Antiepileptogenic effects of Ethosuximide and Levetiracetam in WAG/Rij rats are only temporary

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Abstract

Background: WAG/Rij rats represent a validated genetic animal model of epileptogenesis, absence epilepsy and depressive-like comorbidity. Some treatments (*e.g.* ethosuximide), using specific protocols, prevent the development of spontaneous absence seizures. Accordingly, ethosuximide increases remission occurrence in children with childhood absence epilepsy in comparison to valproic acid. Considering that in this animal model, antiepileptogenic effects are, in some cases, not retained over time, we studied whether the antiepileptogenic effects of both ethosuximide and levetiracetam (which also possesses antiepileptogenic effects in this and other animal epilepsy models) would be retained 5 months after drug suspension. **Methods:** WAG/Rij rats of ~1 month of age were treated long-term with one of the two drugs at a dose of ~80 mg/kg/day for 17 consecutive weeks; 1 and 5 months after drug suspension, the development of absence seizures as well as depressive-like behaviour were assessed by EEG recordings and the forced swimming test (FST). Results: In agreement with a previous report, both drugs continued to show antiepileptogenic effects 1 month after their discontinuation. Furthermore, ethosuximide improved depressive-like behaviour, whereas in contrast, levetiracetam worsened this symptom. However, none of the drugs maintained their antiepileptogenic effects 5 months after suspension, and in addition, animal behaviour in the FST returned to control conditions. **Conclusion:** Overall, these results demonstrate that the antiepileptogenic effects of both ethosuximide and levetiracetam on absence seizure development and associated depressive-like behaviour in this model are only temporary.

Keywords: Ethosuximide; Levetiracetam; Epileptogenesis; Absence epilepsy; Depressive behaviour.

Introduction

Epileptogenesis represents the development of spontaneous recurrent seizures and/or the progression of the pathology once seizures have already been established, together with the development of comorbid pathologies, and is considered one of the major unmet needs in the epilepsy research field [1,2]; this is based on the lack of efficacy of the current therapies, which represent only symptomatic anticonvulsant treatments [1]. Besides the quite large number of preclinical experimental data on the possible prevention of seizure development in post-insult animal epilepsy models, none of these have yet been translated into clinical practice [1]. On the other hand, less is known on the development of spontaneous seizures in genetic epilepsies; however, relevant data have been gathered in some animal models [3–5].

The WAG/Rij rat is a widely recognized animal model of absence seizures accompanied by depressive-like comorbidity and also an animal model of genetically determined epileptogenesis, although the underlying mechanisms are still not completely understood [3]. Interestingly, it has been demonstrated that spike-wave discharges (SWDs) that augment with age in this strain are fundamental for the expression of depressive-like comorbidity. Therefore, epilepsy and depression in this animal model seem directly linked [3,6,7]. However, regarding this bidirectional link, some controversial evidence was also reported. In fact, it has been suggested that drugs suppressing the development of absence seizures are not always able to counteract depressive-like behaviour [3,8–10]. Recently, cognitive impairment was also detected in adult WAG/Rij rats [11,12]. It is noteworthy, that neuropsychiatric comorbidities can frequently occur in childhood absence epilepsy (CAE) [13,14]. It has been hypothesized that absence seizures and depressive-like behaviour can arise independently and separately in a lifetime, from the same underlying network disease [15].

As far as the end of 2007, it was demonstrated that an early long-term treatment with ethosuximide (ETH), started before seizure onset, was able to prevent the development of spontaneous absence seizures in adulthood [16] and these effects, together with an antidepressant-like effect, were later

confirmed by other groups in the same [3,7] and another absence animal model [17]. Contemporarily, other treatments and protocols were tested in this animal model in search of their potential antiepileptogenic and behavioural effects or the mechanisms involved [3,10]. The potential antiepileptogenic effects of ethosuximide were then clinically explored and supported by a more recent study in which it was demonstrated that in children affected by childhood absence epilepsy, remission at 5 or 10 years occurred more often if initially treated with ethosuximide in comparison to valproic acid [18,19].

