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Altered dendritic spine function and integration in a mouse model of Fragile X Syndrome

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1

2 Figures: 9 (+11 Supplementary Materials)

3 Tables: 0 (+1 Supplementary Materials)

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5

6 Cellular and circuit hyperexcitability are core features of Fragile X Syndrome and related autism
7 spectrum disorder models. However, the cellular and synaptic bases of this hyperexcitability
8 have proved elusive. We show in a mouse model of Fragile X Syndrome, glutamate uncaging
9 onto individual dendritic spines yields stronger single-spine excitation than wild-type, with more
10 silent spines. Furthermore, near-simultaneous uncaging at multiple spines revealed fewer
11 spines are required to trigger an action potential. This arose, in part, from increased dendritic
12 gain due to increased intrinsic excitability, resulting from reduced hyperpolarization-activated
13 currents, and increased NMDA receptor signaling. Super-resolution microscopy revealed no
14 change in dendritic spine morphology, indicating no structure-function relationship at this age.
15 However, ultrastructural analysis revealed a 3-fold increase in multiply-innervated spines,
16 accounting for the increased single-spine glutamate currents. Thus, loss of FMRP causes
17 abnormal synaptogenesis, leading to large numbers of poly-synaptic spines despite normal
18 spine morphology, thus explaining the synaptic perturbations underlying circuit hyperexcitability.
19

20 **Introduction:**

21 Cell and circuit hyperexcitability have long been hypothesized to underlie many core symptoms
22 of Fragile X Syndrome (FXS) and autism spectrum disorders more generally, which include
23 sensory hypersensitivity, seizures and irritability ¹. The fundamental role of cellular excitability in
24 circuit function raises the possibility that alterations in neuronal intrinsic physiology may underlie
25 a range of functional endophenotypes in FXS. Despite this potential link, few studies have
26 examined the combined synaptic, dendritic, and cellular mechanisms that lead to generation of
27 neuronal hyperexcitability during early postnatal development.

28

29 Many cellular properties are known to regulate neuronal excitability, such as neuronal
30 morphology, intrinsic physiology, synaptic transmission and plasticity. In FXS, a central
31 hypothesis is that glutamatergic signalling at dendritic spines is impaired ^{2,3} concomitant with
32 changes to intrinsic cellular excitability ⁴. The first major alteration described was a change in
33 dendritic spine density and morphology ^{3,5}, however this observation was not apparent when
34 examined at the nanoscale using super-resolution imaging methods ⁶, despite an increase in
35 synapse and spine density in the neocortex ⁷⁻⁹. Notwithstanding, no study has yet observed a
36 change in synaptic event frequency that would be predicted by a change in spine or synapse
37 density. This has important implications for our understanding of the synaptic aetiology of FXS,
38 as many of the current theories are reliant on altered synaptic function ^{10,11}.

39

40 The rodent somatosensory cortex (S1) is well characterised in terms of its processing of tactile
41 inputs, which, in the case of the barrel cortex arise from the whiskers on the facepad via relay
42 synapses in the brainstem and ventrobasal thalamus ¹². The thalamic inputs arrive predominantly
43 onto layer 4 stellate cells (L4 SCs) which integrate this information within L4, then project to L2/3
44 and L6. Furthermore, L4 SCs undergo a well described critical period for synaptic plasticity, which
45 closes at postnatal day 7-8 (P7-8). For these reasons, L4 of S1 provides a well-described
46 reductionist system to examine sensory processing ^{13,14}. Indeed, hyperexcitability has been
47 observed within S1 of *Fmr1*^{-y} mice, due in part to changes in intrinsic neuronal excitability, axonal
48 morphology, and synaptic connectivity, which together result in increased network excitability ¹⁵⁻
49 ¹⁷. The finding that the critical period for thalamocortical synaptic plasticity is delayed in *Fmr1*^{-y}
50 mice compared to wildtype (WT) gave a suggestion as to how cellular and circuit deficits may
51 arise ¹⁸. How this delay in synapse development delay affects dendritic spine function is not
52 known. Furthermore, no study has directly examined how dendrites integrate synaptic inputs in
53 the absence of FMRP, despite the fact that dendritic integration plays a key role in regulating

54 cellular excitability¹⁹⁻²¹. Of particular relevance are findings that HCN channel expression is
55 altered, leading to changes in intrinsic physiology and dendritic integration^{16,17,22}. Here, we directly
56 test whether there is a functional relationship between dendritic spine function, intrinsic neuronal
57 physiology, HCN channel function, dendritic integration, and ultimately neuronal output. To
58 address this question, we use an integrative approach that combines whole-cell patch-clamp
59 recording from neurons in S1 at P10-14 with 2-photon glutamate uncaging, *post-hoc* stimulated
60 emission-depletion (STED) microscopy, and serial block-face scanning electron microscopy.

61

62 **Results:**

63 *Larger single dendritic spine currents in $Fmr1^{-/y}$ L4 SCs:*

64 To first assess the function of identified dendritic spines in $Fmr1^{-/y}$ mice, we performed single spine
65 2-photon glutamate uncaging. Whole-cell patch-clamp recordings were performed from L4 SCs
66 in voltage-clamp with a Cs-gluconate based intracellular solution containing a fluorescent dye
67 (Alexafluor 488, 100 μ M) and biocytin to allow on-line and *post-hoc* visualization of dendritic
68 spines. Following filling, we performed 2-photon uncaging of Rubi-glutamate (Rubi-Glu) to elicit
69 uncaging excitatory post-synaptic currents (uEPSCs; Figure 1A). From both the concentration-
70 and power-response relationships (Supplementary Figure 1A, B), we determined that 300 μ M
71 [Rubi-Glu] and 80-100 mW laser power (λ 780 nm) were optimal to produce saturating uEPSCs at
72 -70 mV. Analysis of the spatial properties of Rubi-Glu uncaging confirmed that the optimal position
73 for photolysis was 0-1 μ m from the edge of the spine head (Supplementary Figure 1C), and the
74 resulting uEPSCs were blocked with CNQX, confirming that they were produced by AMPA
75 receptors (AMPA receptors, Supplementary Figure 1D). We also found no difference in spine distance
76 from cell soma and uEPSC rise or decay time and amplitude suggesting equal space clamp of
77 the neurons across the dendritic distances examined (Supplementary Figure 1F-H). All details of
78 statistical tests performed can be found in Supplementary Table 1.

79

80 Comparison between genotypes revealed that the single spine uEPSCs in WT mice had an
81 amplitude of 6.9 ± 0.4 pA (n=17 mice), while $Fmr1^{-/y}$ mice (n=14 mice) showed a larger uEPSC
82 amplitude of 9.8 ± 0.5 pA (d.f: 4, 5 $\chi^2 = 8.26$ $p = 0.004$; LMM, Figure 1 and Supplementary Figure
83 2), indicating that spines in $Fmr1^{-/y}$ mice are enriched for AMPAR-mediated currents (Figure 1B,
84 C). This difference appeared to be due to a greater population of uEPSCs at $Fmr1^{-/y}$ spines with
85 amplitudes over 10 pA (Figure 1B). As expected from larger underlying currents, the single spine
86 uncaging excitatory post-synaptic potential (uEPSP) was also larger in $Fmr1^{-/y}$ mice
87 (0.73 ± 0.12 mV, n = 10 mice), when compared to WT littermates (0.47 ± 0.06 mV, n = 16 mice;

88 d.f.: 24; $t = 2.09$; $p = 0.046$; T-test; Figure 1D). In a subset of dendritic spines we observed
89 AMPAR current at -70 mV, however a large NMDA receptor (NMDAR) current was present at
90 +40 mV, indicating the presence of silent dendritic spines (Figure 1E). Quantification of the silent
91 spines revealed an occurrence of $17.6 \pm 3.5\%$ in *Fmr1*^{-y} mice (n=13 mice), almost 3-fold higher
92 than in WT mice ($6.4 \pm 1.6\%$, n=17 mice; d.f.: 27; $t = 3.1$; $p = 0.005$; T-test; Figure 1F). When
93 measured across all spines, the NMDA/AMPA ratio was significantly elevated as both a
94 population average (d.f.: 1, 331; $F = 37.36$; $p < 0.0001$; F-test; Figure 1G) and also as a spine
95 average with *Fmr1*^{-y} mice having a ratio of 1.26 ± 0.05 (n=117 spines) and WT of 0.97 ± 0.03
96 (n=194 spines; $\chi^2 = 6.27$ $p = 0.012$, LMM, Figure 1H and Supplementary Figure 3).

97

98 Given that the majority of L4 SC dendritic spines are formed by cortico-cortical synapses in WT
99 mice²³, and therefore likely comprise the majority of uncaged spines, we next asked whether
100 synapses formed between L4 SCs had larger EPSC amplitudes by performing paired recordings
101 between synaptically-coupled neurons (Figure 2). As previously described in 2-week old mice¹⁶,
102 we observed a low connectivity between L4 SCs in *Fmr1*^{-y} mice of 14.8%, that is significantly
103 lower than that of WT mice which had a connectivity of 33.6% ($p = 0.015$, Fisher's exact test,
104 Figure 2C). Despite this reduced connectivity, there was no difference in either failure rate (d.f.:
105 41; $t = 0.25$, $p = 0.80$; GLMM; Figure 2D) or unitary EPSC amplitude (d.f.: 41; $t = 1.53$, $p = 0.15$;
106 LMM; Figure 2E), suggesting that synaptic strength is unchanged at the majority of synapses in
107 *Fmr1*^{-y} mice.

