Epilepsy & Behavior 105 (2020) 106945

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

# Behavioral and neurochemical characterization of the spontaneous mutation *tremor*, a new mouse model of audiogenic seizures

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ARTICLE INFO

*Article history:*
Received 18 July 2019
Revised 8 January 2020
Accepted 24 January 2020
Available online 25 February 2020

*Keywords:*
Hippocampus
Reflex seizure
Genetic epilepsy
Neurotransmitters
Behavioral phenotype

ABSTRACT

The *tremor* mutant phenotype results from an autosomal recessive spontaneous mutation arisen in a Swiss–Webster mouse colony. The mutant mice displayed normal development until three weeks of age when they began to present motor impairment comprised by whole body tremor, ataxia, and decreased exploratory behavior. These features increased in severity with aging suggesting a neurodegenerative profile. In parallel, they showed audiogenic generalized clonic seizures. Results from genetic mapping identified the mutation *tremor* on chromosome 14, in an interval of 5 cM between D14Mit37 (33.21 cM) and D14Mit115 (38.21 cM), making Early Growth Response 3 (Egr3) the main candidate gene. Comparing with wild type (WT) mice, the *tremor* mice showed higher hippocampal gene expression of Egr3 and Gabra1 and increased concentrations of noradrenalin (NOR; $p = .0012$), serotonin (5HT; $p = .0083$), 5-hydroxyindoleacetic acid (5-HIAA; $p = .0032$), γ-amino butyric acid (GABA; $p = .0123$), glutamate ($p = .0217$) and aspartate ($p = .0124$). In opposition, the content of glycine ($p = .0168$) and the vanillylmandelic acid (VMA)/NOR ratio ($p = .032$) were decreased. Regarding to dopaminergic system, neither dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) contents nor the turnover rate of DA showed statistically significant differences between WT and mutant mice. Data demonstrated that audiogenic seizures of *tremor* mice are associated with progressive motor impairment as well as to hippocampal alterations of the Egr3 and Gabra1 gene expression and amino acid and monoamine content. In addition, the *tremor* mice could be useful for study of neurotransmission pathways as modulators of epilepsy and the pathogenesis of epilepsies occurring with generalized clonic seizures.



## 1. Introduction

Epilepsy is a disease characterized by presenting one of the following conditions: at least two unprovoked (or reflex) seizures occurring more than 24 h apart; one unprovoked (or reflex) seizure with a 10-year period risk of further seizures comparable to the one of patients who have had two unprovoked seizures (at least 60%) or a diagnosis of an epilepsy syndrome [1]. Nearly 50 million people worldwide display epilepsy, and the risk of premature death in people with this disease is up to three times higher than for the general population, obtained from World Health Organization (WHO) (https://www.who.int/news-room/fact-sheets/detail/epilepsy).

At least 81 different epileptic syndromes in humans have a confirmed causal mutation, and these mutated genes have been characterized over the years, but their functions have not been fully

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https://doi.org/10.1016/j.yebeh.2020.106945
1525-5050/

described yet, requiring more detailed studies using animal models [2]. A great variety of animal models, including spontaneous mutants, can be used to study epilepsy syndromes. It is predicted, however, that more than a thousand different genes may be related to genetic epilepsy etiology [3,4]. Many animal species, such as, mice, gerbils, rats, chickens, dogs, and nonhuman primates, have been described as good models for epilepsy. In spite of that, mouse and rat models developed by genetic selection are the most used species to study epilepsy [2].

Within genetic models of epilepsy, susceptibility to generalized tonic–clonic seizures induced by sound (named audiogenic seizures) is frequent and is associated with complex and pathological alterations of synaptic activity mediated by γ-amino butyric acid (GABA), glutamate, dopamine (DA), noradrenalin (NOR), and serotonin [5–7]. Additionally, studies carried out with strains such as the Wistar Audiogenic Rats (WAR) and Genetic Audiogenic Seizure Hamsters (GASH:Sal), for instance, suggest that genes of the Early Growth Response (EGR) family play an important role in mechanisms involved in audiogenic seizure development [8].

Despite the achievement of model development by genetic engineering, spontaneous mutations are still valuable for understanding new functions and structures of genes, considering that their associated phenotype could be similar to the one caused by natural mutations in human genome [9]. Spontaneous mutations seem to occur randomly and in a low frequency in most genes. In laboratory mouse, average occurrence of spontaneous mutations varies from $5 \times 10^{-6}$ to $10^{-7}$ in dominant genes and from $5 \times 10^{-6}$ to $10^{-5}$ in recessive ones [10].