Despite these encouraging results, considering the possibility to test the long-term effects of drug treatment in WAG/Rij rats (as it has been studied for other drugs [3]), we decided to study whether the antiepileptogenic effects of ETH and levetiracetam (LEV) might be retained 5 months after drug suspension in the WAG/Rij rat model. The choice of LEV, among other potential drugs, was based both on its known antiepileptogenic efficacy in this and other models [9,20,21] and on its differential effects on SWD development and depressive-like behaviour. In fact, LEV represents one of the few drugs able to reduce absence seizure development while displaying *pro*-depressant effects [9,10]. Furthermore, based on the link between epilepsy and depressive-like symptoms in WAG/Rij rats, the long-term effects of ETH and LEV treatment on depressive-like comorbidity were also evaluated.

Materials and Methods

Animals

Male WAG/Rij rats (n = 36; n = 6 per group) of 4 weeks of age were obtained from our breeding colony at the University of Catanzaro. Animals were housed four per cage and kept under standard controlled environmental conditions (60 ± 5% humidity; 22 ± 2 °C; 12/12 h reversed light/dark cycle; lights on at 20.00). Animals were allowed free access to standard laboratory chow and tap water. WAG/Rij rats at 27 days of age (P27) were tested, as previously reported [22], to detect their

vulnerability to audiogenic stimuli. Subsequently, only rats without audiogenic seizures were used in experiments, considering that WAG/Rij rats expressing audiogenic susceptibility show anxiety-like behaviour compared with nonaudiogenic rats [6]. The study was approved by the local ethics committee and all procedures involving animals and their care were in compliance with international and national regulations (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept).

Experimental design

Experiment #1 was performed with the aim of evaluating the long-lasting antiepileptogenic effects of an early-long term treatment (ELTT) with ETH or LEV (~80 mg/kg/day for both drugs) at two different time points (1 month and 5 months after drugs withdrawal) in WAG/Rij rats. Likewise, in experiment #2, the effects of these treatments on depressive comorbidity were also evaluated in this model (1 month and 5 months after of drug suspension) (see Fig.1). WAG/Rij rats (n = 18 for each experimental protocol) were randomly divided into 3 subgroups (n = 6): vehicle, ETH-treated and LEV treated.

Drug administration protocol

ETH (Zarontin®; Pfizer Italia Srl, Italy) 250 mg/5 ml syrup was used. LEV was purchased from Sigma-Aldrich Co. Ltd, Poole, U.K. ETH or LEV were administered orally at a dose of ~80 mg/kg/day, by adding 4 ml of ETH syrup (250 mg/5 ml) or solubilising 200 mg of LEV with 300 ml of drinking water respectively, following our previously used protocol [9,23]. Rats (n = 24) started treatment at 4 weeks of age and were kept on drugs for a further 17 weeks up to the age of ~5 months; treatment was then stopped and animals normally housed. Control animals (n = 12) were kept under standard conditions during the same time-window. At the age of ~6 months (1 month after drug

discontinuation), 2 subgroups of treated (1 for each drug treatment) and 1 subgroup of control (vehicle) WAG/Rij rats, after surgery (see below), were experimentally tested by 3 h of EEG recordings over 3 consecutive days. These rats were also studied by EEG recordings at the age of ~10 months (5 months after treatment suspension) to evaluate the potential long-lasting effects ETH and LEV (experiment #1). Furthermore, (experiment #2), other subgroups of treated (1 for each drug treatment) and control (vehicle) WAG/Rij rats, were subjected to the forced swimming test (FST), in order to assess the effects of ETH and LEV, 1 and ~5 months after drug discontinuation, on depressive-like behaviour in this strain (see section on Forced Swimming Test). During the ELTT period, rats were weighed weekly every Monday between 9:00 and 11:00 a.m. and at the same time, a blood sample of about 1 ml was taken through the tail vein for later analysis of drug plasma concentrations from 5 rats per group in a randomized order, as previously reported [23].

