

# *Cannabidiol modulates phosphorylated rpS6 signalling in a zebrafish model of tuberous sclerosis complex*

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1 **TITLE:** Cannabidiol modulates phosphorylated rpS6 signalling in a zebrafish model of  
2 Tuberos Sclerosis Complex

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25

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30

31 **CONFLICT OF INTERESTS:**

32 The original study concept was discussed with GW Research Ltd, although all subsequent  
33 study design, data collection, analysis and interpretation were conducted independently by  
34 the authors. The report was approved by the sponsor company prior to submission, and the  
35 authors retain full control of all primary data.

36

37

38 **ABSTRACT:**

39 Tuberos sclerosis complex (TSC) is a rare disease caused by mutations in the *TSC1*  
40 or *TSC2* genes and is characterized by widespread tumour growth, intractable epilepsy,  
41 cognitive deficits and autistic behaviour. CBD has been reported to decrease seizures and  
42 inhibit tumour cell progression, therefore we sought to determine the influence of CBD on  
43 TSC pathology in zebrafish carrying a nonsense mutation in the *tsc2* gene.

44 CBD treatment from 6 to 7 days post-fertilization (dpf) induced significant anxiolytic  
45 actions without causing sedation. Furthermore, CBD treatment from 3 dpf had no impact on  
46 *tsc2*<sup>-/-</sup> larvae motility nor their survival. CBD treatment did, however, reduce the number of  
47 phosphorylated rpS6 positive cells, and their cross-sectional cell size. This suggests a CBD  
48 mediated suppression of mechanistic target of rapamycin (mTOR) activity in the *tsc2*<sup>-/-</sup> larval  
49 brain.

50 Taken together, these data suggest that CBD selectively modulates levels of  
51 phosphorylated rpS6 in the brain and additionally provides an anxiolytic effect. This is  
52 pertinent given the alterations in mTOR signalling in experimental models of TSC.  
53 Additional work is necessary to identify upstream signal modulation and to further justify the  
54 use of CBD as a possible therapeutic strategy to manage TSC.

55

56 **KEYWORDS:**

57 tuberous sclerosis complex; cannabidiol; cannabinoids; zebrafish; rpS6; mTOR.

58

59 **ABBREVIATIONS<sup>2</sup>**

60

61 **1. INTRODUCTION:**

62 Tuberous sclerosis complex (TSC) is a rare genetic disease caused by a mutation in  
63 the *TSC1* or *TSC2* genes, coding for the proteins hamartin and tuberin, respectively [1]. TSC1  
64 and TSC2 form an inhibitory complex with GTPase-activating protein (GAP) activity. This  
65 keeps Ras homolog enriched in brain (Rheb) bound to GDP and in an inactive form,  
66 preventing downstream phosphorylation of mechanistic target of rapamycin (mTOR). In  
67 humans and animal models of TSC, mutations in TSC1 or TSC2 impair the inhibitory  
68 function of the complex, allowing activation of Rheb by GTP and constitutive activation of  
69 mTOR [2,3].

70 mTOR is a major convergence point for extracellular signalling, through regulation of  
71 anabolic processes such as transcription and translation [4]. This regulation of protein  
72 synthesis by mTOR extends throughout the mammalian lifespan and is crucial for central

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<sup>2</sup> **CBD** cannabidiol; days post-fertilization; **mTOR** mechanistic target of rapamycin; **rpS6** ribosomal protein 6; **TSC** Tuberous sclerosis complex; **TR** Touch-response

73 nervous system (CNS) development, where it controls soma size, dendritic arborisation,  
74 cortical lamination, and plasticity [5,6].

75 Overactivation of mTOR is evident in the majority of TSC patients that present with  
76 benign tumours in several organs, such as skin, kidneys and brain [6,7]. These often require  
77 surgical treatment and are a major source of morbidity for patients [8]. Furthermore,  
78 disruption of mTOR function in TSC also leads to neurological and neuropsychiatric  
79 complications in 85% of patients. Brain lesions such as cortical dysplasia, subependymal  
80 nodules and tubers are present in 70-90% of these patients [1]. Importantly, tubers and the  
81 perituberal tissue have long been associated with epilepsy, the most common neurological  
82 manifestation in TSC. In fact, 80-96% of patients have epilepsy with two thirds refractory to  
83 existent therapies [1,9]. Failure to control seizures in TSC patients is highly correlated with  
84 an early onset of seizures, before the age of 1 year old, in the form of focal epilepsy and  
85 infantile spasms [10]. Epilepsy is frequently associated with tuberous sclerosis associated  
86 neuropsychiatric disorders (TAND), such as autism spectrum disorder, present in 40-50% of  
87 patients, and intellectual disability, present in 30% [9,11]. The importance of seizure control  
88 is further reinforced by its positive impact on developmental outcomes and quality of life  
89 assessments [12,13]. Nonetheless, despite the availability of some treatment options, due to  
90 the high heterogeneity of manifestations and TSC phenotype, not all individuals respond to  
91 the currently available therapies and new options are needed to attend to the patients' needs  
92 [10,14,15].

93 Given that a vast array of different systems and organs are affected by TSC, treatment  
94 typically requires a poly-pharmacological approach. mTOR inhibitors, such as rapamycin and  
95 everolimus, are current first-line treatments to control the growth of asymptomatic lesions.  
96 Epilepsy treatment can include one or multiple anti-epileptic drugs (AEDs), especially if  
97 resistance is present. Vigabatrin, which has been proposed to modulate both GABA levels

98 and the mTOR pathway [16], displays good efficacy in TSC and is the most commonly used  
99 AED in these patients [9,17]. Regarding TAND, no specific therapies are yet approved,  
100 although early-intervention neuropsychiatric programmes, seizure control and mTOR  
101 inhibition have shown to contribute to cognitive improvement [9,18].

102         There is growing evidence to support the use of cannabidiol (CBD), the most  
103 abundant non-euphoric phytocannabinoid derived from *Cannabis sativa* [19], in the  
104 management of seizures [20]. Previous *in vitro* and *in vivo* studies have demonstrated the  
105 efficacy of CBD in reducing the frequency and severity of seizures, in different models of  
106 epilepsy [21,22]. More recently, two clinical trials provided evidence of a CBD induced  
107 reduction in the median frequency of convulsive seizures and of drop seizures, in Dravet  
108 Syndrome (DS) and Lennox-Gastaut Syndrome, respectively [23,24]. Additionally, an  
109 expanded-access study of CBD for patients with TSC also suggested an effect of CBD in  
110 reducing seizure frequency in this population [25]. CBD has also shown beneficial effects in  
111 tumour studies. *In vitro* reduction of cellular viability and proliferation was demonstrated in  
112 both tumour cell lines [26–31] and primary tumour cells [26], whilst reducing tumour volume  
113 [26,28,31] and metastasis *in vivo* [31–33]. Importantly, CBD has been shown to modulate  
114 some components of the mTOR pathway [30,31,34–38], however there is a divergence in the  
115 reported effects with evidence from the cancer field supporting a CBD inhibition of mTOR,  
116 while epilepsy studies indicate an activation of mTOR. Therefore, in the complex pathology  
117 of TSC, the modulation of mTOR signalling via CBD is unclear.

