Fingolimod exerts only temporary antiepileptogenic effects but longer-lasting positive effects on behavior in the WAG/Rij rat absence epilepsy model

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Original research

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Running title: Effects of Fingolimod in WAG/Rij rats.

Abstract

One of the major challenges in the epilepsy field is identifying disease-modifying drugs in order to prevent or delay spontaneous recurrent seizure onset or to cure already established epilepsy. It has been recently reported that fingolimod, currently approved for the treatment of relapsing-remitting multiple sclerosis, has demonstrated antiepileptogenic effects in two different preclinical models of acquired epilepsy. However, to date, no data exist regarding the role of fingolimod against genetic epilepsy. Therefore, we have addressed this issue by studying the effects of fingolimod in WAG/Rij rats, a well-established genetic model of absence epilepsy, epileptogenesis, and neuropsychiatric comorbidity. Our results have demonstrated that an early-long term treatment with fingolimod (1 mg/Kg/day), started before absence seizure onset, has both antiepileptogenic and antidepressant-like effects in the WAG/Rij rats. However, these effects were transitory, since 5 months after treatment discontinuation, both absence seizure and depressive like-behavior returned to control level. Furthermore, a temporary reduction of mTOR signaling pathway activity, indicated by reduced p-mTOR and p-p70S6k levels and by an increased p-AKT in WAG/Rij rats of 6 months of age accompanied the transitory antiepileptogenic fingolimod effects. Surprisingly, fingolimod has demonstrated longer-lasting positive effects on cognitive decline in this strain. This effect was accompanied by an increased acetylation of Lysine 8 of histone H4 (both 6 and 10 months of age). In conclusion, our results support the antiepileptogenic effects fingolimod. However, these antiepileptogenic effects were transitory. Moreover, fingolimod might also have a positive impact on animal behavior and particularly in protecting the development of memory decline.

Keywords: Fingolimod; Epileptogenesis; Absence epilepsy; Behavior; mTOR; Histone deacetylase (HDAC).

Introduction

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

Fingolimod (FTY720), an immunomodulator drug derived from the fungus *Isaria sinclairii*, is the first orally bioavailable disease-modifying drug approved by Food and Drug Administration (FDA), in September 2010, as a first-line treatment of relapsing-remitting multiple sclerosis (MS) [12, 13]. Chemically, it is a synthetic homologous of sphingosine, derived from membrane lipid sphingomyelin, which after its phosphorylation into sphingosine 1-phosphate (S1P) by sphingosine kinases (SphKs) plays a fundamental role in several physiological and pathological functions linked to the immunological, cardiovascular and central nervous system (CNS) [14]. To date, the exact mechanism by which fingolimod acts is

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

Therefore, these multiple relevant effects have prompted the study of fingolimod effects in other brain disorders [16] including Alzheimer's disease [22] and epilepsy [13, 23, 24]. Regarding epilepsy, Gao et al. [23] described the antiepileptogenic and neuroprotective effects of fingolimod in the lithium-pilocarpine rat epilepsy model through its ability to suppress both microglial activation and to decrease IL-1β and TNF-α levels. More recently, fingolimod antiepileptic effects were confirmed in the PTZ-kindling mouse model with pre-treatment reducing seizure development as well as protecting myelin and post-treatment reducing seizure severity as well as inducing remyelination in kindled mice [24]. Considering fingolimod potential effects in epilepsy/epileptogenesis and the lack of data on genetic epilepsy models and epilepsy comorbidities, we aimed in the present study, to evaluate the effects of some fingolimod treatments (acute, sub-chronic and early long-term treatment) in Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats, a well-established genetic model of absence epilepsy, epileptogenesis, and neuropsychiatric comorbidity (dysthymia and decline in learning and memory performance) [25-27]. Despite in this latter model neuroinflamamtion does not seem to play a major role [28]; it has been demonstrated that drugs (e.g. etoricoxib, indomethacin and rapamycin) acting on inflammation can both reduce absence seizures and their development and accordingly, increasing neuroinflammation increases absence seizures

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

Materials and Methods

Animals

All the experiments were carried out in male non-audiogenic WAG/Rij rats. WAG/Rij rat progenitors, weighing about 60 g (4 weeks old), were originally purchased from Charles River Laboratories s.r.l. (Calco, Lecco, Italy) and the rats used in these protocols were all obtained from our breeding colony at the University of Catanzaro animal facility, as previously described [11, 36]. WAG/Rij rats were housed three/four per cage and kept under stable environmental conditions, humidity (60 ± 5%) and temperature (21 ± 2 °C), in a room with 12/12 h reversed light/dark cycle (lights on at 20.00). WAG/Rij rats at 27 days of age (P27) were screened, as previously described [37, 38], to evaluate their susceptibility to audiogenic stimuli. Afterwards, only rats without audiogenic susceptibility were used in experiments, considering that WAG/Rij rats expressing audiogenic seizures display higher levels of anxiety in comparison to non-audiogenic WAG/Rij rats [26].

