Environmental
958 and Safety Committee. Generation of homozygous *Atrx*^{ΔE2} mice is described in ¹⁹. To
959 generate Thy1-Xlr3b TG mice, Xlr3b cDNA was subcloned into the XhoI site of a
960 mouse Thy1.2 expression cassette (**Supplementary Fig. 9a**). The Thy1 promoter
961 construct was a gift of Joshua Sanes (Addgene plasmid # 20736). The Thy1.2-Xlr3b
962 expression cassette was excised with NotI/PvuI and injected into fertilized eggs of B6
963 mice at Oriental Bio Service (Kyoto, Japan). Hemizygous Thy1-Xlr3b TG mice were
964 generated by mating founder mice with B6 mice, and offspring were screened by PCR
965 using genomic tail DNA. Primers used for amplification of Xlr3b sequences were
966 ThXI(FW) (5'-GGTATTCATCATGTGCTCCG-3') and ThXI(RV)
967 (5'-GTTTCTGCCTCTCTTCACAG-3'), and the detected PCR product was 479 bp in
968 founder animals (**Supplementary Fig. 9b**).

969

970 **Cell culture**

971 Neuro-2a mouse neuroblastoma cells (ATCC, CCL-131, authenticated by the
972 provider using short tandem repeat profiling) were grown in Dulbecco's minimal
973 essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum
974 (FBS) and penicillin/streptomycin (100units/100μg/ml) in a 5% CO₂ incubator at 37°C.
975 Cells were routinely tested for mycoplasma contamination. Transfection was performed
976 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent
977 according to the manufacturer's protocol. Primary cultures of neurons were established
978 using previously described methods with slight modifications ⁵⁶. Briefly, hippocampal
979 tissue was dissected from embryonic day 18 mice and dissociated by trypsin treatment
980 and trituration through a Pasteur pipette. Neurons were plated on coverslips coated with
981 poly-L-lysine in Minimum Essential Medium (Invitrogen) supplemented with 10% FBS,
982 0.6% glucose (Wako, Osaksa, Japan), and 1mM pyruvate (Sigma-Aldrich, St. Louis,
983 MO, USA). After cell attachment, coverslips were transferred to dishes containing a
984 glial cell monolayer and maintained in Neurobasal medium (Invitrogen) containing 2%
985 B27 supplement (Invitrogen) and 1% GlutaMax (Invitrogen). 5μM Cytosine

986 β -D-arabinofuranoside (Sigma-Aldrich) was added to cultures at DIV3 after plating to
987 inhibit glial proliferation. Primary neurons were transfected with expression vectors and
988 shRNAs using electroporation (NEPA21; NEPAGENE Co., Ltd. Chiba, Japan) at DIV0.

989

990 **Plasmid constructs and lentiviral transduction**

991 Plasmids expressing cDNAs encoding Xlr3a, Xlr3b, FAM9A, DNMT1,
992 DNMT3A, H3.3 and DAXX were obtained from Kazusa DNA Research Institute
993 (Promega, Madison, WI, USA) and cDNAs were inserted into pCMV vector.
994 GST-Xlr3b was generated by cloning Xlr3b cDNA into pGEX-4T-1 (Pharmacia Biotech,
995 Piscataway, NJ, USA). ATRX cDNA plasmid (pEGFP-C2-ATRX-HA) was kindly
996 provided by Dr. David Picketts (University of Ottawa, Canada). ATRX shRNA
997 (pSUPER-shATRX1) plasmid was kindly provided by Dr. Nathalie Berube (University
998 of Western Ontario, Canada). GFP-MS2-nls and MS2 binding site (MS2bs)-CaMKII α
999 3'UTR constructs were kindly provided by Dr. Kenneth S. Kosik (University of
1000 California Santa Barbara Santa Barbara, USA). Xlr3b shRNA was purchased from
1001 Sigma-Aldrich (MISSION TRC-Mm2.0 TRCN0000255204). The non-targeting hairpin
1002 control SHC002 (Sigma-Aldrich), which containing a sequence that does not target any
1003 known human or mouse gene, was used as a negative control (shControl). FLAG-Xlr3b
1004 (FL, Δ NLS, Δ 74-123, Δ 124-200, and Δ 158-170) and FLAG-FAM9A (FL and Δ 292-304)
1005 were generated using the KOD-Plus Mutagenesis kit (Toyobo, Osaka, Japan) according
1006 to the manufacturer's protocol. For construction of Xlr3b promoter plasmids, genomic
1007 DNA isolated from mouse brain was obtained using a DNeasy Tissue Kit (Qiagen,
1008 Valencia, CA, USA). PCR was carried out to amplify a fragment containing the Xlr3b
1009 promoter region from nucleotides -2085 to +1 (where +1 is the A of the translation
1010 initiation codon). The fragment was then subcloned into the pGL3-basic luciferase
1011 reporter (Promega) vector (pGL3-2K) and sequenced. pGL3-2K Δ G4 and
1012 pGL3-1K Δ CGI were generated using the KOD-Plus Mutagenesis kit (Toyobo). The
1013 CRISPR-Cas9 and GFP fusion protein expression lentivirus, U6gRNA-Cas9-2A-GFP
1014 plus guide Xlr3 sgRNA (Xlr3-Cas9-GFP), was purchased from Sigma-Aldrich. The
1015 Xlr3 guide sequence was TCATCTTTTCAGTGCCATGG. Lentiviral transduction was
1016 performed according to the manufacturer's protocol (Sigma-Aldrich).

1017

1018 **Antibodies and peptides**

1019 The following primary antibodies were used; anti-Xlr3 (1:500, generated
1020 commercially (MBL, Nagoya, Japan) against TDTAGRHSRMDPNLSSDC peptide,
1021 anti-ATRX (1:100, Santa Cruz Biotechnology, H300 #sc-15408, Santa. Cruz, CA, USA),

