

1  
2 **Fingolimod exerts only temporary antiepileptogenic effects but longer-lasting positive**  
3  
4 **effects on behavior in the WAG/Rij rat absence epilepsy model**  
5  
6

7  
8  
9 Antonio Leo<sup>\*</sup>, Rita Citraro<sup>\*</sup>, Nicola Amodio<sup>†</sup>, Caterina De Sarro<sup>\*</sup>, Maria Eugenia Gallo  
10  
11 Cantafio<sup>†</sup>, Andrew Constanti<sup>‡</sup>, Giovambattista De Sarro<sup>\*</sup>, Emilio Russo<sup>\*,§</sup>.  
12  
13  
14

15  
16  
17 <sup>\*</sup>Science of Health Department, School of Medicine, University “Magna Graecia” of  
18  
19 Catanzaro, Italy; <sup>†</sup>Department of Experimental and Clinical Medicine, Magna Graecia  
20  
21 University and Translational Medical Oncology Unit, Salvatore Venuta University Campus,  
22  
23 Catanzaro, Italy; <sup>‡</sup>Department of Pharmacology, UCL School of Pharmacy, 29/39 Brunswick  
24  
25 Square, London, United Kingdom.  
26  
27  
28  
29  
30

31 **Original research**  
32  
33  
34  
35

36 **§ Author for correspondence:**  
37  
38

39 Prof. Emilio Russo  
40

41 Chair of Pharmacology, Department of Science of Health,  
42

43 School of Medicine, University of Catanzaro, Italy  
44

45  
46 Via T. Campanella, 115; 88100 Catanzaro, ITALY.  
47

48  
49 Phone +39 0961 3694191; Fax +39 0961 3694192; e-mail: [erusso@unicz.it](mailto:erusso@unicz.it)  
50  
51  
52  
53  
54  
55

56 **Running title:** Effects of Fingolimod in WAG/Rij rats.  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Abstract

1  
2 One of the major challenges in the epilepsy field is identifying disease-modifying drugs in  
3  
4 order to prevent or delay spontaneous recurrent seizure onset or to cure already established  
5  
6 epilepsy. It has been recently reported that fingolimod, currently approved for the treatment of  
7  
8 relapsing-remitting multiple sclerosis, has demonstrated antiepileptogenic effects in two  
9  
10 different preclinical models of acquired epilepsy. However, to date, no data exist regarding  
11  
12 the role of fingolimod against genetic epilepsy. Therefore, we have addressed this issue by  
13  
14 studying the effects of fingolimod in WAG/Rij rats, a well-established genetic model of  
15  
16 absence epilepsy, epileptogenesis, and neuropsychiatric comorbidity. Our results have  
17  
18 demonstrated that an early-long term treatment with fingolimod (1 mg/Kg/day), started before  
19  
20 absence seizure onset, has both antiepileptogenic and antidepressant-like effects in the  
21  
22 WAG/Rij rats. However, these effects were transitory, since 5 months after treatment  
23  
24 discontinuation, both absence seizure and depressive like-behavior returned to control level.  
25  
26 Furthermore, a temporary reduction of mTOR signaling pathway activity, indicated by  
27  
28 reduced p-mTOR and p-p70S6k levels and by an increased p-AKT in WAG/Rij rats of 6  
29  
30 months of age accompanied the transitory antiepileptogenic fingolimod effects. Surprisingly,  
31  
32 fingolimod has demonstrated longer-lasting positive effects on cognitive decline in this strain.  
33  
34 This effect was accompanied by an increased acetylation of Lysine 8 of histone H4 (both 6  
35  
36 and 10 months of age). In conclusion, our results support the antiepileptogenic effects  
37  
38 fingolimod. However, these antiepileptogenic effects were transitory. Moreover, fingolimod  
39  
40 might also have a positive impact on animal behavior and particularly in protecting the  
41  
42 development of memory decline.  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

56 **Keywords:** Fingolimod; Epileptogenesis; Absence epilepsy; Behavior; mTOR; Histone  
57  
58 deacetylase (HDAC).  
59  
60  
61  
62  
63  
64  
65

## Introduction

1  
2 Despite the presence of many pressing needs in the epilepsy field, one of the major challenges  
3  
4 for modern neurology is identifying disease-modifying drugs. In fact, to date, none of the  
5  
6 available antiepileptic drugs (AEDs) has demonstrated clinical efficacy to prevent or delay  
7  
8 spontaneous recurrent seizure (SRS) onset or to cure already established epilepsy, or even to  
9  
10 prevent the burden of neuropsychiatric comorbidities, including cognitive impairment and  
11  
12 mood disorders, which represent a primary outcome measure for novel AEDs [1, 2].  
13  
14 Therefore, a good antiepileptogenic treatment should not only counteract seizure onset and/or  
15  
16 their course but it should also improve comorbidities related to epilepsy [3, 4]. “Repurposing”  
17  
18 drugs already approved for other diseases could however, lead to new insights into the  
19  
20 epileptogenic process [1]. Accordingly, different commercially available drugs such as those  
21  
22 acting on immune and inflammatory mechanisms have been tested in different preclinical  
23  
24 models of epilepsy [5-7]. Increasing knowledge suggests that the immune system and  
25  
26 inflammation are involved in the pathogenesis of epilepsy, thereby representing potentially  
27  
28 suitable targets to develop novel disease-modifying drugs [8]. However, the clinical efficacy  
29  
30 of anti-inflammatory and immunosuppressant drugs in epilepsy remains to be fully defined  
31  
32 [9-11].  
33  
34  
35  
36  
37  
38  
39

40  
41 Fingolimod (FTY720), an immunomodulator drug derived from the fungus *Isaria sinclairii*, is  
42  
43 the first orally bioavailable disease-modifying drug approved by Food and Drug  
44  
45 Administration (FDA), in September 2010, as a first-line treatment of relapsing-remitting  
46  
47 multiple sclerosis (MS) [12, 13]. Chemically, it is a synthetic homologous of sphingosine,  
48  
49 derived from membrane lipid sphingomyelin, which after its phosphorylation into sphingosine  
50  
51 1-phosphate (S1P) by sphingosine kinases (SphKs) plays a fundamental role in several  
52  
53 physiological and pathological functions linked to the immunological, cardiovascular and  
54  
55 central nervous system (CNS) [14]. To date, the exact mechanism by which fingolimod acts is  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 not yet completely understood. However, being a pro-drug, fingolimod (as well as  
2 sphingosine), is phosphorylated *in vivo*, by SphKs, into the active metabolite fingolimod  
3  
4 phosphate (fingolimod-P or FTY720-P), which acts as a sphingosine 1-phosphate (S1P)  
5  
6 receptor (S1PRs) modulator [12, 13, 15]. Furthermore, fingolimod has also receptor-  
7  
8 independent effects, some of which are mediated by binding to intracellular targets of S1P  
9  
10 [16], including the mammalian target of rapamycin (mTOR) signaling pathway [17-19] and  
11  
12 histone deacetylases (HDACs) [20, 21], whereas other effects could be linked to its ability to  
13  
14 affect the metabolism and signaling of other lipids [16].  
15  
16  
17

18  
19 Therefore, these multiple relevant effects have prompted the study of fingolimod effects in  
20  
21 other brain disorders [16] including Alzheimer's disease [22] and epilepsy [13, 23, 24].  
22  
23 Regarding epilepsy, Gao et al. [23] described the antiepileptogenic and neuroprotective  
24  
25 effects of fingolimod in the lithium-pilocarpine rat epilepsy model through its ability to  
26  
27 suppress both microglial activation and to decrease IL-1 $\beta$  and TNF- $\alpha$  levels. More recently,  
28  
29 fingolimod antiepileptic effects were confirmed in the PTZ-kindling mouse model with pre-  
30  
31 treatment reducing seizure development as well as protecting myelin and post-treatment  
32  
33 reducing seizure severity as well as inducing remyelination in kindled mice [24]. Considering  
34  
35 fingolimod potential effects in epilepsy/epileptogenesis and the lack of data on genetic  
36  
37 epilepsy models and epilepsy comorbidities, we aimed in the present study, to evaluate the  
38  
39 effects of some fingolimod treatments (acute, sub-chronic and early long-term treatment) in  
40  
41 Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats, a well-established genetic model of absence  
42  
43 epilepsy, epileptogenesis, and neuropsychiatric comorbidity (dysthymia and decline in  
44  
45 learning and memory performance) [25-27]. Despite in this latter model neuroinflammation  
46  
47 does not seem to play a major role [28]; it has been demonstrated that drugs (e.g. etoricoxib,  
48  
49 indomethacin and rapamycin) acting on inflammation can both reduce absence seizures and  
50  
51 their development and accordingly, increasing neuroinflammation increases absence seizures  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [11, 29-31]. The role of inflammatory cytokines have been previously studied by Van  
2 Luitelaar et al. [28], IL-1beta and TNF-alpha administration can both increase absence  
3 seizures in this model while their levels were found to be altered in the blood and/or the brain  
4 of WAG/Rij rats at some ages with no clear correlation with SWDs development concluding  
5 therefore that a possible modulatory effect of neuroinflammation is plausible but TNF-alpha  
6 might not have necessarily a negative impact as also suggested by some other previous  
7 articles in other models [32]. Furthermore, considering the likely role of the mTOR pathway  
8 and HDAC in the etiopathogenesis of idiopathic and acquired epilepsy syndromes and  
9 fingolimod mechanism of action [33-35], we have also explored a potential effect of  
10 fingolimod on these targets in this rat strain.  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

## 26 **Materials and Methods**

### 27 *Animals*

28  
29 All the experiments were carried out in male non-audiogenic WAG/Rij rats. WAG/Rij rat  
30 progenitors, weighing about 60 g (4 weeks old), were originally purchased from Charles  
31 River Laboratories s.r.l. (Calco, Lecco, Italy) and the rats used in these protocols were all  
32 obtained from our breeding colony at the University of Catanzaro animal facility, as  
33 previously described [11, 36]. WAG/Rij rats were housed three/four per cage and kept under  
34 stable environmental conditions, humidity ( $60 \pm 5\%$ ) and temperature ( $21 \pm 2$  °C), in a room  
35 with 12/12 h reversed light/dark cycle (lights on at 20.00). WAG/Rij rats at 27 days of age  
36 (P27) were screened, as previously described [37, 38], to evaluate their susceptibility to  
37 audiogenic stimuli. Afterwards, only rats without audiogenic susceptibility were used in  
38 experiments, considering that WAG/Rij rats expressing audiogenic seizures display higher  
39 levels of anxiety in comparison to non-audiogenic WAG/Rij rats [26].  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 Animal care and experimental procedures were conducted in conformity with the  
2 international and national law and policies (EU Directive 2010/63/EU for animal experiments,  
3 ARRIVE guidelines and the Basel declaration including the 3R concept). The experimental  
4 protocols and the procedures reported in this manuscript were approved by the Animal Care  
5 Committee of the University of Catanzaro, Italy. All efforts were made to minimize animal  
6 suffering and to reduce the number of animals used.  
7  
8  
9

### 10 11 12 13 14 15 16 17 *Experimental summary*

18 The aim of these experiments was to evaluate both the potential antiepileptogenic effects of  
19 fingolimod in WAG/Rij rats (**experiment #1**) and its possible acute and/or sub-chronic  
20 effects versus established absence seizures (**experiment #2**) in the same model. This was  
21 accompanied by the analysis of fingolimod effects on different behavioral tasks, which were  
22 performed to study anxiety and depressive-like behavior, motor performance and cognitive  
23 impairment [39]. Finally, the effects of fingolimod on the mTOR signaling pathway as well as  
24 on the acetylation level on histone H4 were explored in order to increase our knowledge on its  
25 likely mechanism of action. A scheme of the experimental protocols is reported in Fig. 1 and  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
2.

