RHEB/mTOR-hyperactivity causing cortical malformations drives seizures through increased axonal connectivity

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13   **ABSTRACT**

14   Dominant-active mutations in *Ras Homolog Enriched in Brain 1* (*RHEB*), such as the recently

15   identified RHEBp.P37L mutation, can cause malformations of cortical development (MCD) with

16   associated epilepsy and intellectual disability through a yet unknown mechanism. We found that

17   focal expression of RHEBp.P37L in mouse somatosensory cortex results in an MCD-like

18   phenotype, with increased mammalian target of rapamycin (mTOR) signaling, ectopic localization

19   of neurons and generalized seizures. In addition, the RHEBp.P37L expressing neurons showed

20   increased axonal length and branching. By temporally controlling RHEBp.P37L expression, we

21   found that the cortical malformation by itself was neither necessary nor sufficient to generate

22   seizures. Rather, seizures were contingent on persistent mTOR activation and enhanced axonal

23   connectivity of RHEBp.P37L expressing neurons, causing hyperexcitability of distally connected

24   neurons. These results provide new evidence of the extent of anatomical and physiological

25   abnormalities caused by mTOR hyperactivity, beyond local malformations, that can lead to

26   generalized epilepsy.

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INTRODUCTION

Malformations of cortical development (MCD) are a heterogenous group of micro- and macroscopic cortical abnormalities, such as focal cortical dysplasia (FCD), megalencephaly, lissencephaly and periventricular nodular heterotopia (Barkovich et al., 2012). MCD arise from disturbances in cortical development during early embryogenesis and are often linked to epilepsy and intellectual disability (ID) (Juric-Sekhar and Hevner, 2019; Leventer et al., 2008; Represa, 2019). It is estimated that up to 40% of intractable or difficult to control childhood seizures are due to MCD, and *vice versa*, at least 75% of the patients with MCD will develop seizures (Leventer et al., 1999).

The mammalian (or mechanistic) target of rapamycin (mTOR) is a kinase that mediates many cellular processes, including neuronal progenitors proliferation and cell growth (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). mTOR forms 2 distinct protein complexes, characterized by different binding partners, mTORC1 and mTORC2 (Bhaskar and Hay, 2007). mTORC1 is regulated by the tuberous sclerosis complex (TSC) and the Ras Homolog Enriched in Brain 1 (RHEB) (Parmar and Tamanoi, 2010). RHEB, a member of the RAS family of small GTPases, is the direct activator of mTORC1 (Bai et al., 2007). The conversion of active GTP-bound RHEB to the inactive GDP-bound form is mediated by the TSC complex, which acts as a RHEB GTPase activating protein (GAP) (Li et al., 2004). In response to nutrients and growth factors the TSC complex is inhibited, allowing activation of mTORC1 by RHEB-GTP (Manning and Cantley, 2003; Sabatini, 2017). Studies in *Rheb* knock-out mice showed that RHEB activity is the rate limiting step for mTOR activation in the brain, and that neuronal functioning in particular is sensitive to increased RHEB-mTOR signaling (Goorden et al., 2015).

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50 Hyperactivation of the mTOR pathway by mutations in genes encoding components of the

51 mTOR pathway (*e.g. AKT3*, *PIK3CA*, *DEPDC5*, *PTEN*, *TSC1, TSC2, RHEB* and *MTOR* itself) has

52 been associated with different types of MCD, such as megalencephaly and FCD, as well as with

53 epilepsy (Crino, 2011; Juric-Sekhar and Hevner, 2019; Moffat et al., 2015). The underlying genetic

54 variability explains the heterogeneity of MCD and illustrates the challenges involved in

55 understanding the mechanisms underlying MCD-associated epilepsy.

56 The discovery of genetic mutations that cause FCD or other types of MCD, allowed the

57 generation of animal models to study the development of MCD and associated epilepsy

58 (Chevassus-au-Louis et al., 1999; Wong and Roper, 2016). In particular, *in utero* electroporation

59 (IUE), that allows for the spatial and temporal control of transgene expression during embryonic

60 development, has been used to generate mouse models with focal malformations and epilepsy

61 (Hanai et al., 2017; Hsieh et al., 2016; Park et al., 2018; Ribierre et al., 2018).

62 One recent FCD mouse model was generated by using IUE to overexpress the

63 constitutively active RHEBp.S16H mutant (Yan et al., 2006). This results in mTOR hyperactivity,

64 FCD and spontaneous seizures (Hsieh et al., 2016). Recently we identified two *de novo* mutations

65 in *RHEB* (c.110C>T (p.P37L) and c.202T>C (p.S68P)) in patients with ID, epilepsy and

66 megalencephaly (Reijnders et al., 2017), providing for the first time a clinically relevant link

67 between RHEB and MCD. IUE of a construct encoding the RHEBp.P37L mutant caused severe

68 focal cortical lesions, resembling periventricular nodular heterotopia, and diffuse neuronal

69 misplacement in the cortex. Furthermore, the mice reliably developed spontaneous seizures

70 starting at three weeks of age (Reijnders et al., 2017). The anatomical and phenotypical features

71 of this novel mouse model, fully recapitulating the most prominent characteristics of MCD (focal

72   lesions and epilepsy), make this a powerful tool and clinically relevant novel model to study the

73   mechanisms underlying mTOR and MCD-related epilepsy.

74   Here we demonstrate that *RHEB* mutations that cause MCD in patients activate mTORC1

75   signaling and that the cortical malformation is not required for the development of mTOR related

76   seizures, in line with previous studies (Abs et al., 2013; Hsieh et al., 2016). Additionally, we

77   provide evidence that the presence of heterotopia by itself is insufficient to cause epilepsy. Instead,

78   we found that persistent activation of the mTOR pathway results in anatomical and functional

79   changes in axonal connectivity, which cause increased excitability of distally connected neurons

80   and the development of generalized seizures.

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82   **RESULTS**

83   **The RHEBp.P37L protein is resistant to TSC complex inhibition and causes aberrant**

84   **cortical development *in vivo***

85   The RHEBp.P37L mutation was identified in patients with ID, megalencephaly and epilepsy, and

86   it was proposed to act as a gain of function mutation (Reijnders et al., 2017). This could potentially

87   be due to resistance to the GAP-function of the TSC complex. To assess whether the TSC complex

88   can convert RHEBp.P37L from its active GTP- to its inactive GDP-bound state, we compared the

89   effects of transient *in vitro* overexpression of the RHEBp.P37L mutant with wild-type RHEB

90   (RHEB WT) and the RHEBp.S16H mutant, a well-known gain of function mutant of RHEB

91   recently used to generate an FCD mouse model (Hsieh et al., 2016; Yan et al., 2006). In the absence

92   of TSC, overexpression of RHEB WT as well as both RHEB mutants caused increased mTORC1

93   activity, as measured by T389-phosphorylation of co-expressed S6K, a direct substrate of the

94   mTORC1 kinase (**Figure 1A**, see **Supplementary table1** for statistics overview). In the presence

95   of the TSC complex, mTORC1 activity was reduced in the RHEB WT and RHEBp.S16H

96   expressing cells, but not in the RHEBp.P37L expressing cells (**Figure 1A**). Here, presence or

97   absence of TSC resulted in similar levels of S6K phosphorylation, confirming that the patient-

98   derived RHEBp.P37L acts as a gain of function mutation which is resistant to inhibitory action of

99   the TSC complex (**Figure 1A**).

100   Using IUE, we have previously shown that overexpression of the RHEBp.P37L mutant,

101   but not RHEB WT, results in the formation of a heterotopic nodule as well as spontaneous epilepsy

102   in 100% of the targeted mice (Reijnders et al., 2017), providing us with a valuable model to study

103   the mechanisms behind mTORC1-dependent and MCD-related epilepsy. To confirm previous

104   results and further characterize the model, we used IUE to introduce the RHEBp.P37L vector or

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105 an empty vector control at E14.5 in progenitor cells that give rise to layer 2/3 (L2/3) pyramidal

106 neurons of the somatosensory cortex (SScx) (**Figure 1B**). As shown previously, overexpression of

107 RHEBp.P37L resulted in a clear migration deficit, with only 20% of the targeted cells reaching

108 the outer layers of the cortex (L2/3) compared to 97% in the empty vector condition (**Figure 1C**,

109 inset). The non-migrated transfected neurons remained in the white matter to form a heterotopic

110 nodule lining the ventricle in the adult brain (**Figure 1C** and **Figure 1-figure supplement 1**).

111 While the general cortical layer architecture remained intact (**Figure 1-figure supplement**

112 **1**, ectopic RHEBp.P37L overexpressing cells showed cytological abnormalities, with dysmorphic

113 appearance and enlarged soma size (**Figure 2A** and **Figure 1-figure supplement 1**). Sholl analysis

114 of biocytin filled cells in the heterotopic nodule of RHEBp.P37L expressing neurons revealed that

115 the cells in the nodule presented a more complex arborization compared to empty vector control

116 cells in L2/3 (**Figure 2B**). Transfected ectopic neurons preserved the molecular identity of mature

117 L2/3 pyramidal cells, being positive for the neuronal marker NeuN and the outer layer molecular

118 marker CUX1 and negative for the deeper layer marker CTIP2 (**Figure 2C** and **Figure 2-figure**

119 **supplement 1A**). Additionally, most neurons in the nodule were SATB2 positive, showing that,

120 despite being mislocalized, they maintained the callosal projection identity (**Figure 2C** and **Figure**

121 **2-figure supplement 1A**). Finally, mice overexpressing RHEBp.P37L showed an overall increase

122 in ribosomal protein S6 phosphorylation, a commonly used readout for mTORC1 activity, in the

123 transfected hemisphere compared to the empty vector condition (**Figure 2D** and **Figure 2-figure**

124 **supplement 1B**).

125 **Overexpression of RHEBp.P37L *in vivo* causes mTORC1-dependent spontaneous**

126 **generalized tonic-clonic seizures and abnormal neuronal network activity**

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127   To assess the reliability of spontaneous seizures development, the RHEBp.P37L mice were

128   continuously monitored from weaning (P21) using wireless electroencephalography (EEG)

129   (**Figure 3A**). Spontaneous seizures started to appear at 3 weeks of age, with an average onset of

130   33 days, confirming previous data (Reijnders et al., 2017) (**Figure 3-figure supplement 1A**).

131   Seizures were highly stereotypical, characterized by the loss of upright posture followed by a tonic-

132   clonic phase with convulsions and twitching behavior. EEG recordings showed that, while control

133   mice did not show any epileptic activity (N = 6), all RHEBp.P37L mice (N = 12) showed clear

134   epileptic events (**Figure 3B** and **Figure 3-figure supplement 1B**). Seizures were characterized by

135   an increase in frequency and amplitude of brain activity (**Figure 3C**, box 3 ictal activity) compared

136   to baseline interictal activity (**Figure 3C**, box 2) and baseline activity of control mice (**Figure 3C**,

137   box 1). The calculated average duration of an epileptic event was 40 seconds (mean ± SEM: 42.6

138   ± 1.33), followed by a post-ictal depression phase of variable length (**Figure 3C**, box 4 post-ictal

139   activity). The frequency of seizures per day was variable between mice as well as per mouse over

140   time (**Figure 3-figure supplement 1C**). Additionally, no correlation was found between the total

141   number of seizures over three consecutive days of recording and the average number of targeted

142   cells per mouse (**Figure 3-figure supplement 1D**).