1022 anti-DNMT1 (1:1000, clone 60B1220.1, Abcam #ab13537, Cambridge, UK),
1023 anti-DNMT3A (1:1000, clone 64B1446, Abcam #ab13888), anti- β -tubulin (1:5000,
1024 clone AC-15, Sigma-Aldrich, #A5441), anti-hnRNP A/B (1:200: Santa Cruz
1025 Biotechnology, G-10 #sc-376411), anti-hnRNP A/B (1:200: Santa Cruz Biotechnology,
1026 M-15 #sc-82628), anti-DYNC1LI2 (1:1000, clone EPR11230(2), Abcam, #ab178702),
1027 anti-DYNLL1 (1:200: Santa Cruz Biotechnology, #sc-136287), anti-histone H3.3
1028 (1:1000, Abcam, #ab62642), anti-DAXX (1:200, Santa Cruz Biotechnology, M-112
1029 #sc-7152), anti-hnRNP D (1:200, clone D6O4F, Cell Signaling Technology, Beverly,
1030 MA, USA, #12382), anti-TIA1 (1:200, Santa Cruz Biotechnology, C-20, #sc-1751),
1031 anti-FLAG (1:1000, clone M2, Sigma-Aldrich, #F1804), anti-synaptophysin (1:1000,
1032 clone SVP-38, Sigma-Aldrich, #S5768), anti-MAP2 (1:10000, Abcam, #ab92434),
1033 anti-RNA polymerase II (1:1000, clone CTD4H8, Millipore, MA, USA, #05-623),
1034 anti-PSD95 (1:500, clone 6G6-1C9, Abcam, #ab2723), anti-phospho-CaMKII
1035 (Thr286/Thr287, 1:5000, ¹⁸) and anti-CaMKII α/β (1:5000, ¹⁸). For immunoblotting, the
1036 following secondary antibodies were used: anti-mouse IgG antibody horseradish
1037 peroxidase conjugated (1:5000, Southernbiotech, Birmingham, AL, USA, #1031-05),
1038 anti-rabbit IgG antibody horseradish peroxidase conjugated (1:5000, Southernbiotech,
1039 #4050-05) and anti-goat IgG antibody horseradish peroxidase conjugated (1:5000,
1040 Rockland Immunochemicals Inc., Limerick, PA, USA, #605-4302). For
1041 immunocytochemistry and immunohistochemistry, the following secondary antibodies
1042 were used: Alexa488-conjugated donkey anti-rabbit (1:500, Invitrogen, #A-21206)
1043 Alexa594-conjugated donkey anti-rabbit (1:500, Invitrogen, #A-21207), Alexa
1044 488-conjugated donkey anti-mouse (1:500, Invitrogen, #A-21202), Alexa
1045 594-conjugated donkey anti-mouse (1:500, Invitrogen, #A-21203), Alexa
1046 488-conjugated donkey anti-goat (1:500, Invitrogen, #A-11055), Alexa 594-conjugated
1047 donkey anti-goat (1:500, Invitrogen, #A-11058) and DyLight 405-AffiniPure donkey
1048 anti-chicken IgY (1:500; Jackson ImmunoResearch, West Grove, PA, USA,
1049 #703-475-155). XIP and ANTP control peptides were obtained from the
1050 peptide-synthetic service at MBL.

1051

1052 **Luciferase assay**

1053 Neuro-2a cells were co-transfected with pGL3 plasmids (pGL3-2K,
1054 pGL3-2K Δ G4 and pGL3-1K Δ CGI) plus a pRL-TK plasmid, which contains Renilla
1055 luciferase under control of the herpes simplex virus thymidine kinase promoter, with or
1056 without other plasmids (ATR_X, ATR_X shRNA, control shRNA, DNMT1, DNMT3A,
1057 H3.3 or DAXX). For *in vitro* DNA methylation assays, pGL3 plasmids were incubated

1058 with 3U CpG methyltransferase (*M.SssI*) (New England Biolabs, Hitchin, UK) for 4h at
1059 37°C in the presence of 1mM S-adenosylmethionine, following the manufacturer's
1060 instructions. Firefly and Renilla luciferase activities were measured using the
1061 Dual-Luciferase Reporter Assay System (Promega) with a luminometer (Gene Light 55
1062 Luminometer, Microtech, Chiba, Japan). The ratio of Firefly to Renilla luciferase
1063 luminescence was calculated.

1064

1065 **Identification of Xlr3 interaction proteins by mass spectrometry**

1066 Mouse brain tissues at P60 were lysed in buffer containing 50 mM Tris-HCl (pH
1067 7.5), 0.15 M NaCl, 0.1% Triton X-100, 4 mM EDTA, 4 mM EGTA, 1 mM Na₃VO₄, 50
1068 mM NaF, 1 mM DTT, and protease inhibitors (trypsin inhibitor, pepstatin A, and
1069 leupeptin), followed by centrifugation at 15,000 × g for 10 min. Supernatants were
1070 collected and incubated with Protein A Sepharose column (Protein A HP SpinTrap, GE
1071 Healthcare Life Sciences, Piscataway, NJ, USA) with TBS buffer (50 mM Tris-HCl,
1072 0.15 M NaCl, pH 7.5) containing an Xlr3 antibody (10 µg) at 4°C for 4 h with constant
1073 rotation. Bound proteins were then washed with TBS and eluted with 2.5% acetic acid.
1074 To confirm specific binding, samples were run on SDS-PAGE and the gel was stained
1075 using a Silver Stain Kit (Wako). All samples were then analyzed by LC-MS/MS at
1076 Oncomics Co., Ltd, (Nagoya, Japan), as a custom service. Proteins identified in control
1077 samples pulled down with rabbit IgG (10 µg) were subtracted from identified proteins.

1078

1079 **Preparation of proteins binding to GST-Xlr3b**

1080 Preparation of proteins was performed using a GST Protein Interaction
1081 Pull-Down Kit (Pierce; Thermo Fisher Scientific, Rockford, IL, USA) according to the
1082 manufacturer's instructions. GST and GST-Xlr3b were prepared in *E. coli* BL21 and
1083 immobilized to glutathione affinity resin. Mouse hippocampal tissues were lysed in
1084 buffer containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 4 mM EDTA, 4 mM EGTA,
1085 1 mM Na₃VO₄, 50 mM NaF, 1 mM DTT, and protease inhibitors (trypsin inhibitor,
1086 pepstatin A, and leupeptin), followed by centrifugation and incubation with
1087 immobilized glutathione affinity resin containing GST fusion proteins at 4°C for 4 h
1088 with constant rotation. Bound proteins were then washed, eluted with glutathione
1089 elution buffer, and run on SDS-PAGE.

1090

1091 **Immunoprecipitation and immunoblotting**

1092 Immunoprecipitation and immunoblotting analysis was performed as described
1093 ⁵⁶. Briefly, tissues or cells were homogenized in buffer containing 50 mM Tris-HCl, pH

1094 7.5, 0.5% Triton X-100, 0.15 M NaCl, 4 mM EDTA, 4 mM EGTA, 1 mM Na₃VO₄, 50
1095 mM NaF, 1 mM DTT, and protease inhibitors (trypsin inhibitor, pepstatin A, and
1096 leupeptin). For immunoprecipitation, lysates were incubated 2 h at 4°C with indicated
1097 antibodies with protein A-Sepharose CL-4B (GE Healthcare Life Sciences) in
1098 homogenization buffer. Subsequently, immunoprecipitates were washed three times
1099 with homogenization buffer, equivalent amounts of protein were electrophoresed on
1100 SDS-PAGE and proteins then transferred to an Immobilon polyvinylidene difluoride
1101 membrane. After blocking with TTBS solution (50 mM Tris-HCl, pH 7.5, 150 mM
1102 NaCl, and 0.1% Tween 20) containing 5% fat-free milk powder for 1 h at room
1103 temperature, membranes were incubated overnight at 4 °C with indicated primary
1104 antibodies. After washing, membranes were incubated with the appropriate horseradish
1105 peroxidase-conjugated secondary antibody diluted in TTBS. Blots were developed using
1106 an ECL immunoblotting detection system (Amersham Biosciences, NJ, USA).
1107 Immunoreactive bands were visualized using a luminescent image analyzer LAS-4000
1108 (Fuji Film, Tokyo, Japan) and quantified using Image Gauge version 3.41 (Fuji Film).