The spontaneous mutation *tremor* was identified in the Swiss–Webster mouse colony of the laboratory animal facility of the Department of Pathology of the School of Veterinary Medicine and Animal Science at University of São Paulo, Brazil (FMVZ-USP). Around three weeks of age, some mice in a litter showed tremors, ataxia, motor incoordination, and audiogenic seizures; therefore, they were labeled *tremor*. These individuals were selected for further studies, which identified the mutation as an autosomal recessive inheritance pattern (Unpublished results).

In order to characterize the spontaneous mutation *tremor* in the present study, a sequence of tests was performed to evaluate postnatal development, behavioral features of seizures, general activity, motor coordination, exploratory behavior, spatial memory, and coping behavior. Additionally, Egr3 and Gabra1 gene expression, neurotransmitter monoamine, and amino acid levels were measured in the hippocampus.

## 2. Material and methods

### 2.1. Mice

Mice were bred at the Department of Pathology, FMVZ-USP, Brazil. Animals were housed in individually ventilated cages (Alesco Indústria e Comércio, Monte Mor, Brazil) with pine shavings for bedding, under controlled room temperature of 22 ± 2 °C, with an air relative humidity of 45–65%. They had unrestricted access to filtered and autoclaved water and to irradiated commercial diet formulated according to the AIN-93M formulation (Nuvilab, Quimtia, Paraná, Brazil). Room was kept on a 12-h light/12-h dark cycle (lights on at 07:00 am) at artificial light. A description of the animals used in each experiment and the number of animals per experimental group are presented in Table 1.

**Table 1**
Description of mice used in each experiment.

| | Testing age | WT | Mutant |
|---|---|---|---|
| Genetic mapping | — | — | 78 males and females |
| DNA sequencing | — | 2 females | 2 females |
| Gene expression | Between 8 to 10-weeks | 6 females | 6 females |
| Postnatal development | At birth | 34 males and females | 34 males and females |
| Open-field test | At 3 and 8-weeks | 8 males, 12 females[a] | 8 males, 12 females[a] |
| T-maze test | At 9-weeks | | |
| Balance beam test (time and number of falls) | At 3 and 8-weeks | 11 males | 8 males |
| Balance beam test (score) | At 8-weeks | 8 males, 8 females | 8 males, 8 females |
| Forced swim test | At 9-weeks | 8 males | 8 males |
| Tail suspension test | At 10-weeks | | |
| Acoustic stimuli | Between 10 to 12-weeks | 10 males, 10 females | 10 males, 10 females |
| Measurement of neurotransmitters | At 10-weeks | 10 males | 10 males |

[a] These animals were the same used for postnatal development.

### 2.2. Ethics statement

The Institutional Animal Care and Use Committee (CEUA/FMVZ/USP) approved the research protocols under numbers 4124150116 and 4864190717, and all procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals of the US National Research Council [11]. The experiments were performed under proper laboratory practice protocols and following quality assurance methods. All efforts were made to minimize animal suffering.

### 2.3. Genetic mapping and candidate gene sequencing

Congenic mutant males on a C57BL/6J background were outcrossed with BALB/cJ females. F1 heterozygous mice were bred to produce F2 offspring that were WT (25%), heterozygous (50%), and homozygous (25%) for the mutation; the latter were selected by phenotype and used for microsatellite mapping. Genomic deoxyribonucleic acid (DNA) was extracted from tail biopsy. Mapping the location of crossovers in these progenies provided information on the recombination events arising in the F2 mutant. Initially, 11 mutant mice were genotyped using a broad panel of 31 polymorphic microsatellite markers showing different PCR products (size in base pairs) between BALB/cJ and C57BL/6J, selected on each autosome. A linkage on chromosome 14 was observed, and 16 additional microsatellite markers were selected, analyzing 78 F2 mutants to establish a candidate region. The genetic positions of markers in centimorgans and the corresponding primer sequences were obtained from the online databases Mouse Genome Informatics (MGI) (www.informatics.jax.org — The Jackson Laboratory) and Ensembl Genome Browser (www.ensembl.org).

Search for functional candidate genes was done based on animal models with similar phenotype reported on databases MGI and Ensembl Genome Browser, suggesting Egr3 gene as the main candidate for the mutation. Genomic DNA was used to sequencing Egr3 exons by Sanger method [12]. Sequence analysis was performed on genomic DNA in 2 mutants and 2 WT mice on a C57BL/6J background using primers designed to amplify the two protein-coding variants of Egr3 gene: Egr3-204 (2 exons, 3940 bp and 387 a.a.) and Egr3-201 (3 exons, 1509 bp and 349 a.a.). The primer sequences are listed in Supplementary Table 1 (S1).