118         Several animal models are available for the study of TSC, although none of these  
119 fully replicates all features of the human disease [39]. Mammalian models include the Eker  
120 rat, with a spontaneous *Tsc2* mutation, and several conditional knockout mice which allow  
121 biallelic inactivation of *Tsc1* or *Tsc2* in a cell specific manner [39,40]. Non-mammalian  
122 models are also widely used, as it is the case of *Saccharomyces cerevisiae*, *Drosophila* and

123 zebrafish [41]. The zebrafish model of TSC used here carries the nonsense *vu242* mutation in  
124 the *tsc2* gene [42]. This renders tuberlin inactive as it lacks the functional GAP domain.  
125 Several human-like disease features, such as increased phosphorylation of rpS6, a protein  
126 downstream of mTOR and often used as a readout of mTORC1's activity [43,44], increased  
127 cell size and early death of homozygotes, are present in this model [42]. More recently, an  
128 mTOR-dependent disruption of locomotor behaviour was also demonstrated in homozygous  
129 *tsc2<sup>-/-</sup>* larvae [45]. Here we use a zebrafish model of TSC to examine the effects of CBD on  
130 the pathogenesis of TSC, including behavioural effects and ribosomal protein 6  
131 phosphorylation. Our data highlights that CBD can modulate the mTOR pathway, through  
132 regulating the phosphorylation status of ribosomal protein 6 in a relevant model of TSC.

133

## 134 **2. MATERIALS AND METHODS:**

### 135 **Zebrafish Husbandry**

136 Zebrafish embryos, heterozygous for the *tsc2<sup>vu242</sup>* mutation backcrossed with Tupfel  
137 longfin wild-type fish, were a generous gift of Malgorzata Wiweger, head of the Zebrafish  
138 Core Facility of the International Institute of Molecular and Cell Biology (Warsaw, Poland).  
139 The zebrafish model of TSC with a *tsc2* nonsense mutation (*tsc2<sup>+/-</sup>*) was previously described  
140 [42,45]. For this study, heterozygous (*tsc2<sup>+/-</sup>*) zebrafish were interbred to generate a mixture  
141 of wild-type (*tsc2<sup>+/+</sup>*), heterozygous (*tsc2<sup>+/-</sup>*) and homozygous (*tsc2<sup>-/-</sup>*) larvae. Adult zebrafish  
142 were maintained at 28.5 °C in UV-sterilized water on a 14 h light/10 h dark cycle under  
143 standard aquaculture conditions. Fertilized eggs were collected via natural spawning.  
144 Embryos and larvae (*tsc2<sup>+/+</sup>*, *tsc2<sup>+/-</sup>* and *tsc2<sup>-/-</sup>*) were raised in embryo medium, containing 1.5  
145 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM MgSO<sub>4</sub> and 0.18 mM  
146 Ca(NO<sub>3</sub>)<sub>2</sub> in an incubator on a 14 h light/10 h dark cycle at 28.5 °C. For all experiments  
147 described, larvae from 0 to 10 days post-fertilization (dpf) were used. All zebrafish



148 experiments were approved by the Ethics Committee of the University of Leuven (Ethische  
149 Commissie van de KU Leuven, approval number 061/2013) and by the Belgian Federal  
150 Department of Public Health, Food Safety and Environment (Federale Overheidsdienst  
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152 LA1210199).

153

#### 154 **Maximum Tolerated Concentration (MTC)**

155 6 dpf larvae were placed in a 24 well-plate (Corning Inc., New York, USA) and  
156 incubated with 396  $\mu\text{L}$  of swimming medium (Danieau's) and 4  $\mu\text{L}$  DMSO (0.1 or 1%) or  
157 CBD (GW Pharmaceuticals, Cambridge, UK) dissolved in DMSO. CBD was serial diluted  
158 and tested in concentrations ranging from 0.3  $\mu\text{M}$  to 125  $\mu\text{M}$ . Plates were then transferred to  
159 a 37 °C incubator, in the dark, and larvae touch-response was tested after 1 and 24 hours of  
160 incubation. The MTC was defined as the highest concentration of CBD in DMSO that did not  
161 induce any observable signs of toxicity, such as necrosis, abnormal heart beat or loss of  
162 posture [46].

163

#### 164 **Locomotor Assay**

165 The locomotor assay was performed as previously published [45]. In brief, 6 dpf  
166 larvae were placed in a 24 well-plate, treated with Danieau's (called *naïve* larvae in the text),  
167 0.1% DMSO or 1.25  $\mu\text{M}$  CBD in 0.1% DMSO, and incubated in dark for 24 hours. Plates  
168 were then removed from the incubator and placed in a Zebrafish box, where movement of the  
169 larvae was automatically tracked and expressed in "*actinteg*" units (which is the sum of pixel  
170 changes detected during tracking). Here, fish were habituated for 10 minutes under both light  
171 and dark condition before being tracked for 5 minutes in both the light and dark conditions.  
172 To measure anxiogenic or anxiolytic effects due to compound administration, changes in the

173 startle response upon light to dark transition were measured as previously published [47,48].  
174 Touch response (TR) can be used to corroborate the MTC data as it is also a measure of  
175 larval peripheral reflexes [49]. Touch-response was tested, by touching the larvae's tail with a  
176 blunt needle, before and after tracking to monitor toxicity. The number of responding and  
177 non-responding larvae was registered. All researchers were blinded to genotype throughout  
178 behavioural testing and data analyses.

179

### 180 **Chronic Treatment and Survivability Assay**

181 Larvae were cultured in 200 µl of Danieau's, in a 96-well plate, and followed from 3  
182 dpf until 10 dpf. Medium was changed every other day and 0.1% DMSO or 1.25 µM CBD in  
183 0.1% DMSO added to the wells. The number of dead larvae was counted daily. At the end of  
184 the assay, the surviving larvae underwent the previously described locomotor assay.