Surgery and EEG recordings

For EEG recordings, WAG/Rij rats at the age of about 6 months were chronically implanted, under anaesthesia (tiletamine/zolazepam 1:1; Zoletil 100®; 50 mg/kg i.p.; VIRBAC s.r.l., Milan, Italy), using a Kopf stereotaxic instrument, with 3 cortical electrodes for EEG recordings attached to a 3-channel rat headmount (8239-SE3, Pinnacle Technology INC), as previously described [8,22]. 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 starting at 9.00 a.m. in order to avoid the confounding effect of circadian changes within groups [24,25]. Blinded quantification of absence seizures was based on number and duration of spike-wave discharges (SWDs) in EEG recordings [22,26].

Forced swimming test

In spite of some limitations, the FST represents a validated tool to investigate the depressive-like behaviour in animals [27,28]. In detail, we carried out an FST protocol previously standardized in our laboratory for WAG/Rij rats [8,29]. Briefly, each rat was placed for 6 min into a glass cylinder (height 47 cm, diameter 38 cm) filled with 38 cm water and maintained at 23 °C to 25 °C. The immobility time (IT), linked to depressive-like behaviour, was recorded during the last 4 min of the 6-min testing period. The condition for immobility time, including passive swimming, was floating vertically in the water while making only those movements fundamental to maintain the head above the surface of the water. At the end of 6 min, the rat was removed and dried with a towel before being housed. Mean swimming velocity and total distance moved were also detected and analysed for every experimental group in order to avoid any possible locomotor deficit. FST was always performed between 09:00 and 11:00 in order to avoid possible circadian alteration of data. This test was performed under controlled environmental conditions, including temperature, humidity, and light intensity (dim illumination), and with the support of video-tracking software (EthoVision XT8; Noldus Information Technology, Wageningen, the Netherlands) [22,30].

Statistics

EEG recordings were subdivided into 30 min epochs, and the duration and number of SWDs were treated separately for every epoch. Such absolute values were averaged and data obtained were expressed as mean ± SEM. for every dose of compound. Results were compared by repeated measures two-way analysis of variance (ANOVA) with age (two levels: 6 or 10 months) and treatment (two levels: vehicle or ETH) as factors, followed by a *post-hoc* Bonferroni test. We also used repeated

measures two-way ANOVA followed by Bonferroni's post hoc test to analyse and compare absolute behavioural data. Data are reported as mean ± SEM. All tests used were two-sided and $p < 0.05$ was considered significant

Results

Effects of early long-term treatment on the development of SWDs

Untreated (control) WAG/Rij rats at 6 months of age showed a mean number of SWDs (nSWDs) of 7.31 ± 0.44, with a mean total duration (dSWDs) of 34.90 ± 1.78 seconds and a mean single duration (sSWD) of 4.83 ± 1.05 seconds for a 30-min epoch (Fig. 2). ELTT with either ETH or LEV (~80 mg/kg/day *per os* for both drugs) significantly ($p < 0.01$) decreased the SWD development in WAG/Rij rats at 6 months of age (1 month after drug discontinuation) in comparison to age-matched untreated rats. In detail, ELTT with ETH significantly decreased all SWD parameters: nSWDs of ~ 54 % ($p < 0.0001$), dSWDs of ~ 57 % ($p < 0.0001$) and sSWD of ~ 36 % ($p < 0.0057$). Likewise, ELTT with LEV significantly reduced all SWD parameters: nSWDs of ~ 42 % ($p < 0.0008$), dSWDs of ~ 55 % ($p < 0.0001$) and sSWD of ~ 30 % ($p < 0.022$). Furthermore, *post-hoc* analysis identified a significant increase in the nSWDs (~ 52 %; $p < 0.0017$) and dSWDs (~ 66 %; $p < 0.0002$) between untreated WAG/Rij rats at the two ages considered, whereas no difference was detected in sSWD between the same rats at the two different ages (Fig. 2). Early long-term treatment with either ETH or LEV at a dose of ~80 mg/kg/day for 17 consecutive weeks, starting at 4 weeks of age, did not have any significant effect on the development of absence seizures when measured at 5 months after drug suspension. Accordingly, a significant increase, for nSWDs and dSWDs, was identified in treated WAG/Rij rats, either with ETH or LEV ($p < 0.0001$ for both drugs), at the two different ages considered in comparison to themselves at 6 months. At odds, no significant difference was detected between the two treatments on SWD parameters in adult WAG/Rij rats at the two different ages

considered (Fig. 2). No significant difference was observed in rats growth during the period of treatment. The plasma concentrations of LEV did not significant change every week, with a total mean concentration of 37.78 $\pm$ 1.24 μg/ml. In contrast, a slight, not significant, reduction of plasma concentration was observed for ETH, in the last 4 weeks of treatment in comparison to the first 3 weeks (total mean concentration 35.94 + 1.05 μg/ml).