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

Experimental summary

The aim of these experiments was to evaluate both the potential antiepileptogenic effects of fingolimod in WAG/Rij rats (**experiment #1**) and its possible acute and/or sub-chronic effects versus established absence seizures (**experiment #2**) in the same model. This was accompanied by the analysis of fingolimod effects on different behavioral tasks, which were performed to study anxiety and depressive-like behavior, motor performance and cognitive impairment [39]. Finally, the effects of fingolimod on the mTOR signaling pathway as well as on the acetylation level on histone H4 were explored in order to increase our knowledge on its likely mechanism of action. A scheme of the experimental protocols is reported in Fig. 1 and 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

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

Experiment #2 assessed the effects of two doses of fingolimod (1 and 3 mg/Kg), acutely and sub-chronically (daily dose) administered in WAG/Rij rats with established seizures at the age of ~6 months (Fig. 2). WAG/Rij rats (n = 90) of ~6 months of age were randomly divided into five groups (n = 18) and each of these into 3 subgroups (n = 6) for the two doses of fingolimod and vehicle (Fig. 2). The first group was used to study (EEGs), both the acute (intraperitoneal injection; i.p.) and the subchronic (7 days oral treatment) effects of fingolimod on established absence seizures. The second and the third groups were used to assess the acute (i.p.) effects of fingolimod on depressive and anxiety-like behavior as well as learning/memory performance, respectively. The fourth and fifth groups were used to test subchronic fingolimod effects on the same parameters of group 2 and 3 (Fig. 2). Six randomly chosen rats from groups 4 and 5 were used to measure the phosphorylation levels of p70S6K in order to study fingolimod (3mg/Kg/day for 1 week) effects on the mTOR pathway.

Surgery and EEG recordings

WAG/Rij rats allocated into the groups or subgroups for EEG recordings and seizure quantification, under general anesthesia (mixture of tiletamine/zolazepam; 1:1; Zoletil 100®; 50 mg/Kg i.p.; VIRBAC Srl, Milan, Italy), were stereotaxically implanted with 3 cortical electrodes for EEG recordings attached to a 3-channel rat headmount (8239-SE3, Pinnacle

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

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

At the age of ~6 months (1 month after drug withdrawal), one subgroup of treated and one of untreated (vehicle) WAG/Rij rats, following surgery (see section Surgery and EEG recordings), were experimentally evaluated by 3 hours of EEG recordings over 3 consecutive days. The same rats were again EEG studied at the age of ~10 months (5 months after treatment discontinuation) to evaluate the potential long-term effects fingolimod. The same recording schedule was used for subchronically treated rats (see Section *Acute and subchronic procedures* for details on treatment protocol) while for the evaluation of potential acute fingolimod effects, rats were subjected to EEG recordings lasting 6h: 1h baseline without drug administration, and 5h after i.p. injection of fingolimod or vehicle.

Behavioral tests

In order to reduce the number of rats used and avoid the influence played by several testings in the same animal, rats (n = 6 for every group) were divided as described in Fig. 1 and 2 and

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

In detail, in order to evaluate the ELTT effects of fingolimod, behavioral tests were performed respectively ~1 and ~5 months after the end of fingolimod treatment. For the acute and sub-chronic effects of fingolimod, behavioral tests were performed at the end of the acute and sub-chronic treatment period, 1h and 7 days respectively. Regarding subchronic administration groups, when the same group of rats was subjected to multiple behavior tests and/or repeated sessions of the same test, it was kept under treatment. Regarding acute administration groups, the rat groups subjected to the passive avoidance test, were injected with fingolimod (1 and 3 mg/Kg/day) 1h before to perform the conditioning session [49], and when multiple tests were required in the same group, the drug was re-injected 1h before.

Forced swimming test

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

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

Elevated plus maze

The elevated plus maze (EPM) consists of two opposing open arms and two opposing closed arms of the same size (45 cm x 10 cm) with walls 10 cm high connected by a central platform (10 x 10 cm) and elevated 80 cm above the floor, as previously described [13, 47]. Rats are positioned in the central platform facing a closed arm and the number of entries into, time spent on each arm and central platform are measured. The maze was systematically cleaned to remove olfactory cues, after each animal was tested. The shorter the time spent in open arms and central platform the higher is anxiety and *vice versa.* Mean velocity and total distance moved were also measured and examined for every experimental group [13, 51].

Passive avoidance test

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

conditioning session (learning trial) was started 15 min after habituation. Rats were individually placed in the light chamber. After 30 sec the sliding door was automatically opened. When the rats entered into the dark chamber, the sliding door was automatically closed and an electrical foot-shock (0.5 mA) was delivered for 3 seconds via the floor grid. Afterwards, rats were housed. The latency to enter into the dark chamber was recorded and analyzed. Each rat was given 300 sec to enter into the dark chamber. If a rat failed to cross from the light to the dark chamber within the cut-off time, it was discarded from the study. Between each training session, the apparatus was systematically cleaned to remove olfactory cues. The retention session (memory trial) was performed 24 h after the conditioning session by re-introducing the rat into the light chamber of the apparatus. Rats' memory was assessed by recording their latency to enter into the dark chamber; however, no foot-shock was delivered in this session. The maximum cutoff time for the step-through latency was 300 s. If a rat failed to cross from the light to the dark chamber within the cut-off time, it was housed and a latency of 300 s was recorded for that rat. Retention memory is directly related to the latency to enter in the dark chamber: the better the memory, the greater the latency [25, 39, 54].

Rotarod test

The rotarod test was used to evaluate any eventual locomotor impairment induced by drug treatment. The test was performed as previously described by Monville et al. [55] with some minor modifications on a Rotarod unit (LE 8500, Panlab, Barcelona, Spain). Briefly, after a habituation session, rats were placed on a rod whose acceleration was increased from 4 to 40 rpm over a period of 300 s. The latency to fall and the number of falls were recorded. Rats were trained for 3 consecutive sessions and the mean of the 3 sessions was analyzed [56].

