

1 **Original Research Paper**

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3 **Basal astrocyte and microglia activation in the central nervous system of Familial Hemiplegic**

4 **Migraine Type I mice**

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20

1 **Abstract**

2 **Background**

3 Gain-of-function missense mutations in the  $\alpha_{1A}$  subunit of neuronal Cav2.1 channels, which define  
4 Familial Hemiplegic Migraine Type 1 (FHM1), result in enhanced cortical glutamatergic  
5 transmission and a higher susceptibility to cortical spreading depolarization. It is now well established  
6 that neurons signal to surrounding glial cells, namely astrocytes and microglia, in the central nervous  
7 system, which in turn become activated and in pathological conditions can sustain  
8 neuroinflammation. We and others previously demonstrated an increased activation of pro-algogenic  
9 pathways, paralleled by augmented macrophage infiltration, in the trigeminal ganglia of FHM1  
10 mutant mice *ex vivo* and *in vitro*. Hence, we hypothesize that astrocyte and microglia activation may  
11 occur in parallel in the central nervous system.

12 **Methods**

13 We have evaluated signs of reactive glia in brains from naïve FHM1 mutant mice in comparison with  
14 wild type animals by immunohistochemistry and Western blotting.

15 **Results**

16 Here we show for the first time in the naïve FHM1 mutant mouse brain signs of reactive astrogliosis,  
17 and microglia activation.

18 **Conclusions**

19 Our data reinforce the involvement of glial cells in migraine, and suggest that modulating such  
20 activation may represent an innovative approach to reduce pathology.

21

22

23 **Keywords**

24 Reactive astrocytes, activated microglia, neuroinflammation, migraine

25

## 1 **Introduction**

2 Familial hemiplegic migraine (FHM) is a monogenic subtype of migraine with aura, characterized by  
3 recurrent attacks of headache that are accompanied by auras consisting of transient neurological  
4 symptoms that include visual, sensory, and motor disturbances [1]. Transgenic FHM1 mutant mice  
5 that express gain-of-function missense mutations in *CACNA1A*, which encodes the  $\alpha_{1A}$  subunit of  
6 voltage-gated  $\text{Ca}_v2.1$  calcium channels, revealed neuronal involvement with increased  $\text{Ca}_v2.1$   
7 channel function and enhanced cortical glutamatergic neurotransmission that can explain the  
8 increased susceptibility to experimentally induced cortical spreading depolarization (CSD) [2, 3]. The  
9 consequences of the mutation may, however, extend to cell types that surround neurons in the brain.  
10 For instance, there is evidence suggesting that neuronal hyperexcitability can trigger so-called  
11 “neurogenic neuroinflammation”, which involves the vascular and glial cell components of brain  
12 tissue, and can recruit immune cells from the bloodstream [4]. Although an homeostatic role for  
13 neuroinflammation has been postulated, it might also lead to further release of pro-inflammatory  
14 mediators by surrounding activated glial cells, namely reactive astrocytes and microglia, which in  
15 turn may trigger or aggravate an underlying pathological condition [4]. Notably, in primary cultures  
16 from from postnatal day 11 trigeminal ganglia tissue (containing a mix of trigeminal ganglion neurons  
17 and satellite glial cells) of FHM1 mutant mice, satellite glial cells exhibit an increased release of  
18 inflammatory mediators and an up-regulation of specific pro-algogenic receptors [5], but it is  
19 questionable whether findings in cultured cells can be translated to the intact trigeminal ganglion as  
20 no such increases were observed in isolated trigeminal ganglia from mice that were 11-14 weeks old  
21 [6]. In addition, trigeminal infiltration of macrophages was observed, pointing to the development of  
22 neuroinflammation in the peripheral nervous system [7]. Hence, both abnormal neuronal and glial  
23 cell function, and likely their interaction, may contribute to FHM pathophysiology. To further  
24 examine the involvement of glial cell activation in FHM, we here investigated whether the FHM1  
25 R192Q missense mutation promotes the development of a reactive glia phenotype in the central  
26 nervous system of naïve mutant mice.

1

## 2 **Methods**

### 3 **Animals**

4 Three- to 4-month-old male homozygous FHM1 R192Q knock-in (KI) (“R192Q”) and wild-type  
5 (“WT”) mice were used. The KI mice were generated by introducing the human FHM1 pathogenic  
6 R192Q missense mutation in the orthologous *Cacnala* gene using a gene targeting approach [8].  
7 Genotyping was performed as described before [8]. Mice were kept under standard conditions  
8 (temperature:  $22 \pm 2^\circ\text{C}$ ; relative humidity:  $50 \pm 10\%$ ; light regimen: 12-hour light/12-hour dark cycle)  
9 with food and water *ad libitum*. All experimental procedures were in strict accordance with the Italian  
10 and EU regulation on animal welfare and were previously approved by the local ethics committee,  
11 and by the Italian Ministry of Health (authorization #736/2015-PR). All results are reported according  
12 to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Researchers were  
13 blinded for genotype information, and animals were randomised. Seven animals for both the WT and  
14 the FHM1 mutant group were analyzed per type of staining.