1109

1110 **Subcellular fractionation.**

1111 Primary hippocampal neurons at DIV21 were homogenized in ice-cold lysis
1112 buffer containing the following (in mM): sucrose 320, HEPES 4, pH 7.4, EGTA 1, and
1113 protease inhibitors. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C.
1114 The supernatant (S1, postnuclear supernatant) was centrifuged again at 12,000 x g for
1115 15 min to obtain crude synaptosomal fraction (P2). For separating synaptosomal cytosol
1116 (LS1) and synaptosomal membrane (LP1), the pellet (P2) was hypo-osmotically lysed
1117 (5% lysis buffer; 95% distilled water containing 5% CHAPS and protease inhibitors) for
1118 30 min and centrifuged at 100,000 x g for 60 min. The pellet (LP1) was resuspended in
1119 lysis buffer containing 1% Triton X-100 and sonicated.

1120

1121 **Immunocytochemistry and immunohistochemistry**

1122 Immunocytochemistry and immunohistochemistry were performed as described
1123 ⁵⁶. Briefly, fixed cells or brain slices with 4% paraformaldehyde in phosphate-buffered
1124 saline (PBS) were treated with PBS containing 0.1% Triton X-100 for 10 min. Samples
1125 were incubated overnight at 4°C with indicated primary antibodies and then washed in
1126 PBS and incubated with appropriate secondary antibodies. Nuclei were stained with 4,
1127 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). To visualize RNA molecules,
1128 living neurons were incubated with 50 nM SYTO14 dye (Life Technologies, Carlsbad,
1129 CA, USA, #S7576). Fluorescence intensities and images were analyzed by confocal

1130 laser scanning microscopy (LSM700, Carl Zeiss, Thornwood, NY).

1131

1132 **Live imaging**

1133 Live images of granule movement were captured in dendrites for 15 min
1134 (proximal dendrites) or 10 min (distal dendrites) at 15-s intervals in primary neurons at
1135 DIV21. Granule movement was analyzed in proximal dendrites (20-100μm) and in
1136 distal dendrites (100-200μm) away from the cell body. Relative frequency of movement
1137 of GFP-CaMKII 3' UTR granules was analyzed for a subset of moving granules.
1138 Granules that changed direction during the tracking period were defined as
1139 bi-directional. Granules were classified as immobile when their movement was < 5 μm
1140 during the 15 min (proximal dendrites) or 10 min (distal dendrites) observation period.
1141 Kymographs were created using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

1142

1143 **RT-PCR and quantitative RT-PCR (RT-qPCR) analysis**

1144 Total RNA was purified from P90 mouse brain using an RNeasy Mini Kit
1145 (Qiagen) according to the manufacturer's protocol. RNA was reverse-transcribed into
1146 single-stranded cDNA using an oligo(dT) primer (Promega) and Moloney murine
1147 leukemia virus-reverse transcriptase (Invitrogen), and then subjected to RT-PCR with
1148 gene-specific primers. RT-qPCR analysis was performed as described¹⁸ in 48-well
1149 plates (Mini Opticon real-time PCR system, Bio-Rad) using iQ SYBR Green Supermix
1150 2× (Bio-Rad). Gene expression was assessed using the differences in normalized Ct
1151 ($\Delta\Delta Ct$) method after normalization to GAPDH. Fold-change was calculated by $2^{-\Delta\Delta Ct}$.
1152 The following primers were used for RT-PCR and RT-qPCR:

1153 In Fig. 1b, Fig. 4d, supplementary Fig. 1e, supplementary Fig. 9c, and supplementary
1154 Fig.10c.

1155 RT-qPCR-Xlr3b(FW) (5'-CAGAAAAAGGAAGGCCACTG-3')

1156 RT-qPCR-Xlr3b(RV) (5'- GTTTTCCTTCCTGGCCTGT -3')

1157 RT-qPCR-GAPDH(FW) (5'-TGTGTCCGTCGTGGATCTGA-3')

1158 RT-qPCR-GAPDH(RV) (5'-CACCACCTTCTTGATGTCATCATAC-3')

1159 In supplementary Fig. 1a,

1160 RT-PCR-Xlr3a(FW) (5'-AGCCGAGACCCGACCAAGTGG-3')

1161 RT-PCR-Xlr3a(RV) (5'-TGTTAGCTGGGTTCTG-3')

1162 RT-PCR-Xlr3b(FW) (5'-AGCCAAGGCCCGACCAAGTGG-3')

1163 RT-PCR-Xlr3b(RV) (5'-TAGCTGCTGCGACTGCACCT-3')

1164

1165 **Bisulphite sequencing**

1166 Genomic DNA isolated from whole brain, medial prefrontal cortex and
1167 hippocampus of WT and Atrx^{ΔE2} mice at P90 were subjected to bisulphite-mediated C to
1168 U conversion using BisulFast (Toyobo, Osaka, Japan) and then used as template for
1169 PCR. The PCR products were ligated into the pCR2.1 vector by using a TOPO-TA
1170 cloning kit (Invitrogen), according to the manufacturer's instruction. The following
1171 primers specific for bisulfite-treated DNA were used for PCR:
1172 Xlr-bisul(FW) (5'-GATTAAGTGGGATGAATTTTGTGAGT-3')
1173 Xlr-bisul(RV) (5'-CCCAAATAAAAATTTTCTCATTC-3')

1174

1175 **Chromatin immunoprecipitation (ChIP) assay**

1176 Chromatin solutions derived from P90 mouse hippocampus were prepared using
1177 the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) and then
1178 immunoprecipitated overnight at 4°C using 2 μg indicated antibodies. Assays included
1179 normal rabbit IgG as an antibody specificity control. The following antibodies and
1180 primers were used:

1181 in Fig. 1

1182 R1(FW) (5'-CCAAGTGGGATGAACCTCTGAGTG-3')

1183 R1(RV) (5'-CCGCGGAGTCCCACTGTGGAACGT-3')

1184 R2(FW) (5'-CACAAGTACTATACTAGCTGAAAC-3')

1185 R2(RV) (5'-GGTTGGTCATACCTATGTAGGAAC-3')

1186 in Fig. 4

1187 amplicon 1(FW) (5'-CCAAGTGGGATGAACCTCTGAGTG-3')

1188 amplicon 1(RV) (5'-CCGCGGAGTCCCACTGTGGAACGT-3')

1189 amplicon 2(FW) (5'-GCCACAGAGGGCAAGGCAAGGGAG-3')

1190 amplicon 2(RV) (5'-AATGATGTCTTGGTTGGGAATTCCA-3')