Effects of early long-term treatment on depressive-like behaviour

As previously reported, WAG/Rij rats, starting from 4 months of age onwards, display depressive-like behaviour, as highlighted by an increased immobility time (IT) in the FST among other possible tests [6,7,9]. ELTT with ETH significantly ($p < 0.0082$) reduced the immobility time in WAG/Rij rats at 6 months of age (1 month after drugs withdrawal) in comparison to untreated control rats. At odds, WAG/Rij rats at 6 months of age, after ELTT with LEV, showed a significant ($p < 0.013$) increase in the IT compared to age-matched untreated rats (Fig. 3). Furthermore, *post-hoc* analysis revealed that there was no significant difference in the IT between untreated WAG/Rij rats at the two different ages (6 and 10 months) considered. Analogously, no significant difference in the IT was noticed between treated (ETH and LEV) and untreated WAG/Rij rats at 10 months of age (5 months after drugs suspension). Moreover, a significant ($p < 0.035$) difference in the IT was detected between LEV-treated groups at the two different ages considered. In detail, the pro-depressant like effects of LEV treatment, observed in WAG/Rij rats at 6 months of age, disappeared in the same rats at 10 months of age. Similarly, a significant ($p < 0.0033$) difference in the IT was also recognized between ETH-treated groups at the two different ages considered (Fig. 3). Mean velocity and total distance moved did not significantly differ ($p > 0.05$) among groups at the two different ages considered (data not shown).

Discussion

WAG/Rij rats represent a validated genetic animal model widely used to study absence epileptogenesis and related neuropsychiatric comorbidities both from a pathophysiological and pharmacological point of view [3,10]. Up to now, several drugs have demonstrated potential antiepileptogenic effects as well as antidepressant properties in this strain. However, for the majority of these drugs, only transitory antiepileptogenic and antidepressant-like effects were reported. In fact, as highlighted by EEG recordings and behavioural tasks performed 5 months after drug suspension, absence seizures and related depressive comorbidity may return to control levels [3,10]. In this study, we have confirmed that an ELTT with ETH or LEV, started before seizure onset (started at P30, whereas seizure onset is at about P60), was able to reduce the development of spontaneous seizures when EEG recordings were obtained in WAG/Rij rats at the age of about 6 months and therefore only 1 month after drug suspension. These results are in line with previously published studies in which, however, it was not evaluated if these antiepileptogenic effects were maintained over time [3]. Surprisingly, we have demonstrated that neither ETH nor LEV antiepileptogenic effects were retained 5 months after suspension. To be noticed, a slight difference in the efficacy among our studies was also observed with a reduced efficacy in the study in which drug effects were measured 45 days after drug suspension [9,23]; furthermore, as previously evidenced, also in the first article by Blumenfeld [16] a progressive increase in the number of absence seizures was observed at 60 and 90 days after drug withdrawal. Furthermore, we have also confirmed the antidepressant-like effects of ETH, which as previously reported, would only seem limited to this strain [7,9,31]. However, this latter effect as well as the antiepileptogenic effect was only temporary. In fact, the depressive-like behaviour appeared again 5 months after ETH suspension, supporting the previously suggested link between absence seizures and depressive-like behaviour in this strain [7,31]. Based on this hypothesis, the antidepressant-like effects of ETH in WAG/Rij rats are secondary to its antiepileptogenic activity [3,7]. In spite of this evidence, the fact that seizures are necessary for the