Western blotting analysis

Rats were decapitated and their brains were quickly removed and submerged in ice-cold artificial cerebrospinal fluid. Subsequently, the cortex was isolated and dissected by an optical microscope as previously described by Russo et al. [29]. The cortex was homogenized using the Gentle MACS dissociator (Miltenyi Biotech) in ice-cold NP40 lysis buffer (Life Technologies) containing a cocktail of protease and phosphatase inhibitors (Life Technologies), and then centrifuged at 12.000 rpm for 30 min at 4°C to remove tissue debris. 50 μg of proteins were electrophoresed through a NuPAGE 4-12% gradient gel (Life Technologies) and electroblotted onto a nitrocellulose membrane (Life Technologies) as previously reported [57]. The membrane was blocked for 1 hour with 5% non-fat dry milk/PBS-tween 0.05% (Biorad), and then incubated over night with the antibodies for p-AKT (S473), AKT, p-mTOR (S2448), mTOR, p-p70S6 Kinase (T389), p70S6 Kinase, HDAC1, histone H4 and acetylated-histone H4 (K8) (all from Cell Signaling); GAPDH (Santa Cruz, DBA) was used as loading control. The levels of proteins and phosphoproteins were detected with horseradish peroxidase-linked secondary antibodies and the ECL (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy). Autoradiographs were scanned to obtain arbitrary densitometric units. Data were normalized against those of the corresponding GAPDH. The experiments were performed in triplicate and the results calculated as means ± SEM and expressed as protein change (%)[57].

Statistical analysis

All statistical procedures were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA 92037, USA). EEG recordings were subdivided into 30 min epochs, and the duration and number of SWDs were evaluated separately, as previously described [42]. Such values were averaged and data were expressed as means ± SEM. EEG data were analyzed and

compared by one-way ANOVA followed by Dunnett's *post hoc* test. Furthermore, we used one-way ANOVA followed by Dunnett's *post hoc* test to analyze and compare behavioral data obtained from acute and subchronic treatments. Data obtained by behavioral tests and Western blotting analysis from the ELTT schedule, were analyzed and compared by two-way ANOVA followed by Bonferroni's *post hoc* test. Data obtained by Western blotting analysis from subchronic treatment, were analyzed and compared by Student's t-test. All tests used were two sided and $P \leq 0.05$ was considered significant.

Results

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

Analysis of EEG recordings of 6 months old WAG/Rij rats showing established absence seizures, indicated that fingolimod, administered acutely i.p. and subchronically o.s. (1 and 3 mg/Kg) was not able to significantly modify ($P > 0.05$) absence seizure parameters in comparison with the control group (data not shown).

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

Untreated control WAG/Rij rats, at the age of 6 months, showed a mean number of SWDs (nSWDs) of 11.06 ± 1.02 with a mean total duration (dSWDs) of 72.03 ± 6.22 s and a mean single duration (sSWD) of 6.31 ± 1.33 for a 30 min epoch (Table 1). ELTT with fingolimod (1 mg/Kg/day; *per os*) significantly decreased ($P < 0.05$) the development of absence seizures (both number and total duration, but not sSWD) in WAG/Rij rats at the age of 6 months (1 month after treatment discontinuation) in comparison to untreated rats of the same age, supporting antiepileptogenic effects (Fig. 3). In particular, ELTT with fingolimod did not significantly (~12 %; $P > 0.05$) reduce mean sSWD duration, whereas it significantly modified

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

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

Effects of fingolimod on depressive- and anxiety like behavior

WAG/Rij rats already at the age of 4 months and even more so at 6 months of age, display an increased immobility time in the FST, which is very likely linked to the development of SWDs [26, 40]. Both fingolimod acute and sub-chronic treatments had no effects on immobility time (IT) in any group tested despite animal age or dose used. On the other hand, fingolimod ELTT (1 mg/Kg/day; *per os*) significantly (P = 0.0054) decreased the IT in 6 months old WAG/Rij rats (Fig. 4a; 1 month after treatment discontinuation), whereas this fingolimod antidepressant-like effect was not observed in WAG/Rij rats where treatment was discontinued 5 months earlier (10 months old rats group; Fig. 4a). Mean velocity and total distance moved did not significantly differ (P> 0.05) between groups (data not shown). Anxiety-like behavior in WAG/Rij rats was evaluated by EPM and, also in this case, it was not affected by both acute and subchronic fingolimod treatment. Similarly, ELTT with fingolimod was not able to modify any measured parameters independently of the time of the test (Fig. 4b).

Effects of fingolimod on learning and memory and motor coordination

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

Effects of fingolimod on the mTOR pathway

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

Western blotting analysis of the rat cortex obtained from animals in the fingolimod (1 mg/Kg/day) ELTT group at 6 months of age revealed an increased (P = 0.0017) phosphorylation of AKT and a reduced (P<0.001) phosphorylation of both mTOR and p70S6K. On the other hand, no differences were found when comparing phosphorylation levels in 10 month old rats previously treated with fingolimod (Fig. 5).

Effects of fingolimod on histone acetylation

No significant (P > 0.05) difference in the acetylation of Lysine 8 of histone H4 was observed between untreated WAG/Rij rats of 6 months of age in comparison to untreated WAG/Rij rats of 10 months of age (Fig. 7b). Fingolimod ELTT significantly increased (P= 0.0009)

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

Discussion

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

The antiepileptogenic effects of fingolimod have also been previously demonstrated in the rat lithium-pilocarpine epilepsy model where, a 1 mg/Kg/day treatment for 14 consecutive days, started 24 h after onset of status epilepticus (SE), was able to decrease both glial activation and associated abnormal expression of IL-1beta and TNF-alpha which are rapidly overexpressed after SE. Furthermore, in this latter study, fingolimod administered during the silent phase was able to decrease the development of spontaneous seizures together with a reduction in their duration and severity [23]. These effects could also be the result of astrocyte S1P receptor modulation, however, further studies are needed to better clarify this hypothesis

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

To date, several animal models and some clinical studies have demonstrated that the mTOR signaling pathway plays a relevant role in the development of some idiopathic and acquired