1191 amplicon 3(FW) (5'-AGTATCAGGCTTAGATTTAAAGGA-3')

1192 amplicon 3(RV) (5'-AAGTCAGGGAGGATGACCAGTCTC-3')

1193 amplicon 4(FW) (5'-CTGTGCGGGACTCCCATGATACCC-3')

1194 amplicon 4(RV) (5'-CGTAAGCCCTTCAGCATAAATACT-3')

1195

1196 **RNA immunoprecipitation (RIP) assay**

1197 Mouse hippocampal lysates were immunoprecipitated with an Xlr3 antibody (15
1198 μg per sample) followed by RNA isolation using the RiboCluster Profiler RIP-Assay kit
1199 (MBL) according to the manufacturer's protocols. Immunoprecipitated RNA was
1200 reverse-transcribed into single-stranded cDNA using an oligo(dT) primer (Promega) and

1201 Moloney murine leukemia virus-reverse transcriptase (Invitrogen), and subjected to
1202 RT-qPCR with the following gene-specific primers:
1203 BDNF(FW) (5'-TGGCCTAACAGTGTTTGCAG-3')
1204 BDNF(RV) (5'-GGATTTGAGTGTGGTTCTCC-3')
1205 CaMKII α (FW) (5'-GACACCAAAGTGCGCAAACAGG-3')
1206 CaMKII α (RV) (5'-GCGAAGCAAGGACGCAGG-3')
1207 Arc(FW) (5'-AGCAGCAGACCTGACATCCT-3')
1208 Arc(RV) (5'-GGCTTGTCTTCACCTTCAGC-3')
1209 GAPDH(FW) (5'-TGTGTCCGTCGTGGATCTGA-3')
1210 GAPDH(RV) (5'-CACCACCTTCTTGATGTCATCATAC-3')

1211

1212 **Gene microarray analysis**

1213 For the oligo-DNA microarray analysis, we used the “3D-Gene” Mouse Oligo
1214 chip 24k and Human Oligo chip 25k arrays (Toray Industries Inc, Tokyo, Japan). Total
1215 RNA was purified from mouse hippocampus using an RNeasy Mini Kit (Qiagen)
1216 according to the manufacturer’s instructions. Total RNA was Cy5-labeled using the
1217 Amino Allyl MessageAMP II aRNA Amplification Kit (Applied Biosystems, CA,
1218 USA). Labeled aRNA pools were then hybridized 16 h in buffer using the supplier's
1219 protocols (www.3d-gene.com). Hybridization signals were scanned using a ScanArray
1220 Express Scanner (PerkinElmer) and processed using the GenePixPro version 5.0
1221 software (Molecular Devices).

1222

1223 **Electrophysiology**

1224 Preparation of hippocampal slices was performed as described¹⁹. Briefly, brains
1225 were rapidly removed from ether-anesthetized mice and chilled in ice-cold oxygenated
1226 artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 2.4
1227 mM CaCl₂, 1.3 mM MgSO₄, 1.26 mM KH₂PO₄, and 10 mM D-glucose). Transverse
1228 hippocampal slices (400- μ m thickness) were cut using a vibratome (Microslicer
1229 DTK-1000, Dosaka EM, Kyoto, Japan) and transferred to a recording chamber where
1230 they were allowed to recover at least 1 h at 34°C before recording. ACSF maintained at
1231 34°C was used during the experiment. A concentric bipolar stimulating electrode was
1232 placed in the stratum radiatum of CA1 to stimulate the Schaffer collateral pathway.
1233 High-frequency stimulation of 100 Hz with 1-s duration was applied twice with a 20-s
1234 interval. Traces were digitized with an A/D converter (PowerLab 200; AD Instruments,
1235 Castle Hill, Australia) and a computer (Windows, Measurement and Analysis System
1236 for LTP: FAL-3000). Paired-pulse responses were measured with inter-stimulus

intervals of 20-500 ms and expressed as the ratio of second stimulated amplitude to the first stimulated amplitude. The hippocampal input/output (I/O) ratio was determined for each group by measuring fEPSP amplitude in response to a series of stimuli with intensities ranging from 0.1 to 1.1 mA. After recording, slices were transferred to a plastic plate on ice to dissect the CA1 region under a microscope. CA1 regions were frozen in liquid nitrogen and stored at -80°C until biochemical analysis.

Drugs

5-ALA (COSMO BIO co., ltd. Tokyo, Japan), and sodium ferrous citrate were kindly provided by SBI Pharmaceuticals Co., Ltd., (Tokyo, Japan). TMPyP4 (5, 10, 15, 20-Tetrakis (1-methyl-4-pyridinio)porphyrin tetra) was purchased from Sigma-Aldrich. For administration to Atrx^{ΔE2} mice, mice were randomized into 7 groups as follows: 5-ALA (3 and 10 mg/kg, p.o. with sodium ferrous citrate (20:1 mol. ratio) dissolved in distilled water) or vehicle (sodium ferrous citrate dissolved in distilled water) was administered daily from P30 to P90. TMPyP4 (10 and 30 mg/kg, i.p. in saline) or vehicle (saline) was administered twice weekly from P30 to P90. For acute administration, 5-ALA (10 mg/kg, p.o.) was administered 3 h before experiments. Measurement of biodistribution in TMPyP4 and 5-ALA was performed as described in ^{47, 49}.

Behavioral analysis

Adult male mice at P90 were used for behavioral analysis. Animals were subjected to behavioral tests including Y-maze, step-through passive avoidance, novel object recognition and social interaction tasks. All behavioral experiments were conducted with the experimenter blind to mouse genotypes and treatments. In the Y-maze task, spontaneous alternation behavior in a Y-maze was assessed as a task of spatial reference memory. The apparatus consisted of three identical arms (50×16×32 cm³) of black plexiglas. Mice were placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The sequence of arm entries was manually recorded. An alternation was defined as entries into all three arms on consecutive choices. The maximum number of alternations was defined as the total number of arms entered minus two, and the percentage of alternations was calculated as actual alternations/maximum alternations×100. The total number of arms entered during the session was also determined. In step-through passive avoidance task, training and retention trials of passive avoidance tasks were conducted in a box consisting of dark (25×25×25 cm³) and light (14×10×25 cm³) compartments. The floor was constructed