15

### 16 **Immunohistochemistry**

17 Mice were anesthetized with 1.5% isoflurane and perfused with 4% paraformaldehyde, as described  
18 [9]. Brains were removed, postfixed in 4% formalin for 60 minutes, cryoprotected in 30% sucrose for  
19 24 hours, embedded in mounting medium (OCT; Tissue Tek, Sakura Finetek, Zoeterwoude, The  
20 Netherlands), and kept at  $-80^\circ\text{C}$  until use. Twenty  $\mu\text{m}$ -thick coronal brain sections were collected for  
21 immunohistochemistry. Rabbit antibodies directed against: (i) astrocyte marker glial fibrillary acidic  
22 protein (anti-GFAP, 1:600; Dako, Milan, Italy), (ii) microglia marker ionized calcium binding adaptor  
23 molecule 1 (anti-Iba1, 1:500; Wako, Richmond, VA, USA), (iii) GPR17 receptor (home-made,  
24 1:20,000); or mouse antibodies directed against mature oligodendrocyte marker glutathione S-  
25 transferase pi (anti-GST $\pi$ , 1:500; MBL International, Woburn, MA, USA) were used. As secondary  
26 antibody, goat anti-rabbit or goat anti-mouse (1:600; Thermo Fisher Scientific, Monza, Italy)

1 conjugated to AlexaFluor®488 or AlexaFluor®555, respectively, were used. Cell nuclei were  
2 counterstained with the Hoechst33258 dye (1:20,000; Sigma-Aldrich, Milan, Italy). Cell staining was  
3 evaluated by a Zeiss Axioskop fluorescent microscope (Carl Zeiss, Milan, Italy), with the aid of the  
4 NIH ImageJ software, as described below.

5

#### 6 *Image analysis*

#### 7 *Cell counting*

8 For Iba1, GPR17, and GST $\pi$  staining, the number of immunopositive cells was counted in whole  
9 sections and in selected brain areas (i.e., the cortex and corpus striatum) acquired at 20X  
10 magnification, and expressed as the number of cells/area in  $\mu\text{m}^2$ .

11

#### 12 *Densitometric analysis*

13 For GFAP staining, a digital image of the immunostained brain sections was acquired at low (10X)  
14 magnification, and the mean values of pixel intensity were automatically evaluated by using the NIH  
15 Image-J software [10], and expressed as integrated density compared to values obtained in WT mice  
16 set to 100%.

17

18

#### 19 *Evaluation of microglia morphology and branch complexity*

20 Fluorescent images of Iba1-positive microglia in the cortex and the corpus striatum were acquired at  
21 higher (40X) magnification, and converted to binary grayscale to better analyze cell morphology by  
22 the “Simple neurite tracer” tool of the Fiji-ImageJ software [11]. In each brain area, 3 randomly  
23 chosen cells in each of 10 randomly chosen optical fields (for a total of 30 cells/animal) were  
24 manually traced (see Figure 2A for representative images) and (i) the area covered by cell processes  
25 (which is proportional to the total cell ramification), (ii) the number of junctions and branches and  
26 the mean and maximal length of the latter (which reflects the complexity and ramification of cells),

1 and (iii) the number of triple and quadruple points (an additional indicator of cell complexity which  
2 indicates junctions where a single branch ramifies in three or four) were evaluated with the Skeleton  
3 analysis tool of the same software [11].

4

## 5 **Western blotting**

6 Western blotting analysis of brain tissues was performed, as described before [12]. Mouse antibodies  
7 directed against: i) GFAP (1:1,000; Cell Signaling, Danvers, MA, USA), or (ii) the marker of mature  
8 oligodendrocyte 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase, 1:250; Millipore, Vimodrone,  
9 MI, Italy) and rat antibody against myelin basic protein (MBP, 1:500; Millipore) as marker of  
10 myelinating oligodendrocytes, were used. Beta-actin (rabbit anti- $\beta$ -actin, 1:1,500; Sigma-Aldrich) or  
11  $\alpha$ -tubulin (mouse anti  $\alpha$ -tubulin, 1:1,000; Sigma-Aldrich) expression was analyzed as internal  
12 loading controls. Next, filters were incubated with species-specific secondary antibodies conjugated  
13 to horseradish peroxidase (goat anti-rabbit, 1:4,000 and goat anti-mouse, 1:2,000; both from Sigma-  
14 Aldrich; goat anti-rat, 1:2,000; Thermo Fisher Scientific). Protein detection was performed by ECL  
15 (BioRad, Milan, Italy). After autoradiography, the relative amount of protein was evaluated by the  
16 NIH Image-J software, normalized for the corresponding  $\beta$ -actin or  $\alpha$ -tubulin values, and expressed  
17 with respect to values obtained in WT mice set to 1.

18

## 19 **Statistical analysis**

20 Scatter plots show single data and their mean $\pm$ standard deviation (S.D.). Continuous variables were  
21 compared by use of Mann-Whitney *U* test. Two-tailed P-value $<$ 0.05 was considered statistically  
22 significant. Testing of hypotheses was performed by the free software R [13].