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

Fingolimod ELTT resulted in a temporary reduction of mTOR signaling pathway activity, indicated by reduced p-mTOR and p-p70S6k levels and by an increased p-AKT in WAG/Rij rats of 6 months of age; this modulatory effect accompanied the transitory antiepileptogenic fingolimod effects. In fact, 5 months after treatment discontinuation, the mTOR pathway activation returned to control level together with the incidence of absence seizures. These results suggest that the inhibitory effects of fingolimod ELTT could be indirect and linked to its antiepileptogenic effects (reduction of absence seizures), which would in turn reduce mTOR pathway activation. This hypothesis is also supported by the fact that a sub-chronic treatment with fingolimod neither reduced mTOR pathway activation nor absence seizures in adult WAG/Rij rats. Furthermore, it was previously demonstrated that the mTOR inhibitor rapamycin has both antiepileptogenic effects (about 50% seizure development decrease vs 30% obtained with fingolimod) and some, albeit limited, anti-absence effects with long-lasting antiepileptogenic effects [29, 31]. Of note, we found an age-dependent increase in the phosphorylation of p-AKT in older control rats [68]; however, the significance of this result still remains controversial [69].

Previous studies have documented the linkage between epilepsy and depression both in

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

At odds with these temporary effects, fingolimod ELTT (but not acutely or subchronically) prevented the development of cognitive decline and this effect was maintained up to 5 months after drug suspension. Memory impairment was only recently demonstrated in WAG/Rij rats [25, 54]. Karson et al. [54] demonstrated that at 5 (range 4-6) months WAG/Rij rats do not differ from Wistar rats while they have an impairment in learning and memory at 13 (range 12-14) months of age; on the other hand, Jafarian et al. [25] reported that WAG/Rij rats of 6 months of age display memory impairment accompanied by hippocampal neuronal death. Accordingly, we found that there was no difference in WAG/Rij rats both aged 6 or 10

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

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

Conclusions

Fingolimod and the related S1P signaling have recently gathered attention in epilepsy [13]; furthermore, fingolimod also possesses other mechanisms of action, which might be relevant for this neurological disease. Our results further extend the current knowledge supporting potential antiepileptogenic effects of this drug; however, in our experiments, these effects were very limited in comparison to previous experiments with other drugs in this model and not permanent. Moreover, fingolimod might also have a positive impact on animal behavior

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

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Figure Legends

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

Fig. 2. Experimental protocol #2 for fingolimod acute/subacute treatment. Graph branches specify the experimental sequence followed and the number of rats used in every test. CTRL, controls; EEGs, electroencephalographic recordings; EPM, elevated plus maze; FST, forced swimming test; PA, passive avoidance; i.p. intraperitoneal administration; mTOR, mammalian target of rapamycin (mTOR).

Fig. 3. Effects of a Early long-term fingolimod treatment on the development of absence seizures. Effects of an early long-term treatment (ELTT; started at P30 and lasting 17 weeks) with fingolimod (Fing) on spike-wave discharges (SWDs) recorded in WAG/Rij rats at 6 (6 m) and 10 (10 m) months of age. Data (means $\pm$ SEM, n = 6 per group) are expressed as percentage change relative to 6-month-old control rats (dotted line; values for control rats were: nSWDs = 11.06 $\pm$ 1.02; dSWDs = 72.03 $\pm$ 6.22; nSWDs = 6.31 $\pm$ 1.33). *Significantly different (P< 0.05) from age-matched control rats. CTRL, control rats; nSWDs, mean number of SWDs for every 30-min epoch; dSWDs, mean cumulative duration of SWDs for every 30-min epoch expressed in seconds(s); sSWD, mean duration of a single SWD expressed in (s).

Fig. 4. a) **Forced swimming test (FST).** Bars indicate the immobility time (IT), expressed in seconds, in the forced swimming test (FST) in WAG/Rij (n = 6 per group) rats at 6 and 10

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

Fig. 5. Western blot analysis after an Early long term fingolimod treatment. a) Representative panel of Western blotting experiments on the effect of an ELTT with fingolimod (Fing) on the expression level in the cortex, of phosphorylated AKT (p-AKT) b), mTOR (p-mTOR) c), and p70S6K (p-p70S6K). Columns represent mean relative protein levels normalized to control (n = 6 per group). Loading was normalized using GADPH levels. Data marked with: * are significantly different ($P< 0.05$) from age-matched control (untreated) WAG/Rij rats; # Significantly different ($P< 0.05$) from age-matched control (untreated) WAG/Rij rats.

Fig. 6. Western blot analysis after a sub-chronic fingolimod treatment. Quantitative Western blot analysis of phosphorylated p70S6K (p-p70S6K) levels in the cortex of WAG/Rij rats of 6 months of age sub-chronically treated with fingolimod. Columns represent mean relative protein levels normalized to control (n = 6 per group). Loading was normalized using GADPH levels.

Fig. 7. Quantitative Western blot analysis of the acetylation of Lysine 8 of histone H4. Data marked with: * are significantly different ($P< 0.05$) from age-matched control (untreated) WAG/Rij rats; # Significantly different ($P< 0.05$) from age-matched control (untreated) WAG/Rij rats.