### 2.4. Egr3 and Gabra1 gene expression

The gene expression of Egr3 and Gabra1 was analyzed in the hippocampus of C57BL/6J mutant and WT mice by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). Briefly, 6 mutant and 6 WT naïve mice (do not submit to acoustic stimuli) were

euthanized by overdose of sevoflurane, and the brains were removed from the skull for dissection of hippocampus as described previously [13].

Total ribonucleic acid (RNA) was isolated and purified using RNeasy Lipid Tissue Mini Kit and RNase-Free DNase Set (Qiagen, Hilden, Germany) according to manufacturer's protocols. The RNA extracts were first analyzed by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). The RNA quality was determined by the A260/A280 (close to 2) and A260/A230 (close to 2) ratios, and qualified RNA samples were tested using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNAs with 28S/18S RNA ratio between 7 and 8 were used for gene expression.

Reverse transcription reactions were carried out with random primers and SuperScript-II reverse transcriptase (Life Technologies Corporation, USA). Complementary DNA (cDNA) for RT-qPCR was prepared from 2 μg of total RNA in 20-μl reactions. Egr3 and Gabra1 gene expressions were analyzed using Taqman® Gene Expression assays Mm00516979_m1 and Mm00439046_m1, respectively (Life Technologies Corporation, CA, USA), performed on the StepOnePlus™ system (Applied Biosystems); 18S rRNA (Hs03003631_g1; Life Technologies Corporation, CA, USA) was used as reference gene. Data for each target gene were normalized to the endogenous reference gene, and the fold change in target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [14].

### 2.5. Evaluation of postnatal development

Mutant males were crossed with Swiss–Webster females to produce heterozygous F1 progeny. Ten F1 females were crossed with homozygous mutant males to produce offspring that were 50% heterozygous and 50% homozygous for mutation. Thus, 34 homozygous mutant and 34 heterozygous littermate WT mice were tested.

Developmental tests adapted from the literature were used to characterize early markers of the mutant mice phenotype [15,16]. The physical development evaluation was done from birth to three weeks and included pinnae detachment, eye opening, lower and upper incisor eruption, fur development, and body weight and length measurement.

A sequence of behavioral tests was chosen to assess developmental reflexology. The surface righting reflex is defined as the motor ability for a mouse pup to turn over from a supine position. Briefly, the pup was placed gently onto its back and hold in this position for 5 s. The time that the pup takes to return to prone position after release was recorded. Three trials were done for each pup in which a maximum time of 60 s was given for each trial.

The negative geotaxis test assesses motor coordination in young mice. Pups are placed on a 45-degree-inclined plane with the head facing downwards and, because of the vestibular cues of gravity, they can turn to face up the slope. The response to stimulus, or taxis, is an innate behavior. The latency period that the pup takes to change its position was recorded, up to a maximum of 60 s [16,17].

The grasping reflex is a motor test in which the pup fore or hind paws is stroked with a rounded side blunt instrument; thus, the fore and hind paws is flexed to grasp the instrument. To perform the test, mouse pups were gently held by the scruff of their neck and fore paws were stroked with a metal paperclip [16]. The presence or absence of grasping was registered.

The startle response test was performed in a room with low level of environmental noise. Mice were exposed to acoustic stimuli by finger snapping at 25 to 30 cm above each pup. A sudden extension of the head and fore and hind limbs should be observed immediately after the acoustic stimuli [16]. The presence or absence of response was registered.

### 2.6. Behavioral phenotyping

The mutant phenotype was assessed by behavior tests as described by Manes et al. [18] with modifications. Testing was performed in a small room with dim lighting. The apparatuses were cleaned with a 5% alcohol/water solution before each animal was placed to prevent possible bias caused by odor cues left by the previous one. All procedures were video-recorded for offline visual evaluation by two experienced observers. In addition, Ethovision XT video tracking system (Noldus Information Technology bv, 7.1.426, The Netherlands) was used for data analyses of distance traveled (cm) and speed (cm/s) in the open-field test (OFT) [19].

The OFT was used to evaluate exploratory behavior and total locomotor activity levels measuring distance traveled and average speed, and the frequencies of rearing and grooming, as described elsewhere [20]. Testing was performed for 5 min with mutant and WT mice at three and eight weeks of age.