23

## 24 **Results**

25 *Activated astrocytes in the naïve FHMI R192Q mutant brain.*

1 We first examined the expression of GFAP, a typical astrocyte marker whose expression is increased  
2 during reactive astrogliosis [14]. Counting cell number was not feasible, due to the complex  
3 interconnesions among cells; thus, we measured the fluorescence intensity of immunostaining (see  
4 Methods). In the WT cortex the highest abundance of stained cells is seen in the subventricular zone  
5 lining lateral ventricles (Figure 1A), similar to what was reported before for that brain region [15]. In  
6 the naïve FHM1 mutant mouse brain, however, GFAP immunoreactivity was typically present in  
7 higher cortical layers (Figure 1A; see magnification in inset), with a significant  $19.3 \pm 4.9\%$  increase  
8 in fluorescence when compared to the WT brain (Figure 1B), suggesting reactive astrogliosis.  
9 Western blotting analysis of cortical tissue confirmed the results by showing a significant  $25.4 \pm 8.7\%$   
10 increase in GFAP expression. In the striatum, GFAP expression was not significantly increased  
11 ( $+26.3 \pm 15.3\%$ ,  $P=0.32$ ; Figure 1C), although the results were quite variable among samples.  
12 Moreover, Western blotting analysis of TG tissue also did not show a genotypic difference  
13 (normalized GFAP expression  $1.00 \pm 0.12$  vs  $1.19 \pm 0.10$  in the TG from WT and R192Q mice,  
14 respectively;  $P=0.144$  Student's t test).

15

#### 16 *Hyper-ramified resident microglia in the naïve FHM1 R192Q mutant brain.*

17 Next, we evaluated the number and morphology of microglial cells in the brains of FHM1 mutant  
18 and WT mice, to assess whether cells are in a resting, activated, or intermediate state [16]. The total  
19 number of Iba1-positive microglia cells in coronal brain sections was found significantly increased  
20 in FHM1 mutant mice (Figure 2A, 2B) suggesting cell proliferation. In both the cortex and corpus  
21 striatum, the increased cell number was paralleled by an increase in the total area covered by  
22 microglia ramifications (Figure 3A, C), as further evidenced by a higher number of long branches  
23 (Figure 3B-D), and of junctions and triple points which are representative of more complex  
24 ramification of cell processes (Figure 3E, F; see images in Figure 2A). The number of quadruple  
25 points was not different between the two groups ( $P=0.165$ ; Figure 3G). Thus, our data show  
26 morphological changes of microglia towards a hyperramified intermediate state [16], which likely

1 reflects cell adaptation in response to changes in the (pathological) environment [16], and suggest an  
2 intensification of surveillance of this cell population.

3

4

5 *No basal changes in markers of mature oligodendrocytes in the FHM1 mutant brain.*

6 Based on the known reciprocal influence of neurons and oligodendrocytes, we have also evaluated  
7 possible changes in the expression of markers of myelination in the brains of FHM1 mutant mice.  
8 Counting the number of CNPase-positive mature and MBP-positive myelinating oligodendrocytes  
9 after immunohistochemistry [17] was not possible due to the high number of cells with a very  
10 complex morphology (not shown). We, therefore, performed semi-quantitative Western blotting  
11 analysis of portions of the cortex from naïve FHM1 R192Q and WT mice. Results show no alteration  
12 in the expression of either protein (CNPase: cortex, P=0.295; c. striatum, P=0.836; MBP: cortex,  
13 P=0.945; c. striatum, P=0.445; Figure 4). Nevertheless, a reduction in the number of immature and  
14 pre-oligodendrocytes expressing the GPR17 receptor [18] was detected in FHM1 mutant brains  
15 (P=0.017; Figure 5A, B). When analyzing specific brain areas, differences in the number of GPR17-  
16 expressing cells were observed in the corpus striatum (P=0.038), with similar values (P=0.465) in the  
17 cortex (Figure 5B), whereas the expression of GST $\pi$ , a marker of intermediate oligodendrocyte  
18 maturation was similar between WT and FHM1 mice ( $65.6 \pm 25.6$  vs.  $59.4 \pm 17.6$  positive  
19 cells/counted field, respectively; P=0.390). Overall, our results suggest that the activity of mutated  
20 neuronal Cav2.1 channels does not alter the basal maturation of oligodendrocytes but reduces the  
21 fraction of immature and pre-oligodendrocytes expressing GPR17.

22

## 23 **Discussion**

24 Here we present evidence for increased glial cell reactivity in the naïve FHM1 R192Q mouse brain,  
25 as evidenced by morphological signs of astrogliosis, and of reactive microglia. Because of the  
26 previously reported increased glutamate neurotransmitter release from excitatory cortical neurons in



1 the FHM1 mutant brain [3], we set out to investigate whether the observed enhanced  
2 neurotransmission could also modify neuron-to-glia cell communication, directly or indirectly, and  
3 promote the basal activation of resident glial cells, thus contributing to the FHM1 phenotype.

4 Given that non-neuronal cells, in particular glia cells, contribute to the initiation and maintenance of  
5 chronic pain [19], which is now referred to as a “gliopathy” [20], one possible role of permanently  
6 activated astrocytes and microglia is to contribute to the pathological brain environment relevant to  
7 FHM1 pathology.