**Experiment #1:** male WAG/Rij rats ( $n=30$ ), after an early long-term treatment (ELTT) with  
fingolimod (1 mg/Kg/day; see section *Early long-term treatment protocol* for details), were  
randomly divided into 5 subgroups ( $n=6$ ; Fig. 1) before being experimentally evaluated [39-  
41]. Identical matched WAG/Rij rat untreated control subgroups were included in the study.  
The first subgroup of rats underwent EEG recordings both at the age of ~6 and ~10 months  
for the quantification of absence seizures and drug effects evaluation (see section *Surgery and  
EEG recordings*). The second subgroup was used to study drug effects on anxiety- and  
depressive-like behavior in ~6 months old rats in the elevated plus maze and forced

1 swimming test, respectively. The third subgroup of 6 months old rats was evaluated in passive  
2 avoidance (learning and memory) test and rotarod (motor performance) test. The same  
3  
4 schedules for subgroup 2 and 3 were applied in the two subgroups (4 and 5) of 10 months of  
5  
6 age in order to evaluate retention of drug effects ~5 months after the end of fingolimod ELTT  
7  
8 (Fig. 1). The brains of rats in subgroups 2-5 were sampled at the end of their respective  
9  
10 behavioral tests to study drug effects on the mTOR pathway and on the acetylation levels of  
11  
12 Lysine 8 of histone H4.  
13  
14  
15  
16  
17

18 **Experiment #2** assessed the effects of two doses of fingolimod (1 and 3 mg/Kg), acutely and  
19  
20 sub-chronically (daily dose) administered in WAG/Rij rats with established seizures at the  
21  
22 age of ~6 months (Fig. 2). WAG/Rij rats ( $n = 90$ ) of ~6 months of age were randomly  
23  
24 divided into five groups ( $n = 18$ ) and each of these into 3 subgroups ( $n = 6$ ) for the two doses  
25  
26 of fingolimod and vehicle (Fig. 2). The first group was used to study (EEGs), both the acute  
27  
28 (intraperitoneal injection; i.p.) and the subchronic (7 days oral treatment) effects of  
29  
30 fingolimod on established absence seizures. The second and the third groups were used to  
31  
32 assess the acute (i.p.) effects of fingolimod on depressive and anxiety-like behavior as well as  
33  
34 learning/memory performance, respectively. The fourth and fifth groups were used to test  
35  
36 subchronic fingolimod effects on the same parameters of group 2 and 3 (Fig. 2). Six randomly  
37  
38 chosen rats from groups 4 and 5 were used to measure the phosphorylation levels of p70S6K  
39  
40 in order to study fingolimod (3mg/Kg/day for 1 week) effects on the mTOR pathway.  
41  
42  
43  
44  
45  
46  
47

#### 48 *Surgery and EEG recordings*

49  
50 WAG/Rij rats allocated into the groups or subgroups for EEG recordings and seizure  
51  
52 quantification, under general anesthesia (mixture of tiletamine/zolazepam; 1:1; Zoletil 100®;  
53  
54 50 mg/Kg i.p.; VIRBAC Srl, Milan, Italy), were stereotaxically implanted with 3 cortical  
55  
56 electrodes for EEG recordings attached to a 3-channel rat headmount (8239-SE3, Pinnacle  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Technology INC), as previously described [11]. After surgery, all animals were allowed at least 1 week of recovery and then connected to pre-amplifiers (Pinnacle Technology's 8400–9000 video/EEG system with Sirenia Software, Kansas, USA) through a flexible recording cable and an electric swivel, attached above the cages, permitting free movements for the animals [39]. Every video-EEG recording was carried out starting at 9.00 am for all groups in order to avoid circadian alterations within groups. EEG signals were amplified and conditioned by analog filters (filtering: below 1 Hz and above 30 Hz at 6 dB/octave) and exposed to an analog-to-digital switching with a sampling rate of 300 Hz. The blinded quantification of absence seizures was based on the number and the duration of EEG spike wave discharges (SWDs), as previously described [42, 43].

#### *Acute and subchronic procedures*

Two doses of fingolimod (1 and 3 mg/Kg/day; gift from Novartis Pharmaceutical Development, Basel, Switzerland) were used in this protocol section and were chosen according to previous studies [13, 21, 23, 24]; the acute effects of fingolimod were always tested 1h after i.p. administration considering its known pharmacokinetic profile [44]. On the other hand, subchronically treated rats received the drug orally as described for the ELTT but only for one week. All these experiments were run on ~6-month-old rats ( $n = 90$  for the entire section) with already established seizures. In order to reduce the number of animal used ( $n = 18$  vs 36), rats in the EEG recording group were initially evaluated after acute administration of fingolimod and then treated orally for 1 week and re-evaluated (see Section *Surgery and EEG recordings*). All other used rats were divided into groups and subgroups as described above (see Section *Experimental summary*) and used in behavioral tests.

#### *Early long-term treatment procedure*



1 WAG/Rij rats ( $n = 30$ ) were administered fingolimod at 1 mg/Kg/day *per os* starting at P30  
2 (before seizure onset) up to ~5 months of age (17 weeks of treatment). The drug was given in  
3  
4 the drinking water by dissolving the desired dose into 120 ml of tap water, as previously  
5 described [40, 45]. The drug dose was calculated on previously evidence that rats drink ~12  
6 ml/100 g/day; this was subsequently confirmed by checking the volume drunk by rats [40].  
7 Water bottles were wrapped in silver foil to exclude light and solutions were freshly prepared  
8 and substituted three times a week [41]. After the end of treatment, WAG/Rij rats were  
9 normally housed (see section Surgery and EEG recordings) up to the age of ~6 months. Age-  
10 matched control (vehicle) rats ( $n = 30$ ) were kept under the same housing conditions over the  
11 same period of time with vehicle (tap water). During the treatment period, animals were  
12 weighed weekly every Monday between 9:00 a.m. and 11:00 a.m.  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26

27  
28 At the age of ~6 months (1 month after drug withdrawal), one subgroup of treated and one of  
29 untreated (vehicle) WAG/Rij rats, following surgery (see section Surgery and EEG  
30 recordings), were experimentally evaluated by 3 hours of EEG recordings over 3 consecutive  
31 days. The same rats were again EEG studied at the age of ~10 months (5 months after  
32 treatment discontinuation) to evaluate the potential long-term effects fingolimod. The same  
33 recording schedule was used for subchronically treated rats (see Section *Acute and subchronic*  
34 *procedures* for details on treatment protocol) while for the evaluation of potential acute  
35 fingolimod effects, rats were subjected to EEG recordings lasting 6h: 1h baseline without  
36 drug administration, and 5h after i.p. injection of fingolimod or vehicle.  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

#### 51 *Behavioral tests*

52  
53 In order to reduce the number of rats used and avoid the influence played by several testings  
54 in the same animal, rats ( $n = 6$  for every group) were divided as described in Fig. 1 and 2 and  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 experimental summary. When two tests were performed on the same animal, at least one day  
2 (range 1-3) was allowed as previously described [13, 46, 47]. Experiments were always  
3  
4 performed between 09:00 and 11:00 a.m. in order to avoid possible circadian alteration of test  
5  
6 results. All behavioral tests were performed under controlled environmental conditions  
7  
8 including temperature, humidity and light intensity (dim illumination) and with the support of  
9  
10 video-tracking software (EthoVision XT8; Noldus Wageningen, Netherlands) [41, 48].  
11  
12  
13

14  
15 In detail, in order to evaluate the ELTT effects of fingolimod, behavioral tests were performed  
16  
17 respectively ~1 and ~5 months after the end of fingolimod treatment. For the acute and sub-  
18  
19 chronic effects of fingolimod, behavioral tests were performed at the end of the acute and sub-  
20  
21 chronic treatment period, 1h and 7 days respectively. Regarding subchronic administration  
22  
23 groups, when the same group of rats was subjected to multiple behavior tests and/or repeated  
24  
25 sessions of the same test, it was kept under treatment. Regarding acute administration groups,  
26  
27 the rat groups subjected to the passive avoidance test, were injected with fingolimod (1 and 3  
28  
29 mg/Kg/day) 1h before to perform the conditioning session [49], and when multiple tests were  
30  
31 required in the same group, the drug was re-injected 1h before.  
32  
33  
34  
35  
36  
37  
38  
39  
40

#### 41 *Forced swimming test*

42  
43 The forced swimming test (FST), despite some limitations [50], is currently used for the  
44  
45 experimental study of depressive-like behavior in animals; we used an FST protocol  
46  
47 previously standardized in our laboratories [31, 48]. Briefly, rats were placed individually for  
48  
49 6 min into a glass cylinder (height 47 cm, diameter 38 cm) filled with 38 cm of water,  
50  
51 maintained at 23-25°C. The total duration of immobility (immobility time; IT) was recorded  
52  
53 during the last 4 min of the 6-min testing period. The criterion for immobility and passive  
54  
55 swimming (IT) was floating vertically in the water while making only those movements  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 essential to keep the head above the surface of the water, which is directly proportional to  
2 depressive-like behavior. At the end of the FST, rats were removed and dried with a towel  
3 before being housed. Mean swimming velocity and total distance moved were also measured  
4 and examined for every experimental group in order to check for any obvious locomotor  
5 impairment [48].  
6  
7  
8  
9

### 10 *Elevated plus maze*

11  
12  
13  
14  
15  
16 The elevated plus maze (EPM) consists of two opposing open arms and two opposing closed  
17 arms of the same size (45 cm x 10 cm) with walls 10 cm high connected by a central platform  
18 (10 x 10 cm) and elevated 80 cm above the floor, as previously described [13, 47]. Rats are  
19 positioned in the central platform facing a closed arm and the number of entries into, time  
20 spent on each arm and central platform are measured. The maze was systematically cleaned to  
21 remove olfactory cues, after each animal was tested. The shorter the time spent in open arms  
22 and central platform the higher is anxiety and *vice versa*. Mean velocity and total distance  
23 moved were also measured and examined for every experimental group [13, 51].  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38

### 39 *Passive avoidance test*

40  
41 In the passive avoidance learning test, used to assess learning and memory, rodents learn to  
42 suppress their innate tendencies: moving from the illuminated chamber to the dark chamber  
43 [52, 53]. The passive avoidance-step through-cage (Ugo Basile, Italy), measuring 57x27x30  
44 cm, consisted of a cage divided into two chambers (light and dark) by a sliding door. The test  
45 was conducted over two consecutive days as previously described [39, 54]. Briefly, during the  
46 first day (habituation), rats were placed individually in the light chamber and they were  
47 allowed to freely explore the apparatus for 5 min with the sliding door, separating the two  
48 chambers, open. At the end of this period, rats were returned to their home cage. The  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 conditioning session (learning trial) was started 15 min after habituation. Rats were  
2 individually placed in the light chamber. After 30 sec the sliding door was automatically  
3 opened. When the rats entered into the dark chamber, the sliding door was automatically  
4 closed and an electrical foot-shock (0.5 mA) was delivered for 3 seconds via the floor grid.  
5  
6 Afterwards, rats were housed. The latency to enter into the dark chamber was recorded and  
7 analyzed. Each rat was given 300 sec to enter into the dark chamber. If a rat failed to cross  
8 from the light to the dark chamber within the cut-off time, it was discarded from the study.  
9  
10 Between each training session, the apparatus was systematically cleaned to remove olfactory  
11 cues. The retention session (memory trial) was performed 24 h after the conditioning session  
12 by re-introducing the rat into the light chamber of the apparatus. Rats' memory was assessed  
13 by recording their latency to enter into the dark chamber; however, no foot-shock was  
14 delivered in this session. The maximum cutoff time for the step-through latency was 300 s. If  
15 a rat failed to cross from the light to the dark chamber within the cut-off time, it was housed  
16 and a latency of 300 s was recorded for that rat. Retention memory is directly related to the  
17 latency to enter in the dark chamber: the better the memory, the greater the latency [25, 39,  
18 54].  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40

#### 41 *Rotarod test*

42  
43 The rotarod test was used to evaluate any eventual locomotor impairment induced by drug  
44 treatment. The test was performed as previously described by Monville et al. [55] with some  
45 minor modifications on a Rotarod unit (LE 8500, Panlab, Barcelona, Spain). Briefly, after a  
46 habituation session, rats were placed on a rod whose acceleration was increased from 4 to 40  
47 rpm over a period of 300 s. The latency to fall and the number of falls were recorded. Rats  
48 were trained for 3 consecutive sessions and the mean of the 3 sessions was analyzed [56].  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