Fig. 1

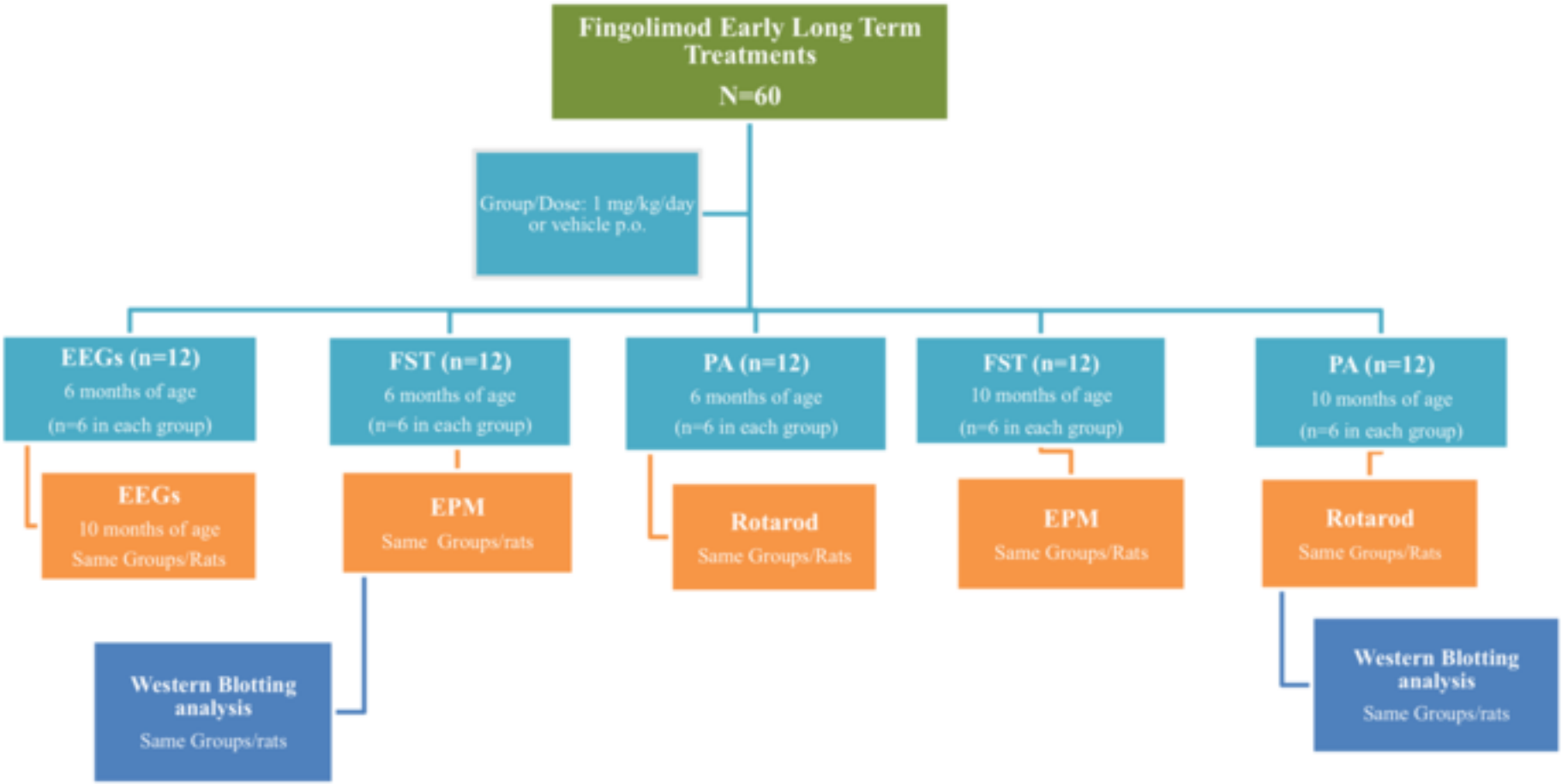


Fig. 2

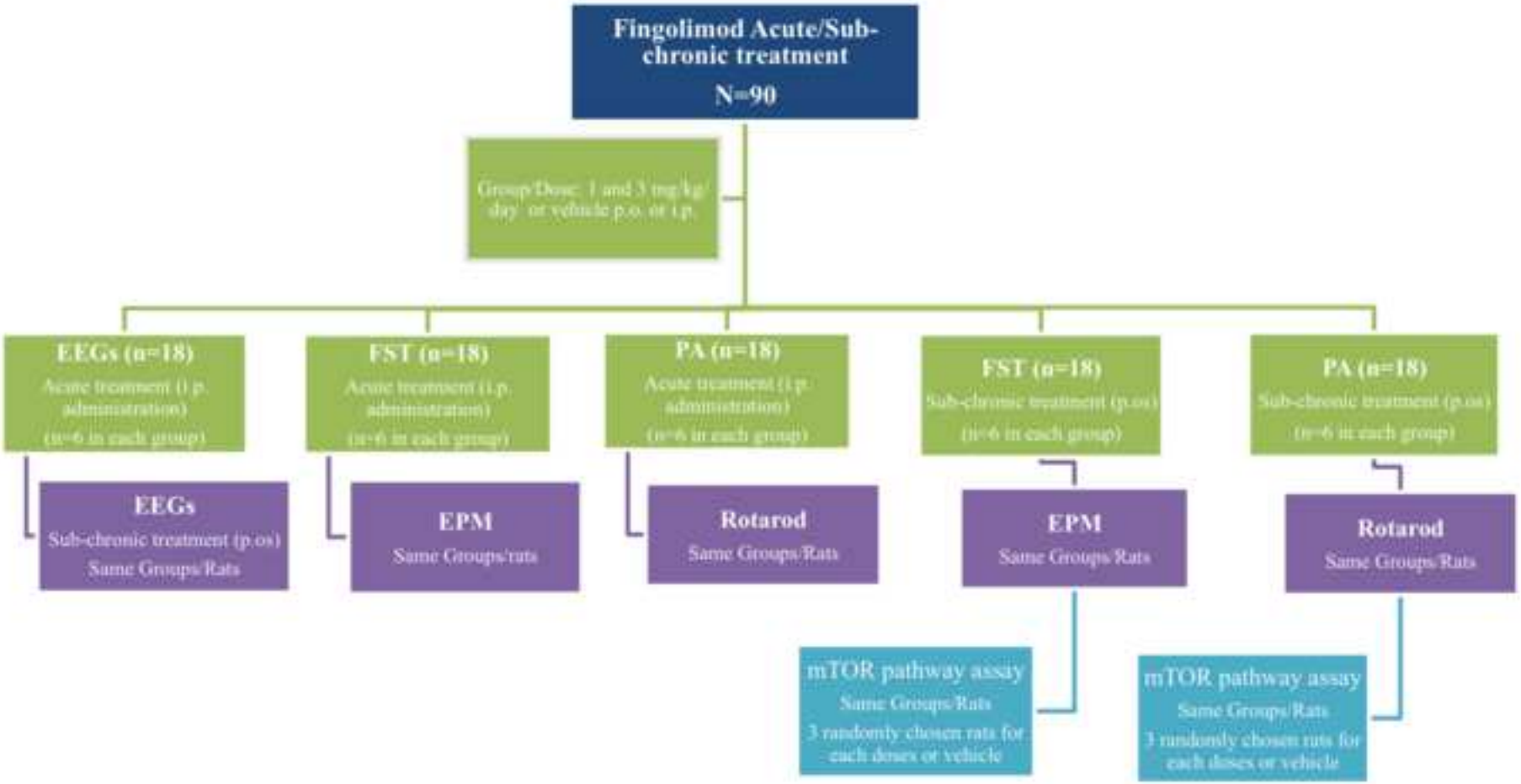