185

### 186 **Genotyping**

187 Genotyping was conducted as previously published [45]. In summary, after sacrifice,  
188 zebrafish tails were collected and lysed for 3 hours, at 55 °C, followed by 10 minutes at 95  
189 °C. PCR was performed on the lysates using *Pfu* polymerase (Thermo Scientific, UK), and  
190 the primers GTAACACAGAATCAGTGAATCGGA (forward primer) and  
191 CACACACAGAAAACACTTGAAGC (reverse primer). After PCR, samples underwent  
192 digestion with *HpyCh4 IV* (New England Biolabs, UK), for 1 hour, at 37 °C, followed by  
193 fragment separation on a 2% agarose/ 0.5 µg/mL ethidium bromide gel, for 1 hour, at 110 V.  
194 Due to post-mortem genotyping, and because a smaller ratio of *tsc2<sup>-/-</sup>* larvae is obtained  
195 compared to *tsc2<sup>+/+</sup>* and *tsc2<sup>+/-</sup>*, variation in group sizes exists throughout experiments.

196

### 197 **Immunohistochemistry (IHC)**

198           After completion of the locomotor assay, 7 dpf zebrafish larvae were sacrificed in ice  
199 cold water. Heads were dissected and placed in 4% PFA for 48 hours, at 4 °C, and then  
200 transferred into a 30% sucrose in PBS solution, at 4 °C. These were then embedded in  
201 optimum cutting temperature (OCT) compound and stored at -80 °C until sectioned. 10 µm  
202 sections were cut and collected onto microscope slides (SuperFrost Plus, Thermo Fisher  
203 Scientific, UK) and stored at -80 °C until used.

204           For IHC, sections were incubated for 2 hours, at room temperature, in a 2% BSA,  
205 10% horse serum and 0.05% TX-100 buffer, followed by an overnight incubation with a  
206 primary antibody against phosphorylated rpS6 (Ser235/236) (1:500; 2211 New England  
207 Biolabs, lot 0023, UK), in a 1 % BSA and 0.05% TX-100 buffer, at 4 °C. The next day, slides  
208 were rinsed in Tris-buffered saline (TBS) and incubated for 2 hours, at room temperature,  
209 with a goat anti-rabbit Alexa Fluor-647 secondary antibody (1:1000; A-21245 Thermo Fisher  
210 Scientific, lot 1805235, UK). After further rinses, sections were counterstained with DAPI  
211 (1:10 000; D1306 Thermo Fisher Scientific, UK) for 10 minutes, rinsed, dried, and mounted.

212           For TUNEL staining (1:10; 12156792910 Roche, lot 26320800, Sigma, UK), slides  
213 underwent the same buffer incubation procedure, excluding antibody incubation. After the  
214 final rinsing step, sections were incubated with TUNEL solution, in the dark, for 1 hour, at 37  
215 °C. Negative control was done by omitting the enzyme solution, while positive control was  
216 performed by previous incubation of sections with 5 mg/mL DNase, for 10 minutes, at 37  
217 °C.

218

### 219 **Imaging and Image quantification**

220           Non-consecutive brain sections were imaged using a Zeiss AxioImager microscope.  
221 Exposure time was constant during image acquisition and background fluorescence was  
222 measured where the primary antibody or enzyme solution was omitted. Pictures were taken

223 with a 20x objective using the AxioVision software. Original images were processed on Fiji  
224 Image J [50], and a region of interest (ROI) was freehand defined around the brain outline.  
225 The average intensity value for each ROI, in each picture, was measured as Intensity  
226 Arbitrary Units (IAU), following background subtraction for each picture. For cell counting,  
227 cells were considered positive if staining for both phosphorylated rpS6 and DAPI were  
228 clearly present. To measure cell size, a similar procedure was used, except five cells per  
229 picture were randomly selected and its cross-sectional area measured. A minimum of three  
230 animals per group and genotype were used, and 3-9 sections per animal were imaged and  
231 counted.

232

### 233 **Statistical Analysis**

234 Statistical analysis was performed in SPSS (IBM SPSS Statistics 22) and GraphPad  
235 Prism 5. Repeated measures two-way ANOVA was used to analyse data from the locomotor  
236 assay (genotype x treatment x exposure to light/dark) and phosphorylated rpS6 positive cell  
237 number (genotype x treatment x number positive cells/section), while a three-way ANOVA  
238 was used to analyse cell size (genotype x treatment x area of cells). Touch response (TR) (yes  
239 x no) was analysed by chi-square test and TUNEL positive cells with the Kruskal- Wallis  
240 test. Normality was assessed with the D'Agostino-Pearson omnibus test. Statistical testing  
241 was followed by Tukey or Bonferroni post-hoc tests.

242 Data are expressed as mean  $\pm$  SEM unless stated otherwise, and significant values were  
243 considered when  $p \leq 0.05$ . All graphs were prepared with GraphPad Prism 5.

244

## 245 **3. RESULTS**

### 246 **3.1. CBD safety profile**

247 To determine the maximum tolerated concentration (MTC) to be used in further  
248 experiments, CBD and DMSO were tested at different concentrations, ranging from 0.3  $\mu$ M  
249 to 125  $\mu$ M CBD in DMSO, and 0.1% to 1% respectively.

250 Concentrations from 5 to 125  $\mu$ M CBD in 1% DMSO, induced varying levels of  
251 toxicity in the larvae, manifested by slow heartbeat, loss of posture and death. We then  
252 compared lower CBD concentrations (0.3 to 2.5  $\mu$ M) in 0.1 or 1% DMSO. Here, the toxicity  
253 of CBD was reduced with the decrease in DMSO concentration from 1% to 0.1%. 1.25  $\mu$ M  
254 CBD in 0.1% DMSO was the highest CBD concentration in which all animals were alive  
255 after 24 hours and showed no signs of gross morphological abnormalities.

256 At a cellular level, toxicity signs were absent from the central nervous system, as  
257 indicated by a non-significant difference ( $p=1.0$ ) in the number of apoptotic cells, as  
258 indicated by positive TUNEL staining (Fig. 1A-C).

259 Regarding touch-response, compared to both the Danieau's and 0.1% DMSO treated  
260 groups, administration of CBD from 6 to 7 dpf did not affect the percentage of responders to  
261 touch ( $\chi^2(2)=2.51$ ,  $p=0.3$ ; 98.0% for Danieau's,  $n=155$ ; 95.8% for 0.1% DMSO,  $n=166$ ;  
262 94.7% for CBD,  $n=171$ ), as manifested by a non-significant difference in the TR of treated  
263 larvae (Fig.2A).