T-maze alternation test is based on the natural proclivity of rodents to alternate between the visited goal-arms in each trial over a series of successive trials, and is very sensitive to assess hippocampal dysfunction [21]. The test was performed to evaluate spatial memory of mutant and WT mice at 9 weeks of age following the protocol previously described [18]. Briefly, mouse was placed in the start area of the apparatus for 10 s. At this point, the barrier was raised, and the mouse was permitted to explore the maze for up to 30 s. Once the mouse entered one of the free choice arms, the barrier was inserted to block the animal inside that arm for 30 s. Thereafter, the mouse was repositioned in the start area, initiating another session. If the mouse did not enter in any of the free choice arms after 30 s, it was replaced in the start area, and the session was restarted. Five sessions were performed for each mouse, and, for each session, the sequence of entries in the free choice arms was evaluated, i.e., whether the mouse first entered in left or right arm. For statistical analysis, data were transformed into scores: 0 = no alternations; 1 = one alternation; 2 = two alternations; 3 = three alternations; and 4 = four alternations.

Motor coordination was assessed in mutant and WT mice at three and eight weeks of age by the balance beam task, as described previously [18]. In brief, the apparatus consisted of a wooden beam 1.5 cm wide and 150 cm long supported above the floor by two platforms of 20 cm high at each end. At the starting end, a light was used as an aversive stimulus, and at the opposite end, a dark box was placed as a shelter and positive stimulus for mice to cross the beam. During the first and second days of training, each mouse was placed on the starting end, and it should walk across the beam from one end to the other for three times. Each mouse underwent three sessions of 5 min. The test was performed on third day, when mice were placed on the starting platform and had to cross the bar. For statistical analysis, data were transformed into scores as follows: 1 — the mouse does not cross the beam; 3 — the mouse crosses the beam with errors; 7 — the mouse crosses the beam without errors. Besides, time taken to cross the beam and number of falls were recorded.

Forced swim test (FST) and tail suspension test (TST) have been used to assess coping behavior and motor function of mutant mice [18,22], and were performed with mutant and WT mice between 9 and 10 weeks of age. The FST was performed as described previously [23]. Shortly, each mouse was individually placed into a vertical glass cylinder with 22 cm in diameter containing water with a depth of 25 cm and temperature of 23–25 °C. After 6 min in the cylinder, animals were removed, dried, and moved to a warm cage. The TST was performed as described [18,24]. In brief, a tape attached to a hook was used to suspend mice by the tail in a single 6-min trial shot with a camera in front of the apparatus. Immobility time was defined as the mouse not struggling. For both tests, latency to immobility and total immobility time was recorded.

### 2.7. Response to acoustic stimuli

Forty mice (20 mutant and 20 WT littermates; 10 males and 10 females of each genotype, 10–12 weeks old) were used. The experimental apparatus consisted of a Plexiglas cylinder (22 cm in

diameter, 30 cm in height) positioned 60 cm beside a 130-Watt ultrasonic processor (Sonic and Materials Inc.), with operating frequency of 20 kHz. During testing, each mouse was placed individually in the cylinder and allowed to explore for 15 s. Then, the ultrasonic processor was turned on for 60 s or until onset of convulsion. Animals were tested only once and the latency to onset of seizure was recorded. One week later, mice that had presented audiogenic seizures received diazepam (UNI-DIAZEPAX®, União Química Farmaceutica Nacional S/A, MG, Brazil) (0.5 mg/kg, subcutaneously) and were submitted to acoustic stimuli 15 min after administration, in the same way as described above.

### 2.8. Measurement of CNS neurotransmitters and its metabolites

Mutant and WT male mice with 10 weeks of age were euthanized by decapitation and the hippocampus was dissected out and placed on an ice-cold plate. Each sample was quickly frozen and stored in an ultrafreezer at −80 °C until assayed. The contents of NOR, vanillylmandelic acid (VMA), DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindole acetic acid (5-HIAA) were determined using high-performance liquid chromatography (HPLC) with a C-18 column and an electrochemical detector (Shimadzu, model 20A, Kyoto, Japan); DA, NOR, and 5-HT turnover (metabolite/neurotransmitter ratio) were calculated. The detection limit for monoamines was 2 pg; the coefficient of variation was less than 15%, and the curve linearity was greater than 0.9. The content of amino acids GABA, glutamate, glycine, and aspartate were measured by HPLC (HP, model 1100) with a Beckman Ultrasphere octadecylsilyl-phenylthiohydantoin (ODS-PTH) column and sample injector (valve for 1.0 ml), and the limit of detection was 20 pg [25].