expression of depressive-like behaviour still remains unclear. In fact, drugs suppressing the development of absence seizures such as zonisamide are not always able to ameliorate depressive-like behaviour in WAG/Rij rats [3,8,10]. Moreover, LEV pro-depressant effects disappear after 5 months of drug discontinuation. LEV as well as zonisamide represent two of the few drugs able to reduce absence seizure development, while displaying pro-depressant and no anti-depressant effects, respectively [3]. Therefore, it could be speculated that these two drugs are only able to partially modify the complex thalamocortical network, which seems to be involved in the genesis of uncorrelated neuropsychiatric conditions, named thalamocortical dysrhythmias, including absence seizures and depression [35,36]. However, to date, no studies were performed with the aim of investigating the effect of these two drugs on the thalamacortical activity or to define which would be the specific networks involved in each phenomenon. Furthermore, despite a link between absence epilepsy and depression in this strain, it has strongly been suggested that the single drug's effects on behaviour should also be considered [3,7,10]. By virtue of this, the reported dual effectiveness of ETH can also be linked to its low risk to give rise to clinical depression [15]. At odds, the pro-depressant effects of LEV could also be associated with its higher risk to induce depression, as previously reported both for patients with epilepsy (PWE) [32,33] and in animal models of epilepsy [9,34].

Furthermore, we have also reported a significant worsening of absence seizures in adult untreated WAG/Rij rats, between 6 and 10 months of age. In fact, as already reported, the incidence and the duration of SWDs increase progressively with age in this strain [3,37,38]. Interestingly, the aggravation of absence seizures between 6 and 10 months age is not apparently related to a significant worsening in IT at the two different ages as it was indicated in previous studies between the ages of 2 and 6 months [9,39]. Several explanations may justify this contradiction and therefore, further experiments are warranted. Overall, no experimental evidence exists regarding this point; from our experiments, it is possible to identify a tendency towards aggravation which is not however

significant. It could be speculated that the outcome in the FST indicates a ceiling effect related to a complete maturation of the network underlying this behaviour at the age of 6 months. At odds, the network underlying absence seizures and also other comorbidities (*e.g.* cognitive impairment) may still undergo further modifciations related to aggravation of symptomatology.

Similarly to the present results, some other effective treatments in this model were found to lose completely (*i.e.* etoricoxib, fingolimod and perampanel) or partially (*i.e.* statins) their antiepileptogenic and/or antidepressant-like effects when tested 5 months after drug suspension [3,6,9].

Our current level of knowledge does not permit a complete explanation of the mechanisms involved both in the antiepileptogenic effects of these drugs and on the mechanisms underlying epileptogenesis in this animal model. To date, there are only some suggestions trying to explain the unpreserved effects of drugs in this model and refer to the lack of ability of these to permanently modify the genetically induced alterations in the brain therefore not modifying the genetic cause which would in turn restart the epileptogenic process after drug suspension. In other words, drugs would only temporary inhibit some of the consequences of the genetic stimulus to become epileptic; indeed, drug effects on established seizures might represent a mechanism related to the probable "*seizure beget seizure*" phenomenon that participates in the epileptogenic process in this strain.

Conclusions

In conclusion, our results in WAG/Rij rats demonstrate that despite the fact that ethosuximide and levetiracetam possess some antiepileptogenic effects, these are not permanent and therefore to maintain such effects, treatment should be continued. However, it cannot be excluded that an earlier intervention (before 21 days of age) in WAG/Rij rats might instead lead to permanent antiepileptogenic effects [10]. Furthermore, these findings also support our recently suggested theory indicating that in genetic epilepsies, it will be more challenging to obtain permanent treatment effects

considering the difficulty to remove the original triggering cause. However, in the future, the determination of a genetic predisposition to develop epilepsy could be used to identify a preventive treatment to stop epileptogenesis or as a biomarker [40].

Declarations of interest

None.

Ethical statement

The authors have reviewed the paper and approved of the content and this manuscript. The authors affirm that this paper has not been submitted for publication elsewhere. The authors confirm that the data in this paper are original. The authors also declare there is no conflict of interest. The experimental protocols and the procedures described in this manuscript were approved by the Animal Care Committee of the University of Catanzaro, Italy and all procedures involving animals and their care were in compliance with international and national regulations (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept).