264

## 265 **3.2.Behavioural effects of CBD**

### 266 **3.2.1. CBD does not induce sedation in this zebrafish TSC model**

267 Next, we analysed larvae locomotor behaviour. To test the reported sedative  
268 properties of CBD [23,25], we quantified the average swimming movement of larvae during  
269 the light period, as a reduction of overall exploratory movement can be used as a measure of  
270 sedation [51,52]. There were no statistically significant differences in the baseline  
271 behavioural exploration, between vehicle (0.1% DMSO, 24h incubation) and CBD (1.25  $\mu$ M,

272 24 hour incubation) treated larvae ( $F(1,322)=2.28$ ,  $p=0.1$ ), in the  $tsc2^{+/+}$  ( $2275.9 \pm 190.3$   
273 *actinteg* units,  $n=63$  vs  $1914.7 \pm 289.1$  *actinteg* units,  $n=49$ ,  $p=0.873$ ),  $tsc2^{+/-}$  ( $2349.3 \pm 241.7$   
274 *actinteg* units,  $n=76$  vs  $1907.3 \pm 158.2$  *actinteg* units,  $n=92$ ,  $p=0.338$ ) and  $tsc2^{-/-}$  ( $906.1 \pm$   
275  $208.3$  *actinteg* units,  $n=26$  vs  $732.5 \pm 159.1$  *actinteg* units,  $n=22$ ,  $p=1.00$ ) groups, indicating  
276 that, under these conditions, CBD does not induce sedation (Fig. 2B).

277

### 278 **3.2.2. CBD reduces startle response of zebrafish larvae during the dark period**

279 Zebrafish larvae respond to changes in light beginning from 5 dpf [53,54], and sudden  
280 changes from light to dark induce a startle response. Decreased locomotion after a startle  
281 stimulus is indicative of an anxiolytic effect [52,53,55–57]. Here, CBD ( $1.25 \mu\text{M}$ , 24 hour  
282 incubation) treatment significantly reduced dark-induced movement compared to 0.1%  
283 DMSO ( $F(1,322)=7.26$ ,  $p=0.01$ ) for all genotypes (Fig.2C).

284

### 285 **3.2.3. CBD does not rescue homozygote behavioural phenotype**

286 One of the features of this model is early death of  $tsc2^{-/-}$  homozygotes, between 9 and  
287 11 dpf [42,45]. This can also be seen in other TSC model organisms, such as mice and rats,  
288 which typically die at embryonic day 10-10.5 [58–60]. Additionally, reduced overall  
289 locomotion was recently shown in  $tsc2^{-/-}$  larvae [45]. We therefore assessed the effects of  
290 long-term CBD incubation, from 3 to 10 dpf, on survivability and locomotion. No difference  
291 in the survival of  $tsc2^{-/-}$  larvae ( $\chi^2(1)=0.27$ ,  $p=0.6$ ;  $n=72$  per group) (Fig.3A) nor swimming  
292 ability ( $t(1)=3.06$ ,  $p=0.2$ ;  $1459.8 \pm 366.8$  *actinteg* units,  $n=28$ , vs  $2875.6 \pm 520.1$  *actinteg*  
293 units,  $n=27$ ) (Fig.3B) was found between 0.1% DMSO and CBD treated groups.

294

### 295 **3.3. CBD modulates phosphorylated rpS6**

296 **3.3.1. CBD reduces the number of phosphorylated rpS6 positive cells in *tsc2<sup>+/+</sup>*,**  
297 ***tsc2<sup>+/-</sup>* and *tsc2<sup>-/-</sup>* larvae**

298 We subsequently assessed the impact of CBD treatment upon rpS6 phosphorylation,  
299 which is increased in *tsc2<sup>-/-</sup>* zebrafish [42,45]. In the Danieau's group, we observed increased  
300 phosphorylated rpS6 immunofluorescence in *tsc2<sup>-/-</sup>* zebrafish brains ( $18.0 \pm 2.0$  IAU),  
301 compared to the *tsc2<sup>+/+</sup>* ( $8.0 \pm 1.1$  IAU) and *tsc2<sup>+/-</sup>* ( $8.4 \pm 1.3$  IAU) groups, confirming what  
302 others had previously shown [42,45]. Unexpectedly, an overall increase in phosphorylated  
303 rpS6 intensity was also observed in sections from 0.1% DMSO incubated larvae ( $10.72 \pm 1.7$ ,  
304 for *tsc2<sup>+/+</sup>*,  $6.0 \pm 0.6$ , for *tsc2<sup>+/-</sup>*,  $12.1 \pm 1.4$  IAU, for *tsc2<sup>-/-</sup>*), while reduced  
305 immunofluorescence was found in the CBD groups ( $4.5 \pm 0.6$ , for *tsc2<sup>+/+</sup>*,  $6.0 \pm 0.9$ , for *tsc2<sup>+/-</sup>*,  
306  $11.09 \pm 1.2$ , for *tsc2<sup>-/-</sup>* IAU) (Fig.4A).

307 Section size was accounted for by prior analysis of total brain section size. This  
308 revealed no significant differences between genotypes or treatments (Fig.1D).

309 Quantification of phosphorylated rpS6 positive cells revealed a significant main effect  
310 of treatment, indicating that 0.1% DMSO on its own increased the number of phosphorylated  
311 rpS6 positive cells per section ( $187.1 \pm 13.6$  cells per section) compared to Danieau's ( $116.1$   
312  $\pm 11.7$  cells per section) and to CBD ( $42.8 \pm 13.0$  cells per section). Considering genotype-  
313 specific effects, further analysis revealed that 0.1% DMSO significantly increased the  
314 number of phosphorylated rpS6 positive cells in the *tsc2<sup>+/+</sup>* group, while this increase was not  
315 evident in the *tsc2<sup>+/-</sup>* and *tsc2<sup>-/-</sup>* group. CBD suppressed the DMSO-induced increase in the  
316 number of phosphorylated rpS6 positive cells across all genotypes ( $184.1 \pm 26.0$ , n=12 vs  
317  $10.7 \pm 19.6$ , n=21,  $p < 0.001$ ;  $97.7 \pm 23.2$ , n=15 vs  $3.2 \pm 26.0$ , n=12,  $p = 0.03$ ;  $279.3 \pm 21.2$ ,  
318 n=18 vs  $114.7 \pm 21.2$ , n=18  $p < 0.001$ ; for *tsc2<sup>+/+</sup>*, *tsc2<sup>+/-</sup>* and *tsc2<sup>-/-</sup>* larvae, respectively)  
319 (Fig.4C).