### *Western blotting analysis*

1  
2 Rats were decapitated and their brains were quickly removed and submerged in ice-cold  
3  
4 artificial cerebrospinal fluid. Subsequently, the cortex was isolated and dissected by an optical  
5  
6 microscope as previously described by Russo et al. [29]. The cortex was homogenized using  
7  
8 the Gentle MACS dissociator (Miltenyi Biotech) in ice-cold NP40 lysis buffer (Life  
9  
10 Technologies) containing a cocktail of protease and phosphatase inhibitors (Life  
11  
12 Technologies), and then centrifuged at 12.000 rpm for 30 min at 4°C to remove tissue debris.  
13  
14 50 µg of proteins were electrophoresed through a NuPAGE 4-12% gradient gel (Life  
15  
16 Technologies) and electroblotted onto a nitrocellulose membrane (Life Technologies) as  
17  
18 previously reported [57]. The membrane was blocked for 1 hour with 5% non-fat dry  
19  
20 milk/PBS-tween 0.05% (Biorad), and then incubated over night with the antibodies for p-  
21  
22 AKT (S473), AKT, p-mTOR (S2448), mTOR, p-p70S6 Kinase (T389), p70S6 Kinase,  
23  
24 HDAC1, histone H4 and acetylated-histone H4 (K8) (all from Cell Signaling); GAPDH  
25  
26 (Santa Cruz, DBA) was used as loading control. The levels of proteins and phosphoproteins  
27  
28 were detected with horseradish peroxidase-linked secondary antibodies and the ECL  
29  
30 (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy). Autoradiographs were  
31  
32 scanned to obtain arbitrary densitometric units. Data were normalized against those of the  
33  
34 corresponding GAPDH. The experiments were performed in triplicate and the results  
35  
36 calculated as means  $\pm$  SEM and expressed as protein change (%) [57].  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47

### *Statistical analysis*

48  
49 All statistical procedures were performed using GraphPad Prism 6.0 (GraphPad Software,  
50  
51 Inc., La Jolla, CA 92037, USA). EEG recordings were subdivided into 30 min epochs, and the  
52  
53 duration and number of SWDs were evaluated separately, as previously described [42]. Such  
54  
55 values were averaged and data were expressed as means  $\pm$  SEM. EEG data were analyzed and  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 compared by one-way ANOVA followed by Dunnett's *post hoc* test. Furthermore, we used  
2 one-way ANOVA followed by Dunnett's *post hoc* test to analyze and compare behavioral  
3 data obtained from acute and subchronic treatments. Data obtained by behavioral tests and  
4 Western blotting analysis from the ELTT schedule, were analyzed and compared by two-way  
5 ANOVA followed by Bonferroni's *post hoc* test. Data obtained by Western blotting analysis  
6 from subchronic treatment, were analyzed and compared by Student's t-test. All tests used  
7 were two sided and  $P \leq 0.05$  was considered significant.  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18

## 19 **Results**

### 20 *Effects of acute and sub-chronic treatment with fingolimod on established absence seizures*

21 Analysis of EEG recordings of 6 months old WAG/Rij rats showing established absence  
22 seizures, indicated that fingolimod, administered acutely i.p. and subchronically o.s. (1 and 3  
23 mg/Kg) was not able to significantly modify ( $P > 0.05$ ) absence seizure parameters in  
24 comparison with the control group (data not shown).  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35

### 36 *Effects of early long-term treatment (ELTT) with fingolimod on absence seizure development*

37 Untreated control WAG/Rij rats, at the age of 6 months, showed a mean number of SWDs  
38 (nSWDs) of  $11.06 \pm 1.02$  with a mean total duration (dSWDs) of  $72.03 \pm 6.22$  s and a mean  
39 single duration (sSWD) of  $6.31 \pm 1.33$  for a 30 min epoch (Table 1). ELTT with fingolimod  
40 (1 mg/Kg/day; *per os*) significantly decreased ( $P < 0.05$ ) the development of absence seizures  
41 (both number and total duration, but not sSWD) in WAG/Rij rats at the age of 6 months (1  
42 month after treatment discontinuation) in comparison to untreated rats of the same age,  
43 supporting antiepileptogenic effects (Fig. 3). In particular, ELTT with fingolimod did not  
44 significantly ( $\sim 12\%$ ;  $P > 0.05$ ) reduce mean sSWD duration, whereas it significantly modified  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 nSWDs as well as dSWDs by about 30% ( $P= 0.003$ ) and 40% ( $P= 0.00029$ ), respectively  
2 (Fig. 3).  
3

4 In contrast, in fingolimod-treated rats at 10 months of age (5 months after drug  
5 discontinuation), absence seizure parameters (nSWDs, dSWDs and sSWD) were no longer  
6 significantly ( $P> 0.05$ ) modified in comparison to the respective untreated control group of  
7 the same age (Fig. 3). Animal growth, over the 17 weeks of treatment, did not significantly  
8 differ between fingolimod-treated and untreated rats (data not shown).  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18

### 19 *Effects of fingolimod on depressive- and anxiety like behavior*

20

21 WAG/Rij rats already at the age of 4 months and even more so at 6 months of age, display an  
22 increased immobility time in the FST, which is very likely linked to the development of  
23 SWDs [26, 40]. Both fingolimod acute and sub-chronic treatments had no effects on  
24 immobility time (IT) in any group tested despite animal age or dose used. On the other hand,  
25 fingolimod ELTT (1 mg/Kg/day; *per os*) significantly ( $P = 0.0054$ ) decreased the IT in 6  
26 months old WAG/Rij rats (Fig. 4a; 1 month after treatment discontinuation), whereas this  
27 fingolimod antidepressant-like effect was not observed in WAG/Rij rats where treatment was  
28 discontinued 5 months earlier (10 months old rats group; Fig. 4a). Mean velocity and total  
29 distance moved did not significantly differ ( $P> 0.05$ ) between groups (data not shown).  
30  
31 Anxiety-like behavior in WAG/Rij rats was evaluated by EPM and, also in this case, it was  
32 not affected by both acute and subchronic fingolimod treatment. Similarly, ELTT with  
33 fingolimod was not able to modify any measured parameters independently of the time of the  
34 test (Fig. 4b).  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

### 56 *Effects of fingolimod on learning and memory and motor coordination*

57  
58  
59  
60  
61  
62  
63  
64  
65

1 Learning and memory decline has been recently identified in WAG/Rij rats older than 6  
2 months [25, 54]. In agreement with our results in other behavioral tests, acute and subchronic  
3 fingolimod treatment did not influence learning and memory evaluated in the passive  
4 avoidance test. Fingolimod ELTT (1 mg/Kg/day; *per os*) however, significantly ( $P = 0.0023$ )  
5 increased the latency time for entering into the dark chamber during the retention session both  
6 in 6 and 10 month old rats; namely, 1 and 5 months after treatment discontinuation (Fig. 4c).  
7 Fingolimod did not influence rotarod test results in any group.  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18

#### 19 *Effects of fingolimod on the mTOR pathway*

20

21 By comparing control rats of 6 months of age with those of 10 months of age, we found that  
22 mTOR and p70S6K phosphorylation levels were not modified by age while AKT was  
23 significantly ( $P = 0.0047$ ) more phosphorylated in older animals (Fig. 5b). The subchronic  
24 administration of fingolimod (3 mg/Kg/day for 1 week) was not able to reduce the amount of  
25 p-p70S6K and the ratio p-p70S6K/p70S6K in the cortex of 6 months old WAG/Rij rats (Fig.  
26 6).  
27  
28  
29  
30  
31  
32  
33  
34  
35

36 Western blotting analysis of the rat cortex obtained from animals in the fingolimod (1  
37 mg/Kg/day) ELTT group at 6 months of age revealed an increased ( $P = 0.0017$ )  
38 phosphorylation of AKT and a reduced ( $P < 0.001$ ) phosphorylation of both mTOR and  
39 p70S6K. On the other hand, no differences were found when comparing phosphorylation  
40 levels in 10 month old rats previously treated with fingolimod (Fig. 5).  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

#### 51 *Effects of fingolimod on histone acetylation*

52

53 No significant ( $P > 0.05$ ) difference in the acetylation of Lysine 8 of histone H4 was observed  
54 between untreated WAG/Rij rats of 6 months of age in comparison to untreated WAG/Rij rats  
55 of 10 months of age (Fig. 7b). Fingolimod ELTT significantly increased ( $P = 0.0009$ )  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1 acetylation of Lysine 8 of histone H4 (H4K8) in WAG/Rij rats both at 6 and 10 months of age  
2 in comparison to respective control groups (Fig. 7b). Furthermore, no significant ( $P > 0.05$ )  
3  
4 difference in HDAC1 expression levels was observed after an ELTT with fingolimod (Fig.  
5  
6  
7 7a).  
8  
9

## 10 11 **Discussion**

12 Our results demonstrate that an early-long term treatment (17 weeks) with fingolimod, started  
13  
14 before absence seizure onset (treatment started at P30 and seizure onset at about P60), has  
15  
16 both antiepileptogenic and antidepressive-like effects in the WAG/Rij rat absence epilepsy  
17  
18 model [27]. However, these effects, as reported for the majority of the drugs tested in this  
19  
20 model so far, were transitory, since 5 months after treatment discontinuation, both absence  
21  
22 seizures and depressive-like behavior (which usually accompanies seizure development in  
23  
24 WAG/Rij rats), returned to control level [26, 27, 31]. Furthermore, comparing fingolimod  
25  
26 effects in WAG/Rij rats of 6 months of age (i.e. 30% absence seizure development reduction)  
27  
28 with the results obtained for other drugs previously tested, it seems that fingolimod is  
29  
30 probably the less effective drug tested so far (compare fingolimod effects vs all other drugs  
31  
32 reported in the review by Russo et al. [27]).  
33  
34  
35  
36  
37  
38  
39  
40  
41

42 The antiepileptogenic effects of fingolimod have also been previously demonstrated in the rat  
43  
44 lithium-pilocarpine epilepsy model where, a 1 mg/Kg/day treatment for 14 consecutive days,  
45  
46 started 24 h after onset of status epilepticus (SE), was able to decrease both glial activation  
47  
48 and associated abnormal expression of IL-1 $\beta$  and TNF- $\alpha$  which are rapidly  
49  
50 overexpressed after SE. Furthermore, in this latter study, fingolimod administered during the  
51  
52 silent phase was able to decrease the development of spontaneous seizures together with a  
53  
54 reduction in their duration and severity [23]. These effects could also be the result of astrocyte  
55  
56 S1P receptor modulation, however, further studies are needed to better clarify this hypothesis  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [13, 23]. More recently, Gol et al. [24] also demonstrated that fingolimod, through  
2 neuroprotective and anti-inflammatory effects, promotes myelin protection and remyelination  
3 improvement in the pentylenetetrazole (PTZ)-kindling mouse model of epilepsy. Particularly,  
4 the pre-treatment with fingolimod, 1h before PTZ-administration, resulted in decreased  
5 seizure onset, microglial activation and neuronal death in hippocampal areas, CA1 and CA3.  
6 Likewise, the post-treatment with fingolimod decreased seizures and promoted the  
7 endogenous remyelination processes in kindled mice. Accordingly, based on this background,  
8 it is not possible to exclude that fingolimod can exert antiepileptogenic effects in this strain  
9 through some kind of antiinflammatory mechanisms, which could also be linked to a  
10 modulation of neuronal S1P receptors [13]. It is known that glial activation and the related  
11 overexpression of proinflammatory cytokines seem to play a crucial role in epileptogenesis  
12 both in humans and in several animal models of epilepsy [58-61]; however, to date, such a  
13 relationship between neuroinflammation and absence seizure development in WAG/Rij rats  
14 remains unclear [27, 28]. Indeed, neuroinflammation and related mediators worsen absence  
15 seizures in this strain [28-30, 62, 63], while cyclooxygenase inhibitors have some partial  
16 antiabsence properties [11, 63, 64] and etoricoxib, a selective COX-2 inhibitor, also possesses  
17 antiepileptogenic effects in this strain, which appear to be more effective than fingolimod  
18 with a reduction in the development of absence seizures of about 45% vs 30% obtained with  
19 fingolimod [11]. Overall, based on the current knowledge, neuroinflammation does not  
20 convincingly seem to take part in the *epileptogenic* process in WAG/Rij rats; rather, it seems  
21 to accompany and participate in seizure generation and synchronization [27] as also supported  
22 by findings in the GAERS model of absence epilepsy [65]; accordingly, these mechanism  
23 may contribute to the limited fingolimod effects in this model.