1273 with stainless steel rods, and rods in the dark compartment were connected to an
1274 electronic stimulator (Nihon Kohden, Tokyo, Japan). Mice were habituated to the
1275 apparatus the day before passive avoidance acquisition. During training, a mouse was
1276 placed in the light compartment, and when it entered the dark compartment, the door
1277 was closed and an electric shock (0.4 mA for 2 s) was delivered from the floor. The
1278 mouse was removed from the apparatus 30 s later. After a 7-day interval, each mouse
1279 was placed in the light compartment and step-through latency was recorded over 300 s
1280 to assess retention. In the novel object recognition task, mice were individually
1281 habituated to an open-field box (35×25×35 cm³) for 2 consecutive days. During
1282 acquisition phase, two objects of the same material were placed symmetrically in the
1283 center of the chamber for 10 min. 24 h later, one object was replaced by a novel object,
1284 and exploratory behavior was analyzed again for 5 min. After each session, objects were
1285 thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an
1286 object was defined as rearing on the object or sniffing it at a distance of less than 1 cm,
1287 touching it with the nose, or both. Successful recognition was reflected by preferential
1288 exploration of the novel object. Discrimination of spatial novelty was assessed by
1289 comparing the difference between exploratory contacts of novel and familiar objects
1290 and the total number of contacts with both, making it possible to adjust for differences
1291 in total exploration contacts. A social interaction test was established using methods
1292 described in ⁵⁷ with slight modifications. WT and Atrx^{ΔE2} mice were housed in new
1293 partitioned cages such that the Atrx^{ΔE2} mouse occupied one compartment and a
1294 weight-matched WT mouse (non-littermate) occupied the other. Forty-eight hours later,
1295 the partition was removed and social interaction between mice was videotaped for
1296 10 min. Scored behaviors were divided into four groups. Active social behavior,
1297 initiated by the experimental mouse toward the partner, includes: (1) *sniffing* of any part
1298 of partner's body including the anogenital area; (2) *following* including direct aggressive
1299 attacks accompanied by bites toward the partner's back. Passive social behavior, which
1300 occurs as a reaction to active behavior of the partner toward the experimental mouse,
1301 includes (3) *receptive responses* when the experimental mouse tolerates sniffing by the
1302 partner but shows no defensive or submissive behavior, (4) *escape* in response to a
1303 partner's following or aggressive act. Data were analyzed as percentage of time spent in
1304 various social behaviors during a 10-min interaction test. The videotapes for all
1305 behavioral analysis were scored by a trained observer blind to genotype and treatment.

1306

1307 **Actin assays**

1308 An *in vitro* F-actin sedimentation assay was performed using the Actin Binding

1309 Protein Spin Down Assay (nonmuscle) kit (BK013; Cytoskeleton, Inc., Denver, CO,
1310 USA). Briefly, recombinant Xlr3b protein (1 μ M) purified from *E. coli* or α -actinin were
1311 mixed with polymerized nonmuscle actin (10 μ M) and incubated 30 min at 22°C.
1312 Mixtures were centrifuged to sediment F-actin, and supernatant and pellet fractions
1313 were analyzed by SDS-PAGE, followed by Silver staining. Actin polymerization was
1314 measured using a pyrene-actin polymerization kit (BK003, Cytoskeleton, Inc.)
1315 following the manufacturer's instructions.

1316

1317 **CD spectra**

1318 All oligonucleotides were purchased from Sigma-Aldrich without further
1319 purification. Oligonucleotides for CD spectra were prepared in Li⁺ solution or K⁺
1320 solution (20 mM lithium cacodylate, pH 7.0, 10 or 100 mM KCl). Annealing was
1321 performed by heating to 95°C for 5 min and cooling down slowly to room temperature.
1322 CD spectra were measured in 0.5-nm steps from 340 to 220 nm using a JASCO
1323 J-805LST Spectrometer and 1-cm quartz cuvettes.

1324

1325 **UV melting experiment**

1326 UV-Vis melting temperature analyses were performed on a V-650
1327 spectrophotometer (JASCO, Japan) with a thermocontrolled PAC-743R cell changer
1328 (JASCO, Japan) and a refrigerated and heating circulator F25-ED (Julabo).
1329 Oligonucleotides (ODN) (5 μ M) in K⁺ solution (20 mM lithium cacodylate, pH 7.0, 5
1330 mM KCl) and 1 eq compounds were added. Annealing was performed by heating to
1331 95°C for 5 min and slowly cooling to room temperature. Melting temperature (T_m) of
1332 each sample was measured at 295 nm from 15 to 95°C at a rate of 1.0 °C /min. ΔT_m was
1333 calculated using T_m values of samples with and without compounds.

1334

1335 **Gel electrophoresis**

1336 DNA samples (ODN) for gel electrophoresis were prepared similarly to CD
1337 spectra samples. Annealing was performed by heating to 95°C for 5 min and cooling
1338 slowly to room temperature. Each sample was analysed by native gel (8%) in 1×TBE
1339 buffer containing 10 or 100 mM KCl at room temperature. Single-stranded DNA was
1340 stained with SYBR® Green I Nucleic Acid Stain (50513, Lonza, Japan). Gels were then
1341 imaged by FLA-3000 (FUJIFILM).

1342

1343 **DMS footprinting**

1344 Samples (ODN) for DMS footprinting were prepared similarly to samples of CD

1345 spectra. DNA samples (10 μ L) were then mixed with 1 μ L of dimethyl sulfate solution
1346 (DMS:ethanol; 4:1, vol/vol). Reactions were quenched with 9 μ L stop buffer (3 M
1347 -mercaptoethanol:water:NaOAc; 1:6:7, vol/vol). After ethanol precipitation and
1348 piperidine cleavage, reactions were separated on 12% denaturing polyacrylamide gels
1349 using a Hitachi DNA sequencer. Electrophoresis was conducted under 1.5 kV, ca. 25
1350 mA, and 40°C.

1351

1352 **Statistical analysis**

1353 To determine the sample size in our experiments, we followed the standard
1354 sample sizes used in similar experiments in each of the relevant fields in the literature.
1355 All values were expressed as means $\pm$ s.e.m. Comparisons between two experimental
1356 groups were made using the two-sided unpaired t test. Statistical significance for
1357 differences among groups was tested by one-way or two-way ANOVA with post-hoc
1358 Bonferroni's multiple comparison test. $P < 0.05$ was considered significant. All the
1359 statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc.,
1360 San Diego, CA, USA). All statistical data were presented in the **Supplementary Table**
1361 **5**.

