

AAV-delivery of diacylglycerol kinase kappa achieves long-term rescue of Fmr1 -KO mouse model deficits of fragile X syndrome

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Karima Habbas, Oktay Cakil, Boglarka Zambo, Ricardos Tabet, Fabrice Riet, et al.. AAV-delivery of diacylglycerol kinase kappa achieves long-term rescue of Fmr1 -KO mouse model deficits of fragile X syndrome. 2021. hal-03450738

HAL Id: hal-03450738 https://hal.science/hal-03450738

Preprint submitted on 29 Nov 2021

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1	Title: AAV-delivery of diacylglycerol kinase kappa achieves long-term
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3	
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24	
25	One sentence summary: DGKk gene therapy in Fmr1-KO mouse model
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27	Keywords:
28	Fragile X syndrome, FMRP, AAV, diacylglycerol kinase, Fmr1-KO
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34

35 Abstract:

36 Fragile X syndrome (FXS) is the most frequent form of familial intellectual disability. It

37 results from the lack of the RNA binding protein FMRP and is associated with the

38 overactivation of signaling pathways downstream of mGluRI receptors and upstream of

39 mRNA translation. We previously found that diacylglycerol kinase kappa (DGKk) is a main

- 40 mRNA target of FMRP in cortical neurons. Here we show that diacylglycerol kinase kappa
- 41 (DGKk), when modified as to become FMRP-independent and delivered into the brain of
- 42 adolescent mice using adeno-associated viral vectors, corrects brain diacylglycerol and
- 43 phosphatidic acid homeostasis and the main phenotypic behaviors of the Fmr1-KO mouse
- 44 model of FXS. Thus, DGKk appears as a key triggering factor of FXS pathomechanism while
- 45 providing a possible means of intervention for FXS gene therapy.
- 46

47 Main Text:

48

49 INTRODUCTION

50 Fragile X syndrome (FXS) is a main cause of familial intellectual disability and autistic

51 spectrum disorder (ASD) with a prevalence in general population estimated as 1 in 5000

52 males and 1 in 8000 females (Hagerman et al., 2017; Kaufmann et al., 2004). FXS is also

53 generally associated with variable behavioral symptoms that can include anxiety,

54 hyperactivity, hypersensitivity, stereotypies, memory deficits and sleeping problems. FXS

55 results from the loss of the fragile X mental retardation protein (FMRP), an RNA binding

56 protein associated to mRNAs and the translation machinery and whose absence in *Fmr1*-

57 deleted mice (*Fmr1*-KO) recapitulates FXS-like phenotypes (1994; Mientjes et al., 2006),

58 with perturbation of neuronal protein translation in hippocampus and cortex (Dolen et al.,

59 2007; Qin et al., 2005). FMRP loss leads to mRNA translation increase of neuronal proteins

60 resulting from an overactivation of metabotropic group 1 glutamate receptor (mGluR)-

61 dependent local mRNA translation (Bear et al., 2004), including phosphatidylinositol 3-kinase

62 enhancer (PIKE) (Gross et al., 2015), matrix metalloproteinase 9 (MMP9) (Gkogkas et al.,

63 2014; Sidhu et al., 2014), glycogen synthase kinase 3 (GSK3) (Guo et al., 2012) and amyloid-

64 β A4 protein (APP) (Pasciuto et al., 2015; Westmark et al., 2011). The mGluR overactivation

- 65 is one well established triggering factor of FXS pathomechanism, and we recently showed
- 66 that mGluR diacylglycerol (DAG) and phosphatidic (PA) dependent signaling upstream of
- 67 local mRNA translation is disrupted by FMRP loss (Tabet et al., 2016a). The diacylglycerol

68 kinase kappa (DGKk) transcript was identified in mouse cortical neurons as the mRNA

- 69 species most bound by FMRP and with highest in vitro binding affinity. DGKk expression
- 70 was found severely reduced in the brain of *Fmr1*-KO mouse and a perturbation of DAG/PA
- 71 acid homeostasis was observed in *Fmr1*-KO cortical neurons and in the brain of FXS
- 72 individuals, suggestive of a decreased DGK activity and altered DAG/PA signaling (Tabet et
- al., 2016a). DGKk knockdown in wild-type mouse brains recapitulated FXS-like behaviors,
- and overexpression of DGKk in *Fmr1*-KO hippocampal slices rescued their abnormal
- 75 dendritic spine morphology (Tabet et al., 2016a). Overall, DGKk appears to play a key role in
- 76 dendritic spine morphology and function and in the determination of FXS-like behaviors.
- 77 DGKk loss of function was proposed to be at the origin of the various forms of abnormal
- 78 synaptic signaling in FXS by causing altered DAG/PA signaling (Tabet et al., 2016b). Thus,
- being the most proximal downstream mediator of FMRP action, DGKk could represent an
- 80 interesting actionable therapeutic target.
- 81 Here we show that DGKk expression is lost in FXS patients' postmortem brains as previously
- 82 shown in *Fmr1*-KO mice (Tabet et al., 2016a). We show that the N-terminal region of DGKk
- 83 is important for its positive translational control by FMRP and the deletion of this region
- 84 renders the protein (ΔN-DGKk) independent of FMRP, suggesting that FMRP alleviates a
- 85 translation blockade within the N-terminal region. ΔN-DGKk is able to modulate protein
- 86 synthesis rate and eIF4E phosphorylation in neurons. Moreover, ΔN-DGKk expression in
- 87 Fmr1-KO mouse brain using adeno-associated virus Rh10 (AAVRh10) is able to correct brain
- 88 lipid profile dysregulations and achieve long term behavioral rescue (over 8 weeks after
- injections) of *Fmr1*-KO mouse, providing a first proof of principle of *DGKk* gene therapy in a
 mouse model of FXS.
- 91

92 **RESULTS**

93

94 DGKk mRNA translation requires FMRP and DGKk N-terminal truncation alleviates 95 FMRP control.

- 96 We previously showed that the expression of diacylglycerol kinase kappa (DGKk) is strongly
- 97 reduced in brain of *Fmr1*-KO mouse (Tabet et al., 2016a) in agreement with the fact that
- 98 DGKk mRNA was identified as the most abundant mRNA species associated with FMRP by
- 99 crosslinking immunoprecipitation in cortical neurons, and with the highest in vitro binding
- 100 affinity. Like in *Fmr1*-KO mouse brain, DGKK expression is also lost in FXS post-mortem
- 101 cerebellum compared to unaffected controls (Fig. 1A). These data suggest that DGKk

102 requires FMRP for its proper expression. DGKk is mostly expressed in neurons and is almost 103 absent in non-neuronal cells (Tabet et al., 2016a). We then tested if it is possible to 104 recapitulate FMRP control in a non-neuronal cell system. We analyzed the influence of 105 FMRP on the expression of HA-tagged mouse and human DGKk (Fig. 1B, Sup Fig. 1A) 106 transfected into two different cell lines, Cos-1 and Hela. Knock-down of endogenous FMRP 107 with siRNA severely reduced the expression level of mouse DGKk (mDGKk) compared to 108 control siRNA treated Cos-1 cells (Fig. 1BC) indicating a strong FMRP requirement for 109 DGKk expression. Human DGKk (hDGKk) level is also affected by FMRP knock-down, 110 indicating that FMRP control is conserved on RNA sequences from different species (Sup 111 Fig. 1A). mDGKk mRNA level is not influenced by the lack of FMRP (Sup Fig. 1B), while 112 the protein level is severely reduced, supporting a control mechanism at the mRNA 113 translational level. This is in agreement with our previous data in mouse brain indicating that 114 DGKk transcript level is not affected by the loss of FMRP, and DGKk mRNA is less associated with polyribosomes (Tabet et al., 2016a). Noticeably, DGKk is the only DGK 115 116 isozyme interacting strongly with FMRP (Tabet et al., 2016a) and bearing a long N-terminal 117 extension constituted of unique proline-rich and EPAP repeated motives (Imai et al., 2005) 118 (Fig. 1B). The N-terminal part of the protein might play a critical role in DGKk expression 119 considering the repetitive nature of the EPAP domain at the beginning of the coding 120 sequence. Thus, we generated a mDGKk construct lacking the first 696 bases following the 121 start codon, encompassing the EPAP domain (Δ N-DGKk), for expression assessment in cells. 122 △N-DGKk lead to a strong increase of expression (about ten-fold higher) (Fig. 1D). FMRP 123 reduction did not affect ΔN -DGKk (Fig. 1D), suggesting that the N-terminal domain of 124 DGKk is required for FMRP control and that Δ N-DGKk expression does no longer depend on 125 the presence of FMRP. Therefore, ΔN -DGKk represents a potential therapeutic target for FXS 126 gene therapy by bypassing the need for FMRP. 127