24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57 To date, several animal models and some clinical studies have demonstrated that the mTOR  
58 signaling pathway plays a relevant role in the development of some idiopathic and acquired  
59  
60  
61  
62  
63  
64  
65

1 epilepsy syndromes [33, 35]. Furthermore, the AKT–mTOR pathway was more active  
2 (hyperphosphorylated) in young WAG/Rij rats, before absence seizures onset, supporting the  
3  
4 role of the mTOR pathway as one of the possible causes of the epileptogenic process in  
5  
6 WAG/Rij rats [27, 66]. However, absence seizures *per se* might also activate the mTOR-  
7  
8 signaling pathway, as also supported by the fact that a single administration of PTZ in rodents  
9  
10 increases mTOR pathway activity [66, 67]. Since it was known that fingolimod is also able to  
11  
12 inhibit the AKT–mTOR pathway both in the experimental autoimmune encephalomyelitis  
13  
14 (EAE) mouse model [17] and in the glioblastoma cell line [19], we investigated its effects on  
15  
16 this pathway.  
17  
18  
19  
20

21 Fingolimod ELTT resulted in a temporary reduction of mTOR signaling pathway activity,  
22  
23 indicated by reduced p-mTOR and p-p70S6k levels and by an increased p-AKT in WAG/Rij  
24  
25 rats of 6 months of age; this modulatory effect accompanied the transitory antiepileptogenic  
26  
27 fingolimod effects. In fact, 5 months after treatment discontinuation, the mTOR pathway  
28  
29 activation returned to control level together with the incidence of absence seizures. These  
30  
31 results suggest that the inhibitory effects of fingolimod ELTT could be indirect and linked to  
32  
33 its antiepileptogenic effects (reduction of absence seizures), which would in turn reduce  
34  
35 mTOR pathway activation. This hypothesis is also supported by the fact that a sub-chronic  
36  
37 treatment with fingolimod neither reduced mTOR pathway activation nor absence seizures in  
38  
39 adult WAG/Rij rats. Furthermore, it was previously demonstrated that the mTOR inhibitor  
40  
41 rapamycin has both antiepileptogenic effects (about 50% seizure development decrease vs  
42  
43 30% obtained with fingolimod) and some, albeit limited, anti-absence effects with long-  
44  
45 lasting antiepileptogenic effects [29, 31]. Of note, we found an age-dependent increase in the  
46  
47 phosphorylation of p-AKT in older control rats [68]; however, the significance of this result  
48  
49 still remains controversial [69].  
50  
51  
52  
53  
54  
55  
56  
57

58 Previous studies have documented the linkage between epilepsy and depression both in  
59  
60  
61  
62  
63  
64  
65

1 preclinical models and in humans [70, 71]. Regarding WAG/Rij rats, it has also been  
2 hypothesized that some common, currently unknown, mechanisms could be responsible for  
3 the appearance of absence seizures and low-grade depression (dysthymia) in this strain, with  
4 seizure activity being required for the expression of depressive-like behavior [26, 72] even  
5 though some exceptions exist [27, 40, 41]. Based on this background, the antidepressant  
6 effects of fingolimod observed after an ELTT in WAG/Rij rats could be linked to its  
7 temporary antiepileptogenic effects, which disappear with seizure reappearance 4 months  
8 later, rather than to a direct effect. Acute and subchronic treatments with fingolimod were not  
9 associated with a reduction of established absence seizures and immobility time in adult  
10 epileptic WAG/Rij rats, thereby supporting this view. However, a direct effect on depressive  
11 symptoms might not be excluded. In fact, the antidepressant effects of fingolimod (3 mg/Kg,  
12 i.p. once a day for 4 weeks) have been reported in mice exposed to chronic unpredictable  
13 stress and in mice chronically treated with corticosterone, both of which represent models of  
14 depression [21] and in patients with relapsing-remitting multiple sclerosis, who switched from  
15 injectable disease-modifying therapy to fingolimod [73, 74]. In our study, fingolimod did not  
16 affect anxiety-like behavior in the EPM test, similar to the findings by di Nuzzo et al. [21] in  
17 mice.

18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42 At odds with these temporary effects, fingolimod ELTT (but not acutely or subchronically)  
43 prevented the development of cognitive decline and this effect was maintained up to 5 months  
44 after drug suspension. Memory impairment was only recently demonstrated in WAG/Rij rats  
45 [25, 54]. Karson et al. [54] demonstrated that at 5 (range 4-6) months WAG/Rij rats do not  
46 differ from Wistar rats while they have an impairment in learning and memory at 13 (range  
47 12-14) months of age; on the other hand, Jafarian et al. [25] reported that WAG/Rij rats of 6  
48 months of age display memory impairment accompanied by hippocampal neuronal death.  
49 Accordingly, we found that there was no difference in WAG/Rij rats both aged 6 or 10  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 months and fingolimod effects were statistically similar at both ages and therefore not  
2 temporally related to its antiepileptogenic effects. Despite these two studies, it still remains to  
3  
4 be clarified whether seizures and learning/memory impairment are independent or related  
5  
6 processes; our results support the hypothesis that they are not dependent, however, further  
7  
8 studies are needed.  
9  
10

11  
12 Considering the ability of fingolimod to inhibit HDAC [20] and the role of HDAC inhibitors  
13  
14 including valproate and lacosamide in enhancing learning and memory processes and the  
15  
16 epigenetic modulation of absence seizure development in this model [27, 75-79], we studied  
17  
18 fingolimod ELTT effects on acetylation of Lysine 8 of histone H4 (both 6 and 10 months of  
19  
20 age) and found a significant increase temporally linked to the preserved cognitive functions.  
21  
22 As previously reported, this epigenetic regulation by fingolimod could also lead to an  
23  
24 augmented expression of growth factors such as BDNF that play a fundamental role in  
25  
26 synaptic plasticity process, which are involved in memory formation and retention [21, 80-  
27  
28 82]. In any case, the role of HDAC modulation in epilepsy and more specifically in WAG/Rij  
29  
30 rats still does not permit us to either support or discard its involvement in fingolimod effects,  
31  
32 also considering the potential of HDAC modulation in epileptogenesis and animal behavior  
33  
34 [34, 83, 84].  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44

#### 45 *Conclusions*

46  
47 Fingolimod and the related S1P signaling have recently gathered attention in epilepsy [13];  
48  
49 furthermore, fingolimod also possesses other mechanisms of action, which might be relevant  
50  
51 for this neurological disease. Our results further extend the current knowledge supporting  
52  
53 potential antiepileptogenic effects of this drug; however, in our experiments, these effects  
54  
55 were very limited in comparison to previous experiments with other drugs in this model and  
56  
57 not permanent. Moreover, fingolimod might also have a positive impact on animal behavior  
58  
59  
60  
61  
62  
63  
64  
65

1 and particularly in protecting the development of cognitive decline associated with epilepsy.  
2 In conclusion, fingolimod might be considered a promising antiepileptogenic treatment on the  
3 basis of the current view of the several unmet needs in this field [4, 5, 85]; however, further  
4 experiments are needed in order to clarify the exact mechanism(s) by which fingolimod exerts  
5 these potentially beneficial effects in this neurological disorder.  
6  
7  
8  
9  
10

### 11 **Acknowledgements**

12  
13  
14 The Novartis Institute for BioMedical Research (NIBR) (Basel, Switzerland) is kindly  
15 acknowledged for the kind gift of fingolimod.  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## References

- 1  
2  
3 [1] Kaminski RM, Rogawski MA, Klitgaard H. The potential of antiseizure drugs and agents  
4 that act on novel molecular targets as antiepileptogenic treatments. *Neurotherapeutics*.  
5  
6 2014;11:385-400.  
7  
8  
9  
10  
11 [2] Franco V, French JA, Perucca E. Challenges in the clinical development of new  
12 antiepileptic drugs. *Pharmacological research*. 2016;103:95-104.  
13  
14  
15  
16  
17 [3] Terrone G, Pauletti A, Pascente R, Vezzani A. Preventing epileptogenesis: A realistic  
18 goal? *Pharmacological research : the official journal of the Italian Pharmacological Society*.  
19  
20 2016;110:96-100.  
21  
22  
23  
24  
25 [4] Brooks-Kayal AR, Bath KG, Berg AT, et al. Issues related to symptomatic and disease-  
26 modifying treatments affecting cognitive and neuropsychiatric comorbidities of epilepsy.  
27  
28 *Epilepsia*. 2013;54 Suppl 4:44-60.  
29  
30  
31  
32  
33 [5] White HS, Loscher W. Searching for the ideal antiepileptogenic agent in experimental  
34 models: single treatment versus combinatorial treatment strategies. *Neurotherapeutics*.  
35  
36 2014;11:373-84.  
37  
38  
39  
40  
41 [6] Kobow K, Auvin S, Jensen F, et al. Finding a better drug for epilepsy: antiepileptogenesis  
42 targets. *Epilepsia*. 2012;53:1868-76.  
43  
44  
45  
46  
47 [7] Loscher W, Klitgaard H, Twyman RE, Schmidt D. New avenues for anti-epileptic drug  
48 discovery and development. *Nature reviews Drug discovery*. 2013;12:757-76.  
49  
50  
51  
52  
53 [8] Rajasekaran K, Goodkin HP. A swell in the armamentarium of antiepileptic drug targets.  
54  
55 *Epilepsy currents / American Epilepsy Society*. 2011;11:172-6.  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [9] Beghi E, Shorvon S. Antiepileptic drugs and the immune system. *Epilepsia*. 2011;52  
2 Suppl 3:40-4.  
3

4  
5 [10] Marchi N, Granata T, Janigro D. Inflammatory pathways of seizure disorders. *Trends in*  
6 *neurosciences*. 2014;37:55-65.  
7  
8  
9

10  
11 [11] Citraro R, Leo A, Marra R, De Sarro G, Russo E. Antiepileptogenic effects of the  
12 selective COX-2 inhibitor etoricoxib, on the development of spontaneous absence seizures in  
13 WAG/Rij rats. *Brain research bulletin*. 2015;113:1-7.  
14  
15  
16  
17

18  
19 [12] Brinkmann V, Billich A, Baumruker T, et al. Fingolimod (FTY720): discovery and  
20 development of an oral drug to treat multiple sclerosis. *Nature reviews Drug discovery*.  
21 2010;9:883-97.  
22  
23  
24  
25

26  
27 [13] Russo E, Leo A, Crupi R, et al. Everolimus improves memory and learning while  
28 worsening depressive- and anxiety-like behavior in an animal model of depression. *Journal of*  
29 *psychiatric research*. 2016;78:1-10.  
30  
31  
32  
33

34  
35 [14] Rosen H, Stevens RC, Hanson M, Roberts E, Oldstone MB. Sphingosine-1-phosphate  
36 and its receptors: structure, signaling, and influence. *Annual review of biochemistry*.  
37 2013;82:637-62.  
38  
39  
40  
41  
42

43  
44 [15] Groves A, Kihara Y, Chun J. Fingolimod: direct CNS effects of sphingosine 1-phosphate  
45 (S1P) receptor modulation and implications in multiple sclerosis therapy. *Journal of the*  
46 *neurological sciences*. 2013;328:9-18.  
47  
48  
49  
50  
51

52  
53 [16] Brunkhorst R, Vutukuri R, Pfeilschifter W. Fingolimod for the treatment of neurological  
54 diseases-state of play and future perspectives. *Frontiers in cellular neuroscience*. 2014;8:283.  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1 [17] Hou H, Cao R, Miao J, et al. Fingolimod ameliorates the development of experimental  
2 autoimmune encephalomyelitis by inhibiting Akt-mTOR axis in mice. *International*  
3 *immunopharmacology*. 2016;30:171-8.  
4  
5  
6

7  
8 [18] Marvaso G, Barone A, Amodio N, et al. Sphingosine analog fingolimod (FTY720)  
9 increases radiation sensitivity of human breast cancer cells in vitro. *Cancer biology &*  
10 *therapy*. 2014;15:797-805.  
11  
12  
13

14  
15 [19] Zhang L, Wang H, Zhu J, Ding K, Xu J. FTY720 reduces migration and invasion of  
16 human glioblastoma cell lines via inhibiting the PI3K/AKT/mTOR/p70S6K signaling  
17 pathway. *Tumour Biol*. 2014;35:10707-14.  
18  
19  
20

21  
22 [20] Hait NC, Wise LE, Allegood JC, et al. Active, phosphorylated fingolimod inhibits  
23 histone deacetylases and facilitates fear extinction memory. *Nature neuroscience*.  
24 2014;17:971-80.  
25  
26  
27

28  
29 [21] di Nuzzo L, Orlando R, Tognoli C, et al. Antidepressant activity of fingolimod in mice.  
30 *Pharmacology research & perspectives*. 2015;3:e00135.  
31  
32  
33

34  
35 [22] Hemmati F, Dargahi L, Nasoohi S, et al. Neurorestorative effect of FTY720 in a rat  
36 model of Alzheimer's disease: comparison with memantine. *Behavioural brain research*.  
37 2013;252:415-21.  
38  
39  
40

41  
42 [23] Gao F, Liu Y, Li X, Wang Y, Wei D, Jiang W. Fingolimod (FTY720) inhibits  
43 neuroinflammation and attenuates spontaneous convulsions in lithium-pilocarpine induced  
44 status epilepticus in rat model. *Pharmacology, biochemistry, and behavior*. 2012;103:187-96.  
45  
46  
47