143   To assess if brain-wide suppression of mTORC1 activity could reduce seizures, we treated

144   a group of mice showing seizures (N=6; 5-6 weeks old) systemically for 7 days with the allosteric

145   mTORC1 inhibitor rapamycin (10 mg/kg). Rapamycin treatment reduced and temporarily

146   abolished the occurrence of seizures within one week from the last day of rapamycin

147   administration. However, seizures reoccurred starting 3 weeks after the last injection of rapamycin

148   in 4 out of 6 mice, indicating that sustained inhibition of mTORC1 is required to fully suppress

149   the seizures (data not shown).

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150   Electrographic frequency dynamics of the interictal phases, and especially *theta*

151   oscillations, have been proven to be good predictors for epilepsy outcome, compared to

152   epileptiform spikes or high-frequency oscillations (HFOs), in several rodent models of epilepsy

153   (Chauvière et al., 2009; Milikovsky et al., 2017). Therefore, using local field potential (LFP)

154   recordings, we assessed the frequency dynamics of cortical brain activity in the interictal periods

155   of RHEBp.P37L expressing mice, starting from 4 weeks of age (**Figure 3D**). The normalized

156   averaged power spectrum of the RHEBp.P37L group did not reveal a significant difference

157   between the targeted and non-targeted cortex (targeting: $F_{(1, 22)}=1.43$, p=0.25, non-significant,

158   Two-way repeated measure ANOVA; data not shown), therefore measurements from both sides

159   were pooled. Whereas the total power across 5 days of recording did not differ between the

160   RHEBp.P37L (N=12) and the control group (N=8) (Mann-Whitney U = 157, p = 0.35, non-

161   significant, two-tailed Mann-Whitney test, data not shown), a significant difference in the *delta*

162   (2-4 Hz), *theta* (4-8 Hz) and *gamma* (30-50 Hz) frequency bands of the normalized power

163   spectrum was seen in the RHEBp.P37L group compared to the control group (**Figure 3E, 3F** and

164   **Figure 3-figure supplement 1E and 1F**; statistics in **Supplementary table2**). The difference in

165   the *theta* and *gamma* frequency bands, but not in the *delta*, could be reverted to the control

166   condition by injecting the RHEB mice with 10 mg/kg rapamycin intraperitoneally for 3

167   consecutive days (**Figure 3E, 3F** and **Figure 3-figure supplement 1E and 1F**; statistics in

168   **Supplementary table2**). Together with the finding that rapamycin abolished seizures, this result

169   indicates that *theta* oscillations, which negatively correlate with *gamma* frequencies (Milikovsky

170   et al., 2017), are a good predictor for epileptogenesis in the RHEBp.P37L mouse model.

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The heterotopic nodule is neither necessary nor sufficient to induce spontaneous seizures

Cortical malformations occur during early embryonic development and are generally associated with the development of epileptic activity (Represa, 2019). Therefore, a transient treatment with mTOR inhibitors during brain development might prevent the formation of a cortical malformation and could consequently reduce the chances of developing epilepsy. To assess if early transient down-regulation of the mTORC1 pathway upon overexpression of RHEBp.P37L could prevent the development of heterotopic nodules, we injected pregnant female mice with 1 mg/kg of rapamycin for 2 consecutive days starting 1 day after IUE of the RHEBp.P37L vector (**Figure 4A**). Prenatal down-regulation of the mTORC1 pathway significantly improved the migration of the targeted neurons, with 75% of the targeted cells successfully migrating out (**Figure 4B**). In addition, prenatal rapamycin treatment successfully prevented the formation of a heterotopic nodule in 9 out of 11 mice. However, 7 out of the 11 targeted mice (58%) still showed spontaneous seizures, including 5 mice that did not develop a discernable heterotopic nodule (**Figure 4C**). Average onset of seizures was comparable to the non-treated RHEBp.P37L mice (mean ± SEM: 32.6 days ± 2.3; Chi square (1) = 0.16, p = 0.69, Log-rank test, data not shown). Hence, the presence of a heterotopic nodule is not required for RHEBp.P37L mediated seizures, and reducing the formation of these nodules does not always prevent epileptogenesis.

When comparing the cortical migration patterns in mice with and without seizures, a clear correlation was observed between the migration pattern of RHEBp.P37L expressing cells and the presence or absence of seizures: RHEBp.P37L-prenatal treated mice with seizures showed a more severe migration deficit of RHEBp.P37L expressing cells compared to prenatal treated RHEBp.P37L expressing mice that were seizure free (**Figure 4D**). In fact, the percentage of cells that reached L2/3 of the SSInx of RHEBp.P37L-prenatal treated mice with seizures (63%), was

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196   significantly lower than RHEBp.P37L-prenatal treated mice without seizures (93%) or control

197   mice (98%) (% targeted cells in L2/3: H(2) = 22.08, p < 0.0001, Kruskal-Wallis test; empty vector

198   *vs* RHEBp.P37L-no seizures, p > 0.99; empty vector *vs* RHEBp.P37L-seizures, p < 0.0001;

199   RHEBp.P37L-no seizures *vs* RHEBp.P37L-seizures, p = 0.002; Dunn's multiple comparisons test,

200   data not shown). These results indicate that ectopic cells do facilitate the process of

201   epileptogenesis.

202   Hyperactivation of mTORC1 is sufficient to cause seizures, independent of the presence

203   of cortical malformations (Abs et al., 2013), even when the mTORC1 activity is increased in a

204   limited set of neurons (Hsieh et al., 2016). Moreover, the cortical malformation by itself, in the

205   absence of continued mTORC1 signaling, does not cause epilepsy, as was also shown by brain-

206   wide inhibition of mTORC1signaling (Hsieh et al., 2016). To further dissect the relationship

207   between increased mTOR activity, cortical malformations and epilepsy, we made use of a genetic

208   approach, that allowed us not only to regulate mTORC1 activity in a temporal fashion, but also to

209   restrict its activity to a limited number of cells. To that end we used IUE to introduce a Lox-Stop-

210   Lox(LSL)-RHEBp.P37Lvector or floxed-RHEBp.P37L vector together with a vector expressing

211   the ERT2CreERT2 fusion protein (**Figure 4E**). This allowed us to switch the RHEBp.P37L gene

212   respectively on or off during different stages of cortical development by means of systemic

213   tamoxifen administration. The use of an additional vector that expresses EGFP in a Cre-dependent

214   manner (CAG-DIO-EGFP), allowed us to assess the efficiency of Cre activation upon tamoxifen

215   administration. IUE of the LSL-RHEBp.P37L construct in the absence of tamoxifen

216   administration, did not result in a migration deficit, or seizures. This indicates that the LSL cassette

217   successfully prevented RHEBp.P37L expression (**Figure 4F**). However, once expression of

218   RHEBp.P37L was induced by administration of tamoxifen either at P7 or P21, a subset of the

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219   mutant mice (38% of the P7 group and 50% of the P21 group) developed spontaneous seizures

220   (**Figure 4G**), albeit with a delayed onset compared to mice that express RHEBp.P37L throughout

221   development (Chi square (2) = 10.18, p =0.006; Log-rank test, data not shown). This indicates that

222   RHEBp.P37L expression in a limited number of cells, can drive seizures in the absence of an

223   observable cortical malformation (migration defects or heterotopic nodules).

224   To investigate if presence of the heterotopic nodule is epileptogenic after normalizing

225   mTORC1 activation only in the targeted cells (instead of brain-wide as others also did in previous

226   studies (Hsieh et al., 2016)), we used IUE to insert the floxed-RHEBp.P37L vector (**Figure 4H**).

227   Upon expression of RHEBp.P37L during early pre- and postnatal development, we normalized

228   mTORC1 activity in these cells by inducing RHEBp.P37L deletion at P14. (**Figure 4I**). Although

229   a clear heterotopic nodule was formed in these mice, none of the mice developed seizures (**Figure**

230   **4I**). Furthermore, inducing deletion of RHEBp.P37L after epileptogenesis, completely abolished

231   the seizures within 10 days from gene deletion (N=4, last EEG measurements performed between

232   day 85 and 90) (**Figure 4I**). Taken together, these results indicate that cortical malformations are

233   neither necessary nor sufficient for the development of spontaneous seizures in our mouse model.

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235   **RHEBp.P37L expression induces aberrant axonal development both *in vitro* and *in vivo* and**

236   **functional increased contralateral L2/3 and L5 connections**

237   The mTOR pathway plays an important role in axonal outgrowth, with functional effects on

238   neuronal network formation (Choi et al., 2008; Gong et al., 2015; Nie et al., 2010). Because

239   increasing mTOR signaling in a limited number of neurons in the brain is enough to cause seizures,

240   independently from cell misplacement, we hypothesized that this could be due to aberrant neuronal

241   connectivity caused by RHEBp.P37L overexpression. Therefore, we investigated the effect of

242   RHEBp.P37L on axonal length and branching. Overexpression of RHEBp.P37L in primary

243   hippocampal neurons *in vitro* caused a significant increase in axonal length and axonal branching,

244   compared to the empty vector control (**Figure 5A**).

245   *In vivo*, axons from callosal projection neurons originating from the superficial layers

246   of the SScx project to the homotopic contralateral hemisphere, where they mostly innervate L2/3

247   and L5 pyramidal neurons (Fenlon et al., 2017; Petreanu et al., 2007). They also send collaterals

248   to L2/3 and, more strongly, L5 pyramidal neurons within the same column ipsilaterally,

249   participating in local circuitry (Fame et al., 2011; Petreanu et al., 2007). Therefore, it is conceivable

250   that *in vivo* overexpression of RHEBp.P37L affects callosal projections to the non-targeted

251   contralateral hemisphere. Analysis of the contralateral callosal axonal growth in matched coronal

252   sections with comparable targeting revealed that upon RHEBp.P37L overexpression, axonal

253   terminals in the contralateral hemisphere, show a broader distribution compared to controls,

254   reaching the primary (S1) and secondary (S2) SScx (**Figure 5B**). Furthermore, a significant

255   difference was found in the distribution of the axonal terminals across the different layers in the

256   contralateral hemisphere. While in the control condition most of the terminals were located in

257   L2/3, with a lower abundance in L5 (Fenlon et al., 2017), in the RHEBp.P37L mice we found that

258   most of the terminals were located in the deeper layers of the cortex, suggesting an improper

259   cortical connectivity (**Figure 5C**). Furthermore, zooming in on the axonal projections on the

260   contralateral cortex revealed the presence of enlarged *boutons* and terminals in the RHEBp.P37L

261   mice that were both Synapsin-1 and VGLUT1 positive, markers for synaptic vesicles and

262   glutamatergic neurons, respectively (**Figure 5-figure supplement 1**).