128 **AN-DGKk counteracts with FXS-like molecular defects.**

129 DGKk deregulation has been proposed to play a key role in FXS pathomechanism by altering 130 DAG/PA signaling and leading to an excess of DAG and lack of PA potentially responsible of 131 excessive protein synthesis, the major molecular hallmark of FMRP defects (Tabet et al., 132 2016a; Tabet et al., 2016b). To test the potential rescuing activity of Δ N-DGKk, we first 133 analyzed its ability to modulate protein synthesis rate. Expression of Δ N-DGKk in Cos-1 cells 134 reduced in a concentration dependent manner the protein translation rate, and this effect was 135 counteracted by pretreatment of cells with DGK specific inhibitors R59022 and R59949

136 (Jiang et al., 2000) (Fig. 2A). These data demonstrate that ΔN -DGKk activity is upstream of 137 mRNA translation and relies on conversion of DAG into PA, as DGK inhibitors prevent ΔN -138 mDGKk from reducing mRNA translation by blocking its enzymatic activity. 139 To assess the biodistribution and efficacy of ΔN -DGKk in a FXS mouse model, we built our 140 expression cassette to be driven by the neuron specific promoter synapsin and packaged into 141 two different adeno-associated viral vectors (AAV), Rh10 and PHP.eB. Both AAV vectors 142 harboring AN-DGKk demonstrated efficient neuronal transduction in *Fmr1*-KO cortical 143 neurons, confirming that ΔN -DGKk can be expressed in the absence of FMRP (Fig. 2B). 144 Phosphorylation of initiation factor eIF4E, which regulates protein synthesis by reducing 145 translation initiation level (Sonenberg, 1994) and is increased after DAG-signaling activation 146 (Wang et al., 1998), is increased in *Fmr1*-KO mouse and FXS patients (Gantois et al., 2017). 147 We show that P-eIF4E is also increased in *Fmr1*-KO mouse cortical neurons compared to WT 148 littermates (Sup Fig. 2A) and ΔN -DGKk is able to reduce their eIF4E phosphorylation (Fig. 149 **2C**). No sign of Δ N-DGKk-specific neuronal toxicity was observed in neuronal cultures 150 transduced with AAV expressing ΔN -DGKk at high multiplicity of infection (MOI) (Sup Fig. 151 2B-E). An overall reduction in NeuN positive cells was observed upon treatment of cells with 152 AAV, independently from Δ N-DGKk expression, as the same effect was observed with AAV-153 FMRP. Such effect was visible with a 10-fold lower amount of AAV-FMRP (10e9 VG/well) 154 (Sup Fig. 2D). Additional tests (caspase 3/7, LDH) did not show signs of toxicity in vitro

- 155 (Sup Fig. 2B, C).
- 156

157 In vivo correction of phosphatidic acid level in adult mice using multiple routes ofadministration

- 159 Δ N-DGKk was administered to 5-week-old *Fmr1*-KO mice by intravenous injection of
- 160 AAVPHP.eB-ΔN-DGKk or intracerebral injection into the striatum and hippocampus of
- 161 AAVRh10-ΔN-DGKk, respectively. Single retro-orbital injection of AAVPHP.eB-ΔN-DGKk
- 162 at 10^11 VG/mouse enabled 0.5-1 VG/cell throughout the brain (**Fig. 3AB**) four or eight
- 163 weeks after injection, with low protein expression as visualized by Western blot (Fig. 3C) and
- 164 immunohistochemistry (**Fig. 3D**, **Sup Fig. 3A**). Intracerebral injection of AAVRh10-ΔN-
- 165 DGKk at 5x10^11 VG/mouse enabled higher brain transduction, with about 100 VG/cell in
- 166 the hippocampus and about 25 VG/cell in rest of brain leading to high protein expression
- 167 levels (Fig. 3BCD). AAVRh10 vector lead to robust ΔN-DGKk expression throughout the
- 168 hippocampus (CA1 and CA2 regions mainly), cortex and striatum (Fig. 3D, Sup Fig. 3A).

169 DGKk deregulation was shown to alter DAG/PA balance in neuronal cultures and human 170 postmortem brains (Tabet et al., 2016a). We confirmed a marked decrease of total PA level 171 $(22\% \pm 8)$ in the cortex of *Fmr1*-KO mice at 13 weeks of age (Fig. 3E). The other mouse brain 172 regions did not show significant differences (Sup Fig. 3B). The decrease in the total pool of 173 PA is reflected by a reduction of most PA species (including abundant species 34:1, 36:2, 174 38:4 or low species 38:3, 40:1) (Sup Fig. 3D-F). Thus, DGKk loss in *Fmr1*-KO cortex led to 175 alteration of PA phosphorylation independent from fatty acid patterns. ΔN -DGKk delivery to 176 the brain alleviated PA reduction in *Fmr1*-KO and led to a PA level comparable to vehicle 177 injected WT mice (Fig. 3E). This is true for all fatty acid PAs with levels undistinguishable 178 from control, demonstrating a complete rescue of PA by ΔN -DGKk expression (Sup Fig. 3D-179 **F**). Intravenous delivery of PHP.eB- Δ N-DGKk led to partial correction of total PA level (Fig. 180 **3E**) that was further confirmed at the single PA level, indicating that ΔN -DGKk is able to 181 balance the PA with only few neurons transduced (Sup Fig. 3D-F). Total DAG level was not 182 significantly altered in cortex (Fig. 3F) and other brain areas tested (Sup Fig. 3C), and at the 183 level of individual DAG species (Sup Fig. 3 G-I). Nine other lipid classes tested did not show 184 significant differences between the groups (Sup Fig. 7J) indicating that the effect of ΔN -

- 185 DGKk is restricted to the correction of DAG/PA balance.
- 186

187 AAVRh10-ΔN-DGKk achieves long-term rescue of *Fmr1*-KO behavioral defects.

Four weeks after injections, a battery of behavioral tests was performed on the AAV injected *Fmr1*-KO mice and their vehicle injected WT (WT-S) or *Fmr1*-KO (Fmr1-S) littermate
controls (Sup table 1). Vehicle injected *Fmr1*-KO mice showed an increase of time spent and

191 in number of entries in open arms of the elevated plus maze (EPM) compared to the WT-

192 vehicle mice (1.9 and 1.8-fold of mean increase, respectively), suggesting decreased anxiety

induced by the genotype. In contrast, AAVRh10-ΔN-DGKk treated mice (Fmr1-Rh10)

194 showed no difference compared to WT-vehicle mice (Fig. 4A). A similar phenotype was

195 observed in the open field arena of novel object recognition test (NOR), where vehicle

196 injected *Fmr1*-KO mice showed an increase of time spent in the center of the arena, while

197 AAVRh10-ΔN-DGKk treated mice were not different from WT-vehicle (**Fig. 4B**).

198 Vehicle injected *Fmr1*-KO mice showed increased locomotor activity compared to WT-

199 vehicle mice. This phenotype was seen in the EPM test (Fig. 4A, number of entries in open

200 and closed arms), in the open field arena of NOR (Fig. 4B, distance travelled in habituation,

201 acquisition and retention phases) and in the habituation phase of three-chambers social

202 recognition test (Fig. 4C, number of entries), and was corrected in all tests with AAVRh10-