1362

1363

1364

1365 **Methods-only References**

1366

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1376

1377 **Figure legends (for Supplementary materials only)**

1378

1379 **Supplementary Fig. 1. Identification of Xlr3b in mouse brain.** **a**, Sequence of
1380 *Xlr3a/Xlr3b* mRNA and position of PCR primers. **b**, *Xlr3a* and *Xlr3b* primer specificity
1381 was confirmed by amplifying respective cDNAs. Serial dilutions of cDNA were
1382 prepared and Q-PCR performed. A negative correlation was found between increasing
1383 concentrations of cDNA and Ct. The R squared value (coefficient of determination, R^2)
1384 was used to determine amplification efficiency. The experiments were repeated twice
1385 with similar results. **c**, *Xlr3a* and *Xlr3b* mRNA levels in mouse brain tissue as
1386 determined by RT-PCR. Xlr3a (lane 1) and Xlr3b (lane 2) cDNAs served as positive
1387 controls. W, wild-type mice; A, *Atrx*^{ΔE2} mice; PC, prefrontal cortex; HP, hippocampus;
1388 HT, hypothalamus; CE, cerebellum. **d**, Cultured hippocampal neurons were transduced
1389 with U6gRNA-Cas9-2A-GFP plus guide Xlr3 sgRNA (Xlr3-Cas9-GFP) lentivirus (top)
1390 or control lentivirus expressing GFP (bottom) and immunostained 21 days later.
1391 Confocal images revealed loss of Xlr3 (red) immunoreactivity in MAP2-positive (blue)
1392 neurons infected with Xlr3-Cas9-GFP but not control GFP virus. In **c** and **d**, the
1393 experiments were repeated three times with similar results. **e**, Quantitative real-time
1394 RT-PCR showing *Xlr3b* mRNA expression in cells shown in **d**. $**P < 0.01$ by
1395 two-sided unpaired t-test. $n = 4$ biologically independent samples. **f**, Immunoblot (left)
1396 and corresponding quantitative (right) analysis of Xlr3 protein in cells shown in **d**.
1397 Densitometric analysis of Xlr3 normalized to β -tubulin (arbitrary units, A.U.). $**P <$
1398 0.01 by two-sided unpaired t-test. $n = 5$ biologically independent samples.

1399

1400 **Supplementary Fig. 2. Bisulfite sequencing evaluation of Xlr3 CGI methylation in**
1401 ***Atrx*^{ΔE2} mouse brain lysates.** **a**, (top) Schematic showing clusters of Xlr genes on the
1402 C57BL/6J X chromosome. See also Raefski and O'Neill, (2005)²³. (bottom) Location of
1403 Xlr3 CpG sites analyzed. Sequences potentially forming G-quadruplex is shaded in gray.
1404 **b**, Methylation status of Xlr3 CpG sites. Open circles, unmethylated CpGs; closed
1405 circles, methylated CpGs. Male P90 mice were used. $n = 3$ mice each. 4 independent
1406 clones of each sample were sequenced. Roman numerals correspond to those of the
1407 CpG sites shown in **a** and **b**.

1408

1409 **Supplementary Fig. 3. G-quadruplex formation by sequences of Xlr3b CGI**
1410 **(Xlr3b-ODN).** **a**, CD spectra of Xlr3b-ODN in Li^+ or K^+ solutions. **b**, Xlr3b-ODN
1411 primarily formed intramolecular, parallel G-quadruplexes based on native gel

1412 electrophoresis. **c**, DMS footprinting of G-quadruplexes formed on Xlr3b-ODN in the
1413 Li^+ or K^+ solutions. In **a-c**, the experiments were repeated twice with similar results.

1414

1415 **Supplementary Fig. 4. ATRX interacts with DAXX, DNMT1, DNMT3A and H3.3**
1416 **in mouse hippocampus. a**, Effect of ATRX shRNA in Neuro-2a cells. Immunoblot
1417 analysis (left) and densitometric quantification (right) of protein expression.
1418 Densitometric analysis of ATRX normalized to β -tubulin (arbitrary units, A.U.). $**P <$
1419 0.01 by two-sided unpaired t-test. $n = 3$ biologically independent samples. **b**, (left)
1420 Representative immunoblot of P90 mouse hippocampal lysates probed with indicated
1421 antibodies, (right) Quantitative densitometry analyses. $**P < 0.01$ by two-sided
1422 unpaired t-test. $n = 6$ mice each. **c**, ATRX was immunoprecipitated (IP'd) from P90 WT
1423 and $\text{Atrx}^{\Delta\text{E}2}$ hippocampal extracts, and western blot analysis performed for indicated
1424 proteins. Control reactions were performed with IgG. Extracts were assessed as 1%
1425 input. The experiments were repeated three times with similar results.

1426

1427 **Supplementary Fig. 5. Purified Xlr3b protein does not bind F-actin or alter actin**
1428 **polymerization. a**, F-actin binding assay. Supernatant (S) and pellet (P) fractions were
1429 collected and samples separated on a SDS-gel subsequently stained using a Silver Stain
1430 Kit. Reactions were set up as indicated at top. Most α -actinin was found in pellet in the
1431 presence of F-actin filaments (lanes 9 and 10), while Xlr3b remained in the supernatant
1432 in presence of F-actin filaments (lanes 5 and 6). **b**, Actin polymerization as measured by
1433 enhanced fluorescence of pyrene-conjugated actin. Xlr3b addition to purified actin did
1434 not alter polymerization. In **a** and **b**, the experiments were repeated twice with similar
1435 results.

1436

1437 **Supplementary Fig. 6. Xlr3b co-localizes with hnRNP A/B. a-c**, Confocal images
1438 showing co-localization of FLAG-tagged Xlr3b constructs with hnRNP A/B (**a**), Dcp1a
1439 (**b**) and ubiquitin (**c**) in Neuro-2a cells. Nuclear DNA is labeled with DAPI (blue). Scale
1440 bars, $10\mu\text{m}$. **d**, Neuro-2a cells were transfected with FLAG-tagged Xlr3b constructs,
1441 cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, and western blot
1442 (WB) was probed with ubiquitin antibody. **e**, (top) Sequence comparison of Xlr3b
1443 residues 158-170 and comparable sequences from the Xlr human orthologs FAM9A and
1444 FAM9B. Identical AAs are in blue and similar in light green. (bottom), Confocal images
1445 show localization of FLAG-tagged FAM9A constructs (green) in Neuro-2a cells.
1446 Nuclear DNA is labeled with DAPI (blue). Scale bars, $10\mu\text{m}$. In **a-e**, the experiments
1447 were repeated three times with similar results.

1448

1449 **Supplementary Fig. 7. Interaction of Xlr3b AA 158-170 and RBPs.** **a**, Diagram of
1450 permeabilization control peptide (antennapedia homeodomain (ANTP)) and Xlr3b
1451 inhibitory peptide (XIP). The latter is a 29-AA peptide that contains Xlr3b AA 158-170
1452 plus ANTP. **b**, FL-Xlr3b-transfected cells were treated with XIP (1 μ M for 4h) and
1453 immunostained for TIA1. Scale bars, 20 μ m. **c**, Pull-down assays with an ANTP
1454 antibody assessing TIA1 and hnRNP A/B in P90 mouse brain lysates. Eluted proteins
1455 and inputs were immunoblotted with indicated antibodies. Extract samples served as 1%
1456 input. IP, immunoprecipitation. In **b** and **c**, the experiments were repeated three times
1457 with similar results.