48  
49 [24] Gol M, Ghorbanian D, Hassanzadeh S, Javan M, Mirnajafi-Zadeh J, Ghasemi-Kasman  
50 M. Fingolimod enhances myelin repair of hippocampus in pentylenetetrazol-induced kindling  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 model. European journal of pharmaceutical sciences : official journal of the European  
2 Federation for Pharmaceutical Sciences. 2017;96:72-83.  
3

4  
5 [25] Jafarian M, Karimzadeh F, Alipour F, et al. Cognitive impairments and neuronal injury  
6 in different brain regions of a genetic rat model of absence epilepsy. Neuroscience.  
7  
8 2015;298:161-70.  
9

10  
11  
12 [26] Sarkisova K, van Luijtelaar G. The WAG/Rij strain: a genetic animal model of absence  
13 epilepsy with comorbidity of depression [corrected]. Progress in neuro-psychopharmacology  
14 & biological psychiatry. 2011;35:854-76.  
15  
16

17  
18 [27] Russo E, Citraro R, Constanti A, et al. Upholding WAG/Rij rats as a model of absence  
19 epileptogenesis: Hidden mechanisms and a new theory on seizure development. Neuroscience  
20 and biobehavioral reviews. 2016;71:388-408.  
21  
22

23  
24 [28] Van Luijtelaar G, Lyashenko S, Vastyanov R, et al. Cytokines and Absence Seizures in a  
25 Genetic Rat Model. Neurophysiology. 2012;43:478-86.  
26  
27

28  
29 [29] Russo E, Andreozzi F, Iuliano R, et al. Early molecular and behavioral response to  
30 lipopolysaccharide in the WAG/Rij rat model of absence epilepsy and depressive-like  
31 behavior, involves interplay between AMPK, AKT/mTOR pathways and neuroinflammatory  
32 cytokine release. Brain, behavior, and immunity. 2014;42:157-68.  
33  
34

35  
36 [30] Kovacs Z, Dobolyi A, Juhasz G, Kekesi KA. Lipopolysaccharide induced increase in  
37 seizure activity in two animal models of absence epilepsy WAG/Rij and GAERS rats and  
38 Long Evans rats. Brain research bulletin. 2014;104:7-18.  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [31] Russo E, Citraro R, Donato G, et al. mTOR inhibition modulates epileptogenesis,  
2 seizures and depressive behavior in a genetic rat model of absence epilepsy.  
3  
4 Neuropharmacology. 2013;69:25-36.  
5  
6

7  
8 [32] Balosso S, Ravizza T, Aronica E, Vezzani A. The dual role of TNF-alpha and its  
9  
10 receptors in seizures. Experimental neurology. 2013;247:267-71.  
11  
12

13  
14 [33] Citraro R, Leo A, Constanti A, Russo E, De Sarro G. mTOR pathway inhibition as a new  
15  
16 therapeutic strategy in epilepsy and epileptogenesis. Pharmacological research. 2016;107:333-  
17  
18 43.  
19  
20

21  
22 [34] Jagirdar R, Drexel M, Kirchmair E, Tasan RO, Sperk G. Rapid changes in expression of  
23  
24 class I and IV histone deacetylases during epileptogenesis in mouse models of temporal lobe  
25  
26 epilepsy. Experimental neurology. 2015;273:92-104.  
27  
28

29  
30 [35] Leo A, Constanti A, Coppola A, Citraro R, De Sarro G, Russo E. mTOR Signaling in  
31  
32 Epilepsy and Epileptogenesis: Preclinical and Clinical Studies. In: Maiese K, editor.  
33  
34 Molecules to Medicine with mTOR: Translating Critical Pathways into Novel Therapeutic  
35  
36 Strategies Elsevier Inc.; 2016. p. 123-42.  
37  
38  
39  
40

41  
42 [36] Citraro R, Leo A, Aiello R, Pugliese M, Russo E, De Sarro G. Comparative analysis of  
43  
44 the treatment of chronic antipsychotic drugs on epileptic susceptibility in genetically epilepsy-  
45  
46 prone rats. Neurotherapeutics. 2015;12:250-62.  
47  
48

49  
50 [37] Kuznetsova GD, Petrova EV, Coenen AM, Van Luijtelaar EL. Generalized absence  
51  
52 epilepsy and catalepsy in rats. Physiology & behavior. 1996;60:1165-9.  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [38] Midzyanovskaya IS, Shatskova AB, Sarkisova KY, van Luijtelaar G, Tuomisto L,  
2 Kuznetsova GD. Convulsive and nonconvulsive epilepsy in rats: effects on behavioral  
3 response to novelty stress. *Epilepsy Behav.* 2005;6:543-51.  
4

5  
6  
7  
8 [39] Citraro R, Leo A, Franco V, et al. Perampanel effects in the WAG/Rij rat model of  
9 epileptogenesis, absence epilepsy, and comorbid depressive-like behavior. *Epilepsia.* 2016.  
10

11  
12  
13 [40] Russo E, Citraro R, Scicchitano F, et al. Effects of early long-term treatment with  
14 antiepileptic drugs on development of seizures and depressive-like behavior in a rat genetic  
15 absence epilepsy model. *Epilepsia.* 2011;52:1341-50.  
16  
17  
18

19  
20  
21 [41] Citraro R, Leo A, De Fazio P, De Sarro G, Russo E. Antidepressants but not  
22 antipsychotics have antiepileptogenic effects with limited effects on comorbid depressive-like  
23 behaviour in the WAG/Rij rat model of absence epilepsy. *British journal of pharmacology.*  
24 2015;172:3177-88.  
25  
26  
27

28  
29  
30 [42] Russo E, Citraro R, Scicchitano F, et al. Vigabatrin has antiepileptogenic and  
31 antidepressant effects in an animal model of epilepsy and depression comorbidity.  
32 *Behavioural brain research.* 2011;225:373-6.  
33  
34  
35

36  
37  
38 [43] Russo E, Constanti A, Ferreri G, Citraro R, De Sarro G. Nifedipine affects the  
39 anticonvulsant activity of topiramate in various animal models of epilepsy.  
40 *Neuropharmacology.* 2004;46:865-78.  
41  
42  
43

44  
45  
46 [44] Meno-Tetang GM, Li H, Mis S, et al. Physiologically based pharmacokinetic modeling  
47 of FTY720 (2-amino-2[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) in rats after  
48 oral and intravenous doses. *Drug metabolism and disposition: the biological fate of*  
49 *chemicals.* 2006;34:1480-7.  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [45] Blumenfeld H, Klein JP, Schridde U, et al. Early treatment suppresses the development  
2 of spike-wave epilepsy in a rat model. *Epilepsia*. 2008;49:400-9.  
3

4  
5 [46] Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF. Factors influencing behavior in  
6 the forced swim test. *Physiology & behavior*. 2013;118:227-39.  
7  
8

9  
10 [47] Russo E, Chimirri S, Aiello R, et al. Lamotrigine positively affects the development of  
11 psychiatric comorbidity in epileptic animals, while psychiatric comorbidity aggravates  
12 seizures. *Epilepsy Behav*. 2013;28:232-40.  
13  
14  
15  
16

17 [48] Citraro R, Gallelli L, Leo A, et al. Effects of chronic sodium alendronate on depression  
18 and anxiety in a menopausal experimental model. *Pharmacology, biochemistry, and behavior*.  
19 2015;129:65-71.  
20  
21  
22  
23  
24  
25

26 [49] Tsuji M, Takeda H, Matsumiya T. Modulation of passive avoidance in mice by the 5-  
27 HT1A receptor agonist flesinoxan: comparison with the benzodiazepine receptor agonist  
28 diazepam. *Neuropsychopharmacology : official publication of the American College of*  
29 *Neuropsychopharmacology*. 2003;28:664-74.  
30  
31  
32  
33  
34  
35  
36  
37

38 [50] Nestler EJ, Hyman SE. Animal models of neuropsychiatric disorders. *Nature*  
39 *neuroscience*. 2010;13:1161-9.  
40  
41  
42  
43

44 [51] Sarkisova KY, Kulikov MA. Behavioral characteristics of WAG/Rij rats susceptible and  
45 non-susceptible to audiogenic seizures. *Behavioural brain research*. 2006;166:9-18.  
46  
47  
48  
49

50 [52] Zovkic IB, Sweatt JD. Epigenetic mechanisms in learned fear: implications for PTSD.  
51 *Neuropsychopharmacology : official publication of the American College of*  
52 *Neuropsychopharmacology*. 2013;38:77-93.  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [53] Sakurai M, Sekiguchi M, Zushida K, et al. Reduction in memory in passive avoidance  
2 learning, exploratory behaviour and synaptic plasticity in mice with a spontaneous deletion in  
3 the ubiquitin C-terminal hydrolase L1 gene. *The European journal of neuroscience*.  
4 2008;27:691-701.  
5  
6  
7  
8  
9

10 [54] Karson A, Utkan T, Balci F, Aricioglu F, Ates N. Age-dependent decline in learning and  
11 memory performances of WAG/Rij rat model of absence epilepsy. *Behavioral and brain*  
12 *functions* : BBF. 2012;8:51.  
13  
14  
15  
16  
17

18 [55] Monville C, Torres EM, Dunnett SB. Comparison of incremental and accelerating  
19 protocols of the rotarod test for the assessment of motor deficits in the 6-OHDA model.  
20 *Journal of neuroscience methods*. 2006;158:219-23.  
21  
22  
23  
24  
25

26 [56] Park HW, Chang JW, Yang YS, et al. The Effect of Donor-Dependent Administration of  
27 Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells following Focal Cerebral  
28 Ischemia in Rats. *Experimental neurobiology*. 2015;24:358-65.  
29  
30  
31  
32  
33  
34

35 [57] Amodio N, Stamato MA, Gulla AM, et al. Therapeutic Targeting of miR-29b/HDAC4  
36 Epigenetic Loop in Multiple Myeloma. *Molecular cancer therapeutics*. 2016;15:1364-75.  
37  
38  
39  
40

41 [58] Gouveia TL, Vieira de Sousa PV, de Almeida SS, et al. High serum levels of  
42 proinflammatory markers during epileptogenesis. Can omega-3 fatty acid administration  
43 reduce this process? *Epilepsy Behav*. 2015;51:300-5.  
44  
45  
46  
47  
48

49 [59] Vezzani A, Aronica E, Mazarati A, Pittman QJ. Epilepsy and brain inflammation.  
50 *Experimental neurology*. 2013;244:11-21.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [60] de Vries EE, van den Munckhof B, Braun KP, van Royen-Kerkhof A, de Jager W,  
2 Jansen FE. Inflammatory mediators in human epilepsy: A systematic review and meta-  
3 analysis. *Neuroscience and biobehavioral reviews*. 2016;63:177-90.  
4  
5

6  
7  
8 [61] van Luijtelaar G, Zobeiri M. Progress and outlooks in a genetic absence epilepsy model  
9 (WAG/Rij). *Current medicinal chemistry*. 2014;21:704-21.  
10

11  
12  
13 [62] Kovacs Z, Czurko A, Kekesi KA, Juhasz G. Intracerebroventricularly administered  
14 lipopolysaccharide enhances spike-wave discharges in freely moving WAG/Rij rats. *Brain*  
15 *research bulletin*. 2011;85:410-6.  
16  
17  
18

19  
20  
21  
22 [63] Kovacs Z, Kekesi KA, Szilagyi N, et al. Facilitation of spike-wave discharge activity by  
23 lipopolysaccharides in Wistar Albino Glaxo/Rijswijk rats. *Neuroscience*. 2006;140:731-42.  
24  
25

26  
27  
28 [64] Rimoli MG, Russo E, Cataldi M, et al. T-type channel blocking properties and  
29 antiabsence activity of two imidazo[1,2-b]pyridazine derivatives structurally related to  
30 indomethacin. *Neuropharmacology*. 2009;56:637-46.  
31  
32  
33

34  
35  
36 [65] Akin D, Ravizza T, Maroso M, et al. IL-1beta is induced in reactive astrocytes in the  
37 somatosensory cortex of rats with genetic absence epilepsy at the onset of spike-and-wave  
38 discharges, and contributes to their occurrence. *Neurobiology of disease*. 2011;44:259-69.  
39  
40  
41

42  
43  
44 [66] Russo E, Follesa P, Citraro R, et al. The mTOR signaling pathway and neuronal  
45 stem/progenitor cell proliferation in the hippocampus are altered during the development of  
46 absence epilepsy in a genetic animal model. *Neurological sciences : official journal of the*  
47 *Italian Neurological Society and of the Italian Society of Clinical Neurophysiology*.  
48 2014;35:1793-9.  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [67] Zhang B, Wong M. Pentylentetrazole-induced seizures cause acute, but not chronic,  
2 mTOR pathway activation in rat. *Epilepsia*. 2012;53:506-11.  
3