203 ∆N-DGKk treatment. Signs of hyperactivity of *Fmr1*-KO mice were also seen in the first hour 204 of light and dark phases of actimetry test (Sup Fig. 4AB), suggesting this phenotype is related 205 to novelty of environment. AAVRh10-ΔN-DGKk treated mice did not show this phenotype. 206 No difference in recognition index was observed between genotypes in the NOR test (Fig. 207 **4B**). Performing the NOR test in a smaller size arena ameliorated the recognition index of 208 objects (Sup Fig. 4D), in correlation with longer object exploration times of objects (Sup Fig. 209 **4CD**, explorations). However, no difference was observed between the genotypes, suggesting 210 an apparent lack of significant memory impairment of the *Fmr1*-KO mice. In small size arena 211 the hyperlocomotor activity phenotype of *Fmr1*-KO mice was not visible (Sup Fig. 4D), 212 potentially because this environment was less anxiogenic than a larger arena. Fmr1-KO 213 vehicle mice showed a trend to dig more, to bury more marbles and to spend less time in 214 grooming compared to the WT-vehicle mice, but these differences were not statistically 215 significant (Sup Fig. 4E). ΔN -DGKk treatment seemed to counteract these effects, but the 216 high variability of these phenotypes between mice precluded drawing conclusions. 217 Vehicle injected *Fmr1*-KO mice showed an increased number of entries in the three-chambers 218 social recognition test and this phenotype was corrected with AAVRh10-ΔN-DGKk (Fig. 4C, 219 Sup Fig. 4F). For social preference over object, no difference was observed between 220 genotype groups (Fig. 4C, Sup Fig. 4F) indicating there was no significant effect of mutation 221 or treatment on social preference. In the social memory test, Fmr1-KO mice failed to 222 recognize previously encountered mice, while Fmr1-KO mice treated with AAVRh10- Δ N-223 DGKk had a higher recognition index relative to untreated mice (Fig. 4C). 224 Vehicle injected *Fmr1*-KO mice also showed a reduced ability to build a nest after 5 and 24h. 225 AAVRh10- Δ N-DGKk treatment rescued this phenotype (Sup Fig. 4G). 226 The phenotypes observed at 4 weeks after injections were generally recapitulated in older 227 animals at 8 weeks after injection (Sup Fig. 5A-F). Consistent with the stability of the 228 treatment effect, no significant variation of ΔN -DGKk protein level was observed between 8 229 and 12 weeks after AAV injection (corresponding to animal groups phenotyped at 4 and 8 230 weeks, respectively) (Sup Fig. 3A). *Fmr1*-KO-vehicle mice tested at 8 weeks showed 231 reduced recognition index in NOR test compared to WT-vehicle and Fmr1-KO-Rh10- Δ N-232 DGKk (Sup Fig. 5D), but within a smaller test arena where *Fmr1*-KO-vehicle mice exhibit 233 less hyperactivity (Sup Fig. 5E) all groups performed equally, suggesting that the memory 234 performance alteration of Fmr1-KO mice might be caused by hyperactivity. Fmr1-KO-235 vehicle mice tested at 8 weeks after injection (13-week-old) showed a strong digging behavior

compared to WT-vehicle and *Fmr1*-KO-Rh10- Δ N-DGKk, a phenotype that was not visible at

- 237 4 weeks (Sup Fig. 5F). An increase in body weight was observed in *Fmr1*-KO-vehicle group
- 238 compared to WT-vehicle. WT-vehicle and *Fmr1*-KO-Rh10-ΔN-DGKk showed no weight
- 239 difference, suggesting the treatment rescued this phenotype (Sup Fig. 5G).
- 240 *Fmr1*-KO-vehicle phenotypes were reproduced in another cohort aimed at testing retro-orbital
- 241 administration of PHP.eB- Δ N-DGKk (Sup Fig. 6A-F). But unlike the mice which received
- 242 AAVRh10-ΔN-DGKk, (and except for distance travelled during retention phase of NOR, Sup
- **Fig. 6D**), *Fmr1*-KO-PHP.eB-ΔN-DGKk mice showed no significant improvement compared
- 244 to Fmr1-KO-vehicle, suggesting that the level of ΔN -DGKk, although well distributed
- throughout the brain, was too low to achieve a sufficient effect.
- 246 Macroorchidism, a well-established phenotype of *Fmr1*-KO model, was not found corrected
- 247 by the treatments (**Sup Fig. 7**), possibly reflecting a non-neuronal origin.
- 248

249 **DISCUSSION**

- 250 FXS is currently uncured as no disease-modifying treatment could be validated despite
- 251 several clinical trials with investigational drugs (Yamasue et al., 2019). Our data provide
- 252 evidence that neuron targeted expression of DGKk enzyme with an AAV-based gene therapy
- approach is able to provide long-term correction of the main behavioral deficits in the young
- adult *Fmr1*-KO mouse model of FXS (**Sup table 2**).
- DGKk is an enzyme whose mRNA was previously found to be the main target of FMRP in cortical neurons (Tabet et al., 2016a). We show that DGKk expression is strongly dependent
- 257 on FMRP and severely altered in FXS brain. In fact, no other protein has been demonstrated
- to be so critically dependent upon FMRP. Loss of DGKk activity could play a critical role in
- 259 manifestation of FXS phenotypes because it is a master regulator of second messenger lipids
- 260 DAG/PA balance and has the potential to be the triggering cause of the many altered neuronal
- 261 signaling pathways observed in FXS (Tabet et al., 2016b). Removal of the N-terminal part of
- 262 DGKk does not impact DGKk activity in vitro (Imai et al., 2005), while it abolishes
- 263 regulation by FMRP. The FMRP-independent Δ N-DGKk protein conserved its ability to
- 264 modulate cell signaling and showed capacity to rescue a fully developed *Fmr1*-KO mouse
- 265 brain. At the dose used, intravenous administration of AAVPHP.eB-ΔN-DGKk was unable to
- 266 rescue the *Fmr1*-KO phenotype, presumably because of insufficient, albeit homogenous
- 267 expression in the brain. In contrast, intracerebral injection of AAVRh10-ΔN-DGKk led to
- 268 higher expression of ΔN -DGKk in the mouse brain and correction of disease phenotypes,
- 269 without affecting survival and with no signs of toxicity several weeks after dosing.
- 270 Consistently, high ΔN -DGKk expression in neuronal cultures was not associated with cellular

271	toxicity. Rescuing Fmr1-KO mouse with AAV-based FMRP administration at an early stage
272	(P0-P5) has provided the first proof of concept of a gene therapy approach for FXS
273	(Gholizadeh et al., 2014; Hampson et al., 2019), but also revealed that inappropriate levels of
274	FMRP expression can lead to worsening of FXS phenotypes, possibly due to the fact that
275	FMRP, like most RNA binding proteins, induces cellular stress when overexpressed (Mazroui
276	et al., 2002). Overall, rescue of $Fmr1$ -KO phenotypes with ΔN -DGKk expression in neurons
277	strengthened the notion that DAG/PA imbalance in neurons is a critical factor of the disease
278	and acting on this imbalance is beneficial for FXS-like condition, including at a late stage of
279	development. Use of ΔN -DGKk could offer potential for FXS gene therapy, representing a
280	very specific target in the complex pathomechanism of the disease.
281	
282	MATERIAL AND METHODS
283	
284	Ethics statement. Animal work involved in this study was conducted according to ARRIVE
285	guidelines and received authorization from relevant national (Comité National de Réflexion
286	Ethique en Expérimentation Animale).
287	
288	Cloning of human and mouse DGKK mRNA. Mouse DGKK was subcloned from clone
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288 289 290 291 292 293 294 295 296 297 298 299 300	 Cloning of human and mouse DGKκ mRNA. Mouse DGKκ was subcloned from clone IMAGE IRAVp968H03163D. The missing 3' UTR and 5' UTR-N-ter region were cloned by PCR from mouse genomic DNA with primer sets (GCAGCTAGCTCCTTGAAAGCTGGAAGGAGA and AATAGAATGCGGCC- GCCAGCTTCAACAGCACTTGTAG) and (CCAgtcgacTTAGACCTCAGAGCTGCGCTAGC and CCAgctagcCCAGGACTCTGGGGCCCTCTCCAT), respectively. The 3' UTR region was introduced at XbaI and NotI sites of the pYX-ΔN DGKκ vector to give pYX-ΔN-DGKκ- 3'UTR, and the 5'-UTR-Nter region at SalI-NheI sites of the pYX-DGKκ-3'UTR, NheI site was subsequently deleted by PCR mutagenesis. pCI-mDGKκ-HA and pCI-HA-ΔN-DGKκ Were obtained by PCR subcloning into pCI vector (GenBank U47119) with addition of the HA sequence before the STOP codon or after ATG, respectively. Human hDGKκ was
288 289 290 291 292 293 294 295 296 297 298 299 300 301	Cloning of human and mouse DGKκ mRNA. Mouse DGKκ was subcloned from clone IMAGE IRAVp968H03163D. The missing 3' UTR and 5' UTR-N-ter region were cloned by PCR from mouse genomic DNA with primer sets (GCAGCTAGCTCCTTGAAAGCTGGAAAGGAGA and AATAGAATGCGGCC- GCCAGCTTCAACAGCACTTGTAG) and (CCAgtcgacTTAGACCTCAGAGCTGCGCTAGC and CCAgctagcCCAGGACTCTGGGGCCCTCTCCAT), respectively. The 3' UTR region was introduced at XbaI and NotI sites of the pYX-ΔN DGKκ vector to give pYX-ΔN-DGKκ- 3'UTR, and the 5'-UTR-Nter region at SalI-NheI sites of the pYX-DGKκ-3'UTR, NheI site was subsequently deleted by PCR mutagenesis. pCI-mDGKκ-HA and pCI-HA-ΔN-DGKκ were obtained by PCR subcloning into pCI vector (GenBank U47119) with addition of the HA sequence before the STOP codon or after ATG, respectively. Human hDGKκ was subcloned from plasmid pAcGFPC1humDGKk (Imai et al., 2005) into Nhe1 of pCI vector with addition of 5' and 3'UTRs by PCR cloning to produce pCI-hDGKκ.
288 289 290 291 292 293 294 295 296 297 298 299 300 301 302	Cloning of human and mouse DGKκ mRNA. Mouse DGKκ was subcloned from clone IMAGE IRAVp968H03163D. The missing 3' UTR and 5' UTR-N-ter region were cloned by PCR from mouse genomic DNA with primer sets (GCAGCTAGCTCCTTGAAAGCTGGAAGGAGA and AATAGAATGCGGCC- GCCAGCTTCAACAGCACTTGTAG) and (CCAgtcgacTTAGACCTCAGAGCTGCGCTAGC and CCAgetageCCAGGACTCTGGGGGCCCTCTCCAT), respectively. The 3' UTR region was introduced at XbaI and NotI sites of the pYX-ΔN DGKκ vector to give pYX-ΔN-DGKκ- 3'UTR, and the 5'-UTR-Nter region at SaII-NheI sites of the pYX-DGKκ-3'UTR, NheI site was subsequently deleted by PCR mutagenesis. pCI-mDGKκ-HA and pCI-HA-ΔN-DGKκ were obtained by PCR subcloning into pCI vector (GenBank U47119) with addition of the HA sequence before the STOP codon or after ATG, respectively. Human hDGKκ was subcloned from plasmid pAcGFPC1humDGKk (Imai et al., 2005) into Nhe1 of pCI vector with addition of 5' and 3'UTRs by PCR cloning to produce pCI-hDGKκ.
288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303	Cloning of human and mouse DGKκ mRNA. Mouse DGKκ was subcloned from clone IMAGE IRAVp968H03163D. The missing 3' UTR and 5' UTR-N-ter region were cloned by PCR from mouse genomic DNA with primer sets (GCAGCTAGCTCCTTGAAAGCTGGAAGGAGA and AATAGAATGCGGCC- GCCAGCTTCAACAGCACTTGTAG) and (CCAgtcgacTTAGACCTCAGAGCTGCGCTAGC and CCAgctagcCCAGGACTCTGGGGCCCTCTCCAT), respectively. The 3' UTR region was introduced at XbaI and NotI sites of the pYX-ΔN DGKκ vector to give pYX-ΔN-DGKκ- 3'UTR, and the 5'-UTR-Nter region at SalI-NheI sites of the pYX-DGKκ-3'UTR, NheI site was subsequently deleted by PCR mutagenesis. pCI-mDGKκ-HA and pCI-HA-ΔN-DGKκ were obtained by PCR subcloning into pCI vector (GenBank U47119) with addition of the HA sequence before the STOP codon or after ATG, respectively. Human hDGKκ was subcloned from plasmid pAcGFPC1humDGKk (Imai et al., 2005) into Nhe1 of pCI vector with addition of 5' and 3'UTRs by PCR cloning to produce pCI-hDGKκ.