Fig. 3

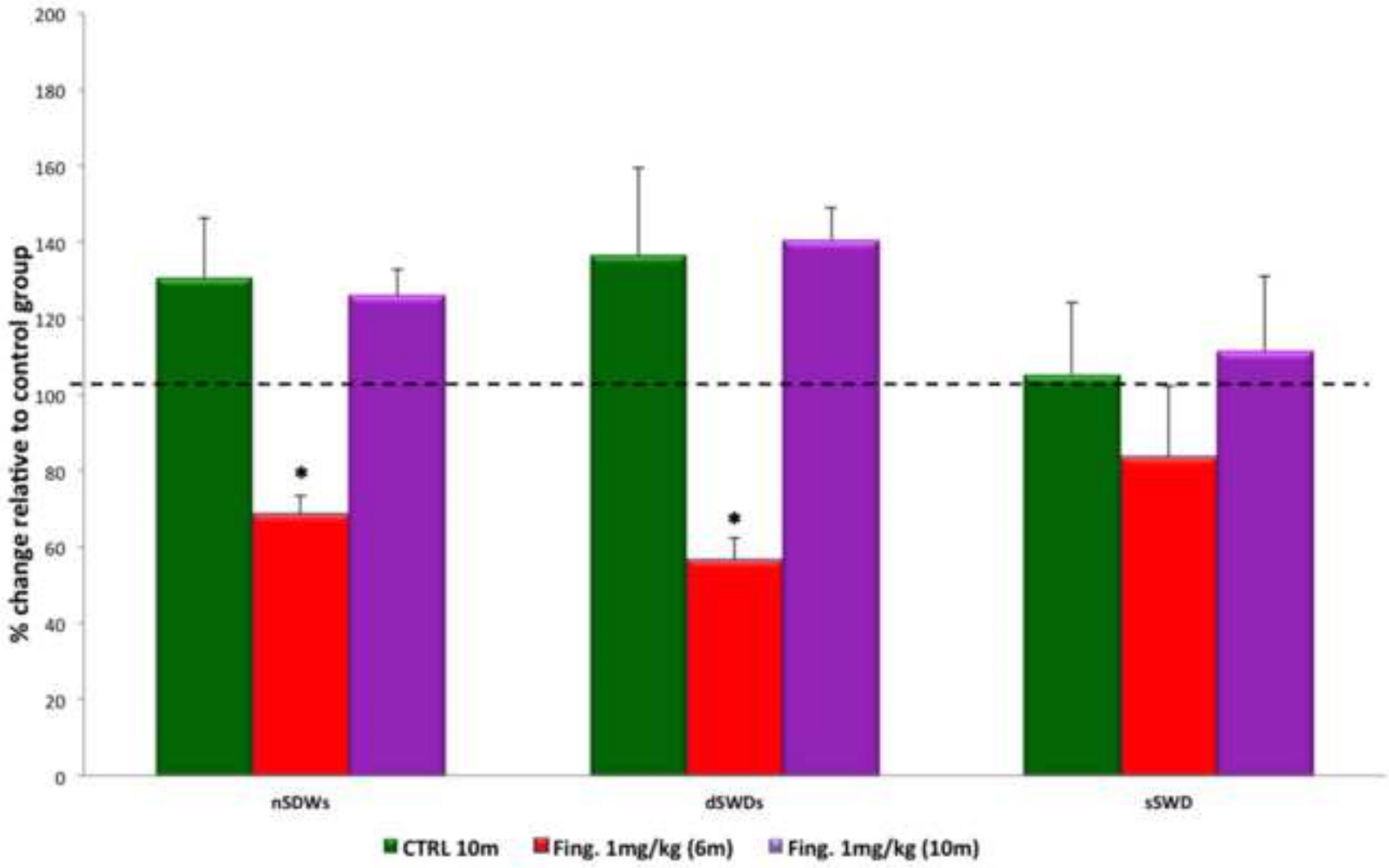


Fig. 4