### 2.9. Statistical analysis

Descriptive statistics was used to analyze gene expression. A two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test was employed to evaluate strain, age, and sex effects on the physical and reflexology development, general activity, T-maze, and balance beam task. Unpaired *t*-test with Welch's correction was used to analyze the TST and FST parameters, and neurotransmitters and its metabolites in the central nervous system (CNS). Statistical analysis was completed with GraphPad Prism 8.2.1 software (GraphPad Software, Inc., 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). The data were expressed as the mean ± standard error of the mean (SEM) or as or the medians and interquartile range, and results were considered significant at $p < .05$.

## 3. Results

### 3.1. Genetic mapping and candidate gene sequencing

The genome scan using polymorphic microsatellite markers distributed through the mouse genome showed that the mutation *tremor* is located on chromosome 14, in the interval of 5 cM between D14Mit37 (33.21 cM) and D14Mit115 (38.21 cM), making Egr3 the main candidate mutant gene. According to the UCSC Genome Browser, Egr3 site has two protein-coding variants, Egr3-204 (2 exons) and Egr3-201 (3 exons). Genomic DNA of all coding regions was sequenced, and DNA sequences from mutant and WT mice on C57BL/6J background were matched using the Clustal application within the BioEdit version 7.2. No mutation was found in the analyzed DNA sequences.

### 3.2. Egr3 and Gabra1 receptor subunit gene expression

In the quantitative RT-qPCR analyses, descriptive statistics demonstrated higher gene expression of Egr3 (25% percentile 1.43; median 3.72; 75% percentile 5.50; mean 3.68 and SEM 0.98) and Gabra1 (25% percentile 1.36; median 1.70; 75% percentile 2.03; mean 1.67 and SEM 0.16) in mutant compared to WT mice (Fig. 1).

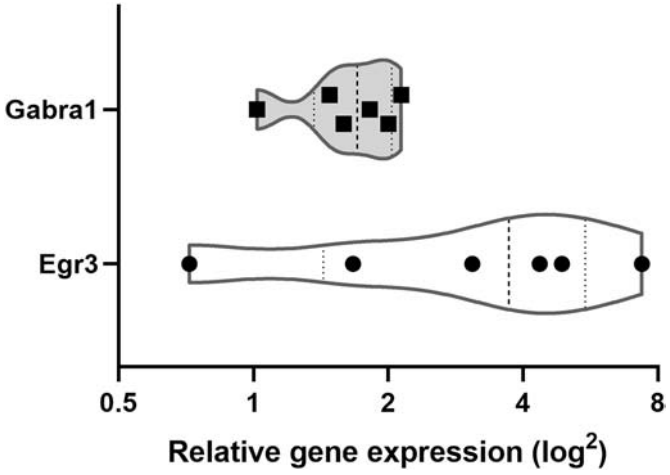


**Fig. 1.** Fold changes in Egr3 and Gabra1 gene expression in the hippocampus of mutant mice (n = 6) on C57BL/6 background measured by RT-qPCR. Data are presented as individual points, medians, and quartiles. Descriptive statistics was used.

### 3.3. Evaluation of postnatal development

The mutant mice did not differ from WT mice in physical and reflexology development from birth to three weeks of age (Fig. 2a–d). After four weeks of age, a two-way ANOVA showed a significant effect of strain ($F(3.28) = 52.56$, $p < .0001$) on weight, such that mutants weighed less than WT mice (Fig. 2e). After three weeks of age, mutants can be easily distinguished from the WT mice by phenotypic characteristics as tremor, ataxia, motor incoordination, and spontaneous seizures (S2. Supplementary Material — Video 1).

### 3.4. Behavioral phenotyping

Effects of strain and age or sex on the distance traveled, average speed, grooming frequency, and rearing frequency were evaluated using two-way ANOVA tests. For the first parameter, a significant main effect of strain was demonstrated on distance traveled ($F[1, 32] = 15.13$, $p = .0005$) and average speed ($F [1, 32] = 15.00$, $p = .0005$), such that mutant mice showed reduced parameters compared to WT mice (Fig. 3a and b). A significant main effect of age was also observed, indicating that eight-week-old mutant and WT mice traveled a longer distance ($F[1, 32] = 28.19$, $p = .0001$) and showed a higher average speed ($F[1, 32] = 28.93$, $p = .0001$) than mice with three weeks of age (Fig. 3a and b).