305 pENN.AAV.hSynapsin.EGFP.RBG (provided by the Penn Vector Core at University of 306 Pennsylvania, Philadelphia) to give pAAV- Δ N-DGKk. Recombinant adeno-associated virus 307 serotype 9 (AAV9), Rh10 (AAVRh10), PHP.eB (AAVPHP.eB) production was carried out 308 by using the AAV Helper-Free system (Agilent Technologies) with some modifications. AAV 309 vectors were generated by triple transfection of 293T/17 cell line using Polyethylenimine 310 (PEI) and plasmids pAAV-hsynapsin-HA-ΔN-DGKκ or pENN.AAV.hSynapsin.EGFP.RBG together with pHelper (Agilent) and pAAV2/9 or pAAV2/Rh10 (provided by J.Wilson and 311 J.Johnston at Penn Vector Core), or pUCmini-iCAP-PHP.eB (provided by V.Gradinaru and 312 313 J.Johnston) for serotypes 9, Rh10 and PHP.eB, respectively. Two days after transfection, cells 314 were collected, lysed by three freeze/thaw cycles in dry ice-ethanol and 37 °C baths, further 315 treated with 100 U/mL Benzonase (Novagen) for 30 min at 37 °C, and clarified by 316 centrifugation at 3,000 x g for 15min. Viral vectors were purified by iodixanol (Optiprep, 317 Axis Shield) gradient ultracentrifugation followed by dialysis and concentration against PBS 318 containing 0.5 mM MgCl₂ using centrifugal filters (Amicon Ultra-15.100 K) and filtered 319 through 0.22u (Zolotukhin et al., 2002). Viral particles were quantified by real-time PCR Q-320 PCR using LightCycler480 SYBR Green I Master (Roche) and primers targeting the flanking 321 sequence of ITR2 (GTAGATAAGTAGCATGGC and CTCCATCACTAGGGGTTCCTTG) 322 or the flanking sequence of rabbit β -globin polyadenylation signal 323 (CCCTTGAGCATCTGACTTCTGG and AGGGTAATGGGTATTATGGGTGGT). To 324 achieve comparable working concentrations, viruses were diluted to a final concentration of 325 $1x \ 10^{13}$ viral genome per ml (VG/ml) and stored at -80° C until use. 326 327 Cell culture and transfections. COS-1 cells were grown in DMEM supplemented with 10% 328 (v/v) FCS and 1 g/L glucose in the presence of antibiotics at 37 °C in 5% CO2. The day 329 before transfection, 4x10⁴ cells were plated into 24-well format plates in 500 µL of antibiotic-330 free medium. Transfections of plasmids were performed in triplicate with Lipofectamine 2000 331 (Invitrogen) as directed by the manufacturer with 10 pmol of siRNA (Control or On Target

- 332 plus Smart Pool mouse *FMR1*; Thermo Fisher Scientific) in a final volume of 600 μL.
- 333 Twenty-four hours later, 300 ng of the reporter pCI-DGKκ-HA or pCI-HA-ΔN-DGKκ and 10
- 334 nM shRNA were cotransfected as above. Twenty-four hours later, cells washed twice in PBS
- 335 were lysed directly in the loading buffer [100 mM Tris·HCl, pH 6.8, 4% (w/v) SDS, 30%
- 336 (v/v) glycerol, 1.4M β -mercaptoethanol, and bromophenol blue] for 3 min at 95 °C.

Primary cortical neuron cultures and treatments. Neuron cultures were performed as

338

339 described in (Tabet et al. 2016). Briefly, cortices from C57BL/6J Fmr1+/y or Fmr1-/y mouse 340 embryos (embryonic day E17.5) were dissected in 1xPBS, 2.56 mg/mL D-glucose, 3 mg/mL 341 BSA, and 1.16 mM MgSO4, incubated for 20 min with 0.25 mg/mL trypsin and 0.08 mg/mL 342 DNase I, and mechanically dissociated after supplementation of medium with 0.5 mg/mL 343 trypsin soybean inhibitor, 0.08 mg of DNase I and 1.5 mM MgSO₄. The cells were plated on 344 poly-L-lysine hydrobromide-coated six-well culture plates for 8 days in Neurobasal Medium 345 (GIBCO) supplemented with B27, penicillin/streptomycin, and 0.5 µM L-glutamine. Where 346 indicated, cultures were treated with addition of puromycin solution at the indicated 347 concentrations and times. DGK inhibitors R59022 (DGK Inhibitor I; Calbiochem) and 348 R59949 (DGK Inhibitor II; Calbiochem) were applied at concentrations of 3 and 0.2 µM each 349 for 15 min at 37 °C. After treatment, cells were immediately washed with ice-cold PBS and 350 lysed in 4X Laemmli buffer. 351 352 Western blot analyses. Immunoblotting was performed as described previously (Tabet et al., 353 2016a). Proteins (equivalent to 15 µg) were denatured 5 min at 95°C and resolved by 10% 354 SDS-PAGE. Separated proteins were transferred onto PVDF Immobilon P membrane 355 (Millipore) using a Mini Trans-Blot (Biorad) cell. Membranes were blocked for 1h with TBS-356 T 1X (Tris-Buffer Saline, pH 7.4 and 0.1% Tween-20 v/v) containing 5% (w/v) BSA or 5% 357 nonfat dry milk. Membranes were incubated overnight at 4°C with primary antibodies diluted 358 in TBS-T buffer containing 5% w/v BSA or milk as follows: mouse anti-FMRP (1C3, 359 1:10,000, IGBMC), purified mouse anti-HA.11 16B12 (1:5000, Biolegend), rabbit anti-360 hDGKk (1:1000, PA5-25046, ThermoFisher), anti-p-EIF4e Ser209 (1:1000, #9741 Cell Signaling), anti-EIF4 (1:1000 BSA, #9742, Cell Signaling), anti-puromycin (12D10, 1:2000, 361 362 Sigma-Aldrich), anti-S6 (5G10, 1:1000 #2217S, Cell Signaling), anti-p-S6 (Ser235/236, 363 1:1000, 2211S Cell Signaling), anti-GAPDH (MAB374, 1:10.000, Merck) was used as an 364 internal standard. Membranes were washed in TBS-T buffer and then incubated for an hour at 365 room temperature with the corresponding horseradish peroxidase-conjugated pre-adsorbed 366 secondary antibody (1:5000, blocking solution corresponding, Molecular Probes). Membranes 367 were washed in TBS-T buffer and immunoreactive bands were visualized with the 368 SuperSignal West Pico Chemiluminescent Substrate (Pierce). Immunoblot pictures were 369 acquired using LAS600 GE Amersham and density of the resulting bands was quantified 370 using ImageJ and statistical significance assessed using repeated measures analysis of 371 variance (ANOVA) with Fisher's post hoc comparisons.