8 Both astrocytes and microglia can respond to external stimuli and to changes in the environment by  
9 exerting either detrimental or protective functions [14, 16]. Although we do not have functional  
10 evidence of the polarization of these cells towards an overall pro- or anti-inflammatory phenotype in  
11 FHM1 brain, it is relevant that here we detected changes in cell morphology, which for glial cells  
12 represent an indicative parameter of altered cell function, in the absence of a CSD trigger. Our data  
13 show that the presence of mutant neuronal  $Ca_v2.1$  channels leads to what we would interpret as an  
14 adaptation of glial cells that have to cope, from time to time, with increased levels of glutamate (and  
15 likely ATP) and  $K^+$  ions, and likely prime cells to be ready to react to possible subsequent triggers.

16 Concerning astrocytes, consistent with the known role of calcium waves in cell-to-cell  
17 communication [21], here we have detected signs of reactive astrogliosis and we can speculate that  
18 this could be a factor that modulates headache pain. Afterall, astrocytes play a key role in the blood-  
19 brain barrier and control of the blood-brain exchanges of chemicals and other potential migraine  
20 triggers, such as certain nutrients [14]. Additionally, glial cell dysfunction has been implicated in  
21 FHM2 mutant mice [22, 23], in which loss-of-function mutations in the  $\alpha 2$  subunit of  $Na^+/K^+$   
22 ATPases expressed in astrocytes resulted in increased cortical glutamatergic neurotransmission, but  
23 in this case as the result of inadequate  $K^+$  and glutamate buffering ability due to abnormal glial  
24 function [24]. Similar to FHM1 mice, FHM2 mice were shown to be more susceptible to  
25 experimentally induced CSD [22, 25]. Thus, data from a different model of FHM suggests that  
26 astrocytes can contribute to migraine pathology.

1 Microglia are the immunocompetent cells in the central nervous system, which constantly scan the  
2 environment and contact surrounding neurons and astrocytes. Under pathological conditions,  
3 microglia acquire an activated phenotype, accompanied by the release of cytokines, increased  
4 proliferation, migration and phagocytic activity [19]. Increased neuronal activity, as seen in epilepsy  
5 or during CSD propagation [26, 27], is another trigger for microglia reaction. Additionally, higher  
6 microglia dynamic movements are detected hours after induction of CSD, and the level of microglia  
7 activation is proportional to the number of CSD waves [28]. The transition between resting and fully  
8 activated (i.e., amoeboid phagocytic microglia) is characterized by the presence of an intermediate  
9 phenotype, which can be recognized by increased branching as in naïve FHM1 brain, representing a  
10 “warning” state of cells to be fully able to respond to subsequent harms [16]. Very recent data show  
11 that elongation of processes is mandatory for microglia to shift from a pro- to an anti-inflammatory  
12 phenotype, which can more efficiently patrol the surrounding brain tissue [29]. Thus, simply based  
13 on changes in cell morphology we cannot definitively assume whether hyper-ramified microglia have  
14 an overall detrimental or beneficial role. Further studies are needed to analyze the expression of  
15 specific markers of microglia polarization towards anti-inflammatory M1 or pro-inflammatory M2  
16 phenotype [16]. Nevertheless, we can conclude that in FHM1 mutant brains under basal conditions,  
17 already after minor disturbances in brain activity, microglia enlarge their area of patrolling by  
18 extending cell processes being therefore able to react more efficiently, if needed, and to release  
19 various signaling molecules involved in the cross-talk with the other cell populations.

20 Oligodendrocytes and their precursor cells (OPCs) are also involved in cell-to-cell communication in  
21 the brain by reacting to neuronal signals and to activated astrocytes and surrounding microglial cells  
22 [30, 31]. To confirm their relevance to migraine pathology, altered patterns of myelination have been  
23 observed in the brains of migraineurs [32], and transient disruption of myelin structure is induced by  
24 CSD [33]. Our data show no basal changes in the expression of mature oligodendrocytes in FHM1  
25 brains, but a significant reduction of GPR17 expression.

1 As GPR17 receptor expression must be down-regulated to allow for the terminal maturation of pre-  
2 oligodendrocytes [34], its reduced expression could accelerate oligodendrocyte maturation.  
3 Nevertheless, since GPR17-expressing OPCs show a higher susceptibility to toxic signals, such as  
4 pathologically elevated concentrations of ATP [18] that are likely to be found in the FHM1 mutant  
5 brain as an additional consequence of the enhanced glutamatergic transmission [35], at present we  
6 cannot exclude that the observed reduction in their number is due to cell death.

7 A recent genetic study, which used genotype data from thousands of migraine patients and controls  
8 tested in genome-wide association studies (GWAS), evaluated gene sets containing astrocyte- and  
9 oligodendrocyte-related genes and found an association with the common forms of migraine [36].  
10 This supports the concept that genetic factors underlie glial cell dysfunction in migraine, so beyond  
11 the correlation presented here in FHM1 mutant mice. Glia cell activation, which is suggestive of  
12 neuroinflammation, may therefore be relevant to various types of migraine, although this has not  
13 firmly been established for common forms of migraine. It might be that glial cell activation is a  
14 relevant reflection of the basal reactive state of a FHM1 mutant brain that might influence how  
15 migraine triggers affect the brain. In that respect it is of interest that experimentally induced CSDs  
16 generate a molecular signature of activated inflammatory pathways specifically linked to interferon-  
17  $\gamma$  signaling that is seen in the FHM1 mutant mice, but not the wild-type control mice [37]. Together  
18 with data in FHM2 mice (see above), all in all the information supports the involvement of glial  
19 dysfunctions eventually leading to neuroinflammation in a migraine-relevant context.