108

109 *Fmr1*^{-y} spines have typical morphology but more synapses

110 The inclusion of biocytin within the internal solution allowed *post-hoc* visualisation of the recorded
111 neurons, following fixation and re-sectioning. We next performed correlated Stimulated Emission-
112 Depletion (STED) imaging of the same dendritic spines we had uncaged upon (Figure 3A-E).
113 Measurement of nanoscale spine morphology revealed that there was no difference in either
114 spine head width (Figure 3B), nor neck-length (Figure 3D), between WT (n=6 mice) and *Fmr1*^{-y}
115 (n=4 mice) mice. Consistent with earlier findings²⁴, we observed a weak positive correlation with
116 spine head width and EPSC amplitude in WT mice (7.8 ± 3.8 pA/ μm , $R^2=0.06$, $F=4.3$, $p=0.042$,
117 F-test), which was not different to that of *Fmr1*^{-y} mice ($F=0.02$, $p=0.89$, Sum-of-Squares F-test;
118 Figure 3C). We observed no correlation with spine neck length and EPSC amplitude (Figure 3E).
119 To confirm that uncaging itself did not result in spine remodelling, we also measured spines from
120 non-uncaged dendrites on filled neurons. Spine density itself was not different between genotypes

121 (Figure 3F), nor were head width (Figure 3G, H) and neck length (Figure 3I, J), in agreement with
122 previous findings from L5 of S1 and CA1 of the hippocampus ⁶.

123

124 Given the strengthening of dendritic spines, but no change in unitary EPSC amplitude or spine
125 morphology, we next asked whether the ultrastructure of dendritic spines was altered. To achieve
126 this, we used serial block-face scanning electron microscopy in L4 of S1 from mice perfusion fixed
127 at P14. In serial stacks (50 nm sections; Figure 4) we identified Type-1 asymmetric synapses on
128 dendritic spines, based on the presence an electron dense post-synaptic density (PSD) opposing
129 an axon bouton containing round vesicles. Following 3-dimensional reconstruction, we identified
130 a subset of dendritic spines that contained more than one PSD, which were each contacted by
131 an independent presynaptic axon bouton (Figure 4A, B), and henceforth referred to as multi-
132 innervated spines (MIS). These MIS were present in both genotypes, however the incidence in
133 *Fmr1^{-/y}* mice was $20.5 \pm 1.6\%$ of all spines (n=7 mice), approximately 3-fold higher than in WT
134 littermates ($7.2 \pm 1.5\%$ of spines, n=3 mice, d.f.: 8; $t = 4.9$; $p = 0.001$; T-test; Figure 4C), which is
135 similar to that observed in organotypic hippocampal cultures from WT mice ²⁵.

136

137 The presence of higher numbers of MIS in *Fmr1^{-/y}* mice, and larger single spines uEPSCs, despite
138 a similar density of spines and similar dendritic morphologies ²⁶, would suggest an increased
139 number of synapses for each L4 SC. The conventional method to assess such a change in
140 synapse number is to perform miniature EPSC (mEPSC) recordings (Figure 5A). AMPAR
141 mEPSCs recorded at -70 mV in *Fmr1^{-/y}* mice were very similar to WT in both amplitude (d.f.: 46;
142 $U = 245$; $p = 0.28$; Mann-Whitney test) and frequency (d.f.: 46; $U = 240$; $p = 0.24$; Mann-Whitney
143 test; Figure 5B). NMDAR mEPSCs, recorded at +40 mV in the presence of CNQX, also had very
144 similar amplitudes (d.f.: 17; $U = 37$; $p = 0.59$; Mann-Whitney test). However, *Fmr1^{-/y}* mice showed
145 a 54% increase in NMDAR mEPSC frequency compared to WT mice (d.f.: 17; $U = 18$; $p = 0.03$;
146 Mann-Whitney test; Figure 5C). These data indicate that while AMPAR-containing synapses
147 number and strength are unaltered in *Fmr1^{-/y}* mice, they possess ~50% more NMDAR containing
148 synapses.

149

150 *Fmr1^{-/y}* L4 SCs are hyperexcitable due to lower HCN currents

151 While these observed changes in synaptic properties reveal differences in dendritic spine
152 function, alone they do not reveal how neurons integrate excitatory inputs leading to
153 hyperexcitability. Dendritic spines act as spatiotemporal filters whose summation is dependent
154 upon synaptic receptor content ²¹ and intrinsic membrane properties ^{20,27}, the latter of which

155 contributes to the cable properties of dendrites ²⁸. To explore the effect of altered synaptic
156 properties on dendritic integration in *Fmr1*^{-/-} SCs, we next measured the intrinsic excitability of L4
157 SCs by assessing their response to hyperpolarising and depolarising current injections (Figure
158 6A, B). In *Fmr1*^{-/-} mice, L4 SC input resistance (R_i) was increased compared to WT mice, as
159 measured from the steady-state current-voltage relationship (Interaction: d.f.: 5, 230; $F = 7.03$;
160 $p < 0.0001$; 2-way RM ANOVA Figure 6C) and smallest current step response (d.f.: 222; $t = 2.21$,
161 $p = 0.023$; GLMM; Figure 6C, inset). This increase in R_i in *Fmr1*^{-/-} mice was associated with an
162 increase in action potential (AP) discharge (Interaction: d.f.: 5, 230; $F = 6.17$; $p < 0.0002$; 2-way
163 RM ANOVA, Figure 6D), resulting from a decreased rheobase currents in the recorded L4 SCs
164 (d.f.: 222; $t = 2.15$, $p = 0.035$; GLMM, Figure 6D, inset). The dynamic response of neurons to
165 modulating current when measured with a sinusoidal wave of current injection (0.2 – 20 Hz,
166 50 pA, 20 s duration, Figure 6E) led to a resonant frequency of 1.1 ± 0.1 Hz in L4 SCs from *Fmr1*^{-/-}
167 mice, which was higher than that of 0.8 ± 0.1 Hz in WT littermates (d.f.: 25; $t = 3.25$; $p = 0.002$;
168 LMM; Figure 6F). Furthermore, there was no change in resonant dampening (Q-factor: WT:
169 1.23 ± 0.07 ; *Fmr1*^{-/-}; 1.13 ± 0.03 ; d.f.: 24; $t = 0.7$; $p = 0.49$; T-test) indicating equally sustained
170 activity at these frequencies between genotypes. Further analysis of passive membrane
171 properties (Supplementary Figure 6B and 6C) did not reveal genotype specific differences. While
172 AP amplitude was minimally reduced (Supplementary Figure 6E), no other parameter was
173 significantly altered, confirming the specificity of R_i leading to altered cellular excitability. These
174 analyses demonstrate that L4 SCs from *Fmr1*^{-/-} mice are intrinsically more excitable than their
175 WT counterparts.

176
177 In S1 L5 pyramidal cells, HCN channel density is reduced leading to reduced I_h as measured
178 indirectly as a voltage-sag in current-clamp ^{17,22}. Therefore, we next asked whether I_h mediated
179 sag is also reduced in L4 SCs and contributes to the genotypic differences in intrinsic excitability
180 we have observed. We first measured the sag and membrane rebound in response to
181 hyperpolarising current steps in current-clamp from -60 mV (0 to -125 pA, 25 pA steps, 500 ms
182 duration; Figure 7A). The voltage sag, as measured as a percentage of the maximum
183 hyperpolarisation (Figure 7B) was significantly reduced in *Fmr1*^{-/-} mice ($7.6 \pm 0.6\%$ of maximum)
184 when compared to WT controls ($10.9 \pm 0.5\%$ of maximum, d.f.: 218; $t = 3.59$, $p = 0.0003$; GLMM),
185 indicating reduced I_h . A further measure of I_h is the rebound potential produced on return to -
186 60 mV ^{22,29}. Consistent with reduced sag, we observed a lower rebound potential in *Fmr1*^{-/-} L4
187 SCs when measured relative to the steady-state potential (Figure 7C). Furthermore, the rebound

188 slope from individual cells was -0.09 ± 0.01 mV/mV in *Fmr1^{-ly}* neurons, lower than that of WT ($-$
189 0.11 ± 0.01 mV/mV d.f.: 207; $t = 2.28$, $p = 0.024$; LMM, Figure 7D).