372

373	Analysis of cortical neuron cultures by immunofluorescence microscopy. Primary cortical
374	neurons grown on poly-L-lysine hydrobromide coated glass coverslips were fixed in 4% (w/v)
375	paraformaldehyde (PFA) in 1xPBS at room temperature (RT) for 20 min, permeabilized with
376	1X PBS and 0.2% Triton X-100 for 10 min at RT, and blocked for 1 h in 1x PBS and 0.1%
377	Triton X-100 with 5% (w/v) BSA. Neurons were incubated with primary antibodies rabbit
378	anti-MAP2 AB5622 (1:500, Merck Millipore), mouse anti-HA.11 16B12 (1:500, Biolegend),
379	mouse anti-NeuN (1:500, MAB377 Merck), rabbit anti-GFAP (1:500, 173002, Synaptic
380	system), overnight at 4 °C. After three washes in 1X PBS and 0.1% Triton X-100 for 10 min,
381	neurons were incubated with secondary goat antibody anti-rabbit (Alexa Fluor 594, 1:1.000,
382	Invitrogen) and anti-mouse (Alexa Fluor 488, 1:1000, Invitrogen), for 1h at RT, and
383	subsequently washed three times in 1xPBS and 0.1% Triton X-100 for 10 min. Coverslips
384	were mounted with antifading medium (Vectashield, Vector) with DAPI and analyzed by
385	fluorescence microscopy. Images were acquired with CellInsight CX7 (Thermo Scientific)
386	using a 10x objective and analyzed with HCS Studio Cell Analysis Software (nuclear
387	segmentation, NeuN and GFAP intensities). Quantification of positive cells for each of
388	these staining was done by applying a threshold manually, based on nuclear segmentation
389	and across 81 fields. Percentage of cells positive for NeuN and GFAP staining was
390	quantified for each well.

391

392 Caspase 3/7 activity detection. Caspase 3/7 positives cells were determined with CellEvent 393 Caspase-3/7 Green Detection Reagent following manufacturer instructions. Briefly, primary 394 neurons were pepared as for LDH assays, treated with reagent diluted at 8 µM in 5% FBS 395 NBM. Positive control wells were treated with apoptotic inducer staurosporine at 0.1 and 396 1uM for 6 hours. Cells were fixed with were fixed in 4% (w/v) PFA and nuclei were 397 counterstained with DAPI. Images were acquired with CellInsight CX7 (Thermo Scientific) 398 using a 10x objective and analyzed with HCS Studio Cell Analysis Software (nuclear 399 segmentation and casp3/7 intensities). Quantification of percentage of caspase-3/7 positive 400 cells was done for each well by applying a threshold manually, based on nuclear 401 segmentation and across 81 fields.

402

403 **Lactate dehydrogenase releasing assay.** Lactate dehydrogenase (LDH) release was 404 determined with the Cytotoxicity Detection KitPLUS kit (Roche) following manufacturer 405 instructions. Briefly, primary neurons from $Fmr1^{+/y}$ or $Fmr1^{-/y}$ E17.5 embryos plated at 406 300,000 cells/well in 24-well plate and transduced at 7 DIV with indicated AAV were tested

- 407 after 7 days. LDH release was measured in microplate reader at 490 nm after 2 hours at 37°C
- 408 with reaction medium. Maximum LDH release was measured in same conditions after 30min
- 409 at RT with stop solution. The % of cell death was determined using the formula: % cell death
- 410 = experimental LDH release /maximum LDH release.
- 411

Animal housing. At weaning age (4 weeks), animals were grouped by 3 or 4 individuals from
same age and genotype in individually ventilated cages (GM500, Tecniplast, UK), with
poplar shaving bedding (Lignocell Select, JRS, Germany), and maintained under standard

- 415 conditions, on a 12-h light/dark cycle (7h/19h), with standard diet food (standard diet D04,
- 416 Scientific Animal Food and Engineering, France) and water available ad libitum. Mice from a
- 417 same cage received the same treatment and were transferred in the animal facility of the
- 418 phenotyping area the next week.
- 419

420 Stereotaxic Surgery and AAV Injections. Five-week old mice C57BL/6J *Fmr1-*/y or

- 421 C57BL/6J *Fmr1*+/y littermates were deeply anesthetized with ketamine/xylazine
- 422 (Virbac/Bayer, 100/10 mg/kg, 13 mL/kg, intraperitoneal) dissolved in sterile isotonic saline
- 423 (NaCl 0.9%) and mounted onto a stereotaxic frame (World Precision Instruments).
- 424 AAVRh10-DGKκ were injected bilaterally into the striatum (coordinates relative to bregma:
- 425 anterior-posterior + 0.5 mm; lateral = ± 2.2 mm; vertical -3.5 mm) and hippocampus
- 426 (coordinates relative to bregma: anterior-posterior 1.7 mm; lateral = ± 1.5 mm; vertical -2.0
- 427 mm) according to the mouse brain atlas (Paxinos G, Franklin KBJ (2001)). A volume of 2.5
- 428 μL of AAV vector (corresponding to 10^e11 Genome copies) or saline solution was delivered
- 429 bilaterally per site of injection with a slow injection rate (0.2 μL/min) through a 32-gauge
- 430 small hub removable needle mounted on a 10 µL Hamilton syringe connected to a micropump
- 431 (World Precision Instruments). After each injection was completed, the injector was left in
- 432 place for an additional 2 min to ensure optimal diffusion and minimize backflow while
- 433 withdrawing the injector.
- 434
- 435 Retroorbital AAV Injections. Five-week old mice C57BL/6J *Fmr1-*/y or C57BL/6J
- 436 Fmr1+/y littermates were deeply anesthetized with ketamine/xylazine (Virbac/Bayer, 100/10
- 437 mg/kg, 10 mL/kg, intraperitoneal) dissolved in sterile isotonic saline (NaCl 0.9%).
- 438 AAVPHP.eB-DGK κ were injected retroorbitaly with a volume of 80 μ L.
- 439

440 Analysis of Δ N-DGKk expression in brain sections. Freshly dissected brains were fixed

441 overnight in PFA 4% and stored in PBS1X prior being processed following Neuroscience

- 442 Associates procedure https://www.neuroscienceassociates.com/technologies/multibrain/.
- 443 Half-brains were washed in PBS1X solution and embedded in gelatin matrix.
- 444 MultiBrain[®] cryosections were prepared with 30µ thickness for free-floating immunolabeling
- 445 with anti-HA (3F10, 1:150, Sigma) and counter stained with hematoxylin/eosin. Adjacent
- 446 sections were immunolabelled with anti-NeuN (1:150, MAB377 Merck).
- 447
- 448 Behavioral Experiments. Behavioral experiments were conducted 4 weeks or 8 weeks after
- 449 AAV injections to allow sufficient time for viral transduction and DGKκ expression.
- 450 Effective gene expression was assessed by q-PCR to measure viral titer, by western blot and
- 451 by immuno-histochemistry in 3 different brain areas (cortex, hippocampus and rest of brain).
- 452 Phenotyping pipeline is described in table 1.
- 453

454 Circadian Activity. Spontaneous locomotor activity and rears are measured using individual 455 cages (20 x 10 x 8 cm) equipped with infra-red captors. The quantity of water and food 456 consumed is measured during the test period using automated pellet feeder and lickometer 457 (Imetronic, Pessac, France). Mice are tested for 32 hours in order to measure habituation to 458 the apparatus as well as nocturnal and diurnal activities. Results are expressed per 1 h periods 459 and/or as a total of the different activities.