263   To investigate if the contralateral axonal projections with these synaptic terminals showing

264   altered morphology are functional, we made use of optogenetics. We used IUE to introduce

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265   channelrhodopsin-2 (pCAGGS-ChR2-Venus) (Petreanu et al., 2007) together with either the

266   empty vector control or the RHEBp.P37L construct in targeted neurons and recorded the

267   postsynaptic responses (EPSCs) to widefield optogenetic stimulation by patch-clamping L2/3 and

268   L5 pyramidal neurons in the (non-targeted) contralateral S1 where axonal terminals could be

269   observed (**Figure 6A and Figure 6-figure supplement 1A**). Analyzing the amplitude of EPSCs

270   following optogenetic stimulation in L5 and L2/3 of the contralateral S1, we observed an overall

271   increase in response in the RHEBp.P37L condition compared to the empty vector control condition

272   (see **Supplementary table 3** for statistics) (**Figure 6B**). When analyzing the total charge of the

273   compound postsynaptic response we observed similar response patterns (**Supplementary table 3**

274   for statistics) (**Figure 6B**). Bath application of tetrodotoxin (TTX) in the RHEBp.P37L group

275   decreased the post-synaptic responses evoked by photo-stimulating ChR2 expressing fibers to

276   noise level, which is indicative of action potential driven neurotransmitter release (**Figure 6-figure**

277   **supplement 1B**). The basic properties (resting membrane potential [Vm] and membrane resistance

278   [Rm]) of L2/3 and L5 contralateral cells in empty vector control and RHEBp.P37L conditions

279   were not different (**Supplementary table 3** for statistics). These data suggest increased synaptic

280   connectivity to the contralateral S1 upon overexpression of RHEBp.P37L.

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282   **Loss of axonal projections or blocking vesicle release of RHEBp.P37L expressing neurons is**

283   **sufficient to stop seizures**

284   Having shown that the RHEBp.P37L expressing neurons show stronger axonal innervation and

285   synaptic connectivity to neurons in the contralateral hemisphere, we investigated whether these

286   altered neuronal projections drive the seizures. To assess this, we made use of the Tetanus toxin

287   light chain, known to specifically cleave the SNARE-complex protein Synaptobrevin/VAMP2

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288   (Syb2) (Schiavo et al., 1992). VAMP2 is part of the SNARE complex that allows synaptic vesicles

289   fusion and the release of neurotransmitters (Gaisano et al., 1994) and recently it has been shown

290   to mediate the vesicular release of Brain Derived Neurotrophic Factor (BDNF) from axon and

291   dendrites, thereby regulating proper cortical connectivity (Shimojo et al., 2015). Intrinsic neuronal

292   activity during early brain development is crucial for axonal growth and branching, and blocking

293   synaptic transmission using Tetanus toxin interferes with proper cortical axonal formation,

294   resulting in the reduction and disappearance of axonal projections (Wang et al., 2007). Indeed,

295   when RHEBp.P37L was co-transfected with a Tetanus toxin construct (TeTxLC) that is active

296   during embryonic development, we observed a complete block of callosal axonal growth (**Figure**

297   **7A, 7B**). Furthermore, the mice targeted with the RHEBp.P37L and TeTxLC constructs did not

298   develop any seizures, suggesting that the abnormal axonal connectivity might mediate the

299   expression of seizures in our mouse model (**Figure 7B**).

300   The complete loss of callosal axonal branching upon embryonic activation of TeTxLC,

301   prevented us from testing whether increased synaptic transmission drives seizure development.

302   Therefore, to enable activation of the Tetanus toxin upon Tamoxifen injection at post-

303   developmental stages, we generated an inducible LSL-TeTxLC construct (**Figure 7C**) and co-

304   transfected this construct with RHEBp.P37L and the CAGG-ERT2CreERT2 vector (see **Figure**

305   **4E**). This allowed us to assess whether, once (abnormal) axonal projections are established,

306   blocking vesicular release either prevents the development of seizures, or stops seizures once they

307   have developed. Activation of the Tetanus toxin during postnatal development, but before seizure

308   onset (P14), completely prevented the development of seizures while allowing the axons to grow

309   and branch to the contralateral side (**Figure 7D**). Administering tamoxifen in 5 weeks old mice,

310   when the cortical connectivity is complete and after the mice showed seizures revealed that

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311   epilepsy is not an irreversible process (**Figure 7D**). Already after 2 days of tamoxifen

312   administration, 3 out of 6 mice (**Supplementary table 4**) stopped showing any seizures and 2

313   weeks after the last tamoxifen injection all mice appeared to be seizure free (**Supplementary table**

314   **4**). These results indicate that inhibiting synaptic transmission by blocking vesicular release from

315   the targeted cells is enough to stop the occurrence of seizures in our mouse model.

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317   **Neurons in the contralateral homotopic cortical area in RHEBp.P37L expressing mice show**

318   **increased excitability**

319   To obtain more insight into the cellular mechanisms that underlie epilepsy in our model, we used

320   whole-cell patch clamp to measure intrinsic physiological properties of the RHEBp.P37L

321   expressing neurons, of (ipsilateral) neurons directly surrounding the targeted cells, and of the

322   contralateral neurons in homotopic cortical areas (**Figure 8A**). Whole cell patch clamp recordings

323   were performed by recording from pyramidal neurons in S1 of 3 weeks old mice. For the

324   RHEBp.P37L expressing neurons (tdTomato positive), we recorded only from neurons that

325   managed to migrate out to L2/3 of S1 to be able to compare their physiological properties with

326   'empty vector' control cells in L2/3 that expressed the tdTomato gene without expressing the

327   RHEBp.P37L protein (**Figure 8A**). RHEBp.P37L expressing neurons showed an increase in the

328   capacitance (Cm) compared to empty vector control cells (**Figure 8B** and see **Supplementary**

329   **table 5** for statistics), which is consistent with the increase in soma size (median of control empty

330   vector cells L2/3: 1.005, n cells=22; median RHEBp.P37L cells L2/3: 1.377, n cells=24; Mann-

331   Whitney U = 105, p=0.0003, Two-tailed Mann-Whitney test, data not shown). Additionally, the

332   membrane resistance (Rm) was decreased, whereas the resting membrane potential (Vm) was

333   unchanged compared to empty vector control cells (**Figure 8B** and see **Supplementary table 5**

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334   for statistics). Depolarizing the neurons with increasing current injections, showed that the

335   excitability of cells expressing the empty vector were not different from non-targeted neurons in

336   the same mice or compared to non-targeted mice (**Figure 8-figure supplement 1**). In contrast,

337   RHEBp.P37L expressing neurons were hypoexcitable compared to control neurons measured in

338   mice expressing the empty vector as well as to non-targeted neurons ipsilateral and contralateral

339   (**Figure 8C** and see **Supplementary table 5** for statistics), without a change in the threshold *Vm*

340   to fire action potentials (F (3, 94) = 0.59, *p*=0.62, non-significant, One-way ANOVA). This result

341   is again in agreement with the observed increased soma size and concomitant increased cell

342   capacitance and decreased membrane resistance. Notably, while ipsilateral non-transfected

343   neurons surrounding the RHEBp.P37L expressing neurons in mice did not show changes in

344   excitability compared to empty vector control, non-transfected neurons in L2/3 on the contralateral

345   hemisphere showed a significant increase in excitability (**Figure 8C** and see **Supplementary table**

346   **5** for statistics), suggesting that the ectopic cells affect long-range connected neurons.

347   To experimentally address if the aberrant connectivity could cause the increase in

348   excitability in neurons on the contralateral cortex, we again made use of the inducible LSL-

349   TeTxLC construct and co-transfected this construct with RHEBp.P37L and the CAGG-

350   ERT2CreERT2 vector to enable activation of the Tetanus toxin upon Tamoxifen injection at post-

351   developmental stages (See **Figure 7C**). Whole-cell patch clamp recordings revealed that activating

352   the Tetanus toxin early during development (P14) (**Figure 8D**), completely reversed the

353   hyperexcitability phenotype of the contralateral non-targeted cells observed in the RHEBp.P37L

354   mice (**Figure 8E**) while the targeted cells co-transfected with the RHEBp.P37L and the Tetanus

355   toxin maintained the hypoexcitable phenotype and the basic properties observed in the

356   RHEBp.P37L group (**Figure 8E-F** and see **Supplementary table 6** for statistics). Taken together,

17

357   these data indicate that the abnormal axonal connectivity caused by RHEBp.P37L overexpression

358   is the primary driver of the hyperexcitability phenotype of contralateral L2/3 pyramidal neurons,

359   which in turn could be the main driver of epilepsy.

18

DISCUSSION

In this study, we investigated the mechanisms behind the spontaneous tonic-clonic seizures in a mouse model that was generated by spatially and temporally restricted overexpression of a mTOR-related ID mutation in *RHEB* (Reijnders et al., 2017). We showed that the RHEBp.P37L mutant is resistant to inhibition by the TSC complex, and that restricted overexpression causes mTORC1 hyperactivity and the development of heterotopia with typical cellular features of human MCD such as enlarged dysplastic neurons with altered morphology and mTORC1 activation. Furthermore, the presence of cortical malformations is accompanied by the development of spontaneous tonic-clonic seizures and alterations of the cortical brain dynamics that are rescued by administration of rapamycin, an mTOR inhibitor. Using a pharmacological and genetic approach we showed that the presence of the cortical malformation by itself is neither necessary nor sufficient to induce epilepsy, while blocking either mTOR activity or vesicle release from these cells is enough to stop or prevent seizures.

Similar to previously generated IUE mouse models of MCD, our model developed clear heterotopia, strikingly resembling focal human cortical malformations, associated with mTORC1 hyperactivity and reliable spontaneous seizures (Hanai et al., 2017; Hsieh et al., 2016; Lim et al., 2015; Park et al., 2018; Ribierre et al., 2018). The malformation in our mouse model is characterized by white matter heterotopia and neuronal misplacement across the different cortical layers, but maintains the molecular fingerprint belonging to L2/3 neurons. However, it is difficult to categorize it as a specific type of MCD because it expresses characteristics of both FCD type I and type IIa (with no Balloon cells observed) (Barkovich et al., 2012). Nonetheless, the targeted cells have features common to several types of mTOR dependent MCD, including enlarged and dysplastic cells with mTORC1 hyperactivation (Crino, 2011).

19

383 Previously it has been shown that brain wide activation of the mTOR pathway is

384 sufficient to induce seizures in the absence of any cortical malformations (Abs et al., 2013).

385 However, these models do not address the role of mTOR signaling in MCD related

386 pathophysiology. To address that, an elegant IUE mouse model was generated which expressed

387 the constitutive active RHEBp.S16H protein. These mice showed a migration deficit resembling

388 FCD and spontaneous epilepsy (Hsieh et al., 2016). Using this mouse model, it was also shown

389 that the presence of a cortical malformation is not necessary to induce seizures (Hsieh et al., 2016).

390 Notably, these mice did not show epilepsy when the SScx was targeted, and hence the investigators

391 suggested that the SScx might be a non-epileptogenic area. This is in contrast with our mouse

392 model using the human ID-related RHEBp.P37L mutant, where targeting the S1 area of the SScx,

393 reliably induces seizures. One reason for these contradictory findings might be the effect of the

394 mutation on RHEB function, as we showed that the RHEBp.S16H mutant could be partially

395 inhibited by the TSC complex, whereas the RHEBp.P37L mutant was insensitive to TSC

396 regulation.