1458

1459 **Supplementary Fig. 8. Dynamics of CaMKII α mRNA transport in distal dendrites.**
1460 **a**, Confocal images of mCherry-Xlr3b Δ 124-200 or Δ 158-170 (red) and GFP-CaMKII α
1461 3' UTR (green) in cultured neurons at day 21 *in vitro*. Scale bars, 10 μ m. The
1462 experiments were repeated three times with similar results. **b**, (top, left) A confocal
1463 image of GFP-CaMKII α 3' UTR in primary mouse cultured neurons at day 21 *in vitro*.
1464 Scale bar, 50 μ m. Images at right is enlarged from corresponding boxed area. Scale bar,
1465 10 μ m. See also **Supplementary Video 2**. (bottom) Relative frequency of movement of
1466 GFP-CaMKII α 3' UTR granules. $**P < 0.01$ by two-way ANOVA with Bonferroni's
1467 post hoc test; n = 5 neurons each, a distal dendrite (100-200 μ m away from the cell
1468 body) per neuron to measure relative frequencies. Cells were treated with XIP (1 μ M) 4
1469 h before imaging. Imm., immobile; Bidirect., bidirectional movement; Antero.,
1470 anterograde movement; and Retro., retrograde movement.

1471

1472 **Supplementary Fig. 9. Generation of Thy1-Xlr3b transgenic mice.** **a**, Genomic
1473 organization of Thy1 gene (top) and the transgenic construct (bottom). The Xlr3b
1474 cDNA was subcloned into a XhoI site of the Thy1.2 expression cassette. Blue boxes,
1475 untranslated exons. **b**, Representative PCR genotyping using tail DNA of transgenic
1476 founders (#13 and #57). M, size marker. **c**, Quantitative real-time RT-PCR showing
1477 *Xlr3b* mRNA expression in P90 mouse hippocampal lysates. $**P < 0.01$ by one-way
1478 ANOVA with Bonferroni's post hoc test; n = 6 mice each. TG, Xlr3b transgenic mouse.
1479 **d**, (top) Representative immunoblot of mouse hippocampal lysates probed with Xlr3
1480 and β -tubulin antibodies, (bottom) Densitometric analysis of Xlr3 normalized to
1481 β -tubulin (arbitrary units, A.U.). $**P < 0.01$ by two-sided unpaired t-test; n = 5 mice
1482 each. **e**, Effect of Xlr3b shRNA in cultured neurons. Immunoblot analysis (top) and
1483 densitometric quantification (bottom) of protein expression. Densitometric analysis of

1484 Xlr3 normalized to β -tubulin (arbitrary units, A.U.). $*P < 0.05$ by two-sided unpaired
1485 t-test; $n = 3$ biologically independent samples. **f**, (left) Method for isolation of
1486 synaptosomal membrane fractions from cultured neurons. The procedure for the
1487 subcellular fractionation is described in Methods. P1, nucleus/cell debris; S1,
1488 postnuclear supernatant; S2, cytosol fraction; P2, crude synaptosomal fraction; LS1,
1489 synaptosomal cytosol fraction; LP1, synaptosomal membrane fraction. Immunoblot
1490 showing CaMKII α and postsynaptic marker, PSD95 between S2, LS1, and LP1 in
1491 cultured neurons from WT mice. (middle and right) Immunoblot and corresponding
1492 quantitative analysis of CaMKII α and PSD95 proteins at LP1 and whole cell lysates in
1493 cultured neurons. Densitometric analyses of CaMKII α normalized to PSD95 (arbitrary
1494 units, A.U.). $**P < 0.01$ by one-way ANOVA with Bonferroni's post hoc test. (In WT
1495 vs. Xlr3b-TG, $**P < 0.01$ by two-sided unpaired t-test.) LP1, $n = 5$ biologically
1496 independent samples; whole cell lysates, $n = 4$ biologically independent samples. **g**,
1497 (top) Representative field excitatory post-synaptic potentials (fEPSPs) were recorded
1498 from the hippocampal CA1 region of mice. (left), Changes in fEPSP slope following
1499 high frequency stimulation (HFS) were attenuated in Xlr3b-TG mice in hippocampal
1500 CA1. (right), Changes in fEPSP slope following HFS at 1 or 60 min. $**P < 0.01$ by
1501 two-way ANOVA with Bonferroni's post hoc test; $n = 8$ mice each. **h**, Paired pulse
1502 facilitation (left) and input-output relationship (right) were recorded. $n = 5$ mice each.
1503 There were no significant changes between the groups. **i**, LTP-induced CaMKII α
1504 phosphorylation in the hippocampus. (left) Representative images of immunoblots
1505 using antibodies against phosphorylated CaMKII α (pCaMKII α) and total CaMKII α .
1506 (right) Densitometric analysis of pCaMKII α normalized to total CaMKII α (arbitrary
1507 units, A.U.). $**P < 0.01$, $*P < 0.05$, vs. WT mice before high-frequency stimulation
1508 (HFS), $###P < 0.01$, $\#P < 0.05$, before HFS vs. after HFS in each group by two-way
1509 ANOVA with Bonferroni's post hoc test. Respective sample sizes are indicated. **j-l**,
1510 Xlr3b-TG mice show memory deficits. Novel-object recognition (NOR) test (**j**),
1511 Latency time in retention trials in a passive avoidance (PA) test (**k**), Alternations in a
1512 Y-maze test (**l**) are shown. $**P < 0.01$, $*P < 0.05$ by two-sided unpaired t-test.
1513 Respective sample sizes are indicated.

1514

1515 **Supplementary Fig. 10. TMPyP4 treatment inhibits Xlr3b expression.** **a**, The
1516 binding effect of Protoporphyrin IX (PpIX), hemin and TMPyP4 on G-quadruplexes
1517 formed by Xlr3b-ODN based on a UV melting experiment. **b**, Luciferase activity of
1518 Neuro-2a cells transfected with pGL3-2K or pGL3-2K Δ G4 and treated with TMPyP4 (1,
1519 3, 10, 30 and 100 μ M) for 48 h. Luciferase activity is shown relative to activity in

vehicle-treated cells. $**P < 0.01$ by one-way ANOVA with Bonferroni's post hoc test; $n = 3$ biological replicates. **c**, Quantitative real-time RT-PCR showing *Xlr3b* mRNA expression in mouse hippocampal lysates. $**P < 0.01$ vs. vehicle-treated WT mice, $##P < 0.01$ vs. vehicle-treated *Atrx*^{ΔE2} mice by one-way ANOVA with Bonferroni's post hoc test; $n = 4$ mice each. **d**, (top) Representative immunoblot of mouse hippocampal lysates probed with Xlr3 and β -tubulin antibodies, (bottom) Densitometric analysis of Xlr3 normalized to β -tubulin (arbitrary units, A.U.). $**P < 0.01$ vs. vehicle-treated WT mice, $##P < 0.01$ vs. vehicle-treated *Atrx*^{ΔE2} mice by one-way ANOVA with Bonferroni's post hoc test; $n = 4$ mice each. **e**, Methylation status of Xlr3b CpG sites. Open circles, unmethylated CpGs; closed circles, methylated CpGs. Male P90 mice were used. $n = 3$ mice each. three independent clones of each sample were sequenced.