320 Taken together, this data suggests that CBD led to a reduction of phosphorylated rpS6  
321 immunoreactivity in larval brain.

322

323 **3.3.2. CBD reduces the size of phosphorylated rpS6 positive cells in *tsc2<sup>+/+</sup>*, *tsc2<sup>+/-</sup>*  
324 and *tsc2<sup>-/-</sup>* larvae**

325 The phosphorylation status of rpS6 has been correlated with cell size [37,61],  
326 therefore we measured the cross-sectional area of phosphorylated rpS6 positive cells in the  
327 brain (Fig.4B).

328 A genotype dependent increase in cell area was seen in the naïve group. That is, cells  
329 from *tsc2<sup>+/-</sup>* sections were larger than *tsc2<sup>+/+</sup>* ( $22.5 \pm 0.6 \mu\text{m}^2$ , n=133 vs  $19.3 \pm 0.5 \mu\text{m}^2$ ,  
330 n=165; p<0.001), while *tsc2<sup>-/-</sup>* cells were also larger compared to *tsc2<sup>+/-</sup>* ( $28.3 \pm 0.6 \mu\text{m}^2$ ,  
331 n=105 vs  $22.5 \pm 0.6 \mu\text{m}^2$ , n=133; p<0.001) (Fig. 4D). Therefore, while a difference in the  
332 number of phosphorylated rpS6 positive cells between the naïve *tsc2<sup>+/+</sup>* and *tsc2<sup>+/-</sup>* larvae was  
333 absent, here, the area of cells from heterozygote and wild-type animals did differ. Regarding  
334 CBD incubated larvae, these had smaller phosphorylated rpS6 positive cells than the ones  
335 present in the 0.1% DMSO group (Fig.4C). This effect was seen across *tsc2<sup>+/+</sup>* ( $22.8 \pm 0.7$   
336  $\mu\text{m}^2$ , n= 95 vs  $18.0 \pm 0.6 \mu\text{m}^2$ , n=129; p<0.001), *tsc2<sup>+/-</sup>* ( $19.9 \pm 0.6 \mu\text{m}^2$  n=115 vs  $16.3 \pm 0.6$   
337  $\mu\text{m}^2$  n=77; p=0.001) and *tsc2<sup>-/-</sup>* larvae ( $27.4 \pm 0.6 \mu\text{m}^2$ , n=115;  $21.7 \pm 0.6 \mu\text{m}^2$ , n=144;  
338 p<0.001) (Fig.4D). Similar to results seen for cell number, where an effect of 0.1% DMSO  
339 was reported, a DMSO-driven increase in cell size was also observed here (Fig.4D). This was  
340 also significantly suppressed by CBD incubation. Altogether, these data indicate that CBD  
341 reduces genotype and DMSO- induced increase of size in *tsc2<sup>+/-</sup>* and *tsc2<sup>-/-</sup>* zebrafish brain  
342 cells.

343

344 **4. DISCUSSION**



345 CBD is a non-psychoactive component of *Cannabis*, that has increasingly been  
346 recognised as the basis for pharmacology intervention in a host of diseases [19,20,62]. Here  
347 we examine the effects of CBD to modulate aberrant mTORC1 signalling in zebrafish  
348 carrying a *tsc2* mutation.

349 Anxiety is a TSC-associated neuropsychiatric disorder and evidence indicates altered  
350 serotonin signalling as a biological mechanism [65-68]. One of the proposed targets for CBD  
351 is the 5-HT<sub>1A</sub> receptor, where it has been shown to bind and to have agonist functions at  
352 concentrations  $\geq 10$   $\mu$ M *in vitro* [73,74]. Several serotonin receptors, orthologues to human  
353 receptors, have been shown to be expressed in zebrafish larvae, including the 5-HT<sub>1A</sub> receptor  
354 [72,79]. This is pertinent to the current study with the 5-HT<sub>1A</sub> receptor a proposed site of  
355 action for CBD [73,74]. However, contrasting with the function of mammalian 5-HT<sub>1A</sub>  
356 receptors, the role of these receptors on anxiety behaviour in zebrafish is less defined. For  
357 example, extracellular serotonin content has been reported to have contrasting effects on  
358 anxiety in the same adult zebrafish species [72,80]. Larvae, in contrast to their adult  
359 counterparts, exhibit a transient elevation in motor activity in response to sudden onset of  
360 darkness [53,64,81]. Dark avoidance was shown to be modulated by anxiolytic drugs such as  
361 the 5-HT<sub>1A</sub> agonist, buspirone, which increased dark preference patterns in zebrafish larvae  
362 [81].

363 Here we demonstrate a CBD induced reduction in startle response across genotypes  
364 However, a limitation of this model is that *tsc2*<sup>-/-</sup> larvae do not reach adulthood and,  
365 therefore, later behavioural testing cannot be performed to confirm an anxiolytic effect of  
366 CBD in this genotype. Nonetheless, further studies in *tsc2*<sup>+/-</sup> larvae could still be beneficial to  
367 elucidate possible effects of CBD in TSC, given the clinical TSC population are heterozygous  
368 [7].

369           Several molecular processes have been proposed to modulate CBD actions [19]. The  
370 serotonergic system is one such example and modulation here could provide control of other  
371 TSC features such as epilepsy, highly prevalent in TSC patients [17]. Evidence from epilepsy  
372 studies indicates that a reduction in serotonin concentration promotes seizures, while reduced  
373 serotonin binding to the 5-HT<sub>1A</sub> receptor has been reported in epileptogenic foci [83,84].  
374 Studies also indicate that TSC patients present with increased tryptophan uptake localised to  
375 epileptic foci [85]. Given that CBD has been shown to reduce seizures in pathologies with  
376 different aetiologies [23,25,89], and that *tsc2*<sup>-/-</sup> zebrafish, and other TSC models, have been  
377 shown to exhibit abnormal brain activity [45,90–92], it would be relevant to further study its  
378 role in the serotonergic system of TSC models.