20 As mentioned above, one limitation of our study is that the claim of glial cell activation in FHM1  
21 mutant brains is only based on changes in cell morphology, i.e., without investigating its functional  
22 consequences. Still, the observed features are regarded as highly representative of the functional state  
23 of the cells, so can be considered sufficient proof that our findings are genuine.

24 Taken together, we provided evidence that FHM1 mutations, at least the R192Q missense mutation,  
25 in a transgenic mouse model, not only impacts neurons, but also glia cells. This study therefore  
26 reinforces the concept that altered neuron-to-glia communication in naïve FHM1 mutant mice might

1 contribute to the disease phenotype, and that normalizing glia cell function could have potential to  
2 treat migraine. We feel that our observations are relevant to the understanding of (hemiplegic)  
3 migraine pathophysiology as one could ask the question whether the same occurs in patients.

4

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11

#### 12 **Key Findings**

- 13 - Reactive astrocytes and activated microglia in the brains of FHM1 mutant mice that express  
14 neuronal Cav2.1 calcium channels with R192Q-mutated  $\alpha_{1A}$  subunits under basal conditions
- 15 - Normalization of glia cell reactivity maybe a promising avenue for drug treatment

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1 **Figure legends**

2 **Figure 1. Reactive astrogliosis in the naïve FHM1 mutant mouse brain.** A: coronal sections from  
3 the brains of wild-type (WT) and R192Q mutant mice stained with anti-GFAP antibody.  
4 Representative pictures are shown with magnified details. Scale bars: 1.5 mm. B: quantification of  
5 GFAP immunoreactivity by densitometric analysis. Scatter plots show the results obtained in seven  
6 animals for each group. The pixel intensity values are expressed as mean  $\pm$  S.D. compared to WT  
7 animals set to 100%. P=0.007 with respect to WT, two-tailed non-parametric Mann-Whitney test. C:  
8 evaluation of GFAP expression in cortical and striatal tissue by Western blotting.  $\beta$ -actin was utilized  
9 as internal loading control (see representative filters at the bottom). Scatter plots show the optical  
10 density of protein bands from seven animals for each group normalized on corresponding  $\beta$ -actin and  
11 expressed as mean  $\pm$  S.D. with respect to WT animals set to 1. P=0.028 (cortex) and P=0.32 (c.  
12 striatum) with respect to WT, two-tailed non-parametric Mann-Whitney test.

13  
14 **Figure 2. Increased number of microglia in the naïve FHM mutant brain.** A: Representative  
15 pictures of coronal sections from the brains of wild-type (WT) and R192Q mutant mice stained with  
16 primary antibody against Iba1 (40X magnification; scale bars: 25  $\mu$ M). Magnified details show  
17 representative cells traced with the «Simple neurite tracer» tool of the Fiji-ImageJ software. Junctions  
18 and branches are indicated. B: the total number of Iba1-positive microglial cells was counted in brain  
19 sections (20X magnification; not shown). Scatter plots show the mean  $\pm$  S.D. of Iba1-positive cell  
20 number/area from seven animals for each group. P=0.002 with respect to WT, two-tailed non-  
21 parametric Mann-Whitney test.