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Supplementary Fig. 11. TMPyP4 treatment rescues cognitive deficits seen in *Atrx*^{ΔE2} mice. **a**, Relative frequency of movement of GFP-CaMKII α 3' UTR granules in distal dendrites. Cells were treated with 5-ALA (1 μ M) for 7 days before imaging. $**P < 0.01$, $*P < 0.05$ vs. vehicle-treated WT neurons, $##P < 0.01$ vs. vehicle-treated *Atrx*^{ΔE2} neurons by two-way ANOVA with Bonferroni's post hoc test; $n = 5$ neurons each, a distal dendrite (100-200 μ m away from the cell body) per neuron to measure relative frequencies. Imm., immobile; Bidirect., bidirectional movement; Antero., anterograde movement; and Retro., retrograde movement. **b**, Paired pulse facilitation (left) and input-output relationship (right) were recorded. $n = 5$ mice each. There were no significant changes between the groups. **c**, (left) Immunoblot and corresponding quantitative analyses of CaMKII α and PSD95 proteins at LP1 (synaptosomal membrane fractions) and whole cell lysates in cultured neurons. Densitometric analyses of CaMKII α normalized to PSD95 (arbitrary units, A.U.). $*P < 0.05$ vs. vehicle-treated WT neurons, $#P < 0.05$ vs. vehicle-treated *Atrx*^{ΔE2} neurons by one-way ANOVA with Bonferroni's post hoc test; $n = 5$ biologically independent samples. (right) LTP-induced CaMKII α phosphorylation in the hippocampus. Representative images of immunoblots using antibodies against phosphorylated CaMKII α (pCaMKII α) and CaMKII α . Densitometric analysis of pCaMKII α normalized to total CaMKII α (arbitrary units, A.U.). $*P < 0.05$, vs. WT mice before high-frequency stimulation (HFS), $##P < 0.01$, before HFS vs. after HFS in each group by two-way ANOVA with Bonferroni's post hoc test; $n = 4$ mice each. **d-f**, TMPyP4 treatment rescued cognitive deficits in *Atrx*^{ΔE2} mice based on memory-related behavioral tests. Novel-object recognition (NOR) test (**d**), latency time in retention trials in a passive avoidance (PA) test (**e**), alternations in a Y-maze test (**f**) are shown. $**P < 0.01$ vs. vehicle-treated WT mice, $#P < 0.05$, $##P <$

0.01 vs. vehicle-treated *Atrx*^{ΔE2} mice by one-way ANOVA with Bonferroni's post hoc test; Respective sample sizes are indicated. **g**, *Atrx*^{ΔE2} mice show withdrawal in social interactions with WT mice. *Atrx*^{ΔE2} mice showed enhanced passivity, higher escape duration and decreased social activity, such as following and sniffing behaviors, in social interactions with WT mice. These behaviors are dramatically improved by 5-ALA treatment. ***P* < 0.01, **P* < 0.05 vs. vehicle-treated WT mice, ## *P* < 0.01, #*P* < 0.05 vs. vehicle-treated *Atrx*^{ΔE2} mice by one-way ANOVA with Bonferroni's post hoc test; Respective sample sizes are indicated. **h**, Measurements of TMPyP4 fluorescence levels. Chronic intraperitoneal injection of TMPyP4 in P90 mice increased fluorescence levels in some tissues, including brain. Respective sample sizes are indicated. **i**, Measurements of body weight following chronic TMPyP4 administration on day 60 (i.p. twice weekly from P30 to P90). Respective sample sizes are indicated. **j**, Measurements of 5-ALA levels in P90 mouse brain after oral administration (3mg/kg, p.o.). Respective sample sizes are indicated.

1570

Supplementary Fig. 12. Schematic showing outcomes following *Atrx* mutation.

ATRAX binds to parallel G-quadruplexes in *Xlr3b* CGIs along with DNMTs, DAXX and H3.3, regulating *Xlr3b* gene expression through DNA methylation in cooperation with DNMTs. CaMKIIα mRNA locates in neuronal dendrites, and its transport is dynamically regulated by the motor protein, dynein. CaMKIIα translation enhances synaptic efficacy postsynaptically, which is critical for learning and memory. In control neurons, ATRAX binds G-quadruplex-forming DNA in *Xlr3b* CGIs, inhibiting *Xlr3b* expression through DNA methylation. In *Atrx* mutant neurons, aberrant expression of *Xlr3b* protein occurs through DNA de-methylation at the site. *Xlr3b* protein has RNA binding capacity and cooperates with RNA binding proteins (RBPs), and inhibits dendritic transport of CaMKIIα mRNA, resulting synaptic dysfunction. Treatment with G-quadruplex (G4) ligand 5-ALA represses *Xlr3b* transcription, antagonizing both synaptic dysfunction and cognitive deficits in *Atrx* mutant mice.

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Supplementary Fig. 13. Full-size scans of western blots shown in figures.

1586

Supplementary Table 1. List of genes exhibiting differential expression in hippocampus of P90 WT and *Atrx*^{ΔE2} mice.

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Supplementary Table 2. *Xlr3* interaction partners identified in proteomic screen using LC-MS/MS analysis.

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1592

1593 **Supplementary Table 3. List of genes exhibiting differential expression in P90 WT,**
1594 **Atrx^{ΔE2} and 5-ALA treated Atrx^{ΔE2} mouse hippocampus.**

1595

1596 **Supplementary Table 4. List of genes exhibiting differential expression in P90 WT,**
1597 **Atrx^{ΔE2} and TMPyP4 treated Atrx^{ΔE2} mouse hippocampus.**

1598

1599 **Supplementary Table 5. Summary of all statistical data.**

1600

1601 **Supplementary Video 1. Time-lapse imaging of GFP-MS2-labeled CaMKIIα**
1602 **mRNA (GFP-CaMKIIα 3' UTR) in a proximal dendrite of a cultured WT neuron.**

1603

1604 **Supplementary Video 2. Time-lapse imaging of GFP-MS2-labeled CaMKIIα**
1605 **mRNA (GFP-CaMKIIα 3' UTR) in a distal dendrite of a cultured WT neuron.**

1606

Fig. 1

Fig. 2

Fig. 3

CaMKIIα 3' UTR

WT vs Atrx ^{ΔE2}		Atrx ^{ΔE2} vs Atrx ^{ΔE2} +5-ALA	
Up	8 genes	Not Rescued	<i>Prdm16, M6prbp1</i>
		Rescued	<i>Xlr3b, Cap1, Xlr3a, S100a4, Fbxo39, D0300131/16Rik</i>
Down	23 genes	Rescued	<i>Plcb4, F2r, Atp7a, Zic1, ENSMUSG00000056615, Inpp4b, Vash2, Arsj, Tmie, Rgs16, Agxt2l1, Zic2, Mrvi1, Ramp3, Slc17a6, Spry2</i>
		Not Rescued	<i>Rora, Cpne9, 4921511K06Rik, Klhdc8a, Atrx, Cbln1, Tac2</i>

Fig. 5