397 Our mouse model offers a good tool to test novel AEDs *in vivo*. However, considering

398 the variability in the number of seizures exhibited, it will be beneficial to focus on different

399 parameters when assessing the potential therapeutic efficiency of AEDs. For this purpose, the *theta*

400 frequency oscillation, which we found to be affected and normalized upon rapamycin treatment,

401 represents a good biomarker for assessing the potential therapeutic value of treatments in our

402 mouse model (Chauvière et al., 2009; Milikovsky et al., 2017).

403 Everolimus and rapamycin (Sirolimus) have been shown in randomized controlled trials

404 to be beneficial for treating TSC associated epilepsy (Iris E Overwater et al., 2019; Overwater et

405 al., 2016), but not for treating the cognitive deficits (Krueger et al., 2017; Iris E. Overwater et al.,

20

406   2019). In this study we investigated the potential of a short prenatal rapamycin treatment in

407   improving both malformation defects and epilepsy, but preventing the possible side effects

408   (developmental delays and poor gain weight) (Tsai et al., 2013). We showed that a 2-day

409   rapamycin treatment during a critical time point of prenatal development can cause a substantial

410   improvement of the cortical malformation defects and prevent the development of seizures in

411   almost 50% of the cases. Future studies will have to assess if a combination of prenatal and

412   postnatal treatment with rapamycin in mice can be sufficient to significantly reduce the epileptic

413   events, as shown for brain malformations, without causing major side effects (Tsai et al., 2013;

414   Way et al., 2012).

415   Surgery is often an alternative to AEDs for treating MCD-related epilepsy. Human

416   electrophysiological findings show that seizures can often have multiple starting points, besides

417   the brain lesion itself (Chassoux et al., 2008; Major et al., 2009). Therefore, from a clinical point

418   of view, it is important to determine whether seizures originate from cells surrounding the cortical

419   malformation. Even though EEG and LFP do not have the spatial resolution to assess the primary

420   epileptogenic zone in our model, we showed that persistent mTORC1 hyperactivation in the

421   targeted cells is the primary cause of epilepsy. In fact, genetically removing the RHEBp.P37L

422   mutant, either before or after seizure development, was sufficient to prevent or stop the epilepsy.

423   Surprisingly, when exploring the causes of epileptogenesis, we observed that the

424   neurons expressing the RHEBp.P37L are hypoexcitable, which is consistent with the increase in

425   soma size but does not provide an obvious physiological explanation for the seizures observed in

426   our mouse model. However, we observed a clear increase of intrinsic excitability and in

427   postsynaptic responses upon optogenetic stimulation of RHEBp.P37L cells in contralateral

428   homotopic S1 cells. This suggests that RHEBp.P37L expressing cells induce cellular changes in

21

429   anatomically connected neurons, which might underlie, or at least exacerbate, the epilepsy

430   phenotype. Notably, these alterations extend well beyond the cells surrounding the cortical

431   malformation, as we found physiological changes were present contralateral to the targeted side.

432   Considering the abnormal axonal connectivity seen in our mouse model, this raises the possibility

433   that other anatomically connected cortical and sub-cortical areas not analyzed in this study might

434   also be affected, thereby providing an explanation for how a small percentage of targeted

435   hypoexcitable cells, independent of their location, can lead to generalized epilepsy. Therefore we

436   propose a model in which subtle microscopic alterations and aberrant connectivity, either through

437   an increase in synaptic connections or an increase in the strength of synaptic contacts caused by

438   mTOR hyperactivity, are sufficient to drive epileptogenesis.

439   By increasing axonal connectivity, RHEBp.P37L expressing neurons could potentially

440   alter synaptically connected neurons through neurotransmitter release. But they can also affect

441   neighboring (including synaptically non-connected) cells through the release of extracellular

442   vesicles such as exosomes (Budnik et al., 2016). The vesicles might mediate pathogenicity as was

443   previously shown *in vitro* (Patel et al., 2015). With the use of Tetanus toxin, we showed that the

444   effects on the contralateral side are directly driven by the abnormal enhanced axonal connectivity,

445   since blocking vesicle release specifically from the RHEBp.P37L expressing neurons, completely

446   rescued the epilepsy and normalized the intrinsic firing properties of the non-targeted contralateral

447   neurons. Tetanus toxin is primarily used to block synaptic transmission due to its effect on

448   neurotransmitter release, acting on the SNARE complex protein VAMP2 (Schiavo et al., 1992).

449   Given the observed increased axonal connectivity and the finding that distally connected cells were

450   physiologically affected, this strongly suggest that neurotransmitter mediated communication is

451   primarily causing the epilepsy phenotype. This notion is further supported by the optogenetics

22

452   experiments that showed increase postsynaptic responses upon stimulating the RHEBp.P37L

453   expressing neurons. While it has been proposed that specific tetanus insensitive VAMP proteins

454   (such as VAMP7) are involved in the release of exosomes into the extracellular space (Fader et

455   al., 2009), we cannot exclude the additional contribution of other types of vesicles to the observed

456   phenotype. Recently it was shown that Tetanus toxin sensitive SNAREs also drive the release of

457   BDNF (Shimojo et al., 2015). Some studies suggest that BDNF might contribute to epileptogenesis

458   (Binder et al., 2001), suggesting that abnormal BDNF signaling could further increase the epileptic

459   phenotype seen in our mouse model. Understanding the contribution of these different signaling

460   pathways is important for the development of targeted therapeutic strategies to treat MCD

461   associated epilepsy.

462   In summary, in this study we show that restricted overexpression of a hyperactive RHEB

463   mutant that was previously identified in patients with ID, megalencephaly and epilepsy, strongly

464   mimics the human MCD-like phenotype with mTOR pathway hyperactivity and seizures. We

465   provided pharmacological and genetic evidence that the cortical malformation *per se* is neither

466   necessary nor sufficient to induce seizures. Furthermore, we show that only a few neurons with

467   increased mTOR activity can be the driving force behind MCD-related epilepsy through aberrant

468   connectivity, resulting in increased excitability of distant non-targeted neurons, which can be

469   reversed by blocking vesicular release.

470   **MATERIALS AND METHODS**

471   **Mice**

472   Unless subjected to a surgical procedure, all experimental mice were kept group-housed in IVC

473   cages (Sealsafe 1145T, Tecniplast) with bedding material (Lignocel BK 8/15 from Rettenmayer)

474   on a 12/12 hr light/dark cycle at 21°C (±1°C), humidity at 40-70% and with food pellets

475   (801727CRM(P) from Special Dietary Service) and water available *ad libitum*. For the neuronal

476   cultures, FvB/NHsD females were crossed with FvB/NHsD males (both ordered at 8-10 weeks old

477   from Envigo). For the IUE, females FvB/NHsD (Envigo) were crossed with males C57Bl6/J

478   (ordered at 8-10 weeks old from Charles River). Both females and males from the *in utero*

479   electroporation litters were included in the experiments and no prescreening for successful

480   electroporation was performed before recruitment in the studies. Young (starting from P7) and

481   adult mice were used and the specific age for each experiment is indicated either in the results

482   section or in the figures' legends. Activation of the ERT2CreERT2 fusion protein (Matsuda and

483   Cepko, 2007) was achieved by intraperitoneal administration of tamoxifen for 4 consecutive days

484   (0.1 mg/g of bodyweight) dissolved in sunflower oil (20 mg/ml) at the ages specified in the results

485   section and in the figures. For inhibition of the mTOR pathway, rapamycin (Sigma-Aldrich) was

486   dissolved in dimethylsulfoxide (10 mg/ml) and injected intraperitoneally in adult mice (> 4 weeks)

487   for postnatal experiments (10 mg/kg) or subcutaneously in pregnant females (E15.5/E16.5) for

488   prenatal experiments (1 mg/kg).

489   All animal experiments were conducted in accordance with the European Commission Council

490   Directive 2010/63/EU (CCD approval AVD1010020172684).

491   **HEK293T cell cultures and transfection**

24

492   HEK293T cells were grown in Dulbecco's modified Eagle medium (DMEM; Lonza, Verviers,

493   Belgium) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml

494   streptomycin in a 5% CO_2 humidified incubator at 37°. Before transfection, 1 x 10^5 HEK293T cells

495   were seeded per well of 6-well culture dishes and transfected 24 hours later with expression

496   constructs encoding the *RHEB* variants (0.2 µg), the S6K reporter (0.2 µg), *TSC1* (0.2 µg) and

497   *TSC2* (0.2 µg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To ensure that a total

498   of 0.8 µg plasmid DNA was added per well, empty pcDNA3 vector was included where necessary.

499   The day after transfection, the growth medium was replaced with DMEM without glucose and

500   incubated for a further 4 hours prior to harvesting and western blot analysis.

Western blotting

502   After transfection, HEK293T cells were transferred on ice, washed with PBS (4 °C) and lysed in

503   70 µl 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 50 mM NaF, 1% Triton X100 in the presence of

504   protease and phosphatase inhibitors (Complete, Roche Molecular Biochemicals, Woerden, The

505   Netherlands). Cell lysates were subjected to immunoblotting using the following primary

506   antibodies: anti-RHEB mouse monoclonal (Groenewoud et al., 2013), anti-TSC1 and TSC2 rabbit

507   polyclonal (Van Slegtenhorst et al., 1998), T389-phosphorylated S6K (1A5, #9206, Cell Signaling

508   Technology), and rabbit anti-myc (#2272, Cell Signaling Technology), all 1:1000. Primary

509   antibody binding was assessed by incubation with goat anti-rabbit (680 nm) and anti-mouse (800

510   nm) conjugates (1:15000, Li-Cor Biosciences, Lincoln, USA) followed by detection on an

511   Odyssey near-infrared scanner (Li-Cor Biosciences).

Neuronal primary hippocampal cultures and transfection

513   Primary hippocampal neuronal cultures were prepared from FvB/NHsD wild type mice according

514   to the procedure described in (Banker and Goslin, 1988). Neurons were transfected at 1 day *in*

25

515   *vitro* (DIV1) with the following DNA constructs: control empty vector (1.8 µg per coverslip) and

516   RHEB p.P37L (2.5 µg per coverslip). Plasmids were transfected using Lipofectamine 2000

517   according to the manufacturer's instructions (Invitrogen).