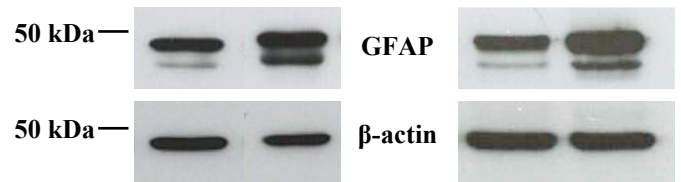
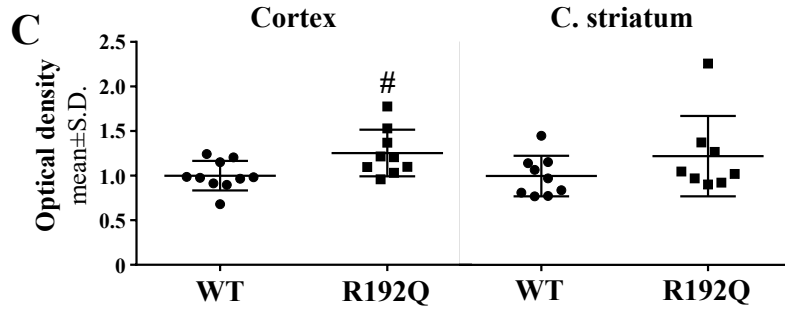
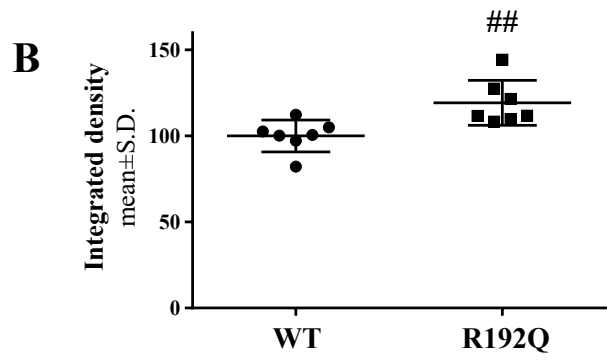
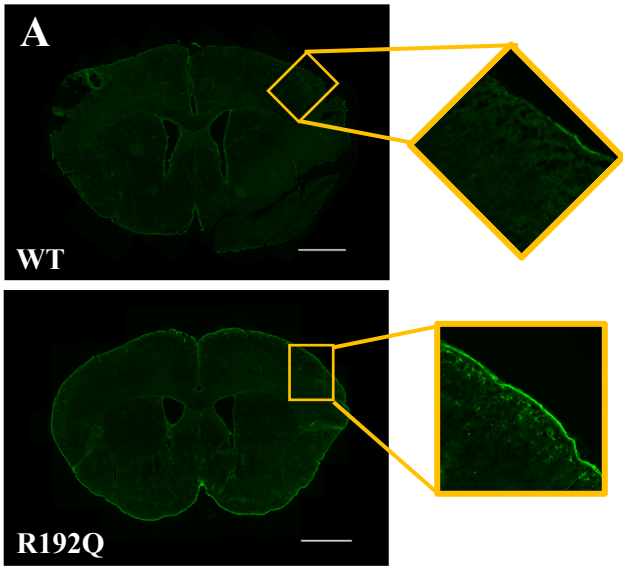
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23 **Figure 3. Increased branching and complexity of microglia in the cortex and corpus striatum**  
24 **of FHM1 mutant mice.** The complexity and ramification of Iba1-positive cells was evaluated by the  
25 Skeleton analysis tool of the Fiji-ImageJ software (see Methods section). In both cortex and c.  
26 striatum from wild-type (WT) and R192Q mutant mice, scatter plots show: (A) the area covered by

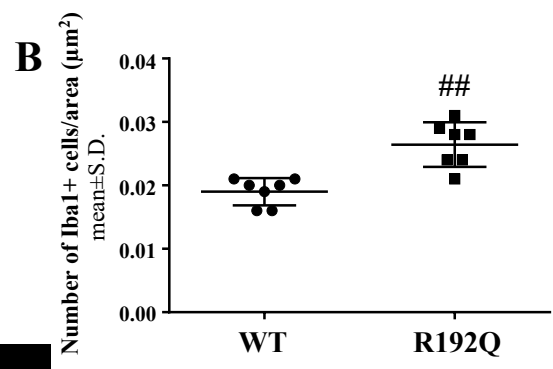
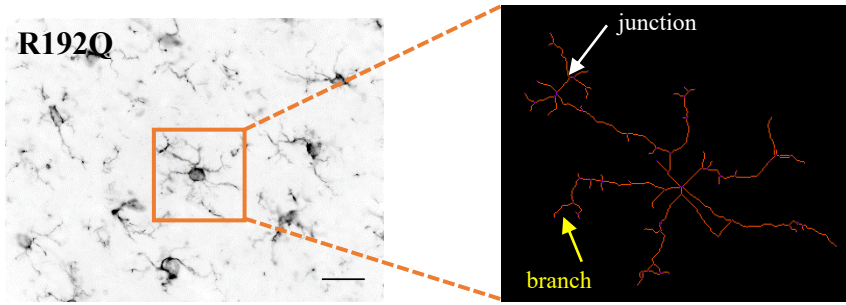
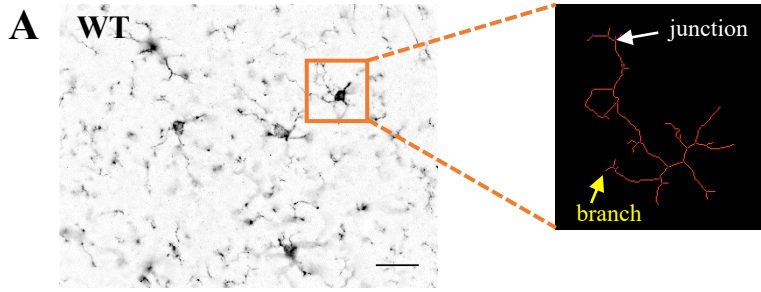
1 the cell projection tree; (B) the total number, (C) the average and (D) the maximal length of branches;  
2 (E) the total number of junctions and of (F) triple and (G) quadruple points. Data are the mean  $\pm$  S.D.  
3 of 30 randomly chosen cells/animal from seven animals for each group (see Methods for details). (A)  
4  $P=0.0006$  (cortex and c. striatum); (B)  $P=0.004$  (cortex) and  $P=0.038$  (c. striatum); (C)  $P=0.053$   
5 (cortex) and  $P=0.026$  (c. striatum); (D)  $P=0.007$  (cortex) and  $P=0.004$  (c. striatum); (E)  $P=0.011$   
6 (cortex and c. striatum); (F)  $P=0.011$  (cortex) and  $P=0.038$  (c. striatum); (G)  $P=0.165$  (cortex and c.  
7 striatum), two-tailed non-parametric Mann-Whitney test.