460

Elevated plus maze. The apparatus used is completely automated and made of PVC (Imetronic, Pessac, France). It consists of two open arms (30 X 5 cm) opposite one to the other and crossed by two enclosed arms (30 x 5 x 15 cm). The apparatus is equipped with infrared captors allowing the detection of the mouse in the enclosed arms and different areas of the open arms. Mice were tested for 5 min during which the number of entries into and time spent in the open arms were measured and used as an index of anxiety. Closed arm entries and total arm entries were used as measures of general motor activity.

468

469 Novel object recognition task. Mice were tested in a circular arena (50cm diameter and 30cm 470 height basin). The locomotor activity was recorded with the EthoVision XT video tracking 471 system (Noldus, Wageningen, Netherlands). The arena was virtually divided into central and 472 peripheral regions and homogeneously illuminated at 40 Lux. Animals were first habituated to

the arena for 15 min. Each mouse was placed in the periphery of the arena and allowed to

474 explore freely the apparatus, with the experimenter out of the animal's sight. The distance 475 traveled and time spent in the central and peripheral regions were recorded over the test 476 session. The percentage of time spent in center area was used as index of emotionality/anxiety. 477 The next day, mice were tested for object recognition in the same arena. They were submitted 478 to a 10-minutes acquisition trial during which they were placed in the arena in presence of a 479 sample objects (A and A') (2.5 cm diameter marble or 2 cm edge plastic dice). The time the 480 animal took to explore the samples (sniffing) was manually recorded. A 10-minutes retention 481 trial was performed 24 h later. During this trial, one of the samples A and another object B 482 (marble or dice depending on acquisition) were placed in the open-field, and the times tA and 483 tB the animal took to explore the two objects were recorded. A recognition index (RI) was 484 defined as $(tB / (tA + tB)) \times 100$.

485

486 Nest building. On the day of test, mice were singly transferred in a standard cage for the 487 duration of nest building measurement. A block of nesting material (5x5cm hemp square, 488 Happi Mats, Utopia) was placed in the cage. Pictures were taken and visual scoring occurred 489 at 2, 5, 24 h without disturbing the animals. The room temperature was noted when the nest 490 was scored, since nest building has a thermoregulatory function and therefore may be 491 influenced by ambient temperatures. We used a 0-5 scale described by (Gaskill et al., 2013) : 492 0 = undisturbed nesting material; 1 = disturbed nesting material but no nest site; 2 = a flat nest 493 without walls; 3 = a cup nest with a wall less than $\frac{1}{2}$ the height of a dome that would cover a 494 mouse; 4 = an incomplete dome with a wall $\frac{1}{2}$ the height of a dome; 5 = a complete dome 495 with walls taller than $\frac{1}{2}$ the height of a dome, which may or may not fully enclose the nest. 496

497 Social recognition test. Social recognition test evaluates the preference of a mouse for a 498 congener as compared to an object placed in an opposite compartment. This test is also used 499 for evaluation of social memory by measuring exploration of a novel congener as compared to 500 a familiar one. Social behavior is altered in several diseases such as autism and mental 501 retardation. The apparatus is a transparent cage composed with a central starting compartment 502 and 2 side compartments where circular grid cup (goal box) is placed at each extremity, and 503 where the congener can be placed during testing. Testing was performed for 2 consecutive 504 days. On the first day, the mouse was placed in central box then allowed to explore freely the 505 apparatus for 10 min in order to attenuate their emotionality. On the second day, a C57Bl/6 506 congener from the same sex was placed in one goal box and an object was placed in the 507 opposite one. The mouse was then placed in the starting central compartment and allowed to

508 explore freely the apparatus for 10 min. The position of the congener and object boxes was 509 counterbalanced to avoid any potential spatial preference. The duration of exploration of each 510 goal box (when the mouse is sniffing the grid delimiting the goal box) was manually 511 measured and the percentage of time the mouse took to explore the congener was used as 512 index of social preference (recognition preference). A 10min retention trial was then 513 performed during which the object was replaced by a novel congener. The duration of 514 exploration of each goal box was manually measured and the percentage of time the mouse 515 takes to explore the congener was used as index of social memory. The social preference 516 index (SR) is defined as (time Congener / (time Object + time Congener)) x100; and the 517 social memory index as (time novel Congener / (familiar congener + time novel Congener))

518

x100.

519

520 Lipidomic analyzes. Nitrogen frozen brain samples (cortex, hippocampus, rest) were let thaw 521 on ice and mechanically homogenized with 1 vol H₂0 with Precellys 24 system during 522 2x15sec at 4°C and 5300 rpm. Protein concentration of sample was adjusted at 5 mg/ml 523 concentration and lipids were analyzed on Lipotype GmbH platform. Lipids were extracted 524 using chloroform and methanol (Sampaio et al., 2011) with Hamilton Robotics STARlet. 525 Samples were spiked with lipid class-specific internal standards prior to extraction. After 526 drying and resuspending in MS acquisition mixture, lipid extracts were subjected to mass 527 spectrometric analysis. Mass spectra were acquired on a hybrid quadrupole/Orbitrap mass 528 spectrometer (Thermo Scientific Q-Exactive) equipped with an automated nano-flow 529 electrospray ion source in both positive and negative ion mode. Lipid identification using 530 LipotypeXplorer (Herzog et al., 2011) was performed on unprocessed (*.raw format) mass 531 spectra. For MS-only mode, lipid identification was based on the molecular masses of the 532 intact molecules. MSMS mode included the collision induced fragmentation of lipid 533 molecules and lipid identification was based on both the intact masses and the masses of the 534 fragments. Prior to normalization and further statistical analysis lipid identifications were 535 filtered according to mass accuracy, occupation threshold, noise and background. Intensity of 536 lipid class-specific internal standards was used for lipid quantification. The identified lipid 537 molecules were quantified by normalization to a lipid class specific internal standard. The 538 amounts in pmol of individual lipid molecules (species of subspecies) of a given lipid class 539 were summed to yield the total amount of the lipid class. The amounts of the lipid classes 540 were normalized to the total lipid amount yielding mol% per total lipids.

- 542 **Statistical analyses.** Quantitative data were analyzed using single or repeated measures
- 543 analysis of variance (ANOVA) and Student Newman Keuls test. For the comparison with
- 544 chance, one group t-test was used. Qualitative parameters (nesting) were analyzed using χ^2
- 545 test. The level of significance was set at p < 0.05.
- 546

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635

636 Acknowledgements:

637 We thank Flora Tassone and Veronica Martinez-Cerdeno for human brain materials. We are

- 638 indebted to Antonina Fedorova, Hélène Puccio, Yann Herault, Sirine Souali-crespo, Tania
- 639 Sorg-Guss and members of Lysogene team for discussions and suggestions. We are grateful
- 640 to Andréa Geoffroy and members of Institut Clinique de la Souris for help with the behavioral
- 641 studies, Pascale Koebel for AAV preparations, Eric Flatter for technical assistance, Nadine
- 642 Banquart-Ott and Chadia Nahy for mice care, Anne Maglott for toxicity analyses, Erwan
- 643 Grandgirard for help with imaging, IGBMC core facilities, Zaiane Schmitt and Fumi Hoshino
- 644 for technical assistance. We specially thank Laura Benkemoun and Fondation Maladies Rares
- 645 for support and suggestions. We are grateful to colleagues of J. Chelly team for discussions.
- 646

647 Funding:

- 648 This work was supported by Satt Conectus program GETEX, ANR-18-CE12-0002-01 and
- 649 Fondation Jérôme Lejeune funding to HM and by Fond Paul Mandel to KH and Fondation
- 650 pour la Recherche Médicale to BZ. This study was also supported by ANR-10-LABX-0030-
- 651 INRT under the frame program Investissements d'Avenir ANR-10-IDEX-0002-02.
- 652 **Competing interests:** HM and RT are listed as inventors on a patent describing the AAV
- 653 construct reported in this manuscript. M.H., and R.L. are full-time employees and hold equity
- 654 in Lysogene. All other authors declare no competing interests.
- 655