518   **Plasmids**

519   cDNA encoding the *RHEB* (NM_005614.3) c.110C>T (p.P37L) mutation was synthesized by

520   GeneCust. The c.46-47CA>TG (p.S16H) variant was generated by site-directed mutagenesis

521   (Invitrogen) using the following primers: Fw 5' – gcgatcctgggctaccggCAtgtggggaaatcctcatt – 3'

522   and Rev 5' – aatgaggatttccccacaTGccggtagcccaggatcgc – 3'. All *RHEB* gene variants were cloned

523   in our dual promoter expression vector using AscI and PacI restriction sites (Reijnders et al., 2017)

524   and the empty vector used as control refers to the dual promoter expression vector without a gene

525   inserted and expressing either tdTOMATO or EGFP (specified in the figures or in the figures'

526   legends). Expression constructs for TSC1, TSC2 and a myc-tagged S6K reporter were as described

527   previously (Dufner Almeida et al., 2019). The following DNA plasmids were obtained from

528   Addgene: pGEMTEZ-TeTxLC (Addgene plasmid #32640; http://n2t.net/addgene:32640;

529   RRID:Addgene_32640) (Yu et al., 2004); RV-CAG-DIO-EGFP (Addgene plasmid #87662;

530   http://n2t.net/addgene:87662; RRID:Addgene_87662) (Ciceri et al., 2013); pCAG-

531   ERT2CreERT2 (Addgene plasmid #13777; http://n2t.net/addgene:13777; RRID:Addgene_13777)

532   (Matsuda and Cepko, 2007); pCAGGS-ChR2-Venus (Addgene plasmid #15753;

533   http://n2t.net/addgene:15753; RRID:Addgene_15753) (Petreanu et al., 2007). The TeTxLC was

534   isolated by PCR using the following primers: Fw 5' – taagcaggcgcgccaccatgccgatcaccatcaacaa –

535   3' and Rev 5' – gccatggcggccgcggggaattcgat – 3' and inserted in our dual promoter expression

536   vector using AscI and NotI restriction sites. To generate the loxP-STOP-loxP (LSL) constructs

537   (loxP-STOP-loxP-*RHEB* p.P37L and loxP-STOP-loxP-TeTxLC) the LSL sequence was obtained

26

538   from the Ai6 CAG-Floxed ZsGreen in Rosa 26 targeting vector (Addgene plasmid #22798;

539   http://n2t.net/addgene:22798; RRID:Addgene_22798) using multiple cloning sites and inserted

540   just after the CAGG promoter and before the beginning of the gene in our dual promoter expression

541   vector containing either *RHEB*p.P37L or TeTxLC. The floxed *RHEB* p.P37L construct was

542   generated by introducing two loxP site sequences before the CAGG promoter and at the end of the

543   *RHEB*p.P37L gene, with the same orientation to ensure proper deletion. To achieve this, the

544   following   couples   of   oligonucleotides   were   used   for   annealing: Fw   5'-

545   cgcgtATAACTTCGTATAGCATACATTATACGAAGTTATg   -   3',   Rev: 5'-

546   ctagcATAACTTCGTATAATGTATGCTATACGAAGTTATa   -   3';   Fw: 5'-

547   taaATAACTTCGTATAGCATACATTATACGAAGTTATg   -   3',   Rev: 5'-

548   tcgacATAACTTCGTATAATGTATGCTATACGAAGTTATttaat - 3'.

In utero electroporation

549

550   IUE was performed as described previously (Saito and Nakatsuji, 2001). Pregnant FvB/NHsD

551   mice at E14.5 of gestation were used to target the progenitor cells giving rise to pyramidal cells of

552   the layer 2/3. Each *RHEB* DNA construct (including the LSL and floxed conditions) was diluted

553   to a final concentration of 0.5 µg/µl in fast green (0.05%), while other plasmids were diluted to a

554   concentration of 1.5-2 µg/µl. The DNA solution was injected into the lateral ventricle of the

555   embryos while still *in utero*, using a glass pipette controlled by a Picospritzer ® III device. When

556   multiple constructs were injected, a mixture of plasmids was prepared to achieve a final

557   concentration of 1.5-2 µg/µl, keeping the *RHEB* concentration constant throughout all the

558   experiments. To ensure proper electroporation of the injected constructs (1-2 µl) into the

559   progenitor cells, five electrical square pulses of 45V with a duration of 50 ms per pulse and 150

560   ms inter-pulse interval were delivered using tweezer-type electrodes connected to a pulse generator

27

561 (ECM 830, BTX Harvard Apparatus). The positive pole was placed to target the developing

562 somatosensory cortex. Animals of both sexes were used to monitor seizure development, for *ex*

563 *vivo* electrophysiology experiments, or for histological processing with no exclusion criteria

564 determined by a postnatal screening of the targeting area.

565 **Immunostainings**

566 For immunocytochemistry analysis, neuronal cultures were fixed 3 days post-transfection with 4%

567 paraformaldehyde (PFA)/4% sucrose, washed in PBS and incubated overnight at 4°C with primary

568 antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30mM phosphate buffer,

569 pH7.4). Mouse pan anti-SMI312 (1:200, BioLegend, #837904) was used to stain for the axon and,

570 after several washings in PBS, donkey anti-mouse-Alexa488 conjugated was used as secondary

571 antibody diluted in GDB buffer for 1 hour at room temperature (1:200, Jackson ImmunoResearch).

572 Slides were mounted using mowiol-DABCO mounting medium.

573 For the staining of brain tissue sections, mice were deeply anesthetized with an overdose

574 of Nembutal and transcardially perfused with 4% PFA in PB. Brains were extracted and post-fixed

575 for 1 hour in 4% PFA. They were then embedded in gelatin and cryoprotected in 30% sucrose in

576 0.1 M Phosphate Buffer (PB) overnight, frozen on dry ice, and sectioned using a freezing

577 microtome (40 μm thick). Immunofluorescence was performed on free-floating sections that were

578 first washed multiple times in PBS and blocked in 10% normal horse serum (NHS) and 0.5%

579 Triton X-100 in PBS for 1 hour at room temperature. Primary antibodies diluted in PBS containing

580 2% NHS and 0.5% Triton X-100 were added at room temperature overnight. The day after,

581 sections were washed three times with PBS and secondary antibodies were added diluted in PBS

582 containing 2% NHS and 0.5% Triton X-100. After washing in PBS and 0.05 M PB, sections were

583 counterstained with 4′,6-diamidino-2-phenylindole solution (DAPI, 1:10000, Invitrogen) before

28

584   being washed in PB 0.05 M and mounted on slides using chromium (3) potassium sulfate

585   dodecahydrate (Sigma-Aldrich) and left to dry. Finally, sections were mounted on glass with

586   mowiol (Sigma-Aldrich).

587   Biocytin labelling was achieved by fixating the patched slices overnight in 4% PFA in PB

588   at 4°. Slices were then washed multiple times in PBS and incubated with Alexa488-Streptavidin

589   (1:200; #016-540-084, Jackson ImmunoResearch) or AlexaCy5-Streptavidin (1:200; #016-170-

590   084, Jackson ImmunoResearch) overnight at 4°. The next day, after washing in PBS and 0.05 M

591   PB, sections were counterstained with DAPI (1:10000, Invitrogen) and mounted on glass with

592   mowiol (Sigma-Aldrich).

593   When performing Nissl stainings, few selected free floating sections corresponding to the

594   Somatosensory cortex were mounted on glass using chromium (3) potassium sulfate

595   dodecahydrate (Sigma-Aldrich) and left to dry overnight. Slides were stained in 0.1 % Cresyl

596   Violet for 4-10 minutes, then rinsed briefly in tap water to remove excess stain, dehydrated in

597   increasing percentages of alcohol, cleared with xylene and covered using Permount (Fisher

598   Scientific).

599   The primary antibodies used in this study to stain for the specific targets indicated for each

600   experiment in the figures' legends were: anti-rabbit pS6 (Ser 240/244), 1:1000; Cell signaling,

601   catalog #5634; anti-rabbit RFP, 1:2000; Rockland, catalog 600-401-379; anti-rabbit RHEB,

602   1:1000, Proteintech Group Inc., catalog 15924-1-AP; anti-rabbit CUX1, 1:1000; Proteintech

603   Group Inc., catalog 11733-1-AP; anti-rat CTIP2, 1:200; Abcam, catalog ab18465; anti-rabbit

604   NeuN, 1:2000; Millipore catalog ABN78 (RRID: AB_10807945); anti-mouse SATB2, 1:1000;

605   Santa cruz, catalog sc-81376; anti-rabbit synapsin 1, 1:1000; Merck Millipore, catalog #AB1543P;

606   anti-guinea pig VGLUT1, 1:1000; Merck Millipore, catalog #AB5905; Secondary antibodies used

29

607   were: donkey anti rabbit 488, catalog #711-545-152; donkey anti rabbit 647, catalog #711-605-

608   152; donkey anti rabbit Cy3, catalog #711-165-152; donkey anti mouse 488, catalog #715-545-

609   150; donkey anti mouse 647, catalog #715-605-150; donkey anti rat Cy5, catalog #712-175-150;

610   donkey anti guinea pig 647, catalog #706-605-148; all from Jackson ImmunoResearch, 1:200.

611   **LFP and EEG recordings**

612   Starting from 3 weeks of age surgeries were performed according to the procedures described in

613   (Koene et al., 2019; Kool et al., 2019). After at least three days of recovery from the EEG surgical

614   procedure, mice were connected to a wireless EEG recorder (NewBehavior, Zurich, Switzerland)

615   for 24 hours per day for at least two consecutive days (one session of recordings). EEG recordings

616   were manually assessed by two different researchers blind for the genotypes to check for

617   occurrence of seizures, defined as a pattern of repetitive spike discharges followed by a progressive

618   evolution in spike amplitude with a distinct post-ictal depression phase, based on the criteria

619   described in (Kane et al., 2017). If no seizures were detected during the first week *post*-surgery,

620   mice were recorded for another session of 48-56 hr for a maximum of four sessions over four

621   weeks *post*-surgery. During the days in which no EEG recordings were performed, mice were

622   monitored daily to assess for the presence of behavioural seizures and discomfort.

623   For the LFP recordings, two days after the surgical procedure, mice were head-fixed to a

624   brass bar suspended over a cylindrical treadmill to allow anaesthesia-free recording sessions and

625   placed in a light-isolated Faraday cage as described in (Kool et al., 2019). Mice were allowed to

626   habituate to the set-up before proceeding to the recording. LFP measurements were acquired every

627   day in sessions of 20-30' for five or eight consecutive days, using the Open Ephys platform with

628   a sampling rate of 3 kS/s and a band pass filter between 0.1 and 200 Hz. For the power spectrum

629   analysis, the average power density spectrum of all the days of recording was obtained using

30

630   MATLAB software (MathWorks; RRID:SCR_001622). The mean relative power was calculated

631   over four frequency bands relative to the total power: delta (2–4 Hz), theta (4–8 Hz), beta (13–30

632   Hz), and gamma (30–50 Hz).

633   At the end of each experiment, mice were sacrificed for immunohistological analysis to

634   assess electrodes' positioning, amount of targeting and efficiency of cre-dependent recombination

635   when tamoxifen was administered.