Relative to grooming frequency, a significant main effect of strain ($F [1, 38] = 4.69$, $p = .0366$) was observed, according to which mutants showed increased frequency of grooming at three weeks of age and decreased frequency at eight weeks of age (Fig. 3c) compared to WT animals. A main effect of age ($F[1, 38] = 9.72$, $p = .0035$) and the interaction between age and strain ($F [1,38] = 17.74$, $p = .0001$) were also demonstrated, showing that mutants at eight weeks of age present decreased grooming frequency when compared to mutants at three weeks of age (Fig. 3c). In addition, a significant main effect of sex ($F[1, 36] = 12.51$, $p = .0011$) at eight weeks was demonstrated, indicating that males presented decreased grooming frequency when compared to females (Fig. 3d).

Regarding the rearing frequency, a significant main effect of strain ($F [1, 38] = 21.92$, $p < .0001$) was demonstrated, in which mutants showed decreased frequency at three and eight weeks of age when compared to WT mice (Fig. 3e). Additionally, a significant main effect of age by strain ($F[1, 38] = 15.04$, $p = .0004$) and the interaction of age between strains

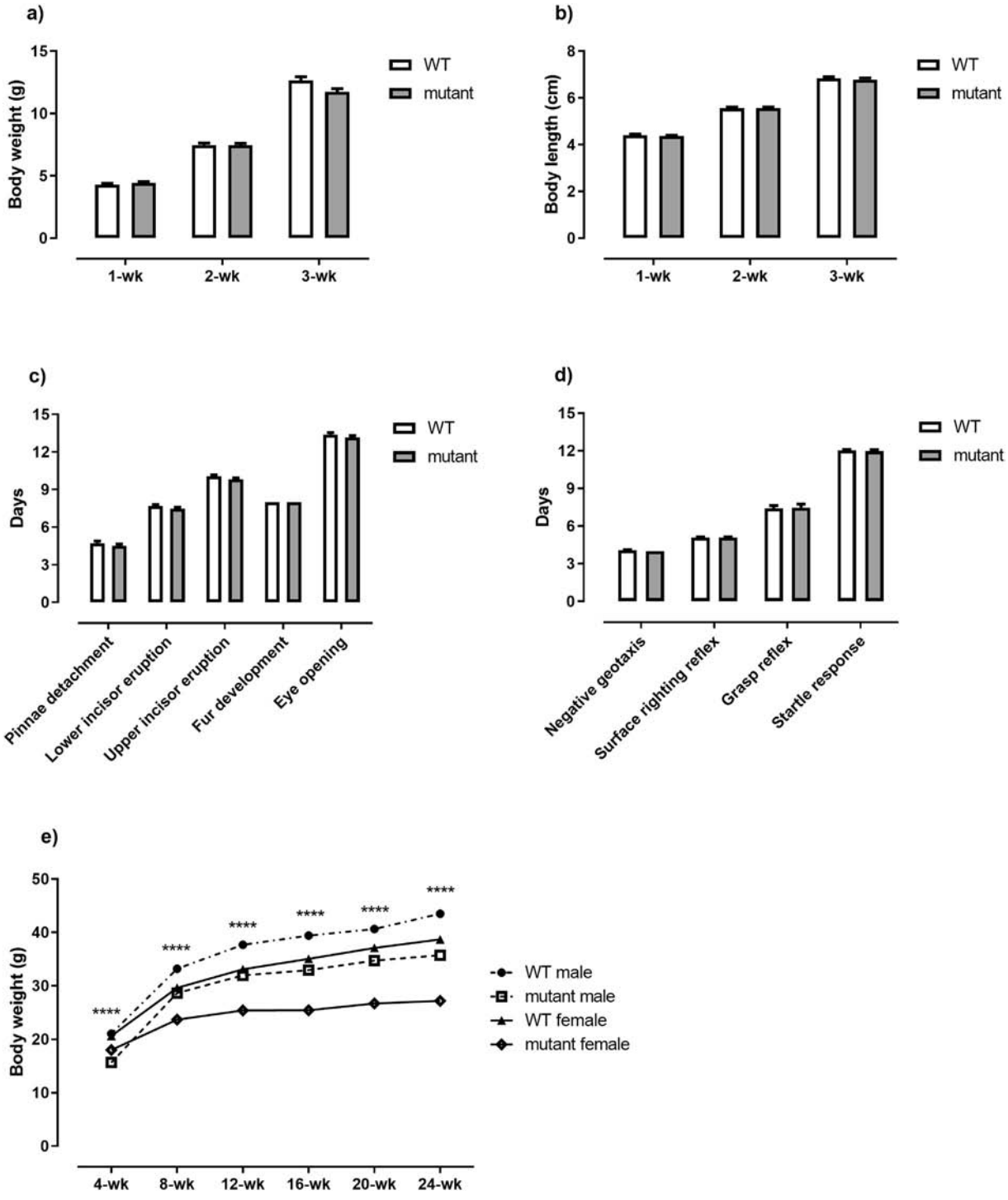


**Fig. 2.** Body weight from one to three weeks of age (a); body length (b); parameters of physical (c) and reflexology development (d); and body weight from 4 to 24 weeks of age (e) in mutant (n = 34) and WT (n = 34) mice. Data are presented as the means ± SEM. Two-way ANOVA followed by Sidak's multicomparison test was employed to evaluate strain and sex differences.

($F[1, 38] = 4.61$, $p = .0381$) were also demonstrated, such that mutants presented decreased rearing frequency at eight weeks of age when compared to mutants at three weeks of age (Fig. 3e). Finally, a significant main effect of sex ($F[1, 36] = 19.24$, $p < .0001$) was also observed, according to which WT males presented decreased rearing frequency when compared to WT females (Fig. 3f).

In the balance beam test, WT mice presented higher scores than mutants when crossing the bar ($F[1, 28] = 504003$, $p < .0001$) (Fig. 4a). No differences were observed between males and females. Mutant mice took a longer time to cross the bar ($F[1, 32] = 55.96$, $p < .0001$) and had a greater number of falls ($F[1, 34] = 13.69$, $p = .0008$) compared to the controls (Fig. 4b and c). With a two-way ANOVA, a significant main effect of age and the interaction between strain and age ($F[1, 34] = 7.70$, $p = .0089$) on the number of falls were observed, while mice were crossing the beam.

In the T-maze alternation task, mutant and WT mice presented similar behavior, but males showed higher scores relative to females ($F[1, 36] = 4.43$, $p = .0423$) (Fig. 4d).

Finally, in the FST and TST, the latencies for the first immobility of mutant and WT mice were not significantly different. The total