190
191 We next applied the I_h blocker ZD-7,288 (ZD; 20 μ M) to a subset of cells to assess the effect of I_h
192 on intrinsic excitability. We observed a tendency to greater R_i in *Fmr1^{-ly}* than in WT mice (d.f.: 57;
193 $t = 1.85$, $p = 0.078$; LMM; Figure 7E), similar to that we had observed previously (Figure 6C).
194 Following ZD application in WT L4 SCs, R_i increased by 49% (d.f.: 28; $t = 6.05$, $p = 1.99 \times 10^{-7}$;
195 LMM; Figure 7E), while *Fmr1^{-ly}* L4 SCs only showed a 14% increase (d.f.: 28; $t = 1.28$, $p = 0.20$;
196 LMM; Figure 7E). The ZD effect on R_i was significantly lower *Fmr1^{-ly}* L4 SCs compared to WT
197 (d.f.: 57; $t = 4.37$, $p = 6.3 \times 10^{-5}$; LMM; Figure 7F). Given the observed differences in AP discharge
198 between genotypes (Figure 6D), we next tested whether ZD normalised this genotypic difference.
199 In WT L4 SCs, ZD application significantly increased AP firing (d.f.: 5, 80; $F = 3.2$; $p = 0.011$ for
200 interaction; 2-way RM ANOVA; Figure 7G). However, ZD had no effect on the AP discharge of
201 *Fmr1^{-ly}* L4 SCs (d.f.: 5, 174; $F = 0.23$; $p = 0.95$ for interaction; 2-way ANOVA; Figure 7H),
202 consistent with reduced sag. Finally, we examined the effect ZD had on the resonance of L4 SCs.
203 In WT L4 SCs, ZD increased the impedance at low frequencies by 33% (d.f.: 15; $t = 2.66$, $p =$
204 0.017 ; GLMM; Figure 7I, K), whereas ZD had no effect on impedance in *Fmr1^{-ly}* neurons (d.f.: 13;
205 $t = 0.83$, $p = 0.41$; GLMM; Figure 7J, K). These data show that the intrinsic excitability of L4 SCs
206 is increased in *Fmr1^{-ly}* mice, with WT L4 SC excitability increased by ZD application, potentially
207 explaining genotype specific differences in cellular intrinsic excitability.

208
209 Voltage sag and rebound are indicative of altered I_h . To directly measure I_h in L4 SCs we next
210 performed dedicated voltage-clamp experiments using a paradigm described previously³⁰. I_h was
211 recorded from L4 SCs in the presence of sodium channel, potassium channel, calcium channel,
212 and GABA_A receptor blockers, as well as AMPA and NMDA antagonists, from -50 mV with
213 hyperpolarising steps (10 mV steps, 5 second duration, Figure 8A). I_h had a half-maximal
214 activation potential ($V_{1/2 \text{ max}}$) in WT L4 SCs of -86 mV, which in *Fmr1^{-ly}* was more hyperpolarised
215 at -92 mV (d.f.: 4, 584; $F = 4.58$, $p = 0.001$; F-test; Figure 8B). Despite this difference, I_h elicited at
216 the most hyperpolarised voltage steps was similar (d.f.: 1, 370; $F = 0.001$, $p = 0.97$; F-test),
217 suggesting a normal complement of HCN channels (these currents in both WT and *Fmr1^{-ly}* L4
218 SCs were sensitive to ZD, Figure 8B, inset). As the activation of I_h is directly associated to the
219 intracellular cyclic-AMP concentration³¹, we next asked if increasing intracellular cyclic-AMP
220 could rescue I_h activation in *Fmr1^{-ly}* neurons. To increase cyclic-AMP levels, we bath applied the
221 adenylyl cyclase activator forskolin (50 μ M) to the bath. Forskolin significantly increased the

222 activation of I_h in both WT and *Fmr1*^{-y} L4 SCs (Figure 8C), normalising the I_h activation curves
223 between genotypes (d.f.: 4, 310; $F = 0.2$, $p = 0.94$; F-test, Figure 8D). This data indicates that the
224 decrease in I_h and hence increase in intrinsic excitability, in *Fmr1*^{-y} L4 SCs results from a reduced
225 cAMP-mediated shift in HCN activation.

226

227 *Enhanced dendritic summation in L4 SCs from Fmr1^{-y} mice*

228 Given that NMDARs and HCN channels are a key determinants of dendritic integration^{19,20}, we
229 next assessed both spatial and temporal dendritic summation in the *Fmr1*^{-y} L4 SCs. To address
230 spatial summation in L4 SC dendrites we performed near-simultaneous glutamate uncaging at
231 multiple spines (Fig. 9A), by focal puff application of Rubi-Glu (10 mM) and rapidly uncaged on
232 dendritic spines (0.5 ms/spine). We first performed a sequential uncaging (i.e. each spine
233 individually), then near simultaneous uncaging of spine ensembles (i.e. groups of spines; Figure
234 9B).

235

236 Summating EPSPs ultimately resulted in a AP discharge from L4 SCs. *Fmr1*^{-y} L4 SCs required
237 activation of fewer spines on average to initiate an AP (d.f.: 23; $t = 2.3$; $p = 0.03$, T-test; Figure
238 9C), which was more pronounced when silent-spines excluded from analysis (d.f.: 18; $t = 3.2$; $p =$
239 0.005). In five *Fmr1*^{-y} L4 SCs, uncaging at spines individual was not performed, thus were not
240 included in further analysis. Measurement of the summated EPSP, with respect to number of
241 spines near-simultaneously uncaged showed that both WT and *Fmr1*^{-y} L4 SC dendrites showed
242 an increase in EPSP amplitude with increasing number of spines (Figure 9D), which was
243 significantly greater in the *Fmr1*^{-y} L4 SCs (d.f.: 1, 170; $F = 8.98$; $p = 0.003$; F-test). This measure
244 will include effects due to increased spine synaptic strength and input resistance, in addition to
245 dendritic integrative properties. Therefore, we next compared the expected linear sum of single
246 spine EPSPs to that of the observed summated EPSP (Figure 9E), thereby excluding individual
247 spine strength and input resistance effects on EPSP amplitude. We observed sublinear
248 integration in WT and *Fmr1*^{-y} L4 SCs, however WT neurons showed low levels of integration
249 (Slope: 0.50 ± 0.09), while *Fmr1*^{-y} neurons presented over 50% higher summation (Slope:
250 0.79 ± 0.08 ; d.f.: 1, 195; $F = 3.18$; $p = 0.044$; F-test). These data clearly show that the dendrites
251 of *Fmr1*^{-y} L4 SCs undergo excessive dendritic summation of synaptic inputs. To confirm that
252 dendritic summation is altered in response to endogenous synaptic transmission, we next
253 provided extracellular stimulation to thalamocortical afferents (TCA) from the ventrobasal
254 thalamus, whilst recording from L4 SCs (Figure 9F). Stimulus intensity was titrated so that an
255 EPSC of ~ 150 pA was produced, then trains of EPSPs were elicited in current-clamp at either 5

256 or 10 Hz. At these stimulation intensities summing EPSPs in L4 SCs in WT mice never produced
257 a somatic AP, however in *Fmr1^{-ly}* mice 5 Hz stimulation resulted in an AP in $19 \pm 7\%$ of recordings
258 (d.f.: 16; $t = 2.57$ & 3.81 ; $p = 0.02$ & 0.002 , T-test) and 10 Hz stimulation $55 \pm 13\%$ of the time
259 (d.f.: 16; $t = 3.81$; $p = 0.002$, T-test), confirming that dendritic integration properties alter the output
260 of L4 SCs, to promote hyperexcitability (Figure 9G).

261
262 As I_h has known effects on dendritic summation¹⁹, we next asked whether ZD altered summation
263 properties. First, we determined whether inhibition of HCN channels altered amplitude or kinetics
264 of synaptic events. Application of ZD itself had no effect on spontaneous EPSC amplitudes,
265 frequencies, or kinetics (Supplementary Figure 8). However, spontaneous EPSCs were of higher
266 frequency in *Fmr1^{-ly}* L4 SCs, potentially indicating underlying circuit hyperexcitability (d.f.: 25; $t =$
267 2.99 , $p = 0.016$; GLMM). Summing uEPSPs from WT mice (normalised to the initial uEPSP)
268 displayed long decay times at low summation, which were more rapid at higher summation levels
269 (Supplementary Figure 9A and 9B). By comparison, in *Fmr1^{-ly}* mice we did not observe this
270 relationship and the genotype-specific log(EPSP summation) was divergent (d.f.: 1, 109; $F = 32.1$,
271 $p < 0.0001$; F-test). The summation-dependent temporal sharpening of EPSPs in WT neurons was
272 abolished following application of ZD (Comparing slope: d.f.: 1, 85; $F = 6.4$, $p = 0.01$; F-test;
273 Supplementary Figure 6D) and also prolonged decay times of the first EPSP (Figure 9F, d.f.: 15;
274 $t = 2.34$; $p = 0.034$; T-test; Supplementary Figure 9C). ZD had no observable effect on summing
275 EPSPs in *Fmr1^{-ly}* L4 SCs (Supplementary Figure 9E). Finally to confirm that altered I_h and NMDAR
276 function contribute to the observed aberrant dendritic summation, in a subset of experiments we
277 examined the effects of both ZD and AP-5 on EPSP summation during multispine uncaging.
278 Application of either ZD or AP-5 to near-simultaneous uncaging of uEPSPs in WT L4-SCs had
279 minimal effect on the observed summation when compared to the expected linear sum
280 (Supplementary Figure 10A), consistent with an absence of non-linear summation. However, bath
281 application of either ZD or AP-5 significantly reduced the summation of *Fmr1^{-ly}* L4 SCs
282 (Supplementary Figure 10B). These findings confirm that both reduced HCN activation and
283 increased NMDARs contribute to the enhanced summation in dendrites of *Fmr1^{-ly}* L4 SCs relative
284 to WT cells.