379           A hallmark of TSC across all experimental models is an increase in mTOR activity  
380 [1,3,5,6,42,45]. Activation of mTOR leads to an increase in the ratio of downstream targets  
381 phosphorylated rpS6 /total rpS6, both in *in vitro* and *in vivo* models [88,91,95,96]. Therefore,  
382 phosphorylated rpS6 is often used as a read out of mTOR activation [97–99]. The reduction  
383 of rpS6 phosphorylation presented here is in line with published work where CBD treatment  
384 was found to modulate the mTOR pathway. In breast cancer cells, incubation with CBD has  
385 been observed to modulate Akt, a kinase upstream of mTOR, as well as 4E-BP1 and cyclin D  
386 [30,31]. Another study also reported the reduction of ERK phosphorylation, a kinase  
387 upstream of mTOR [31]. The effects observed here with 0.1% DMSO may indicate a  
388 proinflammatory response [101-103] with subsequent activation on mTOR [104,105].

389           The mTOR-S6K-S6 axis is also known to have a major role in controlling cell size  
390 [98,106]. In fact, we saw that Danieau's incubated animals showed a mutation-dependent  
391 increase in cell size, with *tsc2*<sup>-/-</sup> brain cells bigger than *tsc2*<sup>+/-</sup>, followed by *tsc2*<sup>+/+</sup>. These  
392 results are similar to previous work on this model, where a difference in size was found  
393 between *tsc2*<sup>+/+</sup> and *tsc2*<sup>-/-</sup>, in liver, brain, and spinal cord cells [42]. In accordance with a

394 reduction in phosphorylated rpS6 positive cells there was a corresponding CBD effect on the  
395 cross-sectional area of brain cells. A comprehensive analysis of mTOR activity in  
396 phosphorylated rpS6 cells through enzymatic assay would definitely link the disruption in  
397 phosphorylated rpS6 to mTOR activity which remains unresolved in this current study.

398         However, contrasting effects on the mTOR signalling pathway by CBD have also  
399 been reported. For example, in amphetamine-sensitized rats, CBD reduced levels of pGSK-  
400  $3\beta$  and pAkt, but importantly it induced an increase of pmTOR and pS6K [35]. To further  
401 demonstrate the effect of CBD on mTOR specifically, this effect was reversed with the  
402 mTOR inhibitor, Torin 2 [35]. Additionally, administration of 10 mg/kg CBD to a mouse  
403 model of multiple sclerosis revealed increased pPI3K, pAkt, pmTOR and pS6K in spinal cord  
404 tissue. Importantly, in this model, basal levels of mTOR pathway activation were shown to be  
405 decreased [36]. Regarding the zebrafish model used here, we observed a decrease in  
406 phosphorylated rpS6, which could result from a reduction in mTOR activation. However, the  
407 survival and locomotion data regarding the chronically CBD-treated *tsc2*<sup>-/-</sup> larvae highlight  
408 that this modulation of mTOR was insufficient to impact these whole system outputs.

409

## 410         **5. CONCLUSION**

411         In the current study, using a TSC zebrafish model, we demonstrate that CBD was  
412 tolerable, while behavioural testing showed that CBD exhibited an anxiolytic profile without  
413 sedative effects. Additionally, we showed modulation of rpS6 manifested by the reduction of  
414 the number and size of phosphorylated rpS6 positive cells in the brain. Altogether, these data  
415 demonstrate that CBD modulates aberrant mTOR signalling in a model of TSC. It provides a  
416 rationale for further investigation into CBD as a therapeutic agent in diseases where mTOR  
417 signalling is disrupted.

418

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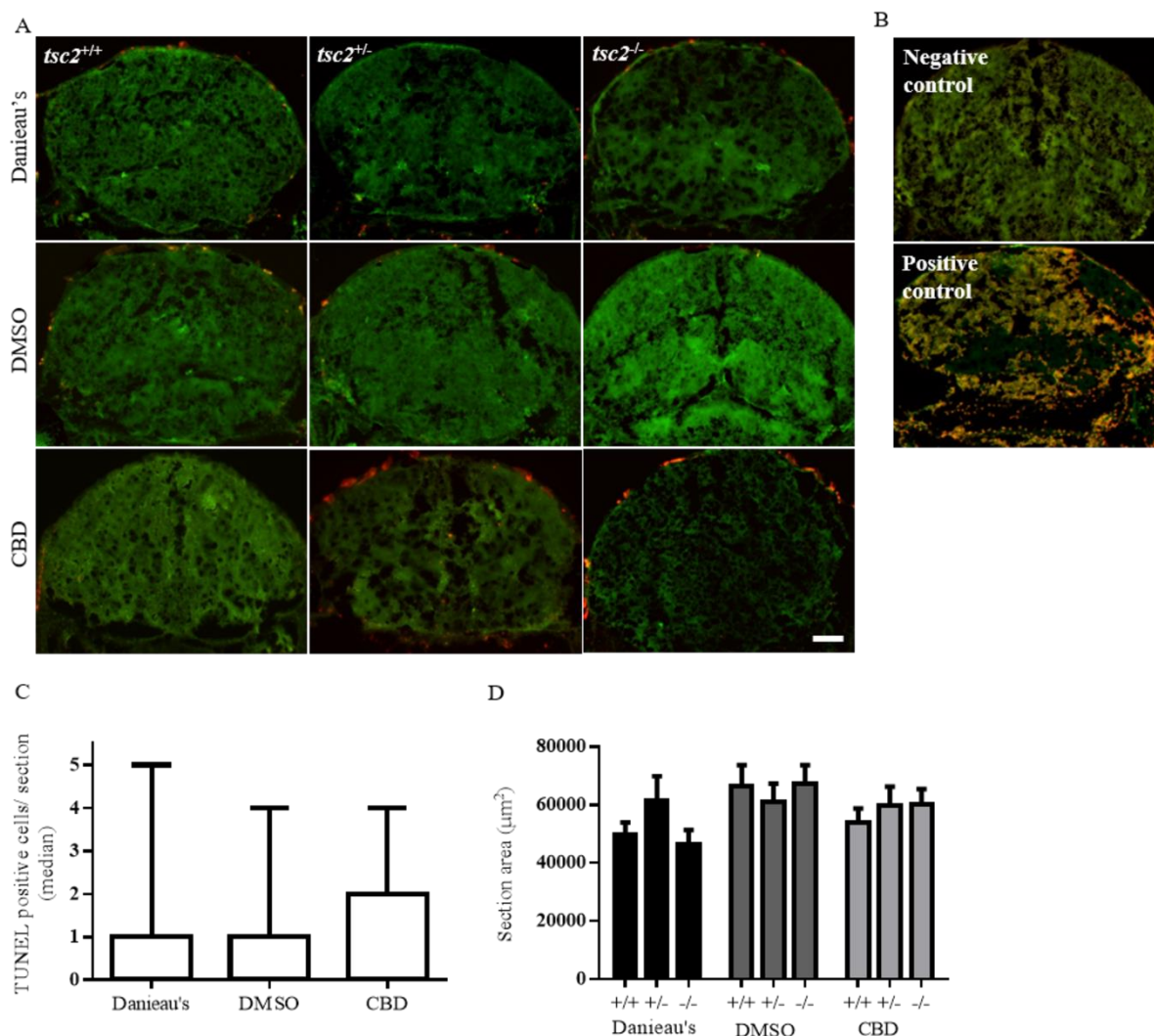
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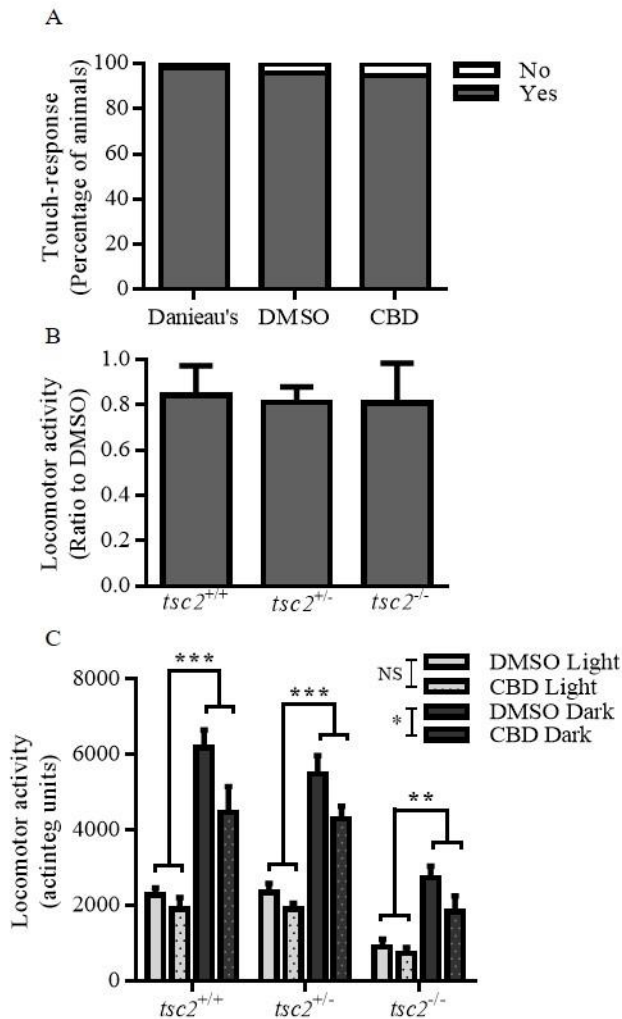
## Figures