4  
5 [68] Song GY, Kang JS, Lee SY, Myung CS. Region-specific reduction of Gbeta4 expression  
6 and induction of the phosphorylation of PKB/Akt and ERK1/2 by aging in rat brain.  
7  
8 *Pharmacological research*. 2007;56:295-302.  
9

10  
11 [69] Orellana AM, Vasconcelos AR, Leite JA, et al. Age-related neuroinflammation and  
12 changes in AKT-GSK-3beta and WNT/ beta-CATENIN signaling in rat hippocampus. *Aging*  
13 (Albany NY). 2015;7:1094-111.  
14  
15

16  
17 [70] Sankar R, Mazarati A. Neurobiology of Depression as a Comorbidity of Epilepsy. In:  
18 Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, editors. *Jasper's*  
19 *Basic Mechanisms of the Epilepsies*, Bethesda (MD)2012.  
20  
21

22  
23 [71] Kanner AM, Mazarati A, Koepp M. Biomarkers of epileptogenesis: psychiatric  
24 comorbidities (?). *Neurotherapeutics*. 2014;11:358-72.  
25  
26

27  
28 [72] Sarkisova KY, Kuznetsova GD, Kulikov MA, van Luijtelaar G. Spike-wave discharges  
29 are necessary for the expression of behavioral depression-like symptoms. *Epilepsia*.  
30 2010;51:146-60.  
31  
32

33  
34 [73] Fox E, Edwards K, Burch G, et al. Outcomes of switching directly to oral fingolimod  
35 from injectable therapies: Results of the randomized, open-label, multicenter, Evaluate Patient  
36 OutComes (EPOC) study in relapsing multiple sclerosis. *Mult Scler Relat Disord*.  
37 2014;3:607-19.  
38  
39  
40  
41  
42  
43  
44



1 [74] Hunter SF, Agius M, Miller DM, et al. Impact of a switch to fingolimod on depressive  
2 symptoms in patients with relapsing multiple sclerosis: An analysis from the EPOC (Evaluate  
3 Patient Outcomes) trial. *Journal of the neurological sciences*. 2016;365:190-8.  
4  
5  
6

7 [75] Bang SR, Ambavade SD, Jagdale PG, Adkar PP, Waghmare AB, Ambavade PD.  
8 Lacosamide reduces HDAC levels in the brain and improves memory: Potential for treatment  
9 of Alzheimer's disease. *Pharmacology, biochemistry, and behavior*. 2015;134:65-9.  
10  
11  
12

13 [76] Nalivaeva NN, Belyaev ND, Lewis DI, et al. Effect of sodium valproate administration  
14 on brain neprilysin expression and memory in rats. *Journal of molecular neuroscience : MN*.  
15 2012;46:569-77.  
16  
17  
18

19 [77] Kilgore M, Miller CA, Fass DM, et al. Inhibitors of class 1 histone deacetylases reverse  
20 contextual memory deficits in a mouse model of Alzheimer's disease.  
21 *Neuropsychopharmacology : official publication of the American College of*  
22 *Neuropsychopharmacology*. 2010;35:870-80.  
23  
24  
25

26 [78] Sitnikova E, Rutsikova EM, Raevsky VV. Reduction of epileptic spike-wave activity in  
27 WAG/Rij rats fostered by Wistar dams. *Brain research*. 2015;1594:305-9.  
28  
29  
30

31 [79] Sitnikova E. Neonatal sensory deprivation promotes development of absence seizures in  
32 adult rats with genetic predisposition to epilepsy. *Brain research*. 2011;1377:109-18.  
33  
34  
35

36 [80] Deogracias R, Yazdani M, Dekkers MP, et al. Fingolimod, a sphingosine-1 phosphate  
37 receptor modulator, increases BDNF levels and improves symptoms of a mouse model of Rett  
38 syndrome. *Proceedings of the National Academy of Sciences of the United States of America*.  
39 2012;109:14230-5.  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 [81] Calfa G, Chapleau CA, Campbell S, et al. HDAC activity is required for BDNF to  
2 increase quantal neurotransmitter release and dendritic spine density in CA1 pyramidal  
3 neurons. *Hippocampus*. 2012;22:1493-500.  
4  
5  
6

7  
8 [82] Palleria C, Leporini C, Maida F, et al. Potential effects of current drug therapies on  
9 cognitive impairment in patients with type 2 diabetes. *Frontiers in neuroendocrinology*.  
10 2016;42:76-92.  
11  
12  
13  
14

15  
16 [83] Jagirdar R, Drexel M, Bukovac A, Tasan RO, Sperk G. Expression of class II HDACs in  
17 two mouse models of temporal lobe epilepsy. *Journal of neurochemistry*. 2015.  
18  
19  
20

21  
22 [84] Morris MJ, Karra AS, Monteggia LM. Histone deacetylases govern cellular mechanisms  
23 underlying behavioral and synaptic plasticity in the developing and adult brain. *Behavioural*  
24 *pharmacology*. 2010;21:409-19.  
25  
26  
27  
28

29  
30 [85] French JA, White HS, Klitgaard H, et al. Development of new treatment approaches for  
31 epilepsy: unmet needs and opportunities. *Epilepsia*. 2013;54 Suppl 4:3-12.  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Figure Legends

1  
2  
3  
4  
5 **Fig. 1. Experimental protocol #1 for fingolimod ELTT.** Graph branches specify the  
6 experimental sequence followed and the number of rats used in every test. CTRL, controls;  
7 EEGs, electroencephalographic recordings; EPM, elevated plus maze; FST, forced swimming  
8 test; PA, passive avoidance; i.p. intraperitoneal administration.  
9

10  
11  
12  
13  
14  
15  
16  
17 **Fig. 2. Experimental protocol #2 for fingolimod acute/subacute treatment.** Graph  
18 branches specify the experimental sequence followed and the number of rats used in every  
19 test. CTRL, controls; EEGs, electroencephalographic recordings; EPM, elevated plus maze;  
20 FST, forced swimming test; PA, passive avoidance; i.p. intraperitoneal administration;  
21 mTOR, mammalian target of rapamycin (mTOR).  
22  
23  
24  
25  
26  
27

28  
29  
30  
31 **Fig. 3. Effects of a Early long-term fingolimod treatment on the development of absence**  
32 **seizures.** Effects of an early long-term treatment (ELTT; started at P30 and lasting 17 weeks)  
33 with fingolimod (Fing) on spike-wave discharges (SWDs) recorded in WAG/Rij rats at 6 (6  
34 m) and 10 (10 m) months of age. Data (means  $\pm$  SEM,  $n = 6$  per group) are expressed as  
35 percentage change relative to 6-month-old control rats (dotted line; values for control rats  
36 were: nSWDs =  $11.06 \pm 1.02$ ; dSWDs =  $72.03 \pm 6.22$ ; sSWDs =  $6.31 \pm 1.33$ ). \*Significantly  
37 different ( $P < 0.05$ ) from age-matched control rats. CTRL, control rats; nSWDs, mean number  
38 of SWDs for every 30-min epoch; dSWDs, mean cumulative duration of SWDs for every 30-  
39 min epoch expressed in seconds(s); sSWD, mean duration of a single SWD expressed in (s).  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

56 **Fig. 4. a) Forced swimming test (FST).** Bars indicate the immobility time (IT), expressed in  
57 seconds, in the forced swimming test (FST) in WAG/Rij ( $n = 6$  per group) rats at 6 and 10  
58  
59  
60  
61  
62  
63  
64  
65

1 months of age following an Early Long Term Treatment (ELTT; started at P30 and lasting 17  
2 weeks) with fingolimod at 1 mg/Kg/day (Fing). \*Significantly different ( $P < 0.05$ ) from age-  
3 matched control rats (CTRL). b) **Elevated plus maze (EPM)**. Bars indicate the time spent in  
4 open arms, expressed in seconds, in the elevated plus maze (EPM), in WAG/Rij ( $n = 6$  per  
5 group) rats at 6 and 10 months of age following an ELTT with fingolimod at 1 mg/Kg/day  
6 (Fing). \*Significantly different ( $P < 0.05$ ) from age-matched control rats (CTRL). c) **Passive**  
7 **avoidance (PA) test**. Bars indicate the time spent to enter into the dark chamber. Data  
8 marked with: \* are significantly different ( $P < 0.05$ ) from age-matched fingolimod-treated  
9 rats; # significantly different ( $P < 0.05$ ) from age-matched control (untreated) WAG/Rij rats.  
10  
11 Data are means  $\pm$  S.E.M.;

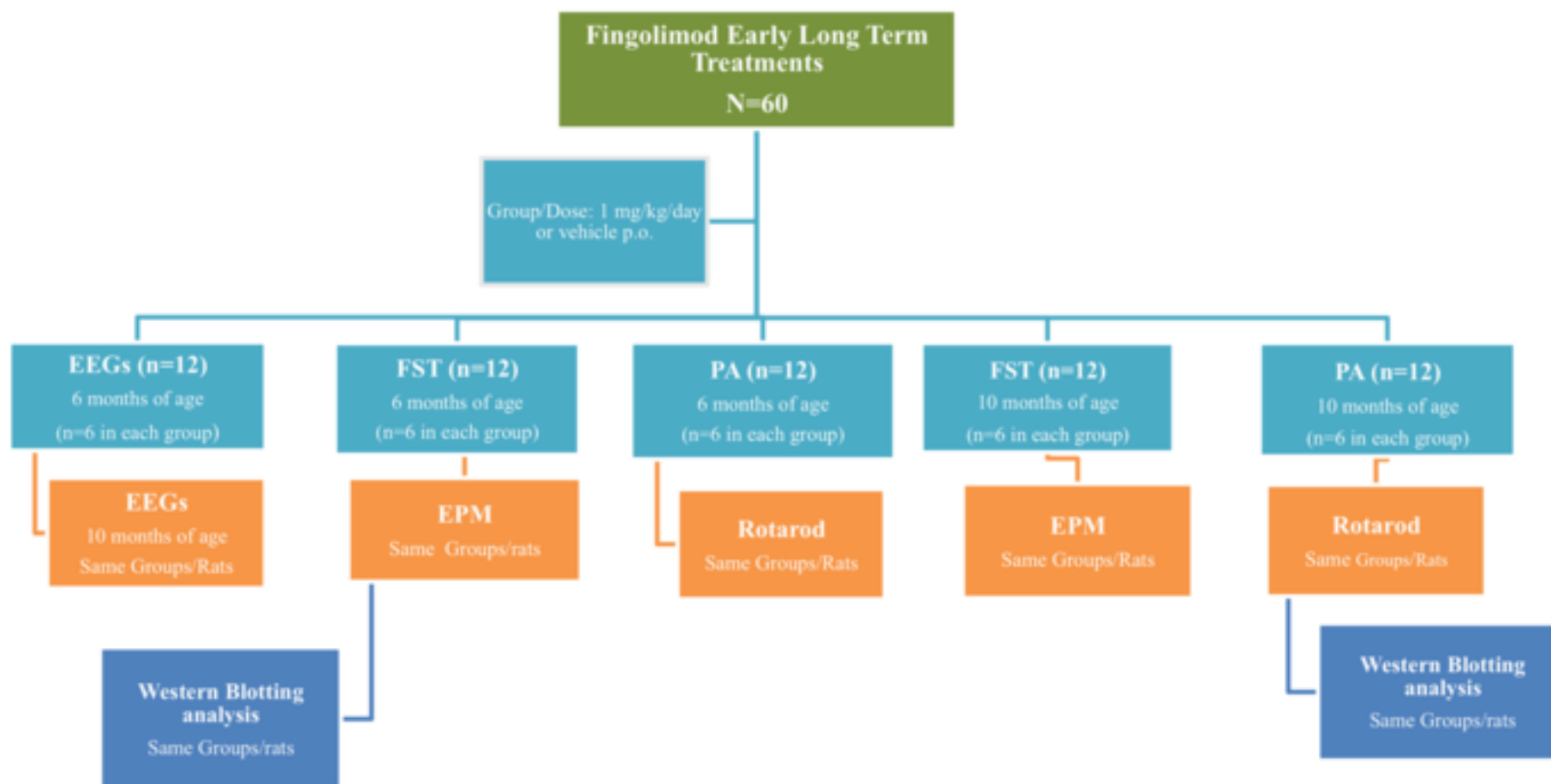
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26 **Fig. 5. Western blot analysis after an Early long term fingolimod treatment.** a)  
27 Representative panel of Western blotting experiments on the effect of an ELTT with  
28 fingolimod (Fing) on the expression level in the cortex, of phosphorylated AKT (p-AKT) b),  
29 mTOR (p-mTOR) c), and p70S6K (p-p70S6K). Columns represent mean relative protein  
30 levels normalized to control ( $n = 6$  per group). Loading was normalized using GADPH levels.  
31 Data marked with: \* are significantly different ( $P < 0.05$ ) from age-matched control  
32 (untreated) WAG/Rij rats; # Significantly different ( $P < 0.05$ ) from age-matched control  
33 (untreated) WAG/Rij rats.  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47