656 Author contributions

- 657 K.H., O.C, B.Z., and F.P. designed and performed experiments and helped writing the
- 658 manuscript. D.D. performed statistical analyses. R.T., M.H., R.L. helped designing the
- experiments and writing manuscript. H.M. supervised the project, designed experiments and
- 660 wrote the manuscript.
- 661
- 662
- 663 Figure

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664

665 Fig. 1 : DGKk expression is altered in FXS and FMRP is required for translation control via its N-666 terminal domain. A) Western blot analysis of lysates from cerebellum of control (n = 3) and FXS patients 667 "FXS" (n = 4). Hela cell extract transfected with pCI-hDGK κ was used as antibody specificity control and size 668 marker. Representative images of immunoblots probed with antibodies against the indicated proteins are shown. 669 GAPDH was the loading control. Quantification of Western blots is shown on right. Protein amounts of DGKk 670 are normalized to GAPDH and presented as fold change relative to control. B) Schematic map of DGKk 671 constructs used for the transfection experiments and subsequent vector preparations. The different domains of 672 the protein are indicated and represented at scale (repeated EPAPE, Pleckstrin Homology PH domain, phorbol 673 ester/diacyl glycerol binding C1 domain, catalytic split C4 a and b domains, putative PDZ binding motive, HA-674 tag, grey bars interval 1kB), 5' and 3' UTR regions are represented with blue line, 3'UTR not at scale (3.8 kB). 675 C) Immunoblots and quantification of lysates from Cos-1 cells transfected with plasmid pCI-mDGKK-HA or 676 mock transfected (NT) and pre-transfected 24h before with siRNA control (siC) or against FMRP (siFMR1). 677 GAPDH was used as a loading control. For quantification, the DGKk and FMRP signals were normalized 678 against GAPDH signal and presented relative to the signal for siC treated cells (n = 5 in each group). **D**) 679 Immunoblots and quantification of lysates from Cos-1 cells transfected with plasmid pCI-HA- Δ N-DGK κ or pCI-680 mDGKk-HA and pre-treated with siRNA control (siC) or against FMRP (siFMR1). Quantifications as in C. 681 Each point represents data from an individual culture, and all values are shown as mean \pm SEM ***P < 0.001, 682 **P < 0.01, *P < 0.05 calculated by unpaired Student T test.





684 Fig. 2 : AN-DGKk expression impacts cellular signaling. A) Immunoblots and quantification of lysates from Cos-1 cells transfected with plasmid pCI-HA- Δ N-DGK κ , 685 untransfected (NT), or plasmid pCI control, with the indicated amount of plasmid (µg) and 686 687 incubated with puromycin to measure basal rates of protein synthesis. GAPDH was used as a loading control. 0 indicates no puromycin treatment, -/+ indicates treatment without or with 688 689 DGK inhibitor (DGKi) 3 µM R59022 and 0.2 µM R59949 at 6 µM, 15 min. Densitogram of 690 puromycin incorporation is presented as change relative to mock transfected conditions (n =3). B) Representative immunofluorescence staining of cortical neuron cultures transduced at 8 691 DIV (days in vitro) with 10e9 VG/ml culture volume AAVRh10 GFP or Δ N-DGKk and 692 693 assessed after 5 days using anti-MAP2 and anti-HA for Δ N-DGKk or direct 488 nm excitation for GFP, Dapi was used to visualize nuclei on merged images. Scale bar, 40 µm. C) 694 Representative immunoblots of lysates from cortical neurons transduced with AAVRh10- Δ N-695 696 DGKk (AAV Δ N-DGKk), at the indicated titers (VG/ml culture volume) and quantification of phosphorylation and total levels of eIF4E. GAPDH was used as a loading control. For 697 698 quantification, the phospho-protein signal was normalized against total protein signal and is 699 presented relative to the signal for vehicle-treated cultures. Each point represents data from an individual culture, and all values are shown as mean \pm SEM *P < 0.05 calculated by One 700 way-ANOVA with Tukey's multiple comparison test (n = 3 individual cultures). 701







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720

721 Fig. 4 : AAVRh10-ΔN-DGKk rescues behavior alterations of *Fmr1*-KO mouse 4 weeks

722 **after injections.** A) Elevated Plus Maze. Percentage of time spent in open arms and number

- of entries in open, closed and total (open+closed) arms. **B**) Novel object recognition.
- Locomotor activity (distance in m) in the whole arena and percentage of time spent in the
- center during 15 min habituation. Locomotor activity during the acquisition and retention
 trials. Recognition index. C) Stereotypies. Number of digging. D) Social recognition. Number
- of entries in the two side compartments during preference session (left), social preference
- 728 (percentage of exploration of a congener vs an object) (center) and social memory (percentage
- 729 of exploration of a novel vs familiar congener) (right). Data are expressed as median with
- 730 interquartile range with minimum and maximum values and analyzed using one-way
- ANOVA and Tukey's multiple comparisons test and one group t-test. * p<0.05, **p<0.01,
- 732 ***p<0.001, ****p<0.0001 vs WT-S; # p<0.05, ## p<0.01, ### p<0.001 vs Fmr1-S; @
- 733 p<0.05, @@ p<0.01, @@@@ p<0.0001 vs chance (50%).

734

735 Supplementary material



736

Supplementary Fig. 1 : Influence of FMRP protein on human DGKk protein expression 737 738 and on mouse DGKk mRNA level. A) Immunoblots and quantification of lysates from Hela 739 cells transfected with plasmid pCI-hDGKk encoding human DGKk and pre-transfected 24h 740 before with siRNA control (siC) or against FMRP (siFMR1). GAPDH was used as a loading control. For quantification, DGKk and FMRP signals were normalized against GAPDH signal 741 742 and presented relative to the signal for siC treated cells. Each point represents data from an 743 individual culture, and all values are shown as mean \pm SEM ****P* < 0.001, calculated by 744 unpaired Student T test. B) Quantification of mouse DGKK mRNA by qRT-PCR in RNA extracts of Cos-1 cells transfected with plasmid pCI-mDGKk-HA and pre-transfected with 745 746 siRNA control (siC) or siRNA against FMRP (siFMR1) or mock transfected (NT). Data are 747 means of fold change \pm SEM, determined using $\Delta\Delta$ Ct method with Actb as normalizer, n = 3

748 biological replicates.



750 Supplementary Fig. 2 : Phosphorylation of eIF4E is increased in Fmr1-KO cortical 751 neurons compared to WT, and Δ N-DGKk expression in neurons does not lead to 752 toxicity. A) Representative immunoblots of lysates from WT and Fmr1-KO cortical neurons and quantification of phosphorylation and total levels of eIF4E. GAPDH was used as a 753 754 loading control. For quantification, the phospho-protein signal was normalized first against 755 total protein signal and is presented relative to the signal for WT culture. Each point 756 represents data from an individual culture, and all values are shown as mean \pm SEM ***P* < 757 0.01 by Student T test (n = 3 individual cultures). Quantification of caspase 3/7 activity (**B**), 758 release of lactate dehydogenase (LDH) (C), percentage of NeunN (D) and GFAP (E) positive 759 cells, in WT and Fmr1-KO cortical neurons untreated (NT) or transduced at 8 DIV for 8 DIV 760 with indicated titers of AAV (viral genome copies) AAVRh10-ΔN-DGKk or AAVRh10-FMRP by immunofluorescence high throughput cell imaging. Positive control wells were 761 762 treated with apoptotic inducer staurosporine (STS) at 0.1 and 1µM for 6 hours. Data are mean 763 \pm SEM and analyzed using one-way ANOVA and Tukey's multiple comparisons test. * 764 p<0.05.

Sup Fig. 3

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777 Supplementary Fig. 3 : ΔN-DGKk expression in brain with AVV vectors is stable over

- 778 time and normalizes phosphatidic acid level. A) Representative coronal brain sections
- processed for detection of ΔN -DGKk at 8 and 12 weeks post-injections using
- 780 immunohistochemistry on Fmr1-KO mice treated with indicated treatment, counter stained
- 781 with eosin hematoxylin. Three regions a, b, c, are shown with their corresponding position on
- brain sagital map. Scale bar 2mm. **B**) Measure of total phosphatidic acid (PA) level by mass
- 783 spectrometry in hippocampus and rest of brain of WT mice treated with saline solution (WT)
- and Fmr1-KO mice treated with saline (Fmr1-S), AAVPHP.eB- Δ N-DGKk (Fmr1-PHP.eB),
- 785 AAVRh10- Δ N-DGKk (Fmr1-Rh10) 8 weeks after injections. Data are expressed as mean \pm
- 786SEM of mol % of total lipids and analyzed using one-way ANOVA and Tukey's multiple
- 787 comparisons test, *p<0.05, **p<0.01, n=8 individual animals, except for WT n=7. C) Total
- 788 diacylglycerol (DAG) level in cortex measured as in B). PA and DAG individual species level
- respectively in cortex (**D**, **G**), hippocampus (**E**, **H**), and rest of brain (**F**, **I**). **J**) lipid
- composition (mol % of total lipid) for cholesterol esters (CE), diacylglycerol (DAG),
- 791 phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE),
- 792 phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin
- 793 (SM), triacylglycerol (TAG), measured as in A. Data are expressed as mean \pm SEM and
- 794 analyzed using two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs WT-S,
- 795 #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs Fmr1-S.