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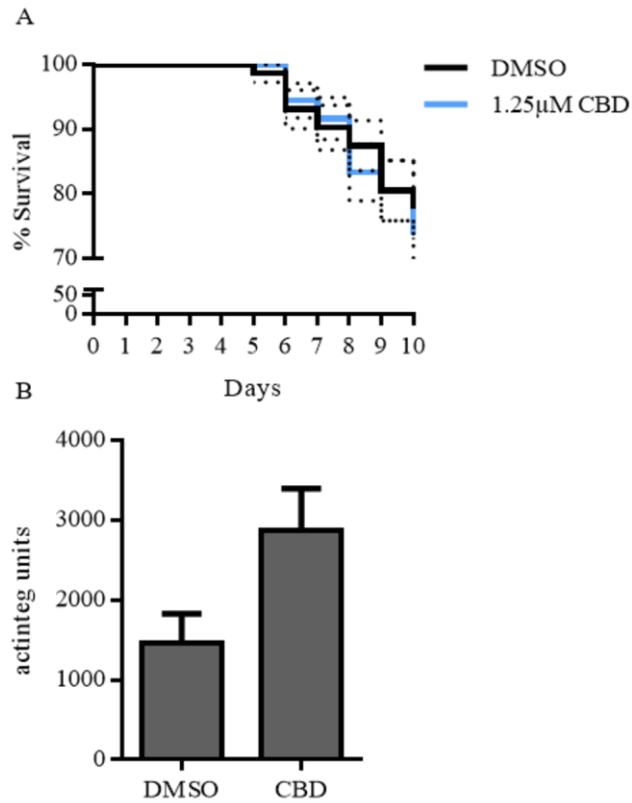
796 **Figure 1: TUNEL labelling in the larval brain.** (A) Representative pictures of the midbrain, for each  
797 treatment (Danieau's, 0.1% DMSO and 1.25  $\mu\text{M}$  CBD) and genotype (*tsc2*<sup>+/+</sup>, *tsc2*<sup>+/-</sup> and *tsc2*<sup>-/-</sup>), showing  
798 TUNEL positive cells in red and tissue autofluorescence in green. (B) Representative pictures of negative  
799 control, where enzyme solution was omitted, and of positive control, incubated with DNase. (C) Median  
800 number of TUNEL positive cells in each section analysed, showing no statistical differences between treatment  
801 groups. (n=3 animals per group; p=1.0. Data shown as median and minimum to maximum values) (D)  
802 Measurement of brain sections' area showed no significant differences in cross-sectional size, regardless of  
803 genotype (F(2,209)=0.30, p=0.7) or treatment (F(2,209)=2.92, p=0.06) (n= 3-4 animals per group, 15-30  
804 sections per group measured). Scale=50  $\mu\text{m}$ .

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807 **Figure 2: Treatment and genotype effects on larval locomotor behaviour:** (A) Zebrafish touch-response is  
 808 not altered in the presence of 0.1% DMSO nor CBD. Pooled data demonstrating no significant differences  
 809 ( $\chi^2(2)=2.5$ ,  $p=0.3$ ) between the percentage of zebrafish responding to touch in each treatment group ( $n=152$  for  
 810 Danieau's,  $n=165$  for 0.1% DMSO,  $n=163$  for CBD). Values are shown as percentage of "Yes" or "No"  
 811 response. (B) CBD has no effect on locomotor activity during light phase. *Actinteg* units normalised to vehicle  
 812 (0.1% DMSO) values demonstrate lack of effect on swimming activity under light ( $F(1,322)=2.28$ ,  $p=0.1$ ),  
 813 following exposure to CBD, indicating the absence of sedating properties ( $n=49-63$  for *tsc2*<sup>+/+</sup>,  $n=76-92$  for  
 814 *tsc2*<sup>+/-</sup>,  $n=22-26$  for *tsc2*<sup>-/-</sup>). (C) CBD reduces zebrafish locomotor activity after a dark startling stimulus.  
 815 Exposure to CBD during the light period (light bars) did not alter the average larval movement in any genotype.  
 816 In the presence of a dark startling stimulus (dark bars), CBD induced a reduction of the average swimming  
 817 activity ( $F(1,322)=7.26$ ,  $p=0.01$ ) Values are shown as mean *actinteg* units  $\pm$  SEM, \*\*\* $p<0.001$ , \*\* $p<0.01$ ,  
 818 \* $p<0.05$