285
286 **Discussion:**
287 L4 of the primary somatosensory cortex is the first layer to receive and integrate incoming sensory
288 information, which is integrated and relayed within the cortex. As such, L4 SCs play a crucial role
289 in sensory perception¹⁴. Individuals with FXS show altered sensory processing^{32,33} and mouse

290 models show altered circuit processing in primary sensory areas ^{1,15,17,18,34,35}. Furthermore, while
291 FMRP has been shown repeatedly to regulate synapse function and plasticity, little is known about
292 how these alterations affect dendritic spine function and dendritic integration to sensory input. To
293 address these questions, we used glutamate uncaging at L4 SC dendritic spines to examine how
294 they integrate and generate action potentials following synaptic stimulation. We show that L4 SCs
295 in S1 have dendritic and synaptic properties that result in increased action potential generation in
296 *Fmr1^{-y}* mice relative to WT controls. Specifically, we show increased excitatory synaptic currents
297 at individual spines resulting from increased AMPAR and NMDAR content. Despite this, we
298 observed no change in spine morphology using STED microscopy and there was little correlation
299 between spine structure and function, indicating that spine morphology is not an effective proxy
300 for spine function, at least at the age used in this study. However, electron microscopic analysis
301 revealed an increase in multiply-innervated spines which likely accounts for the increase in single-
302 spine synaptic currents. Interestingly there was also an increase in silent spines which agrees
303 with the increase in NMDAR mEPSC frequency, but not AMPAR mEPSC frequency. The overall
304 increase in dendritic spine currents was accompanied by enhanced dendritic integration likely
305 resulting, at least in part, from a ~50% reduction in I_h . This reduced I_h was causal to the altered
306 intrinsic physiology of L4 SCs at P12-14. Finally, TCA stimulation at frequencies that fail to elicit
307 AP discharge from L4 SCs in WT mice, in the presence of intact synaptic inhibition, reliably elicits
308 APs in *Fmr1^{-y}* neurons, indicating that the local inhibitory circuit cannot compensate for the
309 increase in synaptic and dendritic excitability. Together these findings demonstrate that aberrant
310 dendritic spine function and dendritic integration combine to result in cellular hyperexcitability in
311 L4 SCs. As the first cortical cells to receive input from the sensory periphery, the resultant
312 hyperexcitability likely contributes previously reported circuit excitability in *Fmr1^{-y}* mice and the
313 sensory hypersensitivities in individuals with FXS.

314

315 Our study quantifies the incidence of MIS in intact tissue and implicates their presence in
316 pathological states associated with disease models. Indeed, the mean increase in spine uEPSC
317 amplitude, but not miniature, spontaneous or unitary EPSCs, in *Fmr1^{-y}* mice is likely caused by
318 the increase in the number of MIS. Indeed, the presence of MIS in both WT and *Fmr1^{-y}* mice
319 disagrees with the one spine/one synapse hypothesis ³⁶. A potential mechanistic link between
320 loss of FMRP and the increase in MIS may come from its ability to regulate PSD-95. *Psd-95*
321 mRNA is a known FMRP target ³⁷ and an increase in PSD-95 puncta in L4 of S1 has been
322 observed ⁷, with no change in cell number, dendritic morphology, or spine density in *Fmr1^{-y}* mice
323 ²⁶. Furthermore, transient overexpression of PSD-95 results in increased MIS incidence through

324 nitric oxide synthase, as well as NMDARs and other LTP mechanisms ^{22,25,38-40}. Future
325 experiments exploring the effect of NOS blockade, PSD-95, and NMDAR function in *Fmr1*^{-y} mice
326 should test the mechanism of MIS formation and influence on dendritic protein synthesis, as well
327 as potential therapeutic targeting.

328
329 Interestingly the increase in spines with increased uEPSC amplitudes and MIS was mirrored by
330 an increase in silent spines, though their number was insufficient to compensate for the overall
331 increase in dendritic currents in other spines. An increase in silent TCA synapses at P7 ¹⁸ was
332 previously reported in *Fmr1*^{-y} mice. However, this study also reported a delay in the critical period
333 for inducing LTP at these synapses which terminated at P10. Therefore, the period of synaptic
334 potentiation at TCA synapses is complete by the age we tested in this study. Hence the
335 percentage of silent spines receiving TCA input would be expected to be low ⁴¹. Furthermore, the
336 reduced connectivity between L4 SCs at P12-14, despite no change in spine density (Till et al.,
337 2012), strongly indicates that SC to SC synapses are preferentially silent at this developmental
338 stage in the *Fmr1*^{-y} mouse. Together, these findings suggest that silent spines measured in our
339 study reflect cortico-cortical, rather than TCA, synapses. Given the hierarchical nature of sensory
340 system development, it would not be surprising if a delay in intra-cortical synapse development in
341 *Fmr1*^{-y} mice follows the aforementioned delay in TCA synapse development, but this remains to
342 be directly tested.

343
344 While dendritic spines are functionally disrupted in the *Fmr1*^{-y} mouse, using super resolution
345 microscopy we found no evidence of a genotypic difference in spine morphology of L4 SC
346 neurons. This is in good agreement with our previous findings that spine morphology is unaffected
347 in hippocampal CA1 and layer 5 S1 neurons ⁶. Furthermore, we find only a weak correlation
348 between dendritic spine structure and function, demonstrating the pitfalls of using spine structure
349 as a proxy for synaptic function, especially in young animals and genetic models of disease.
350 These findings are in stark contrast to those observed from post-mortem human tissue ³ or from
351 other mouse studies ⁵; however these studies were only performed with diffraction-limited
352 microscopy, suggesting that super-resolution imaging techniques should be the gold-standard for
353 dendritic spine morphological studies in future. Single dendritic spines do not typically produce
354 AP discharge from neurons, rather they require co-activation and summation of multiple synaptic
355 inputs arriving with high temporal precision ⁴². L4 SCs have been previously been shown to
356 possess linear integration of Ca²⁺ influx in their dendrites ⁴³. We show that synaptic potentials
357 sublinearly integrate in L4 SCs of WT mice, and that this integration is strongly enhanced in *Fmr1*⁻

358 ^{ly} mice, leading to more efficient discharge of APs, due in large part to a combination of increased
359 NMDARs and reduced I_h . The latter has been implicated in the altered neuronal excitability of
360 FXS ^{17,22}, with the HCN1 channel expression dictating whether the current is increased or
361 decreased. Unlike these former studies, we provide evidence that I_h is not reduced in L4 SCs, but
362 rather displays shifted activation properties, likely due to reduced cyclic-AMP levels. This finding
363 is in agreement with previous work implicating altered cAMP levels in the aetiology of FXS ⁴⁴⁻⁴⁸.
364 Whether the altered I_h currents in the absence of FMRP reported in other cell types ^{17,22} could
365 also be explained by altered cAMP levels is not known; however, at least for layer 5 neurons in
366 somatosensory cortex, a reduced level of HCN channels has also been reported ¹⁷. Future
367 experiments will be needed to determine the developmental and cell-specific nature of cellular
368 hyperexcitability in *Fmr1*^{-ly} mice.

369
370 Our observations showing sublinear dendritic integration in layer 4 SCs are at odds with reported
371 NMDAR-dependent non-linear (supra-linear) summation of cortical cells reported from many
372 laboratories ^{20,21,49,50}. However, many factors may account for this discrepancy, including
373 recording conditions, stimulation paradigms, cell type and developmental age. Furthermore, the
374 somatosensory cortex has a well described developmental profile of membrane properties,
375 notably decreasing membrane resistance as a function of age ⁵¹. This combined with the compact
376 dendritic arbour of L4 SCs ²⁶, will lead to these neurons at the age of ~14 days likely having very
377 uniform cable properties ²⁸. It is possible that as L4 SCs mature, their dendrites may develop non-
378 linear properties. Irrespective of the differences between studies, we provide the first direct
379 evidence in *Fmr1*^{-ly} neurons for a functional deficit at excitatory synapses onto dendritic spines
380 and that these alterations contribute to an increase in dendritic integration. The summation of
381 synaptic responses contributes to hyperexcitability of sensory neurons in the *Fmr1*^{-ly} mouse,
382 which along with changes in intrinsic excitability, may underlie pathophysiology associated with
383 altered sensory function.