В







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Supplementary Fig. 4 : Behavioral analyses of AAVRh10-AN-DGKk treated *Fmr1*-KO 801 mice (4 weeks after injection). A) Circadian activity (locomotor activity). Evolution of 802 803 locomotor activity per hour over the 32h testing (upper panel) and total locomotor activity for the habituation, dark and light phases (mid panel) and for the first habituation hour, first dark 804 805 hour and total duration (lower panel). B) Circadian activity (rearing activity). Evolution of 806 rearing activity per hour over the 32h testing (upper panel) and total rearing activity for the habituation, dark and light phases (mid panel) and for the first habituation hour, first dark 807 808 hour and total duration (lower panel). C) Novel object recognition in 50cm diameter arena 809 (30cm height). Duration of objects exploration during the acquisition and retention trials. **D**) 810 Novel object recognition in 30cm diameter arena (30cm height). Locomotor activity 811 (distance) in the whole arena during the 15min habituation, acquisition and retention trials. 812 Duration of objects exploration during the acquisition and retention trials and recognition 813 index. E) Digging, marble burying and grooming duration tests. F) Social recognition. 814 Number of entries and locomotor activity (total traveled distance in cm) in the two side 815 compartments during habituation (up), social preference (middle) and social memory (bottom) sessions. Social preference was determined as percentage of exploration of a 816 817 congener vs an object (middle right) and social memory as percentage of exploration of a 818 novel vs familiar congener (bottom right). G) Nest building. Scoring of nests at 2, 5 and 24h. 819 0-5 scale as described by Gaskill et al (2013): 0 = undisturbed nesting material; 1 = disturbed 820 nesting material but no nest site; 2 = a flat nest without walls; 3 = a cup nest with a wall less than $\frac{1}{2}$ the height of a dome that would cover a mouse; 4 = an incomplete dome with a wall $\frac{1}{2}$ 821 the height of a dome; 5 = a complete dome with walls taller than $\frac{1}{2}$ the height of a dome, 822 823 which may or may not fully enclose the nest. Data are expressed as median with interquartile 824 range with minimum and maximum values for A-F, mean \pm SEM for G, and analyzed using 825 one-way ANOVA and Tukey's multiple comparisons test. * p<0.05 vs WT-S; # p<0.05 vs 826 827 (50%); and χ^2 test. *p<0.05 vs WT-S.



Α





Sup Fig. 5 FG

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831 832 Supplementary Fig. 5 : Behavioral analyses of AAVRh10-∆N-DGKk treated Fmr1-KO 833 mice (8 weeks after injection). Circadian activity analysis of locomotor (A) and rearing (B) 834 activity per hour over the 32h testing (upper panel) and total locomotor and rearing activity 835 for the habituation, dark and light phases (mid panel) and for the first habituation hour, first 836 dark hour and total duration (lower panel). C) Elevated Plus Maze. Percentage of time spent 837 in open arms and number of entries in open, closed and total (open+closed) arms. D) Novel 838 object recognition in 50cm diameter arena (30cm height). Percentage of time spent in the 839 center during the habituation, locomotor activity (distance) in the whole arena during the 840 habituation, acquisition and retention trials. Duration of objects exploration during the 841 acquisition and retention trials and recognition index. E) Novel object recognition in 30cm 842 diameter arena (30cm height). Locomotor activity (distance) in the whole arena during the 843 habituation and retention trials. Duration of objects exploration during the acquisition trials 844 and recognition index. F) Digging, marble burying and grooming duration tests. G) Body 845 weight of mice. Data are expressed as median with interquartile range with minimum and maximum values and analyzed using one-way ANOVA, Tukey's multiple comparisons test 846 847 and one group t-test. *p<0.05, **p<0.01, ***p<0.001 vs WT-S, # p<0.05, ## p<0.01, ### p<0.001 vs Fmr1-S, @p<0.05, @@p<0.01, @@@ p<0.001 vs chance (50%). 848 849







Sup Fig. 6 F

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855 Supplementary Fig. 6 : Behavioral analyses of AAVPHP.eB- Δ N-DGKk treated *Fmr1*-

856 **KO mice (4 weeks after injection).** Circadian activity analysis of locomotor (A) and rearing

(B) activity per hour over the 32h testing (upper panel) and total locomotor and rearing
activity for the habituation, dark and light phases (mid panel) and for the first habituation

hour, first dark hour and total duration (lower panel). C) Elevated Plus Maze. Percentage of

time spent in open arms and number of entries in open, closed and total (open+closed) arms.

B(1) Novel object recognition in 50cm diameter arena (30cm height). Percentage of time spent

in the center during the habituation, locomotor activity (distance) in the whole arena during
 the habituation, acquisition and retention trials. Duration of objects exploration during the

acquisition and retention trials and recognition index. E) Nest building. Scoring of nests at 2,

865 5 and 24h as in Sup. Fig. 4. F) Social recognition. Number of entries and locomotor activity

866 in the two side compartments during habituation (up), social preference (middle), social

867 memory (bottom) sessions. Social preference was determined as percentage of exploration of

868 a congener vs an object (middle right) and social memory as percentage of exploration of a 869 novel vs familiar congener (bottom right). Data are expressed as median with interquartile

range with minimum and maximum values for A-F, as mean \pm SEM for E and analyzed using

one-way ANOVA, Tukey's multiple comparisons test and one group t-test (recognition index)

872 or $\gamma 2$ test (nesting). *p<0.05 vs WT-S. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs

873 WT-S, # p<0.05 vs Fmr1-S.

Sup Fig. 7

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875 876

876 Supplementary Fig. 7. Testes weight of AAVRh10-AN-DGKk (left) and AAVPHP.eB-

877 Δ N-DGKk (right) treated *Fmr1*-KO mice (12 weeks after injection). Data are means of the 878 two testes of each animal and expressed as median with interquartile range with minimum and

879 maximum values and analyzed using one-way ANOVA, Tukey's multiple comparisons test

- 880 ***p<0.0001 vs WT-S.
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883 Supplementary table 1: Phenotyping pipeline

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ype of test locomodor activity other types of activity memory weight weight	Body weight Weight	 Social memory – distance travele Social prelevence – sin^{fi}fing duration Social memory – sniffing duration 	- Habibuation - Nb entrase - Habibuation - distance traveled (c - Social preference - Ab entras - Social preference - distance trave - Social memory - Nb entres	-Nesting 2h -Nesting score 2h 5h Social Memoor 24h	Stereotypies - Marble burying (%) - Grooming duration (s) - Digging (Nb)	- Acquisition – exploration (s) - Relention – exploration (s) - Recognition Index (%) 50 cm arer - Recognition Index (%) 30 cm arer	Novel Object Recognition - Habiluation - distance traveled - Habiluation - Stime in coner - Acquisition - Stime traveled - Retention - distance traveled - Retention - distance traveled	Elevated Plus maze State in open ams Open arm entries Closed arm entries Total arm entries	- Habituation lightphase - Dark phase - Lightphase - Tst Habituation H - Tst Bark hour - total (32h)	Locomolor activity (cm) - Habibuidion lightphase - Dark phase - Lightphase - 1st Habibuation H - 1st Dark hour - 1st Dark hour - 1st Dark hour - 1st Dark hour	nentary table 2 Name of test Actimetry
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886 Supplementary table 2: Summary of the phenotypic comparisons between WT mice

887 treated saline (WT-S), Fmr1-KO treated saline (KO-S) and Fmr1-KO treated AAV

888 (KO-Rh10 or KO-PhPeB) 4 and 8 weeks post-treatment. *, **, ***, **** p<0.05, 0,01,

889 0,001, 0,0001 KO-S vs WT-S, or KO-AAV vs WT-S. #, ##, ####, #### p<0.05, 0,01, 0,001,

890 0,0001 KO-AAV vs KO. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs WT-S, # p<0.05

- 891 vs Fmr1-S.
- 892