48 **Fig. 6. Western blot analysis after a sub-chronic fingolimod treatment.** Quantitative  
49 Western blot analysis of phosphorylated p70S6K (p-p70S6K) levels in the cortex of WAG/Rij  
50 rats of 6 months of age sub-chronically treated with fingolimod. Columns represent mean  
51 relative protein levels normalized to control ( $n = 6$  per group). Loading was normalized using  
52 GADPH levels.  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

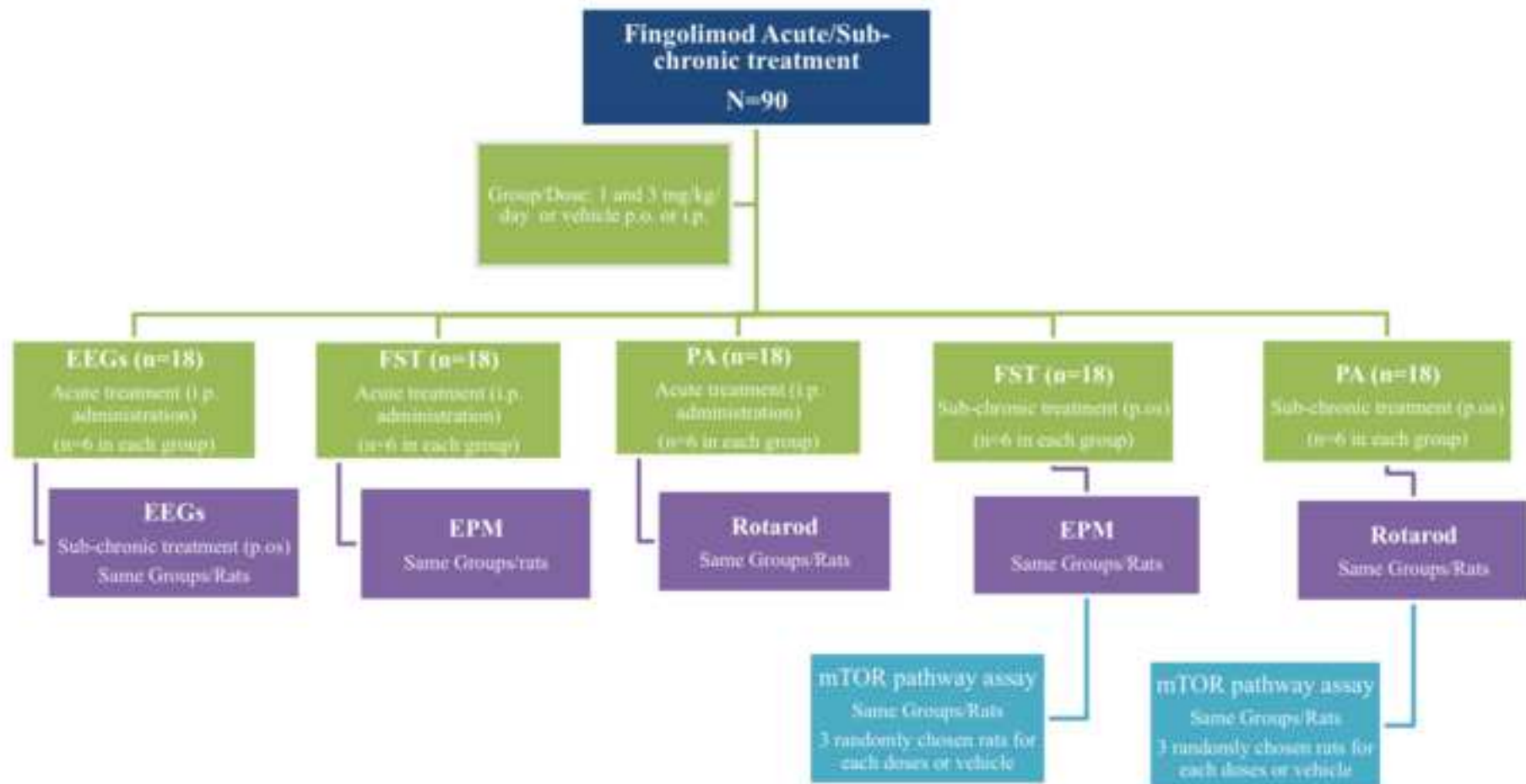
1  
2 **Fig. 7. Quantitative Western blot analysis of the acetylation of Lysine 8 of histone H4.**  
3

4 Data marked with: \* are significantly different ( $P < 0.05$ ) from age-matched control  
5 (untreated) WAG/Rij rats; # Significantly different ( $P < 0.05$ ) from age-matched control  
6 (untreated) WAG/Rij rats.  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**Fig. 1**



**Fig. 2**



**Fig. 3**

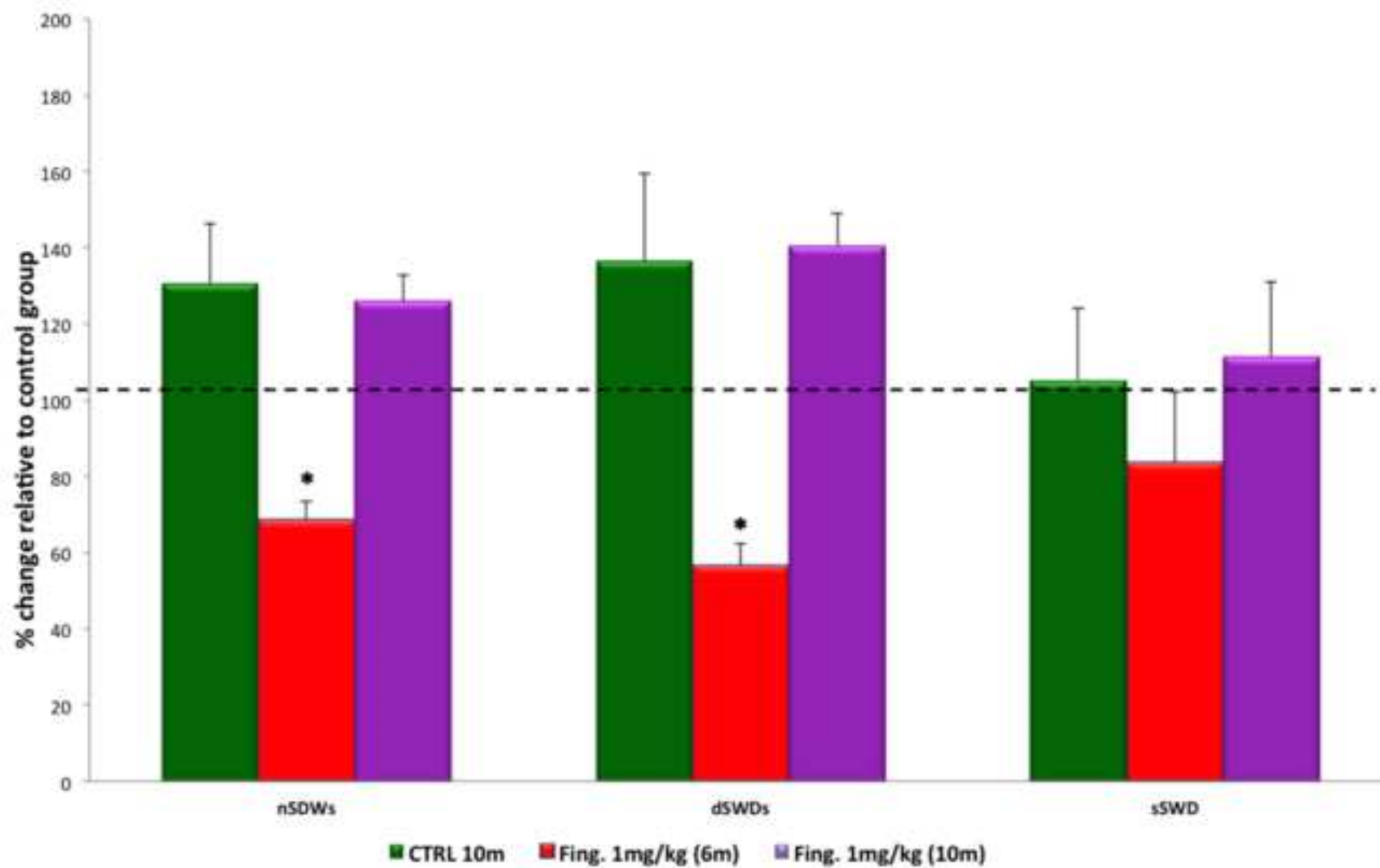
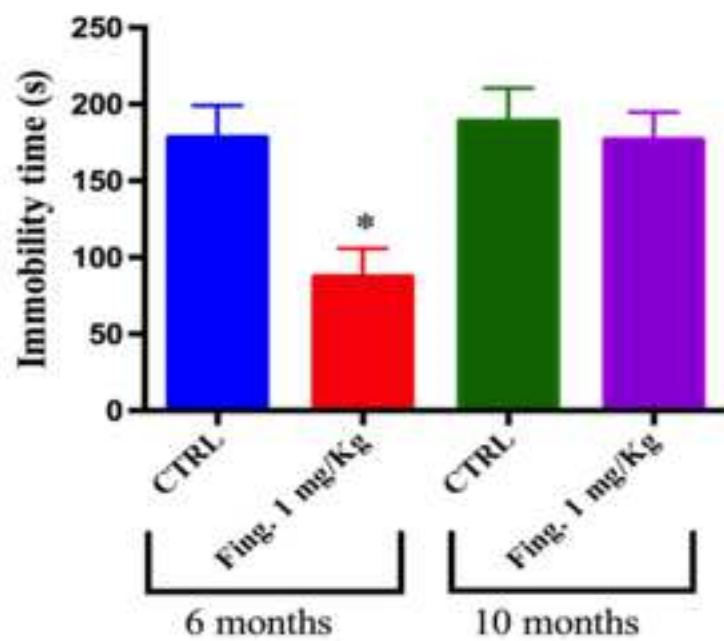