384

385

386 **Methods:**

387 *Animals and ethics:*

388 All procedures were performed in line with Home Office (ASPA, 2013; HO license: P1351480E)
389 and institutional guidelines. All experiments were performed on C57/Bl6J mice, bred from *Fmr1^{+/-}*
390 mothers, cross-bred with *Fmr1^{+ly}* male mice, giving a Mendelian 1:1 ratio of *Fmr1^{+ly}* and *Fmr1^{-ly}*
391 amongst male offspring. Only male mice were used for the present study and all mice were killed
392 at P10-15, before separation from the mother. Mothers were given *ad libitum* access to food and
393 water and housed on a 12 hr light/dark cycle. All experiments and analysis were performed blind
394 to genotype.

395

396 *Acute slice preparation:*

397 Acute brain slices were prepared similar to previously described^{52,53}. Briefly, mice were
398 decapitated without anaesthesia and the brain rapidly removed and placed in ice-cold
399 carbogenated (95 % O₂/5 % CO₂) sucrose-modified artificial cerebrospinal fluid (in mM: 87 NaCl,
400 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 25 glucose, 75 sucrose, 7 MgCl₂, 0.5 CaCl₂). 400 µm thick
401 thalamocortical (TC) slices were then cut on a Vibratome (VT1200s, Leica, Germany) and then
402 stored submerged in sucrose-ACSF warmed to 34°C for 30 min and transferred to room
403 temperature until needed.

404

405 *Whole-Cell Patch-Clamp Recordings:*

406 For electrophysiological recordings slices were transferred to a submerged recording chamber
407 perfused with carbogenated normal ACSF (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO₃,
408 1.25 NaH₂PO₄, 25 glucose, 1 MgCl₂, 2 CaCl₂) maintained at near physiological temperatures
409 (32 ± 1°C) with an inline heater (LinLab, Scientifica, UK) at a flow rate of 6-8 ml/min. Slices were
410 visualized with IR-DIC illumination (BX-51, Olympus, Hamburg, Germany) initially with a 4x
411 objective lens (N.A. 0.1) to position above a L4 barrel, and then with a 20x water-immersion
412 objective (N.A. 1.0, Olympus). Whole-cell patch-clamp recordings were made with a Multiclamp
413 700B amplifier (Molecular Devices, USA). Recording pipettes were pulled from borosilicate glass
414 capillaries (1.7 mm outer/1mm inner diameter, Harvard Apparatus, UK) on a horizontal electrode
415 puller (P-97, Sutter Instruments, CA, USA), which when filled with intracellular solution gave a
416 pipette resistance of 4-5 MΩ. Unless otherwise stated, all V-clamp recordings were performed at
417 V_M = -70 mV. All signals were filtered at 10 kHz using the built in 4-pole Bessel filter of the amplifier,
418 digitized at 20 kHz on an analogue-digital interface (Digidata 1440, Axon Instruments, CA, USA),
419 and acquired with pClamp software (pClamp 10, Axon Instruments, CA, USA). Data was analysed

420 offline using the open source Stimfit software package ⁵⁴ (<http://www.stimfit.org>). Cells were
421 rejected if the I_{hold} was >150pA in voltage-clamp, membrane potential more depolarised than -
422 50 mV in current-clamp, series resistance >30 M Ω , or the series resistance changed by more
423 than 20% over the course of the recording.

424

425 *Sequential dendritic spine 2-photon glutamate uncaging:*

426 Slices were transferred to the recording chamber, which was perfused with normal ACSF,
427 containing 50 μM picrotoxin (PTX) and 300 nM tetrodotoxin (TTX). For voltage clamp recordings
428 of dendritic spine uncaging neurons were filled with an internal solution containing (in mM):
429 140 Cs-gluconate, 3 CsCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 2 Na₂-ATP, 0.3 Na₂-GTP, 1
430 phosphocreatine, 5 QX-314 chloride, 0.1% biotinoylated-lysine (Biotin, Invitrogen, UK), and 0.1
431 AlexaFluor 488 or 594 (Invitrogen, UK), corrected to pH 7.4 with CsOH, Osm = 295 – 305 mOsm.
432 Whole-cell patch clamp was then achieved and cells allowed to dye fill for 10 minutes prior to
433 imaging. During this period, we collected 5 minutes of spontaneous recording, to analyse
434 mEPSCs from recorded neurons at -70 mV voltage clamp. For all imaging and uncaging
435 experiments we used a galvanometric scanning 2-photon microscope (Femto2D-Galvo,
436 Femtonics, Budapest, Hungary) fitted with a femtosecond aligned, tuneable wavelength
437 Ti:Sapphire laser (Chameleon, Coherent, CA, USA), controlled by a Pockel cell (Conoptics, CT,
438 USA). Following dye filling, a short, low zoom z-stack was collected (2 μm steps, 2-3 pixel
439 averaging, 512 x 512 pixels) over the whole dendritic extent of the cell at low laser power (<5 mW)
440 with a high numerical aperture 20x lens (N.A. 1.0, Olympus, Japan). Then a short section of spiny
441 dendrite, 50-100 μm from the cell somata, within the top 50 μm of the slice, and running parallel
442 to the slice surface was selected and imaged at higher zoom. Between 7-10 spines were then
443 selected based on being morphologically distinct from neighbouring spines, ordered distal to
444 proximal to soma, and then 300 μM Rubi-Glutamate (Rubi-Glu; Ascent Scientific, Bristol, UK) was
445 applied to the bath, and recirculated (total volume: 12.5 ml; flow rate: 6-8 mls/minute). Following
446 wash-in of Rubi-Glu (<2 minutes), short duration, high power laser pulses (1 ms, λ 780 nm, 80-
447 100 mW, 0.2 μm diameter) local photolysis was performed \sim 1 μm adjacent to individual spines.
448 In a subset of recordings from WT mice, we confirmed spatial, quantal release, and
449 pharmacological properties of Rubi-Glu uncaging under our recording conditions (Supplementary
450 Figure 1). Individual spines were sequentially uncaged at 2 second intervals followed by a 40
451 second pause; therefore each spine receiving Rubi-Glu photolysis every 60 seconds. All spines
452 underwent photolysis at least 3 times and the average uncaging-EPSC (uEPSC) at -70 mV
453 measured. In a subset of experiments we confirmed that these uEPSCs were mediated by direct

454 activation of AMPARs by subsequent application of 10 μ M CNQX to the perfusing ACSF
455 (Supplementary Figure 1D). Following each 3 repetition cycle, the focal plane and dendritic health
456 was checked with short scans, at low power (<5 mW) to prevent background photolysis.
457 Following successful recording of AMPA uEPSCs, we increased the holding potential to +40 mV
458 and recorded the outward mixed AMPA/NMDA currents. In a subset of experiments we confirmed
459 the AMPAR and NMDAR dependence of these outward currents by bath applying 10 μ M CNQX
460 and then 50 μ M D-AP5 (Supplementary Figure 1E). AMPA uEPSCs were measured over the first
461 10 ms following the uncaging stimulus (0.5 ms peak average) at both -70 and +40 mV. NMDA
462 currents were measured from 20-50 ms post-photolysis, which was confirmed to be following
463 complete decay of the AMPA uEPSC at -70 mV. All sequential spine uncaging experiments were
464 performed as quickly as possible following dye filling, to prevent phototoxic damage to the
465 recorded neurons, and L4 SCs resealed with an outside-out patch. Cells were rejected if
466 photolysis resulted in blebbing of dendrites or depolarisation of the membrane potential.

467

468 In a subset of experiments, we performed mEPSC analysis of L4 SCs independent of Rubi-Glu
469 photolysis, under the same conditions as above (with no AlexaFluor dye), recording 5 minutes of
470 mEPSCs at -70 mV voltage clamp. Cells were then depolarised to +40 mV voltage-clamp and
471 mixed AMPA/NMDA mEPSCs recorded for 1 minute, after which 10 μ M CNQX was applied to the
472 bath. Following full wash in of CNQX (~2-3 minutes) a further 5 minutes of pure NMDA mEPSCs
473 were recorded. In all experiments 50 μ M AP-5 was then bath applied, to confirm that the mEPSCs
474 recorded were NMDAR-mediated. All mEPSC data was analysed using a moving-template
475 algorithm⁵⁵, with templates made from the tri-exponential non-linear fit to optimal mEPSCs at
476 each holding potential using the event-detection interface of Stimfit. For mEPSCs at -70 mV, the
477 minimum time between EPSCs was set to 7.5 ms, and 25 ms for those at +40 mV. Detected
478 events were analysed if they had an amplitude greater than 3x the SD of the 5 ms preceding
479 baseline of the mEPSC.