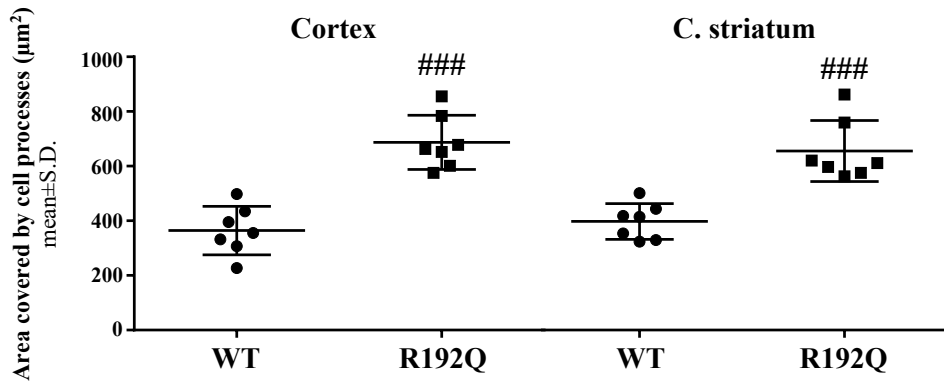
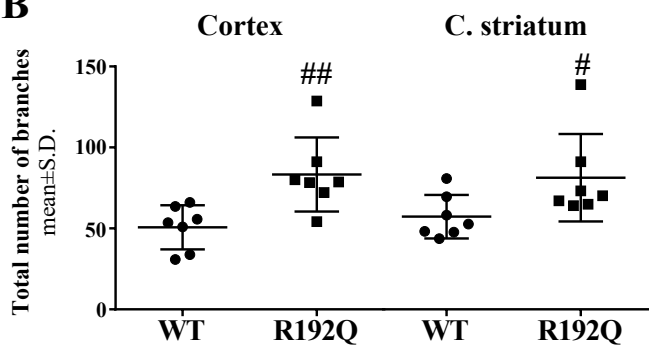
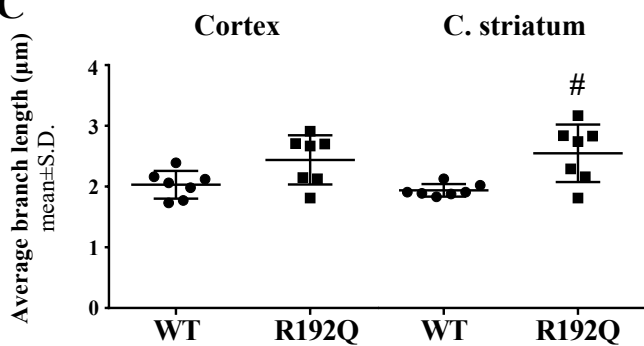
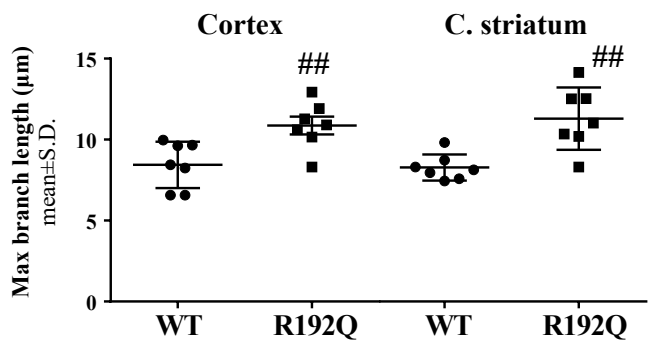
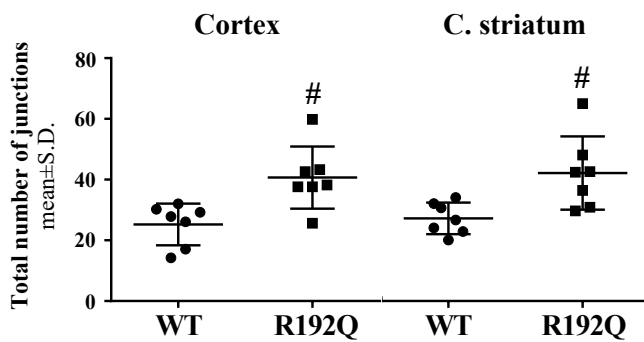
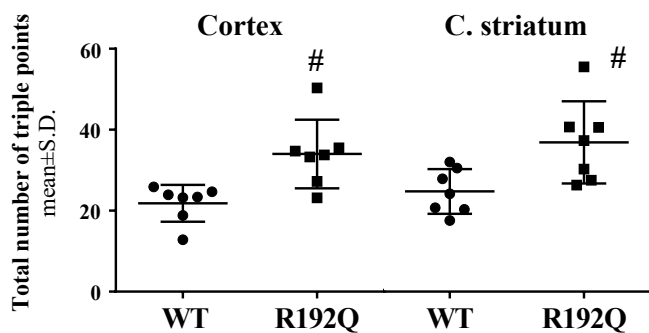
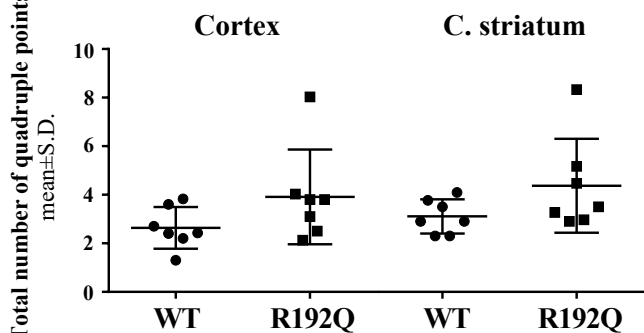
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10 **Figure 4. No changes in the expression of markers of mature oligodendrocytes in the brains of**  
11 **FHM1 mutant mice.** A: Western blotting evaluation of CNPase and MBP expression in cortical and  
12 striatal tissues from wild-type (WT) and R192Q mutant mice. Alpha-tubulin was utilized as internal  
13 control for protein loading (see representative filters of cortex samples in B). The three MBP bands  
14 at different molecular weights (some 15-16-18 kDa) were analyzed together. Scatter plots show the  
15 optical density of protein bands from seven animals for each group expressed as mean  $\pm$  S.D. with  
16 respect to WT animals set to 1. In (A) CNPase:  $P=0.295$  (cortex) and  $P=0.836$  (c. striatum); MBP:  
17  $P=0.945$  (cortex) and  $P=0.445$  (c. striatum), two-tailed non-parametric Mann-Whitney test.