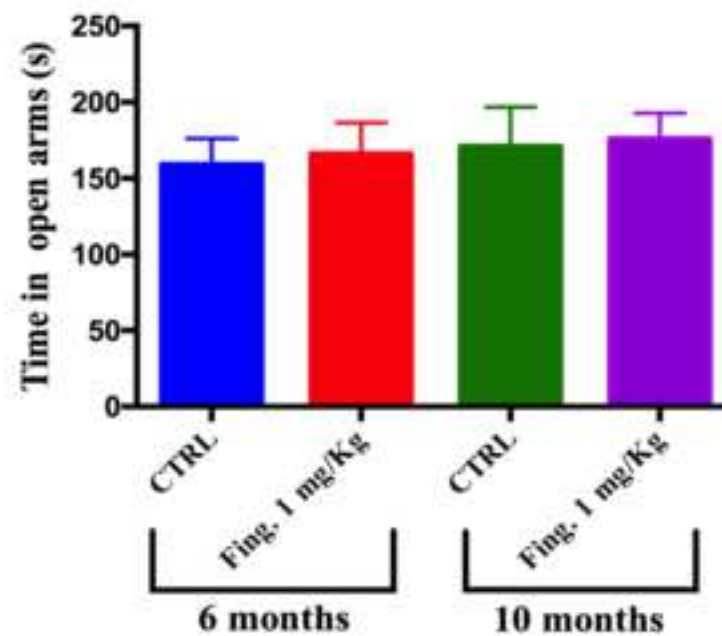


Fig. 4

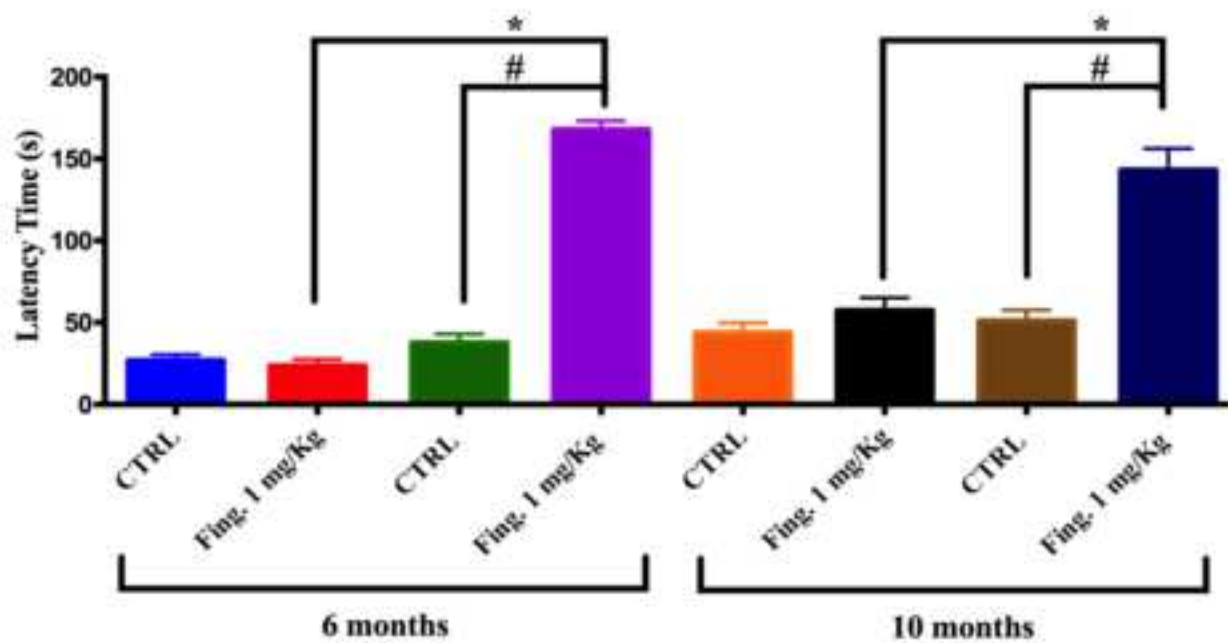
a)



b)



c)



**Fig. 5**

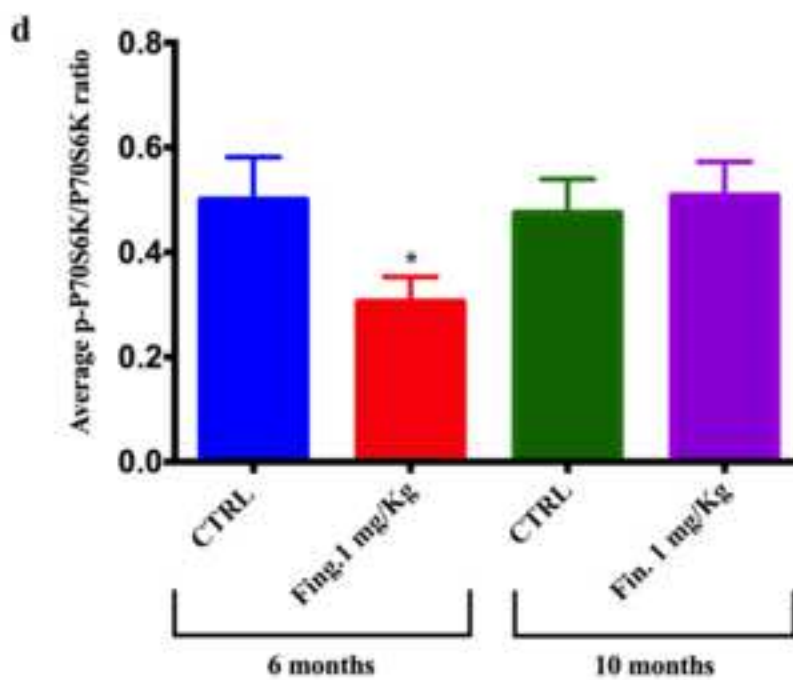
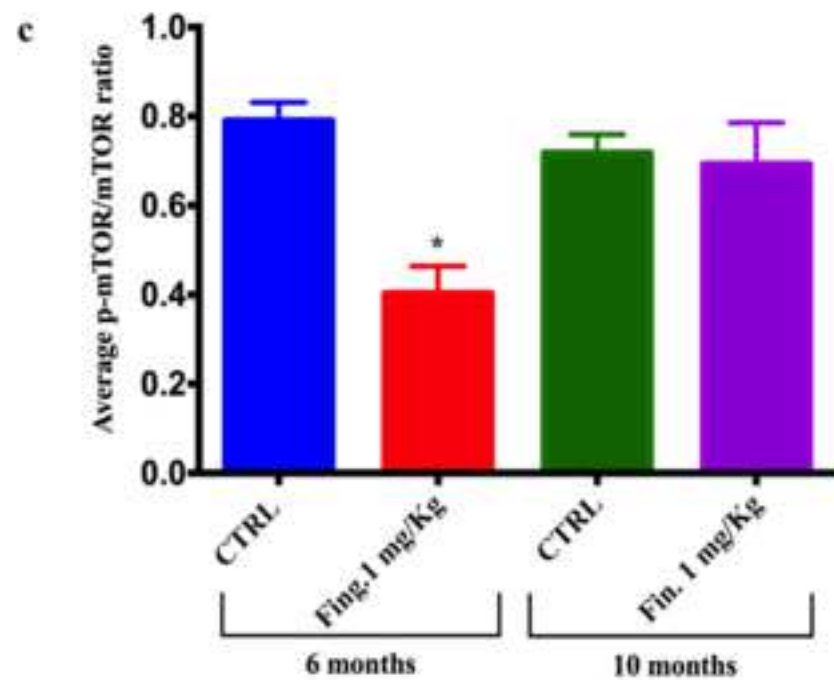
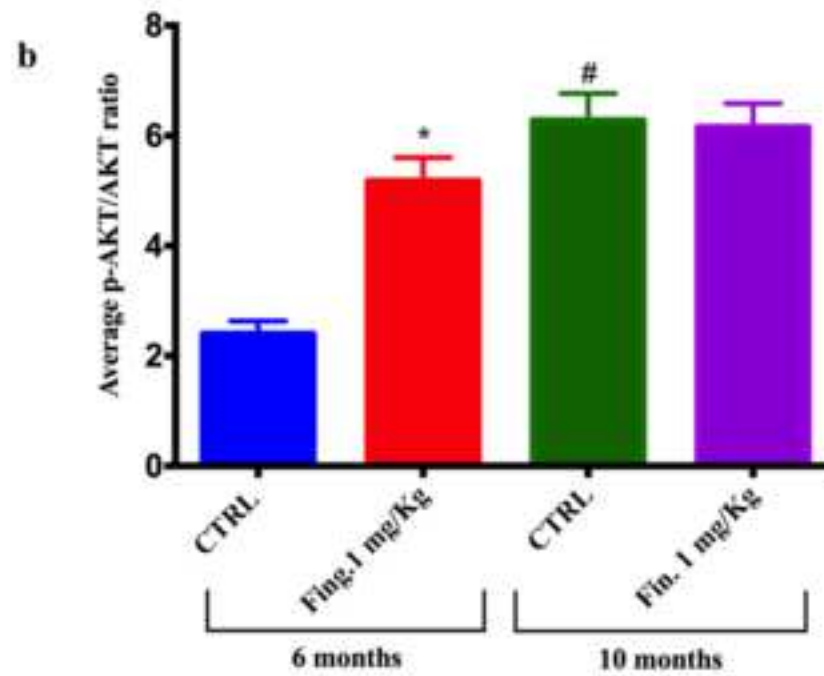
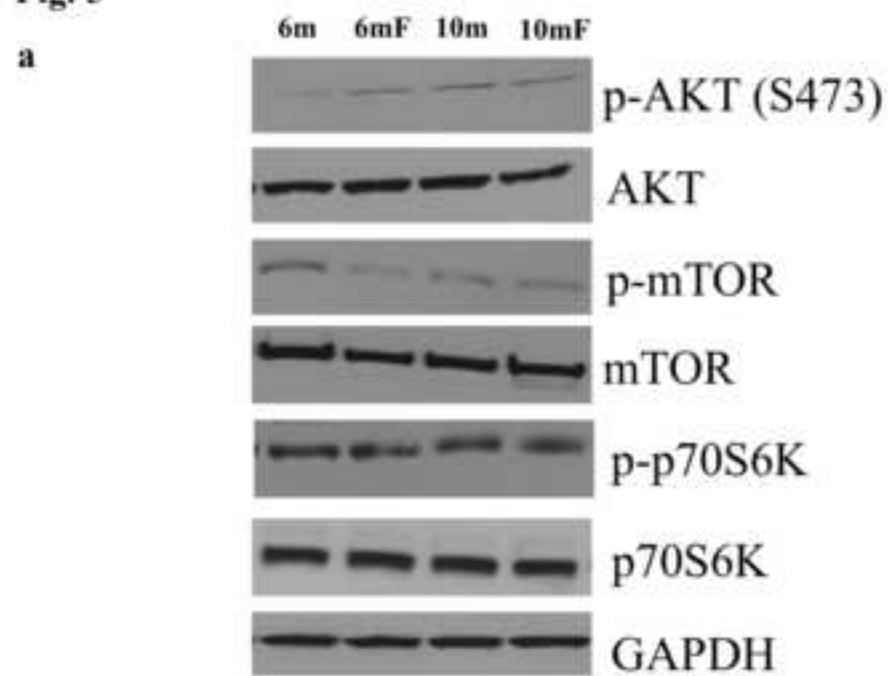
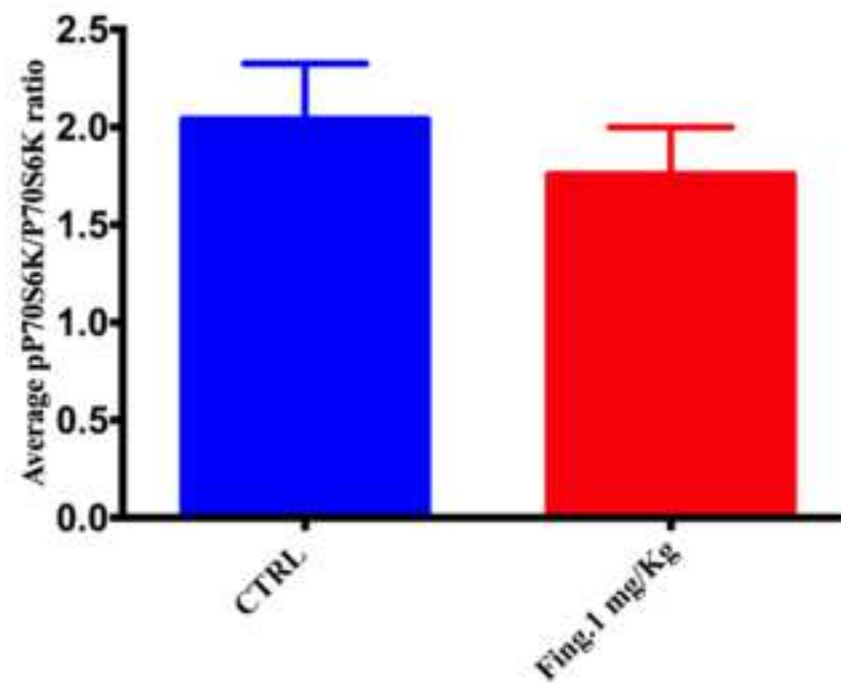
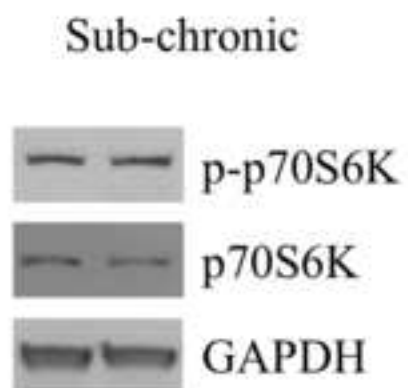
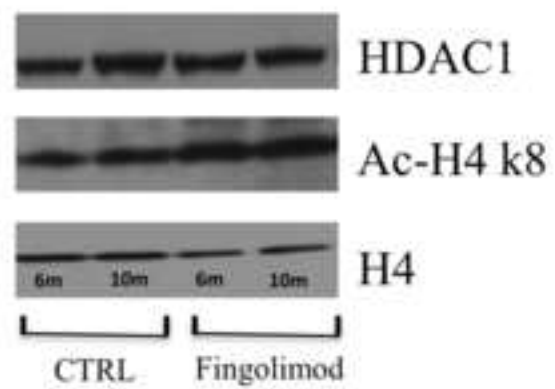


Fig. 6



**Fig. 7**

**A**



**B**