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Figure legends

Fig. 1. Experimental design for ethosuximide (ETH) or levetiracetam (LEV) early long-term treatment. Blue arrow specifies the experimental sequence used to perform EEG recordings in control and treated (ETH or LEV) WAG/Rij rats (*n* = 6 per group) at the ages of 6 and 10 months (1 month and 5 months after treatment discontinuation). Red arrow specifies the experimental sequence used to perform the forced swimming test in control and treated (ETH or LEV) WAG/Rij rats (*n* = 6 per group) at the ages of 6 and 10 months (1 month and 5 months after treatment discontinuation). CTRL = control (untreated) rats; ETH = Ethosuximide treated rats; LEV = Levetiracetam treated rats.

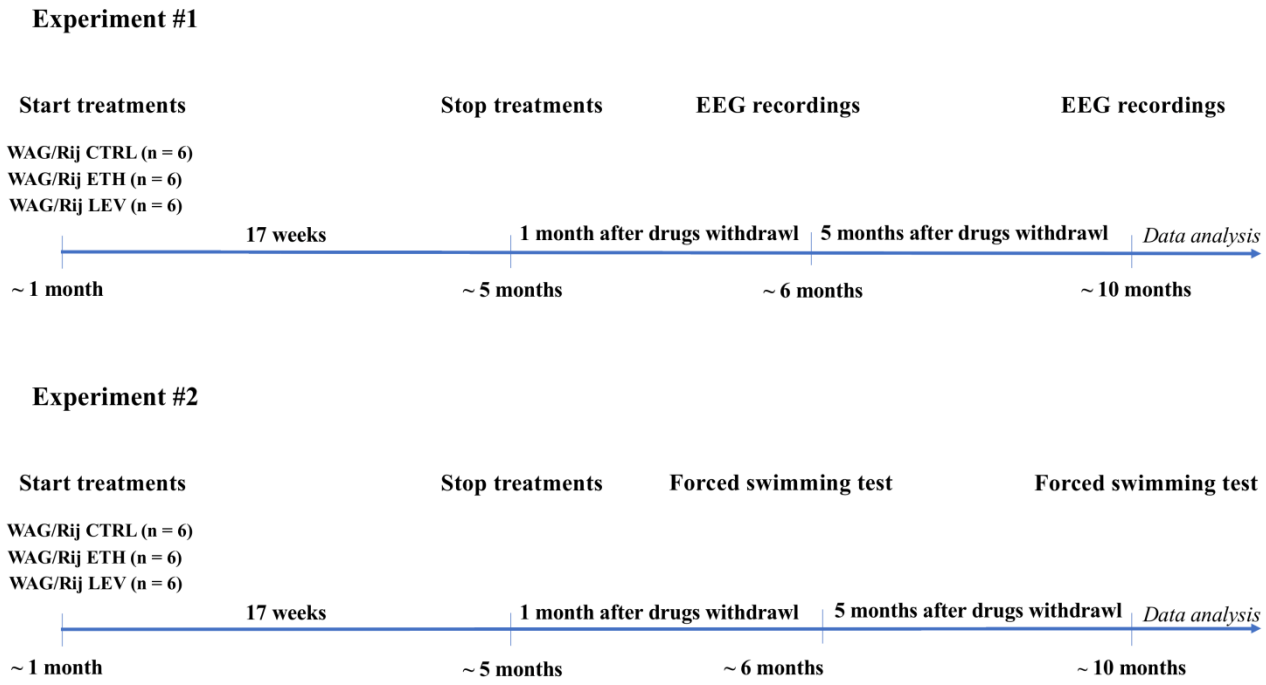

Fig. 2. Effects of early long-term chronic treatment with ethosuximide (ETH) or levetiracetam (LEV) on spike-wave discharges (SWDs) recorded in WAG/Rij rats at 6 and 10 months of age (1 month and

5 months after treatment discontinuation). Data (means ± SEM, n = 6 per group) are expressed as percentage change relative to 6-month-old control rats (dotted line). *****Significantly different ($p < 0.01$) from age-matched control rats (CTRL). [#]Significantly different ($p < 0.01$) from control rats at 6 months of age. [§]Significantly different ($p < 0.01$) from treated (ETH or LEV) rats at 6 months of age. CTRL = control (untreated) rats; ETH = Ethosuximide treated rats; LEV = Levetiracetam treated 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).