480

481 HCN-mediated currents were measured as previously reported³⁰. Briefly, slices were transferred
482 to the recording chamber perfused with modified recording ACSF (in mM: 115 NaCl, 5 KCl,
483 25 NaHCO₃, 1.2 NaH₂PO₄, 2 glucose, 1 MgCl₂, 2 CaCl₂) which was supplemented with channel
484 blockers TEA (5 mM), CdCl₂ (0.1 mM), BaCl₂ (1 mM), 4-aminopyridine (1 mM), and TTX (300 nM);
485 and blockers for ionotropic receptors CNQX (10 μ M), AP-5 (50 μ M), and picrotoxin (50 μ M), with
486 a flow rate of 4-6 ml/minute at room temperature. Cells were recorded with K-gluconate based
487 intracellular solution (in mM: 142 K-gluconate, 4 KCl, 0.5 EGTA, 10 HEPES, 2 MgCl₂, 2 Na₂-ATP,

488 0.3 Na₂-GTP, 10 phosphocreatine, 0.1% Biocytin, corrected to pH 7.4 with KOH,
489 Osm = 295 – 305 mOsm). I_h was recorded in voltage-clamp from a holding potential of -50 mV
490 and activated by applying hyperpolarising voltage steps (-10 mV, 5 s duration). I_h was measured
491 as the difference in peak to steady state current during the hyperpolarising step over the full range
492 of potentials. In subsets of experiments, the HCN channel blocker ZD-7,288 was bath applied
493 (20 μM) to confirm the identity of the current or the adenylyl cyclase activator forskolin (50 μM)
494 was bath applied. Currents were plotted and fitted with a variable slope sigmoidal function to
495 determine the 50% maximum activation. Representative traces are shown as P/N subtractions of
496 the -10 mV from the -50 mV step.

497

498 Summation of thalamic inputs to L4 SCs was measured by electrical stimulation of the ventrobasal
499 thalamus with a twisted bipolar Ni-Chrome wire. Synaptically coupled barrels were identified by
500 placing a field electrode (a patch electrode filled with ACSF) in visually identified barrels and
501 stimulating the thalamus. When a field response was observed, then a L4 SC was recorded in
502 whole-cell patch clamp with K-gluconate internal solution, as described above. Trains of 5 stimuli
503 were then delivered at 5-10 Hz, with a stimulation intensity sufficient to produce an EPSC of large
504 amplitude similar between genotypes (20 to 540 pA; WT: 181 ± 35 pA; *Fmr1*^{-/-}: 159 ± 34pA; D.F.
505 = 23, t=0.44, P=0.66, T-test). In current clamp the EPSP summation was assessed as the ability
506 of the recorded cell to fire an AP in response to this stimulus. Data are show as the average P_{spike}
507 from 10 trials.

508

509 *Near-simultaneous dendritic spine 2-photon glutamate uncaging:*

510 To determine the summation properties of dendrites in L4 SCs we performed near simultaneous
511 photolysis of Rubi-Glu at multiple dendritic spines^{20,49}. Using a current-clamp optimized K-
512 gluconate based internal solution supplemented with 0.1 AlexaFluor 488 (Invitrogen, UK) we dye
513 filled neurons as for sequential photolysis described above, in normal ACSF containing PTX and
514 TTX, but not Rubi-Glu. Once dye filling was complete (<10 minutes) we imaged the L4 SC (as
515 above) at low zoom, then identified a superficial spiny dendrite 50-100 μm from the soma. At this
516 point we placed a wide puff pipette (borosilicate patch pipette with tip broken to ~20 μm diameter)
517 just above the surface of the slice, adjacent to the dendrite of interest. The puff pipette was filled
518 with 10 mM Rubi-Glu in a HEPES buffered ACSF (in mM: 140 NaCl, 2.5 KCl, 10 HEPES,
519 1.25 NaH₂PO₄, 25 glucose, 1 MgCl₂, 2.5 CaCl₂; adjusted to pH 7.4 with HCl). At this point the
520 dendrite was imaged at high magnification and 7-10 spines chosen and a very low pressure
521 stimulus given to the puff-pipette (3-5 mBar), sufficient to cause dialysis of the Rubi-Glu, but not

522 powerful enough to cause obvious movement of the tissue. The dialysis of Rubi-Glu was
523 maintained throughout the remainder of the recording. The cell was then switched to current-
524 clamp mode, membrane potential held at -60 mV with a bias current, and spines 1-7 sequentially
525 uncaged (0.5 ms laser duration, 80 mW power) to give the individual spines uEPSP amplitude.
526 Following 3 repetitions and correction of focus, a line scan was created, with 0.5 ms dwell time at
527 each spine ROI in order from distal to proximal. Spines were then uncaged in a cumulative
528 manner, with 1, 2, 3 ... n spines uncaged near simultaneously. The total duration of uncaging was
529 5.5 ms for 10 spines and there was a 10 second delay between each run of photolysis, with the
530 total protocol lasting minimally 4-5 minutes. At least 3 repetitions of this protocol were run and
531 focus re-checked. In a subset of experiments the HCN inhibitor ZD was applied to the perfusing
532 ACSF and a further 3 repetitions collected. All uEPSP data was analysed as peak amplitude
533 measured over the 20 ms directly following beginning of the photolysis stimuli. Data was either
534 normalised to the first EPSP amplitude, or measured as the absolute simultaneous uEPSP, as
535 plotted against the summed individual uEPSP amplitude for the same spines.

536

537 In a set of experiments (without PTX, TTX or AlexaFluor 488), intrinsic electrophysiological
538 properties of L4 SCs were measured, also in current-clamp mode. From resting membrane
539 potential a hyper- to depolarizing family of current injections (-125 to +125 pA, 500ms duration)
540 were given to the recorded neuron. The input resistance, rheobase current, and action potential
541 discharge frequency were all measured from triplicate repetitions. In a further subset of
542 experiments, 3x series of voltage steps were given (in voltage-clamp) from -60 mV to -110 mV
543 (10 mV steps, 500 ms duration) to estimate the amplitude of I_h in the recorded L4 SCs. ZD was
544 then applied to the bath and the same steps repeated. I_h was estimated as the amplitude of the
545 current produced in response to hyperpolarizing voltage steps.

546

547 *Visualisation and STED microscopy of recorded neurons*

548 Following completion of experiments and resealing of the neuron, slices were immediately
549 immersion fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Slices were then transferred to
550 phosphate buffered saline (PBS; 0.025 M phosphate buffer + 0.9% NaCl; pH: 7.4) and kept at
551 4 °C until processed (<3 weeks). Slices were then cryoprotected in a solution containing 30%
552 sucrose in PBS overnight at 4 °C and then freeze-thaw permeabilised on IN_2 , and returned to
553 cryoprotectant solution for 1 - 2 hrs. The slices were then mounted, recording side up, on the
554 stage of a freezing microtome; which had been prepared with a plateau of OCT medium and
555 slices embedded within OCT prior to sectioning. The OCT block containing the recorded slice was

556 trimmed to the slice surface and then 50 μm sections taken from the top 200 μm . The sections
557 were rinsed 3 times in PBS and then incubated with streptavidin conjugated to AlexaFluor488
558 (1:500, Invitrogen, UK) at 4 $^{\circ}\text{C}$ for 3-5 days. The slices were then washed for 2 hours in repeated
559 washes of PBS and then desalted with PB and mounted on glass slides with fluorescence
560 protecting mounting medium (Vectorshield, Vector Labs, UK).

561
562 Sections were imaged on a gated-Stimulated emission depletion (STED) microscope (SP8
563 gSTED, Leica, Germany). Cells were found using epifluorescent illumination (488 nm excitation)
564 under direct optics at low magnification (20x air immersion objective lens, N.A. 0.75) and then
565 positioned under high magnification (100x oil-immersion objective lens, N.A. 1.4, Olympus,
566 Japan) and then switched to gSTED imaging. Sections were illuminated with 488 nm light,
567 produced by a continuous-wave laser, and short sections of non-uncaged dendrite used to
568 optimize acquisition parameters, first under conventional confocal detection, then by gSTED
569 imaging. The 488 nm illumination laser was set to 60-70% of maximum power, and the continuous
570 wave STED laser (592 nm) set to 25% and gated according to the best STED-depletion
571 achievable in the samples (1.5 – 8 ms gating). Once optimized, a region of interest (ROI) was
572 selected over the uncaged dendrite, which at 1024x1024 pixel size, gave a pixel resolution of 20-
573 30 nm. Short stacks were taken over dendritic sections containing uncaged and non-spines
574 (0.5 μm steps) with STED images interleaved with confocal images for confirmation of STED
575 effect. STED images were deconvolved (Huygen's STED option, Scientific Volume Imaging,
576 Netherlands) and uncaged spines identified by comparison to live 2-photon images (see Figure
577 2A). Measurements of head width and neck length were then made on the deconvolved images
578 in FIJI (ImageJ)⁵⁶.