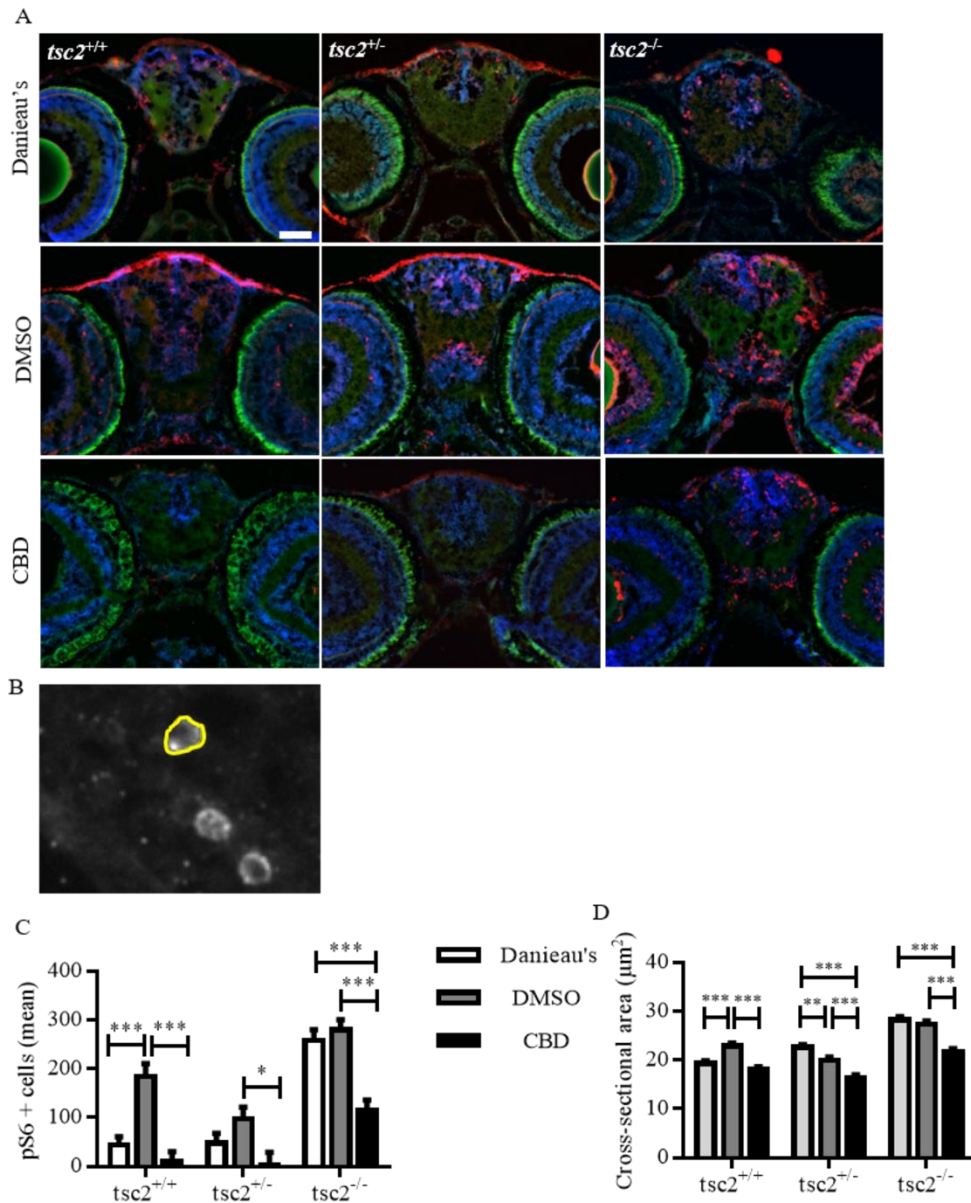


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821 **Figure 3: CBD does not improve *tsc2<sup>-/-</sup>* larvae survival nor rescues movement deficits.** (A) Treatment with  
 822 1.25 μM CBD, from 3-10 dpf, did not alter *tsc2<sup>-/-</sup>* larvae survival compared to vehicle (n=72 per group). (B)  
 823 CBD treatment from 3-6 dpf CBD did not modulate movement deficits in *tsc2<sup>-/-</sup>* larvae (t(1)=3.06, p=0.2; 1459.8  
 824 ± 366.8 vs 2875.6 ± 520.1 *actinteg* units, n= 28 for 0.1% DMSO and n=27 for CBD, data presented as mean ±  
 825 SEM).

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828 **Figure 4: CBD reduces the number and size of phosphorylated rpS6 (Ser235/236) positive cells.** (A)

829 Representative pictures of the forebrain of Danieau's, 0.1% DMSO and CBD incubated larvae. Blue represents

830 DAPI, green tissue autofluorescence and red phosphorylated rpS6 (Ser235/236) positive cells. (C)

831 Quantification of the number of phosphorylated rpS6 (Ser235/236) positive cells in larval brain sections. 0.1%

832 DMSO incubation increased the number of phosphorylated rpS6 (Ser235/236) positive cells in the *tsc2*<sup>+/+</sup> group.

833 CBD reduced the number of positive cells in all genotypes compared to 0.1% DMSO but only in the *tsc2*<sup>-/-</sup>

834 group, compared to the Danieau's incubated larvae (F(4,44)=3.14, p=0.02; n= 3-9 sections analysed, from 3-4

835 animals per group). (B) Magnification of a 0.1% DMSO-treated *tsc2*<sup>+/+</sup> brain section exemplifying how the

836 cross-sectional area of phosphorylated rpS6 (Ser235/236) positive cells was measured. (D) CBD incubation



837 induced a reduction of the average cross-sectional area of phosphorylated rpS6 (Ser235/236) positive cells in all  
838 genotypes, compared to 0.1% DMSO ( $F(4,1050)=9.06$ ,  $p<0.001$ ;  $n= 77-115$  cells per genotype and treatment  
839 from 3-4 animals per group). Values are shown as mean  $\pm$  SEM. \*\*\* $p<0.001$ , \* $p<0.05$ . Scale=50  $\mu$ m.

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