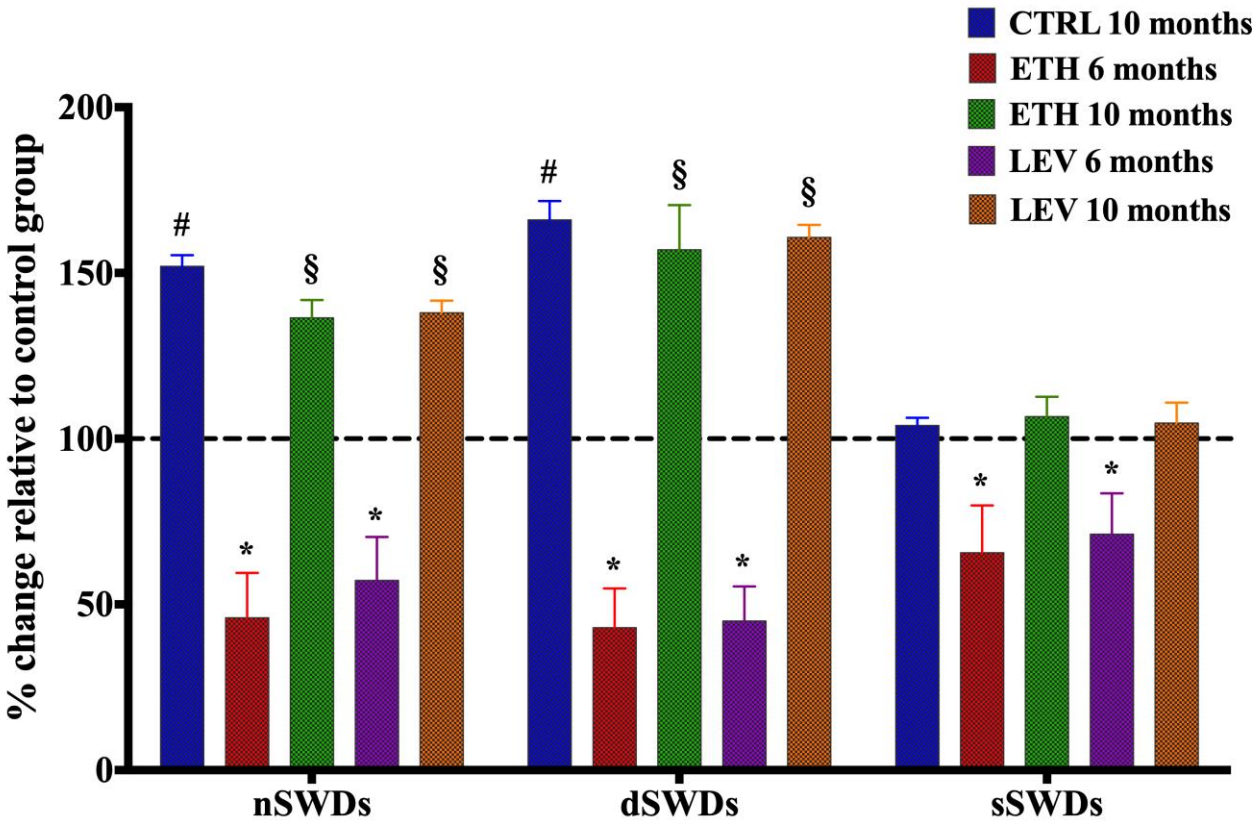

Fig. 3. Forced swimming test (FST). Bars indicate the immobility time, expressed in seconds, in the FST in WAG/Rij rats *(n* = 6 per group) at 6 and 10 months of age following an early long-term

treatment with ethosuximide or levetiracetam. Data are means ± SEM. *Significantly different (p < 0.05) from age-matched control rats (CTRL). #Significantly different (p < 0.05) from treated rats at 6 months of age. CTRL = control (untreated) rats; ETH = Ethosuximide treated rats; LEV = Levetiracetam treated rats.