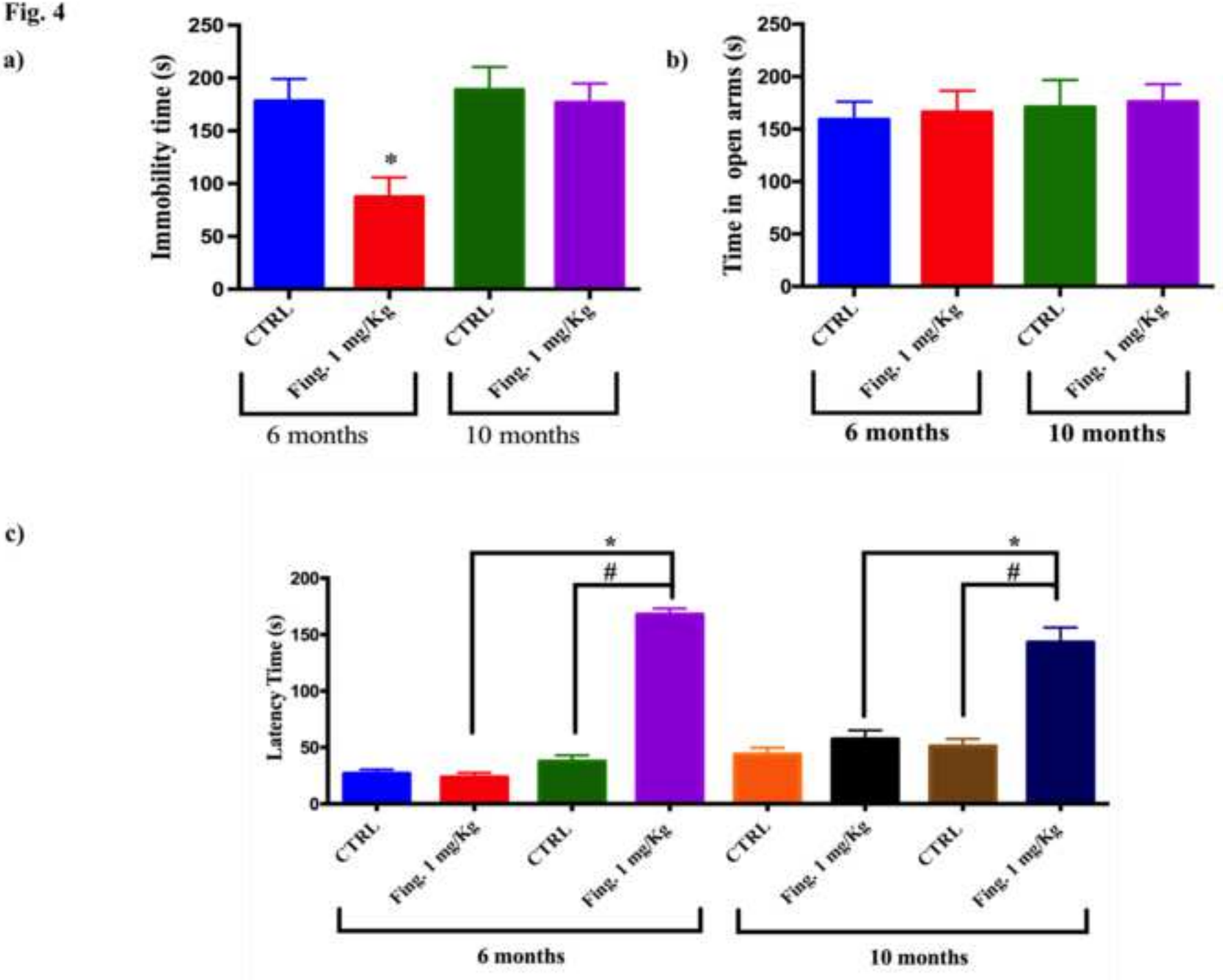


Fig. 5

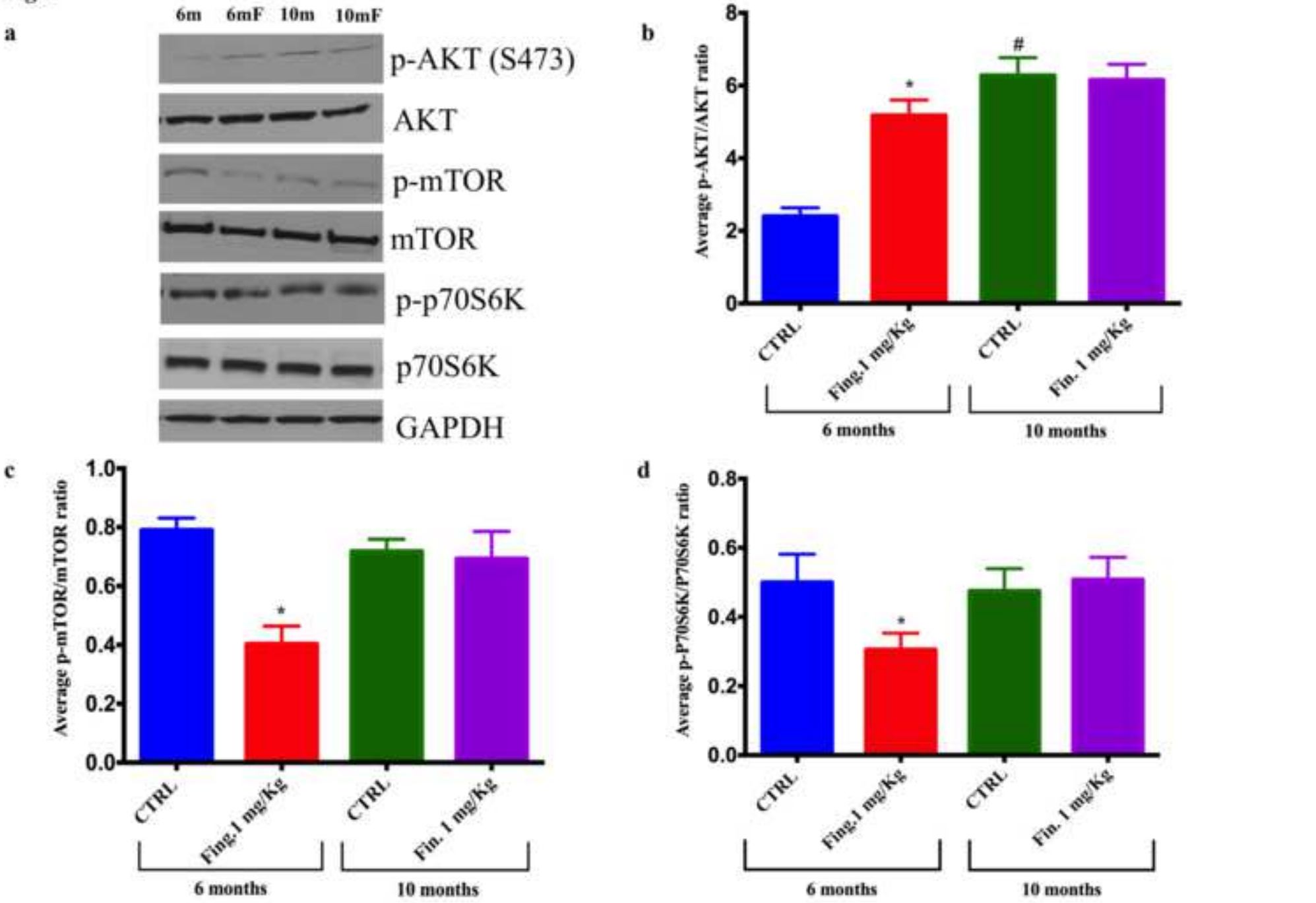


Fig. 6