636   *Ex vivo* **slice electrophysiology for excitability**

637   P21-P25 mice of both sexes *in utero* electroporated with the plasmids specified in the figures and

638   in the legends for each experiment were anaesthetized with isoflurane before decapitation. The

639   brain was quickly removed and submerged in ice cold cutting solution containing (in mM): 110

640   Choline Chloride, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, 0.5 $CaCl_2$, 10 $MgSO_4$. Acute

641   300 μm coronal slices were made of the somatosensory cortex using a vibratome (HM650V,

642   Microm) while being saturated with 95% O_2/5% CO_2. The slices were immediately transferred to

643   a submerged slice holding chamber and incubated at ±34°C for 5 min before being transferred to

644   a second slice holding chamber also kept at ±34°C. The second holding chamber contained the

645   same artificial cerebrospinal fluid (ACSF) as was used during all recordings and contained (in

646   mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2 $CaCl_2$, 1 $MgSO_4$. During the

647   slicing procedure and experimental recordings, slices were saturated with 95% O_2/5% CO_2. Slices

648   recovered for an hour at room temperature before starting the experiment. After the experiment,

649   slices were fixed in 4% PFA overnight and then transferred to PBS until further processing. Whole-

650   cell patch clamp recordings were obtained from the soma of visually identified L2/L3 pyramidal

651   neurons from the S1 cortex with an upright microscope using IR-DIC optics (BX51WI, Olympus,

652   Tokyo, Japan). Targeted cells in the ipsilateral side were identified by the presence of either

31

653   tdTomato or GFP, depending on the experiment, elicited by an Olympus U-RFL-T burner. All

654   recordings were done under physiological temperatures of 30± 1 °C. Patch clamp pipettes were

655   pulled from standard wall filament borosilicate glass to obtain electrodes with a tip resistance

656   between 2-4 MΩ. All recordings were performed using a Multiclamp 700B (Molecular Devices,

657   Sunnyvale, CA, USA) and digitized by a Digidata 1440A (Molecular Devices, Sunnyvale, CA,

658   USA). For the current clamp recordings, pipets were filled with a K-gluconate internal solution

659   containing (in mM): 125 K-gluconate, 10 NaCl, 10 HEPES, 0.2 EGTA, 4.5 MgATP, 0.3 NaGTP

660   and 10 Na-phosphocreatine. For analysis of cell morphology, biocytin (5%) was added to the

661   intracellular solution. The final solution was adjusted to a pH of 7.2–7.4 using KOH and had an

662   osmolarity of 280 ± 3. After getting a seal of at least 1 GΩ, whole cell configuration was obtained

663   by applying brief negative pressure together with a short electric pulse. Prior to breaking in, cell

664   capacitance was compensated. Series resistance was monitored but not corrected. Recordings with

665   an unstable series resistance and higher than 20 MΩ were rejected. Membrane potentials were not

666   corrected for liquid junction potential. Resting membrane potential was measured immediately

667   after break in.

668   Each sweep started with a small and short hyperpolarizing step (-50 pA, 50 ms) to monitor

669   access resistance. Action potentials were triggered by square step current injections into the

670   patched neurons while holding them at -70 mV. Steps were 750 ms long and started at -300 pA

671   with increments of 20 pA. The number of action potentials and action potential properties were

672   analyzed using Clampfit 10.7.0.3 (Molecular Devices, LLC, USA). For each cell, the first action

673   potential at rheobase was analyzed. The threshold was calculated by plotting the first derivative of

674   the trace. The threshold was defined when the first derivative was lower than 10 mV/ms. Series

675   resistance was calculated offline for each cell by plotting the difference in voltages between

32

676   baseline and the hyperpolarizing steps. A linear line was plotted to visualize passive current only.

677   The tau was calculated by fitting a standard exponential on the end of the hyperpolarizing steps.

678   From tau and series resistance, capacitance was calculated.

679

680   ***Ex vivo* slice electrophysiology for optogenetics**

681   P21-P25 mice of both sexes *in utero* electroporated either with the RHEBp.P37L and pCAGGS-

682   ChR2-Venus plasmids or the empty vector and pCAGGS-ChR2-Venus plasmids (Petreanu et al.,

683   2007), were anaesthetized with isoflurane before decapitation. The brain was quickly removed and

684   submerged in ice cold cutting solution containing (in mM): 93 NMDG, 93 HCl, 2.5 KCl, 1.2

685   $NaHPO_4$, 30 $NaHCO_3$, 25 glucose, 20 HEPES, 5 Na-ascorbate, 3 Na-pyruvate, 2 Thiourea, 10

686   MgSO4, 0.5 CaCl2, 5 N-acetyl-L-Cysteine (osmolarity 310 ± 5; bubbled with 95% O_2 / 5% CO_2)

687   (Ting et al., 2014). Next, 250 μm thick coronal slices were cut using a Leica vibratome (VT1000S).

688   For the recovery, brain slices were incubated for 5 min in slicing medium at $34 \pm 1°C$ and

689   subsequently for ~40 min in ACSF (containing in mM: 124 NaCl, 2.5 KCl, 1.25 Na_2HPO_4, 2

690   $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 20 D–glucose, osmolarity 310 ± 5mOsm; bubbled with 95%

691   O_2 / 5% CO_2) at $34 \pm 1°C$. After recovery brain slices were stored at room temperature. For all

692   recordings, slices were bathed in $34 \pm 1°C$ ACSF (bubbled with 95% O_2 / 5% CO_2). Whole-cell

693   patch-clamp recordings were recorded with an EPC-10 amplifier (HEKA Electronics, Lambrecht,

694   Germany) and sampled at 20 kHz. Resting membrane potential and input resistance were recorded

695   after whole-cell configuration was reached. Recordings were excluded if the series resistance or

696   input resistance (RS) varied by >25% over the course of the experiment. Voltage and current clamp

697   recordings were performed using borosilicate glass pipettes with a resistance of 3-5 MΩ that was

698   filled with K-gluconate-based internal solution (in mM: 124 K-gluconate, 9 KCl, 10 KOH, 4 NaCl,

33

699   10 HEPES, 28.5 sucrose, 4 Na_2ATP, 0.4 Na_3GTP (pH 7.25-7.35; osmolarity 290 ± 5mOsm)).

700   Recording pipettes were supplemented with 1 mg/ml biocytin to check the location of the patched

701   cells with histological staining. Current clamp recordings were corrected offline for the calculated

702   liquid junction potential of -10.2 mV.

703   Full-field optogenetic stimulation (470 nm peak excitation) was generated by the use of a

704   TTL-pulse controlled pE2 light emitting diode (CoolLED, Andover, UK). Light intensities at 470

705   nm were recorded using a photometer (Newport 1830-C equipped with an 818-ST probe, Irvine,

706   CA) at the level of the slice. To trigger neurotransmitter release from targeted axons we delivered

707   a 1 ms light pulse with an intensity of 99.8 mW/mm^2 at a frequency of 0.1 Hz. To ensure that we

708   recorded action potential-driven neurotransmitter release most experiments were concluded by

709   bath application of 10 μM tetrodotoxin (TTX), which blocked all post-synaptic responses in the

710   recorded pyramidal neurons.

711

712   **Imaging and analysis**

713   Images of Nissl stained sections were acquired in brightfield with a Nanozoomer scanner

714   (Hamamatsu, Bridgewater, NJ) at a 40X resolution using the NDP.view2 software. All

715   immunofluorescent images were acquired using a LSM700 confocal microscope (Zeiss). For the

716   analysis of the axons *in vitro*, at least ten distinct confocal images from two different neuronal

717   batches were taken from each coverslip for each experiment (20X objective, 0.5 zoom, 1024x1024

718   pixels; neurons were identified by the red immunostaining signal). The simple neurite tracer plugin

719   from the FIJI ImageJ software was used for the analysis of the axonal length and branches.

720   Overview images of the coronal sections were acquired by tile scan with a 10X objective. Zoom

721   in images of the targeted area (ipsilateral) and contralateral S1 were taken using a 10X objective.

722   For the migration analysis, confocal images (10X objective, 0.5 zoom, 1024x1024 pixels) were

34

723   taken from 2 – 3 non-consecutive sections from at least 2/3 electroporated animals per condition.

724   Images were rotated to correctly position the cortical layers, and the number of cells in different

725   layers were counted using the 'analyze particles' plugin of FIJI. The results were exported to a

726   spreadsheet for further analysis. Cortical areas from the pia to the ventricle were divided into 10

727   bins of equal size and the percentage of tdTomato-positive cells per bin was calculated. The soma

728   size analysis was performed on z-stacks images acquired using a 20X objective, 1 zoom,

729   1024x1024 pixels, of the targeted cells in both empty vector control and *RHEB*p.P37L coronal

730   sections. A ROI around each targeted cell in maximum intensity projection pictures was defined

731   using the FIJI software and the area of the soma was measured using the 'Measure' option in

732   ImageJ. For the analysis of pS6 intensity levels, confocal images (10X objective, 0.5 zoom,

733   1024x1024 pixels) of the ipsilateral and contralateral S1 cortex were acquired with the same master

734   gain from both control and RHEB groups previously stained together against pS6 (240/244). The

735   overall intensity level of the staining for each picture was measured using the 'RGB measure'

736   plugin of FIJI and the values of each ipsilateral side were normalized against the corresponding

737   contralateral side and plotted as averaged values. The analysis of the fluorescent intensity of the

738   axonal branches over the contralateral cortical layers, was obtained from 3-4 matched coronal

739   sections from at least 3 different animals per group with comparable amount of targeting. The

740   axonal arborization was measured selecting the S1/S2 border, drawing a straight segmented line

741   with adjusted width and length and resized in 1000 bins, and using the 'plot profile' option of the

742   analyze section of FIJI to measure the fluorescent intensity of the tdTomato signal over the

743   different layers. The values obtained for each section were exported to a spreadsheet were they

744   were normalized against the mean background fluorescent intensity calculated on a non-targeted,

745   cortical area of fixed size and plotted as averaged values over 10 bins of equal size. For the analysis

35

746   of the morphology of biocytin filled pyramidal cells and ectopic cells in the nodule labelled with

747   streptavidin-488 or streptavidin-Cy5, z-stacks images were taken using a 20X objective, 0.5 zoom,

748   1024x1024 pixels, to include the dendritic tree. Maximum intensity projection pictures were

749   analyzed using the SynD software for the MATLAB platform to automatically detect the dendritic

750   morphology and perform Sholl analysis (Schmitz et al., 2011).

751

752   **Statistics**

753   Normality of the distribution for the different experiments was determined using either the Wilk–

754   Shapiro test (Figures 1A, 1C, 2D, 3E-F, 4B, 5A, 6B, 8F and Supplementary Figure 5B,) or the

755   Kolmogorov-Smirnov test (Figures 2A, 2B, 5C, 8B-C-E). Statistical analysis was performed using

756   a one-way ANOVA (or corresponding non-parametric Kruskal-Wallis test), two-way repeated-

757   measures ANOVA or mixed-effects analysis, Student's t test (or corresponding non-parametric

758   Mann-Whitney test) and correlation/association analysis. The specific test used for each

759   experiment and relative significance are specified in the figures' legends, in the supplementary

760   tables or in the results section (when data are not shown in a figure). For all statistical analyses α

761   was set at 0.05. Values are represented as average ± SEM or as median, minimum and maximum

762   values (specified in the figures' legends). No samples or mice were excluded from the final

763   analysis. Group sizes, biological replicates, number of cells, samples or brain sections are indicated

764   in the figures and their corresponding legends. All statistical tests were performed either using

765   GraphPad Prism 8.0 (RRID: SCR_002798) or SPSS Statistics v25.0 (RRID:SCR_002865).

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36

768 **ACKNOWLEDGMENTS**

774 **COMPETING INTERESTS**

775 The authors declare no competing interests.

776 **AUTHOR CONTRIBUTIONS**

777 Conceptualization, M.P.O., Y.E. and G.M.v.W.; Methodology, M.P.O. , Y.E. and G.M.v.W.;

778 Investigation, M.P.O., L.M.C.K., C.B.S., M.N.; Formal Analysis: M.P.O., L.M.C.K., C.B.S.,

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979

980 **FIGURE LEGENDS**

981

982 **Figure 1. The RHEBp.P37L protein is resistant to TSC complex inhibition and causes**
983 **aberrant cortical development *in vivo*.**