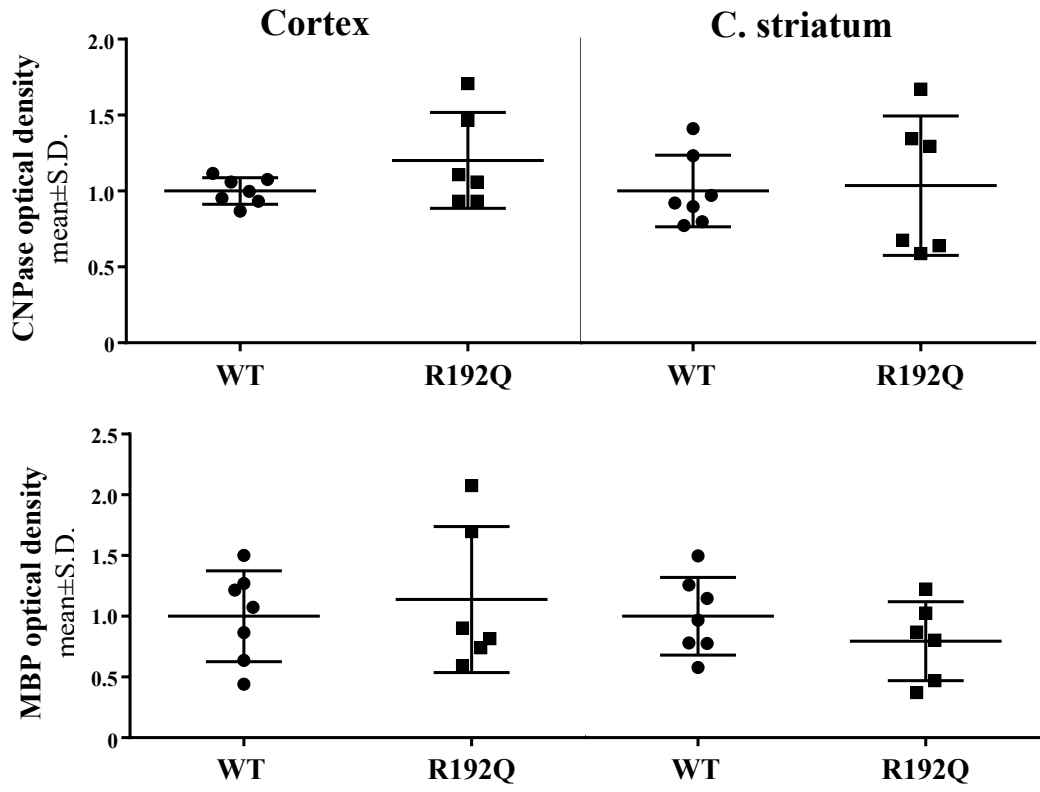
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20 **Figure 5: Reduced number of GPR17-positive cells in the FHM mutant brain.** A: representative  
21 pictures of coronal sections from the brains of wild-type (WT) and R192Q mutant mice stained with  
22 an home-made antibody against the membrane receptor GPR17. Cell nuclei were counterstained with  
23 the Hoechst33258 dye. The number of GPR17-positive cells was counted in the entire coronal section  
24 and in selected brain areas, i.e., the cortex and the corpus striatum. Scale bars: 1.5 mm. B: scatter  
25 plots show the mean  $\pm$  S.D. of GPR17-positive cell number/counted field in the different brain areas.  
26 Data were obtained by counting ten sections from seven independent animals/group. \* $P=0.017$  (total

- 1 number of cells),  $P=0.456$  (*cortex*) and  $P=0.038$  (*c. striatum*) with respect to WT, two-tailed non-
- 2 parametric Mann-Whitney test.





**A****B****C****D****E****F****G**

**A****B**