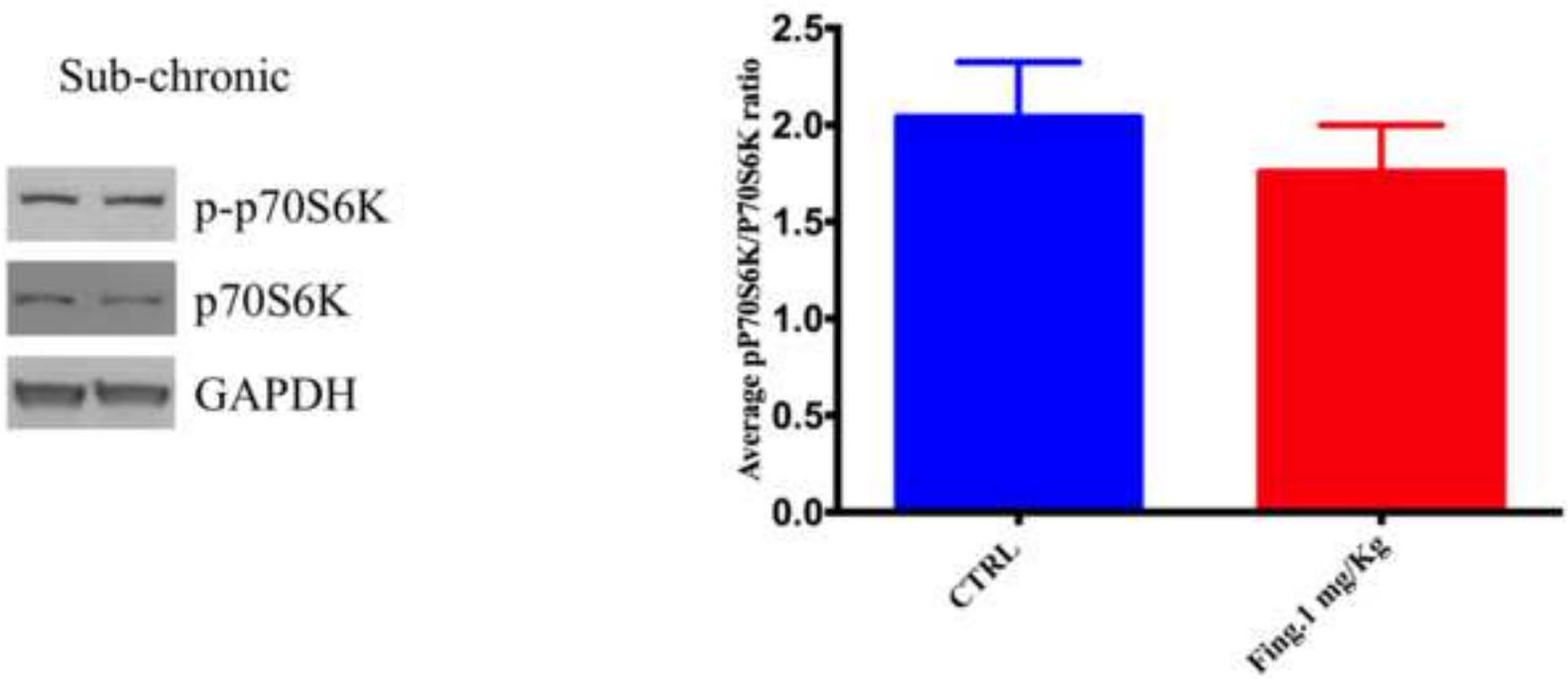
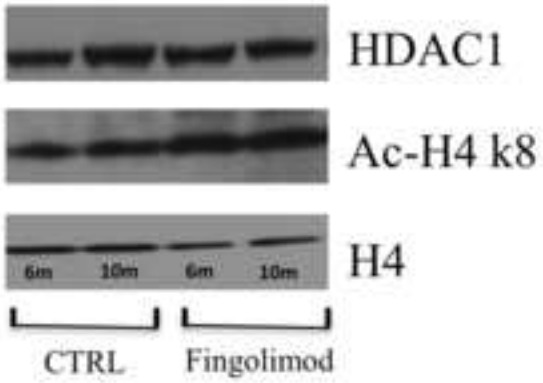


Fig. 7

A



B