984 **(A)** Wild-type RHEB (WT), p.S16H or p.P37L constructs were transiently co-expressed with an

985 S6K reporter construct and the TSC complex in HEK 293T cells to assess the effect on mTORC1

986 activity. Quantification of the ratio of T389-phosphorylated S6K to total S6K was calculated

987 relative to control condition, in absence or presence of the TSC complex (control indicates empty

988 vector pcDNA3); dashed lines indicate where an irrelevant lane in the original scan was excluded

989 from the picture; bar graph represents mean ± SEM and single data points represent the number of

990 independent biological samples per condition; for statistics see **Supplementary table 1**. **(B)**

991 Schematic representation of the main constructs used throughout the experiments and overview of

992 the experimental design. MCS indicate the multiple cloning site with specific restriction sites (AscI

993 and PacI in this case) to insert the gene of interest. Each construct was delivered by *in utero*

994 electroporation (IUE) at E14.5 to target the progenitor cells of layer 2/3 pyramidal neurons in the

995 somatosensory cortex (SScx); ctx=cortex; GE=ganglion eminence. **(C)** Representative confocal

996 images of the targeted SScx counterstained with DAPI showing the transfected cells (tdTomato+)

997 in red (see also **Figure 1-figure supplement 1**) and quantification of tdTomato+ cells across the

998 different layers of the SScx with percentage of cells reaching layer 2/3 (L2/3) in the inset (bins 1-

999 5 from the top). Dotted lines indicate the border of the intermediate zone (IZ, bottom) and delineate

1000 L2/3. Numbers in the legend indicate number of targeted mice (N=5) and total number of pictures

1001 analyzed (n=11, n=10); results are represented as mean ± SEM and single data points in the bar

48

1002   graph indicate the number of pictures analyzed; inset analysis: Mann-Whitney U = 0, $p<0.0001$,

1003   two-tailed Mann-Whitney test.

1004   * $p<0.05$, ** $p<0.01$, **** $p<0.0001$; scale bars: 50 μm.

1005

1006   **Figure 2. Ectopic RHEBp.P37L cells display aberrant morphology and show mTOR**

1007   **hyperactivity while still preserving the molecular identity of L2/3 neurons.**

1008   **(A)** Soma size quantification of L2/3 empty vector expressing cells and RHEBp.P37L expressing

1009   cells in the nodule; box plots represent minimum and maximum values with median, dashed lines

1010   represent the mean values for empty vector (black) and for RHEBp.P37L (red); numbers indicate

1011   number of targeted mice (N=2, N=3) and number of cells analyzed (n=275, n=366); Mann-

1012   Whitney U = 1940, $p<0.0001$, two-tailed Mann-Whitney test. **(B)** Reconstruction and Sholl

1013   analysis of dendritic morphology of biocytin filled cells in L2/3 of the SScx (for empty vector

1014   control) and RHEBp.P37L cells in the nodule; numbers in the legend indicate number of targeted

1015   mice (N=3, N=2) and number of cells analyzed (n=17, n=11); data are presented as mean ± SEM;

1016   interaction group condition/distance from the soma: $F(44, 1144) = 15.69$, mixed-effects analysis;

1017 $p<0.0001$. **(C)** Representative images of the nodule stained with CUX1 (L2/3 marker), CTIP2 (L5

1018   marker), SATB2 (cortical projection neurons marker) or NeuN (mature neurons marker); arrows

1019   in the zoomed pictures point at examples of targeted cells; for an overview see **Figure 2-figure**

1020   **supplement 1**. **(D)** Representative images of the targeted L2/3 (SScx) of empty vector control and

1021   nodule showing increased pS6-240 levels for the ipsilateral targeted cortex in RHEBp.P37L

1022   targeted mice; for an overview see **Figure 2-figure supplement 1**; bar graph represents mean ±

1023   SEM and single data points indicate the values of each normalized ipsilateral/contralateral pS6

49

1024   intensity; numbers in the bars indicate number of targeted mice (N=5) and number of pictures

1025   analyzed (n=10, n=11); Mann-Whitney U = 13, *p* = 0.002, two-tailed Mann-Whitney test).

1026   Histological analysis for **(A) (C)** and **(D)** was performed on 5 weeks old mice.

1027   ** *p*<0.01, **** *p*<0.0001; scale bars: 20 μm (A), 50 μm (B-D).

1028

1029   **Figure 3. Overexpression of RHEBp.P37L *in vivo* causes mTORC1-dependent spontaneous**

1030   **generalized tonic-clonic seizures and abnormal neuronal network activity**

1031   **(A)** Timeline and experimental design indicating the cortical area targeted with the IUE and

1032   position of the electrodes placed during the EEG surgery (LSScx = left SScx; RSScx = right SScx;

1033   B = bregma; L= lambda; r = reference electrode). **(B)** Example EEG traces and spectrogram of 5

1034   weeks old control mouse (N=6, non-targeted mice from the same litters as the RHEBp.P37L mice)

1035   and RHEBp.P37L mouse (N=12); see also **Figure 3-figure supplement 1**; colored boxes are

1036   zoomed in panel **(C)**. **(C)** Highlighted EEG traces showing: box 1. the baseline activity of a control

1037   mouse; box 2. the interictal activity, box 3. the ictal (seizure) activity and box 4. the post-ictal

1038   phase of a RHEBp.P37L targeted mouse. **(D)** Timeline and experimental design indicating the

1039   cortical area targeted with the IUE, the position of the electrodes for the local field potential (LFP)

1040   recordings and the IP rapamycin injections. **(E)** Example LFP traces for each group condition and

1041   normalized power spectrum density (PSD) averaged bilaterally over the overall consecutive days

1042   of recording (for the PSD until 50 Hz see **Figure 3-figure supplement 1**); N indicates number of

1043   mice analyzed for each group; data are represented as mean (thick line) ± SEM (shading area). **(F)**

1044   Calculation of the *delta* (2-4 Hz) and *theta* (4-8 Hz) frequency bands over the total power of the

1045   PSD presented in **(E)**, and relative ratio *theta*/*delta* (see also **Figure 3-figure supplement 1**); box

50

1046 plots represent minimum and maximum values with median, dashed lines represent the mean

1047 values for each group; for statistics see **Supplementary table 2**; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

1048

1049

1050 **Figure 4. The heterotopic nodule is neither necessary nor sufficient to induce spontaneous**

1051 **seizures**

1052 **(A)** Schematic representation of the timeline of the IUE, SC rapamycin injections, EEG surgery

1053 and measurements. **(B)** Quantification of the percentage of tdTomato+ cells that managed to

1054 migrate out to L2/3 in mice prenatally exposed to rapamycin; data are presented as mean ± SEM,

1055 single data points represent the values of each picture analyzed and dashed lines indicate the mean

1056 value of cells reaching L2/3 in empty vector control mice (black line) and in RHEBp.P37L mice

1057 (red line); numbers in the graph indicate number of mice (N=11, N=5) and number of pictures

1058 analyzed (n=21, n=11, n=10); (% targeted cells in L2/3: H(2) = 25.97, $p < 0.0001$, Kruskal-Wallis

1059 test; EV *vs* RHEBp.P37L-prenatal rapamycin, $p = 0.05$; RHEBp.P37L *vs* RHEBp.P37L-prenatal

1060 rapamycin, $p = 0.002$, RHEBp.P37L *vs* EV, $p < 0.0001$, Dunn's multiple comparisons test; *

1061 $p<0.05$, ** $p<0.01$. **(C)** Percentage of targeted mice showing spontaneous seizures; control mice

1062 are non-targeted mice from the same litters as the RHEBp.P37L mice prenatally exposed to

1063 rapamycin; numbers in the bar plots indicate the number of mice per group. **(D)** Representative

1064 images of RHEBp.P37L mice prenatally exposed to rapamycin that showed or did not show

1065 seizures and degree of association between the migration phenotype (mean value of % of targeted

1066 cells in L2/3, dependent scale variable, for each mouse shown in figure B) and the absence or

1067 presence of seizures (independent nominal variable) in RHEBp.P37L mice (N=4 and N=5,

1068 respectively, with the exclusion of the mice that showed heterotopia); the dashed line represents

51

1069   the mean value of the empty vector control group already shown in B, as comparison; η_p=0.821,

1070 η_p^2=0.674, Eta measure of association, with values of η_p close to one indicating strong association.

1071   **(E)** Schematic representation of the DNA plasmids used in the experiment. The Lox-Stop-Lox

1072   (LSL) or the floxed construct was expressed in combination with the CAGG-ERT2CreERT2 and

1073   a CAGG-DIO-EGFP constructs. The EGFP in the CAGG-DIO-EGFP construct is expressed only

1074   upon tamoxifen injection, providing a measure of efficient cre-dependent recombination. **(F)** and

1075   **(H)** Representative images showing the effect of tamoxifen administration in adult mice injected

1076   *in utero* with either the LSL construct **(F)** or the floxed construct **(G)**; mice were injected 4 times

1077   with tamoxifen starting from P7 in **(F)** and starting from P14 in **(G)** and sacrificed at P50. **(G)** and

1078   **(I)** Timeline of the experimental design and bar graphs indicating the percentage of targeted mice

1079   for each group measured with EEG until 12 weeks of age showing spontaneous seizures; numbers

1080   in the bar plots indicate the number of mice.

1081   Scale bars: 100 μm.

1082

1083   **Figure 5. RHEBp.P37L overexpression induces an increase in axon length and branching**

1084   **both *in vitro* and *in vivo***

1085   **(A)** Representative images of primary hippocampal cultures transfected at day *in vitro* 1 (DIV1)

1086   with either empty vector control or RHEBp.P37L constructs (tdTomato, in red) stained at DIV4

1087   with a pan axonal marker SMI312 (in green); arrowheads indicate the axons; bar graphs represent

1088   mean ± SEM and single data points indicate the number of cells analyzed; numbers indicate

1089   number of neuronal cultures (N=2) and total number of cells analyzed (n=24, n=20); axonal

1090   length: Mann-Whitney U = 32, p<0.0001, Mann-Whitney test; axonal branches: Mann-Whitney U

1091   = 53, p<0.0001, Mann-Whitney test. **(B)** Overview coronal sections in grey scale stained with anti-

52

1092 RFP antibody of an empty vector and a RHEBp.P37L mouse brain *in utero* electroporated on the

1093 left S1 and magnification of the axon terminals on the contralateral S1; scale bars: 500 μm. **(C)**

1094 Representative images of ipsilateral and contralateral S1 area of an empty vector and a

1095 RHEBp.P37L mouse coronal section (P50) with quantification of the axonal projections across the

1096 different layers in the contralateral cortex measured as normalized fluorescent intensity of the

1097 tdTomato signal; numbers in the legend indicate number of targeted mice (N=3, N=4) and number

1098 of contralateral pictures (n=10, n=23) analyzed; data are presented as mean (thick line) ± SEM

1099 (shading area); interaction group condition/cortical layers: $F(9, 279)=13.96$, $p<0.0001$, mixed-

1100 effects analysis; control *vs* RHEBp.P37L L2/3 (bin2-3 from the top): $p<0.0001$; control *vs*

1101 RHEBp.P37L L5-L6: bin7, $p=0.0074$, bin8, $p<0.0001$, bin 9, $p=0.002$; Bonferroni multiple

1102 comparisons test.