579
580 *Serial block face scanning-electron microscopy (SBF-SEM) of L4 SCs*

581 For SBF-SEM, 10 P14 mice (3 WT / 7 *Fmr1*^{-y}) were perfusion fixed. Briefly, mice were sedated
582 with isoflurane and terminally anaesthetized with I.P. sodium pentobarbital (50 mg/mouse). The
583 chest was opened and 10 mls of PBS (pH 7.4, filtered) transcardially perfused (~0.5 mls/second);
584 once cleared the PBS was replaced with ice-cold fixative solution containing (3.5% PFA, 0.5%
585 glutaraldehyde, and 15% saturated picric acid; pH 7.4), and 20 mls perfused. Brains were then
586 removed and post-fixed overnight at 4 $^{\circ}\text{C}$ in the same fixative solution. 60 μm coronal sections
587 were cut on a vibratome (Leica VT1000) and S1 identified based on visual identification. Sections
588 were then heavy-metal substituted: first sections were rinsed in chilled PBS (5 x 3 mins) and then
589 incubated with 3% potassium ferrocyanide and 2% w/v OsO₄ in PBS for 1 hr at 4 $^{\circ}\text{C}$. Sections

590 were rinsed liberally in double distilled (dd) H₂O and then incubated with 1% w/v
591 thiocarbohydroxide for 20 minutes at room temperature. Sections were rinsed again in ddH₂O,
592 and then incubated with 2% w/v OsO₄ for 30 minutes at room temperature, rinsed in ddH₂O and
593 contrasted in 1% w/v uranyl acetate overnight at 4 °C. Sections were rinsed in ddH₂O and then
594 contrasted with 0.6% w/v lead aspartate for 30 mins at 60 °C. Sections were then rinsed in ddH₂O,
595 dehydrated in serial dilutions of ethanol for 30 minutes each at 4 °C, then finally dehydrated twice
596 in 100% ethanol and then 100% acetone both at 4 °C for 30 minutes. Sections were then
597 impregnated with serial dilutions (25%, 50%, 75%, diluted in acetone) of Durcupan ACM (Sigma
598 Aldrich, UK) at room temperature for 2 hours per dilution, followed by 100% Durcupan ACM
599 overnight in a dissector at room temperature. Sections were transferred to fresh Durcupan ACM
600 for 1 hour at room temperature and then flat-embedded on glass slides, coated with mould-
601 release agent, cover-slipped, and then cured for 12 hours at 60 °C.

602 For SFB-SEM imaging, small pieces of L4 of S1 were dissected from flat-embedded sections,
603 with aid of a stereo microscope and glued with cyanoacrylate to stage mounting pins. The
604 mounted tissue was then trimmed and gold-plated prior to insertion imaging. Initially, semi-thin
605 sections trimmed from the surface of the block, and imaged under transmission electron
606 microscopy at low power to confirm tissue ultrastructure and ROI selection for SBF-SEM. Next
607 the tissue blocks were mounted in an SBF-SEM (3View, Gatan, CA, USA) and 3 x ~10 μm² ROIs
608 chosen on the surface of the block, avoiding blood vessels or L4 SC somata, and imaged at 50 nm
609 steps at 8000x magnification (1024x1024, 10 nm pixel size). Approximately 100 sections were
610 collected from each block, giving a total depth of 5 μm. SBF-SEM images were analysed offline
611 using the TrakEM module of FIJI⁵⁷. Dendrites and spines were traced as surface profiles and
612 then PSDs identified on dendritic spines as electron dense regions within 25 nm of the lipid
613 bilayer. 6-11 dendrites were reconstructed from each mouse, which possessed a total of 38-49
614 spines (average= 4.4 spines/dendrite). The incidence of PSDs was calculated as an average
615 within each mouse, and final averages produced as an animal average.

616

617 *Data analysis*

618 All data is presented as the mean ± SEM. Where appropriate, data were analysed with a linear
619 (LMM) or generalised linear mixed-effects model (GLMM). Probability distributions for models
620 were chosen by goodness of fit to normal, log-normal or gamma distributions (**Figures S2 and**
621 **S3**). Appropriate to the particular experiment and statistical model, genotype, drug treatment and
622 potentially their interaction were used as fixed effects, while litter, animal and slice were used as
623 random effects. Statistical significance was assessed by likelihood ratio tests with models in which

624 the parameter of interest had been dropped and expressed as a p -value. When animal or paired
625 cell data is shown and not modelled, datasets were tested for normality (d'Agostino-Pearson test)
626 and either Student's t-test, Mann-Whitney non-parametric U-test, or Wilcoxon signed-rank tests
627 performed. Comparison of linear and non-linear regression was performed with a Sum-of-Squares
628 F-test. Statistically significant differences were assumed if $p < 0.05$. Which statistical test employed
629 is indicated throughout the text. Either GraphPad Prism or R was used for all statistical analyses.
630 All statistical tests performed are presented in supplementary materials (**Table S1**).

631

632 **Data availability:**

633 All datasets will be made available upon reasonable request.

634

635

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805 **Author Contributions:**

806 SAB – designed and performed experiments, analysed/interpreted data and wrote the manuscript;
807 APFD - designed and interpreted, performed experiments, analysed data and wrote the
808 manuscript; ORD – analysed/interpreted data and wrote the manuscript; JTRI - designed
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813

814 **Competing Interests:**

815 The authors declare no competing interests.

816

817 **Figure Legends:**

818 Figure 1: L4 SC dendritic spines have larger uEPSCs with more silent synapses in *Fmr1^{-ly}* mice.
819 **A** 2-photon image of a L4 SC (left) with selected spines and AMPAR uEPSCs from WT and *Fmr1⁻*
820 *ly* mice. Scale bars: 20 μ m (left), 5 μ m (right). **B** Single spine uEPSCs from WT (black) and *Fmr1⁻*
821 *ly* (red) mice shown as a histogram, with spine average shown (inset). Note that spines with no
822 AMPA response, silent spines have not been included. **C** Animal average uEPSC amplitudes,
823 excluding silent spines. Number of animals tested shown in parenthesis. **D** Animal average of
824 uEPSP amplitudes. **E** AMPAR (upper) and NMDAR (lower) uEPSCs, illustrating silent spines.
825 Scale: 5 μ m. **F** Incidence of silent spines in WT and *Fmr1^{-ly}* mice. **G** AMPAR and NMDAR uEPSCs

826 for all spines, with NMDA/AMPA ratio (WT: 0.76 ± 0.03 ; *Fmr1*^{-/-}; 1.05 ± 0.04 ; d.f.: 1, 331; $F = 37.4$;
827 $p < 0.0001$; F-test). **H** Average NMDA/AMPA ratio plotted for all spines. Statistics shown: * -
828 $p < 0.05$, ** - $p < 0.01$, from LMM (B, D, H), unpaired t-test (C, F) and sum-of-least-squares F-test
829 (G). Plots of individual spine data for panel 1C (inset) and 1H can be found in Supplementary
830 Figure 4. All data is shown as mean \pm SEM and source data for all plots are provided as a Source
831 Data file.

832

833 Figure 2: Typical EPSC amplitude at unitary connections between L4 SCs. **A** Schematic paired
834 recordings between synaptically coupled L4 SCs. **B** Representative presynaptic action potentials
835 (top) produced unitary EPSCs in the second L4 SC (lower), from WT (black) and *Fmr1*^{-/-} (red)
836 mice. **C** Synaptic connectivity is reduced between L4 SCs in the *Fmr1*^{-/-} mouse (d.f.: 162; $p =$
837 0.015 ; Fisher's exact test; 110 pairs from 13 mice for WT mice and 54 pairs from 7 mice in *Fmr1*^{-/-}
838 mice were tested. **D** Failure rate was not different between genotypes when a connection was
839 present. **E** Unitary EPSC amplitudes from L4 SC synapses were not different between genotypes.
840 Statistics shown: ns – $p > 0.05$, * - $p < 0.05$ from Fisher's exact test (C) and LMM (D, E). All data is
841 shown as mean \pm SEM and source data for all plots are provided as a Source Data file.

842

843 Figure 3: Dendritic spines show no difference in nanoscale morphology, or structure-function
844 relationship. **A** Dendrites from WT (left) and *Fmr1*^{-/-} (right) mice under 2-photon microscopy (top),
845 then *post-hoc* STED imaging (bottom). Scale bar: 5 μm . **B** Average spine head width in WT (black)
846 and *Fmr1*^{-/-} (red) mice (WT: 0.43 ± 0.05 ; *Fmr1*^{-/-}; 0.45 ± 0.04 ; d.f.: 8; $t = 0.29$; $p = 0.78$, T-test).
847 Number of mice is indicated. **C** Comparison of spine head-width and uEPSC amplitude
848 (comparing slope: d.f.: 1, 100; $F = 0.02$; $p = 0.89$). WT spines showed a positive correlation (d.f.
849 70, $F = 4.27$, $p = 0.042$, F-test). **D** Average spine neck length (WT: 1.52 ± 0.22 ; *Fmr1*^{-/-}; $1.31 \pm$
850 0.20 ; d.f.: 8; $t = 0.66$; $p = 0.53$, T-test). **E** Comparison of spine neck-width and uEPSC amplitude
851 (Slope: WT: 2.1 ± 0.8 ; *Fmr1*^{-/-}; 0.8 ± 1.4 ; d.f.: 1, 101; $F = 0.84$; $p = 0.36$; F-test). **F** Spine density
852 on L4 SCs (WT: 6.8 ± 0.7 spines/10 μm ; *Fmr1*^{-/-}: 6.1 ± 0.80 spines /10 μm ; d.f.: 13; $t = 0.60$; $p =$
853 0.56 ; T-test). **G** Distribution of non-uncaged spine head-widths, as an average of all mice (bold)
854 and individual mice (dashed). **H** Average head-width of non-uncaged spines (WT: 0.48 ± 0.05
855 μm ; *Fmr1*^{-/-}: 0.48 ± 0.04 μm ; d.f.: 13; $U = 20.0$; $p = 0.59$; Mann-Whitney U-test). **I** Distribution of
856 spine neck-length of non-uncaged spines. **J** Average of spine neck-length in non-uncaged spines
857 (WT: 1.36 ± 0.12 μm ; *Fmr1*^{-/-}: 1.27 ± 0.14 μm ; d.f.: 13; $U = 20.0$; $p = 0.55$; Mann-Whitney U-test).
858 Statistics shown: ns – $p > 0.05$ from unpaired t-test (B, D, F, H, J) and sum-of-least-squares F-

859 test (C, E). All data is shown as mean \pm SEM and source data for all plots are provided as a
860 Source Data file.