1103 ** $p<0.01$, **** $p<0.0001$; scale bars: 50 μm **(A)**, 500 μm **(B)**, 100 μm **(C)**.

1104

1105 **Figure 6. Overexpressing RHEBp.P37L increases synaptic connectivity on the contralateral**
1106 **hemisphere.**
1107 **(A)** Schematic representation of the timeline and experimental conditions of the IUE and *ex vivo*

1108 whole-cell patch clamp recordings in contralateral L2/3 and L5 upon wide-field optogenetic

1109 stimulation. **(B)** Example traces and analysis of the compound postsynaptic responses after

1110 photostimulation (blue light), showing the postsynaptic response amplitudes and total charge in

1111 contralateral L2/3 and L5 in empty vector (EV) and RHEBp.P37L expressing slices; numbers in

1112 the graph indicate number of targeted mice (N=5) and number of cells (n=8, n=13, n=10, n=15)

1113 analyzed; data are presented as mean ± SEM and single data points indicate the values of each

1114 cell; for statistics see **Supplementary table 3**; * $p<0.05$, *** $p<0.001$.

1115

53

1116 **Figure 7. Loss of axonal projections or blocking vesicle release of RHEBp.P37L expressing**

1117 **neurons is sufficient to stop seizures.**

1118 **(A) (C)** Schematic representation of the timeline of the IUE, EEG surgery and measurements and

1119 timepoints of tamoxifen administration in **(C)**. **(B)** Example figures of ipsilateral targeted S1,

1120 corpus callosum and contralateral S1 of an adult mouse (12 weeks) *in utero* electroporated with

1121 the RHEBp.P37L (construct expressing EGFP, in green) and a Tetanus toxin construct (TeTxLC,

1122 construct expressing tdTomato, in red). Note the absence of axonal projections on the contralateral

1123 side. The bar graph shows percentage of seizure-free targeted mice measured with EEG until 12

1124 weeks of age. Numbers in the bar graph indicate number of mice. **(D)** Example figures of ipsilateral

1125 targeted area (left) and contralateral cortex of an adult mouse (12 weeks) *in utero* electroporated

1126 with the RHEBp.P37L (in green) and a LSL-Tetanus toxin construct (LSL-TeTxLC, in red) and

1127 injected with tamoxifen starting at P14. The bar graph shows percentage of targeted mice

1128 developing seizures upon early tamoxifen injection (P14) or upon tamoxifen administration after

1129 detecting seizures and measured with EEG until 12 weeks of age (see **Supplementary table 4** for

1130 details on the timeline of the experiment). Numbers in the bar graphs indicate number of mice.

1131 Scale bars: 100 μm.

1132

1133 **Figure 8. Neurons in the contralateral homotopic cortical area in RHEBp.P37L mice show**

1134 **increased excitability that can be reversed by blocking vesicles release.**

1135 **(A)** Schematic representation of the timeline and experimental conditions of the IUE and *ex vivo*

1136 whole-cell patch clamp recordings showing the targeted cells patched in the targeted S1, L2/3 and

1137 non-targeted L2/3 cells in the ipsilateral and contralateral sides. **(B)** Analysis of the passive

1138 membrane properties (capacitance [Cm], membrane resistance [Rm] and resting membrane

54

1139 potential [Vm]) of pyramidal cells in L2/3 (targeted and non-targeted) of control empty vector

1140 mice and targeted and non-targeted pyramidal cells in L2/3 of RHEBp.P37L mice; numbers in the

1141 legend indicate number of targeted mice (N=3, N=6) and number of cells (n=37, n=20, n=22,

1142 n=21) analyzed; data are presented as mean ± SEM and single data points indicate the values of

1143 each cell; for statistics see **Supplementary table 5**). **(C)** Example traces and number of action

1144 potentials in response to increasing depolarizing currents; number of mice and cells is as indicated

1145 in **(B)**; data are presented as mean ± SEM and the red dashed line represents the pooled mean value

1146 ± SEM of targeted and non-targeted cells in empty vector control mice (N=3) shown separately in

1147 **Figure 8-figure supplement 1**, for comparison; for statistics see **Supplementary table 5**). **(D)**

1148 Schematic representation of the timeline and experimental conditions IUE, tamoxifen injections

1149 and *ex vivo* whole-cell patch clamp recordings in L2/3 of ipsilateral and contralateral S1 cortex.

1150 **(E)** Number of action potentials in response to increasing depolarizing currents of cells expressing

1151 both RHEBp.P37L and LSL-TeTxLC in L2/3 ipsilateral S1 and non-targeted cells in L2/3

1152 contralateral S1; data are presented as mean ± SEM and dashed lines represent the mean values ±

1153 SEM of the pooled control cells from empty vector mice shown in **Figure 8-figure supplement 1**

1154 and of the RHEBp.P37L mice from **Figure 8C**, for comparison; N=number of mice, n=number of

1155 cells analyzed; for statistics see **Supplementary table 6**. **(F)** Analysis of passive membrane

1156 properties (Cm, Rm and Vm) of pyramidal cells in L2/3 of mice targeted with RHEBp.P37L and

1157 LSL- TeTxLC in ipsilateral S1 and non-targeted cells on the contralateral side; data are presented

1158 as mean ± SEM and the dashed lines indicate the mean values of capacitance, membrane resistance

1159 and resting membrane potential of RHEBp.P37L targeted cells in L2/3 and contralateral cells

1160 shown in **Figure 8B**, for comparison; for statistics see **Supplementary table 6**.
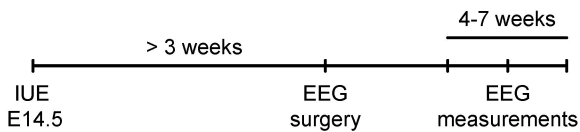
1161 * *p*<0.05, ** *p*<0.01, *** *p*<0.001, **** *p*<0.0001.
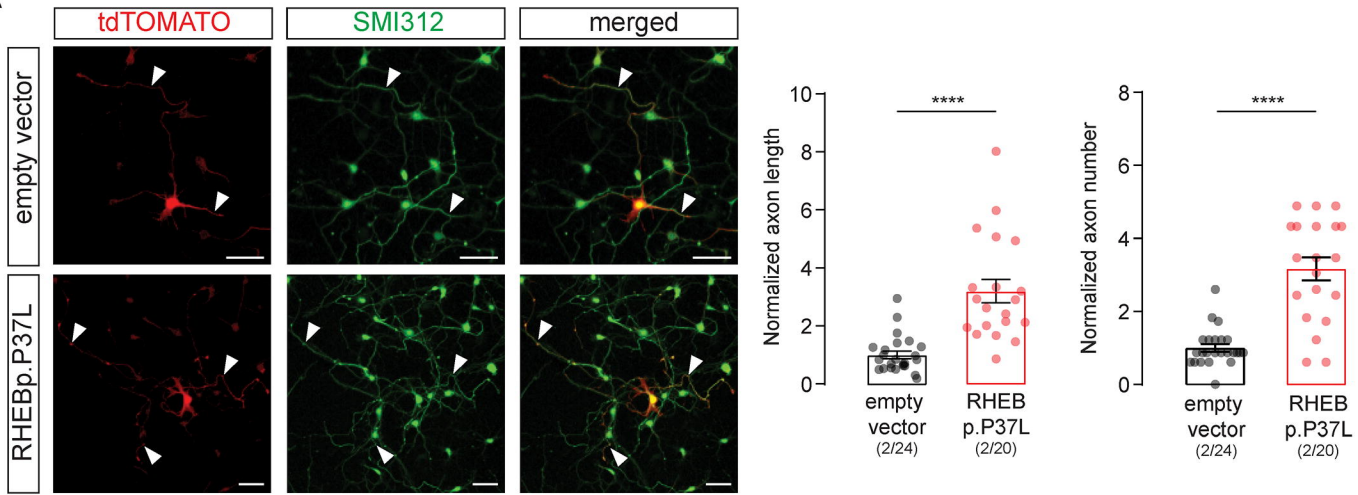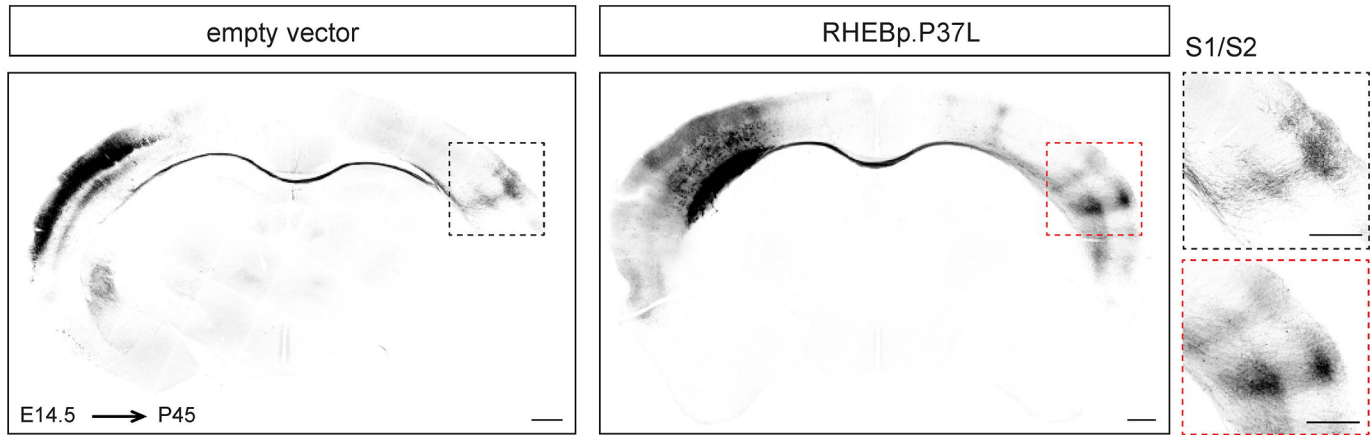
55

Figure 1

Figure 2

A

B

C

D

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

A

> 3 weeks

4-12 weeks

IUE
E14.5

EEG
surgery

EEG
measurements

ctx

RHEBp.P37L+
TeTxLC

GE

B

DAPI/tdTOMATO/EGFP

ipsilateral | corpus callosum | contralateral

L2/3

L2/3

IZ

IZ

Seizure free

% targeted mice

150
100
50
0

7

RHEBp.P37L
+TeTxLC

C

tamoxifen
P14-P17

E14.5
IUE

4 weeks

EEG
surgery

5-12 weeks

EEG
measurements

tamoxifen
after seizures

ctx

RHEBp.P37L+
LSL-TeTxLC

GE

D

DAPI/tdTOMATO/EGFP

ipsilateral | contralateral

L2/3

L2/3

IZ

IZ

Seizure free   Spontaneous seizures

% targeted mice

150
100
50
0

Tamoxifen
P14

13

150
100
50
0

Before
tamoxifen

6

Tamoxifen
post-seizure

6

RHEBp.P37L+LSL-TeTxLC

Figure 8