861
862 Figure 4: L4 spines in *Fmr1^{-ly}* mice form multiple synaptic contacts. **A** Serial electron micrographs
863 in L4 from WT and *Fmr1^{-ly}* mice, indicating spines (asterisk) contacted by multiple presynaptic
864 boutons (b) each with a PSD (arrows); scale bar: 500 nm. **B** Reconstructed dendrites from WT
865 (grey) and *Fmr1^{-ly}* (red) mice, with PSDs (blue) and MIS indicated (arrows). **C** Incidence of MIS
866 in WT and *Fmr1^{-ly}* mice. Statistics shown: ** - $p < 0.01$ from unpaired t-test. All data is shown as
867 mean \pm SEM and source data for all plots are provided as a Source Data file.

868
869 Figure 5: mEPSCs in *Fmr1^{-ly}* L4 SCs show enrichment of NMDAR synapses. **A** mEPSCs recorded
870 from L4 SCs for AMPAR at -70 mV (top), NMDAR at +40 mV with CNQX (10 μ M, middle), and
871 following application of the NMDAR antagonist D-AP5 (50 μ M, bottom) in the same cell; from WT
872 (left) and *Fmr1^{-ly}* (right) mice. **B** Quantification of AMPAR mEPSC amplitude (WT: 13.1 ± 0.8 pA;
873 *Fmr1^{-ly}*; 12.7 ± 1.3 pA) and frequency (WT: 3.9 ± 0.5 Hz; *Fmr1^{-ly}*; 4.9 ± 0.6 Hz) in WT (black) and
874 *Fmr1^{-ly}* (red) mice. Number of mice indicated in parenthesis. **C** NMDAR mEPSC amplitude (WT:
875 16.9 ± 2.6 pA; *Fmr1^{-ly}*; 14.4 ± 1.6 pA) and frequency (WT: 1.7 ± 0.17 Hz; *Fmr1^{-ly}*; 2.6 ± 0.3)
876 measured in WT and *Fmr1^{-ly}* mice. Statistics shown: ns – $p > 0.05$, * - $p < 0.05$ from unpaired t-test.
877 All data is shown as mean \pm SEM and source data for all plots are provided as a Source Data file.

878
879 Figure 6: Altered intrinsic physiology of L4 SCs in *Fmr1^{-ly}* mice. Voltage responses to hyper- and
880 depolarizing current steps (-125 to +125 pA, 25 pA steps, 500 ms duration) led to AP discharge
881 in WT (**A**) and *Fmr1^{-ly}* (**B**) mice. **C** The current-voltage response to hyperpolarizing currents with
882 linear fit (dashed lines) in WT (black) and *Fmr1^{-ly}* (red) mice. **C (inset)** R_i measured from all L4
883 SCs tested. **D** Current-frequency plot showing AP discharge. **D (inset)** Average rheobase current
884 measured in all cells. **E** subthreshold membrane chirps (0.2 -20 Hz, 50 pA, 20 s duration) in L4
885 SCs from WT (black) and *Fmr1^{-ly}* mice. Right, frequency-impedance plot for both genotypes
886 \pm SEM, shown on a logarithmic frequency scale. **F** resonant frequency of L4 SCs from both
887 genotypes. Statistics shown: * - $p < 0.05$, ** - $p < 0.01$, *** - $p \pm < 0.001$, from LMM (C and D insets,
888 F) and 2-way ANOVA (C and D, main). Summary plots of all cells recorded for 6C (inset) and
889 6D(inset) can be found in Supplementary Figure 5. All data is shown as mean \pm SEM and source
890 data for all plots are provided as a Source Data file.

891

892 Figure 7: I_h is reduced in L4 SCs from *Fmr1^{-ly}* mice, resulting in hyperexcitability. **A** hyperpolarizing
893 steps in L4 SCs (0 to -125 pA, 25 pA steps, 500 ms duration) with voltage “sag” and rebound
894 potential indicated, as measured in WT (black, left) and *Fmr1^{-ly}* mice (red, right). **B** quantification
895 of voltage sag expressed as % of maximum voltage for WT and *Fmr1^{-ly}* L4 SCs **C** plot of rebound
896 potential, as a function of steady state voltage for WT and *Fmr1^{-lyS}* L4 SCs, fitted with linear
897 regression and with fit values displayed. **D** quantification of the rebound slope of individual L4
898 SCs for both genotypes. **E** R_i measured before and after bath application of the I_h blocker ZD-
899 7,288 (ZD; 20 μ M) in WT and *Fmr1^{-ly}* L4 SCs. **F** change in R_i change following ZD application (as
900 100% of control levels). **G (left)** hyper- to depolarising current steps (-125 to +125 pA, 25 pA
901 steps, 500 ms duration) in WT L4 SCs before and after ZD application. **G (right)** current-frequency
902 plot of AP discharge before (solid lines) and after (dashed lines) ZD application. **H** the same
903 analysis as in G, but in *Fmr1^{-ly}* L4 SCs. **I**, subthreshold membrane chirps (0.2-20 Hz, 50 pA, 20 s
904 duration) and current-impedance plot for WT L4 SCs before (black) and after (grey) ZD
905 application. **J**, The same data as in F, but in *Fmr1^{-ly}* mice. **K**, Impedance measured at peak
906 resonant frequency in WT and *Fmr1^{-ly}* L4 SCs before and after ZD (+ZD) application. Statistics
907 shown: ns – $p > 0.05$ * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, from LMM (B, D, E, F, K). Summary
908 plots of all data shown in Figure 7B and 7D can be found in Supplementary Figure 7. All data is
909 shown as mean \pm SEM and source data for all plots are provided as a Source Data file.

910
911 Figure 8: Altered I_h voltage-sensitivity in *Fmr1^{-ly}* L4 SCs, due to reduced cyclic-AMP. **A** subtracted
912 I_h traces recorded during a -50 mV step from -50 mV holding potential for WT (black) and *Fmr1^{-ly}*
913 (red) L4 SCs, and following ZD application (grey, light red, respectively). **B** I_h measured over the
914 range of -50 to -120 mV for both WT and *Fmr1^{-ly}* L4 SCs fitted with a sigmoidal curve (dashed
915 lines). $V_{1/2 \text{ max}}$ is indicated. **Inset**, I_h was blocked to a similar degree by ZD in both genotypes when
916 tested on steps to -100 mV. **C** I_h recorded before (top) and after (bottom) application of forskolin.
917 **D** quantification of I_h responses over the range of -50 to -100 mV, fitted with a sigmoidal curve.
918 All data is shown as mean \pm SEM and source data for all plots are provided as a Source Data file.
919

920 **Figure 9:** Enhanced dendritic integration of L4 SCs in *Fmr1^{-ly}* mice. **A** schema of near-
921 simultaneous glutamate uncaging (Rubi-Glu) at multiple spines (blue dots/numbers). **B** Near-
922 simultaneous glutamate uncaging produced subthreshold (inset, right) and suprathreshold
923 uEPSPs (inset, left) along dendrites. **C** The number of spines required to evoke an AP, from all
924 spines (left; WT: 8.8 ± 0.7 ; *Fmr1^{-ly}*; 6.6 ± 0.6) and excluding “silent spines” (right; WT: 8.7 ± 0.7 ;

925 *Fmr1^{-y}*; 5.6 ± 0.7). **D** Summation of near-simultaneous subthreshold uEPSPs normalized to the
926 first EPSP in WT (black) and *Fmr1^{-y}* (red) L4 SCs (Slope: WT: 1.1 ± 0.13 ; *Fmr1^{-y}*; 1.9 ± 0.2 ; d.f.:
927 1, 170; $F = 8.98$; $p = 0.003$; F-test). **E** Summating uEPSPs plotted against the expected linear-
928 sum. Unity is indicated (grey). **F** Electrical stimulation of TCA at low frequency 10 Hz is shown. **G**
929 Average spike probability in response to 5 Hz and 10 Hz stimulation. Statistics shown: * - $p < 0.05$,
930 ** - $p < 0.01$. All data is shown as mean \pm SEM and source data for all plots are provided as a
931 Source Data file.

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