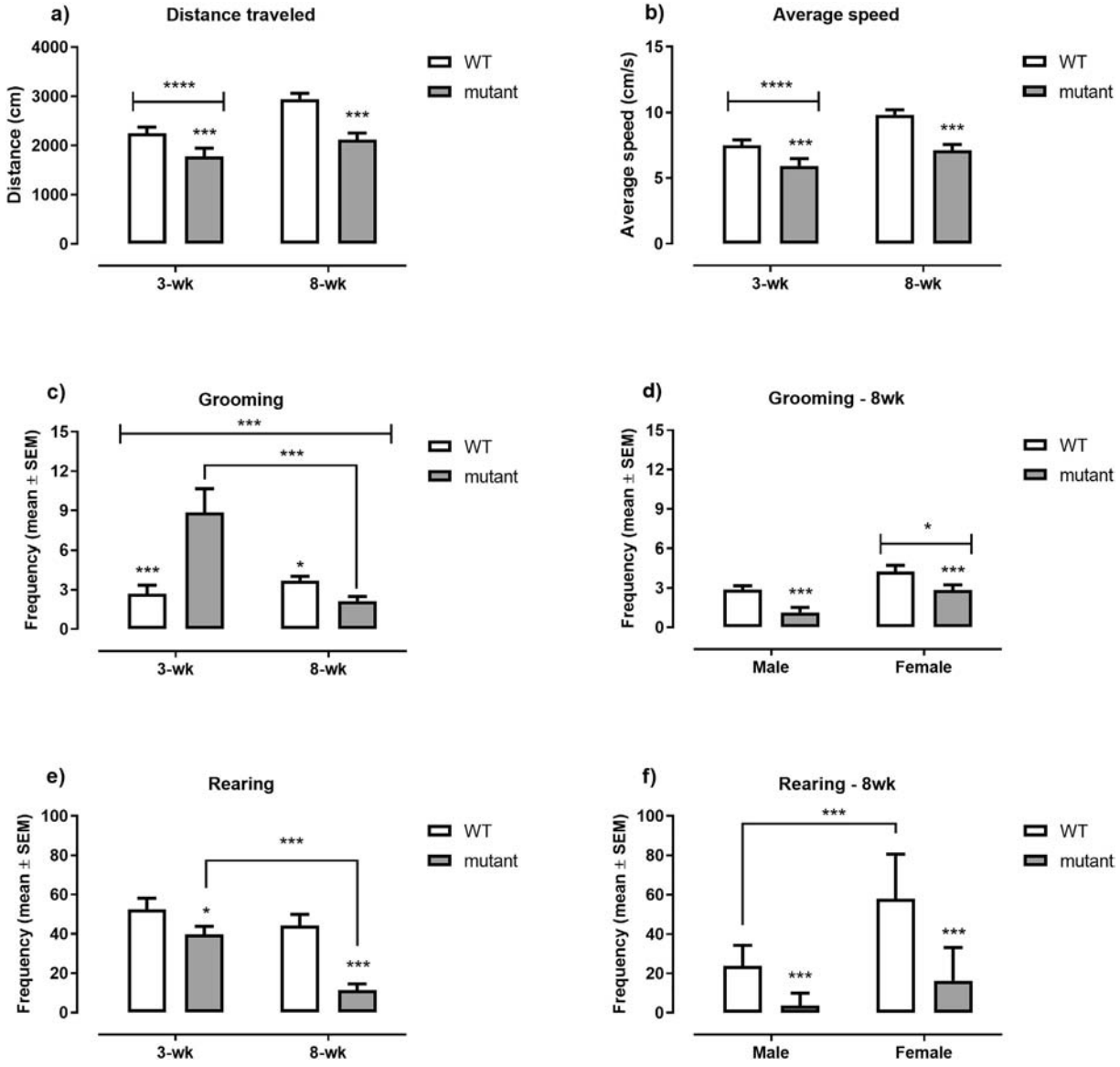


**Fig. 3.** General activity in the open field: distance traveled (a); average speed (b); grooming (c and d); and rearing (e and f) in mutant (n = 20) and WT (n = 20) mice. Data are presented as the means ± SEM. Two-way ANOVA followed by Sidak's multicomparison test was employed to evaluate strain and sex differences.

immobility time of mutants was significantly higher than that of WT mice, in the FST ($t = 6.89$, $df = 6.64$, $p = .0003$) as well as in the TST ($t = 6.68$, $df = 13.73$, $p < .0001$) (Fig. 4e and f).

### 3.5. Response to acoustic stimuli

During acoustic stimulation, WT littermates of mutant mice displayed normal behavior with exploration and walking around the cylinder. In contrast, beginning between 1 and 2 s after the stimulus onset, all female and 80% of male *tremor* mice showed monophasic wild running followed by jumping, atonic falling with generalized clonic seizures, sometimes with body rolling, for a period of nearly 40 s. In the sequence, mice remained in ventral decubitus with occasional clonus and dyspnea for an additional interval of 90 to 120 s. Finally, mice recovered walking and exploring behavior with similar features to that displayed in the basal prestimulus condition. Mutant mice treated with diazepam did not express audiogenic seizures after stimuli. A video recording of a representative seizure can be seen in Supplementary Material – S3 Video 2.

### 3.6. Measurement of CNS neurotransmitters and its metabolites

Comparing mutants with WT mice, the hippocampal concentrations of GABA ($t = 2.52$, $df = 14$, $p = .0123$), glutamate ($t = 2.22$, $df = 14$, $p = .0217$), aspartate ($t = 2.51$, $df = 14$, $p = .0124$), NOR ($t = 3.95$, $df = 15.88$, $p = .0012$), 5-HT ($t = 2.97$, $df = 17.66$, $p = .0083$), and 5HIAA ($t = 3.56$, $df = 13.88$, $p = .0032$) were significantly increased. In contrast, VMA/NOR ratio ($t = 2.38$, $df = 13.90$, $p = .032$) and glycine levels ($t = 2.36$, $df = 14$, $p = .0168$) were decreased. Regarding to dopaminergic system, neither DA and DOPAC contents nor the turnover rate of DA (the DOPAC plus HVA to DA ratio) showed statistically significant differences between WT and mutant mice (Fig. 5, Table 2).

## 4. Discussion

In the present study, *tremor* mice displayed audiogenic seizures with behavioral features similar to the ones detected in rodents such as dilute brown agouti coat color (DBA) mice [26], WAR [27], and genetically epilepsy-prone rat (GEPR) [26], and GASH:Sal hamsters [28]. Seizures in *tremor* mice were associated with hippocampal alterations of Egr3

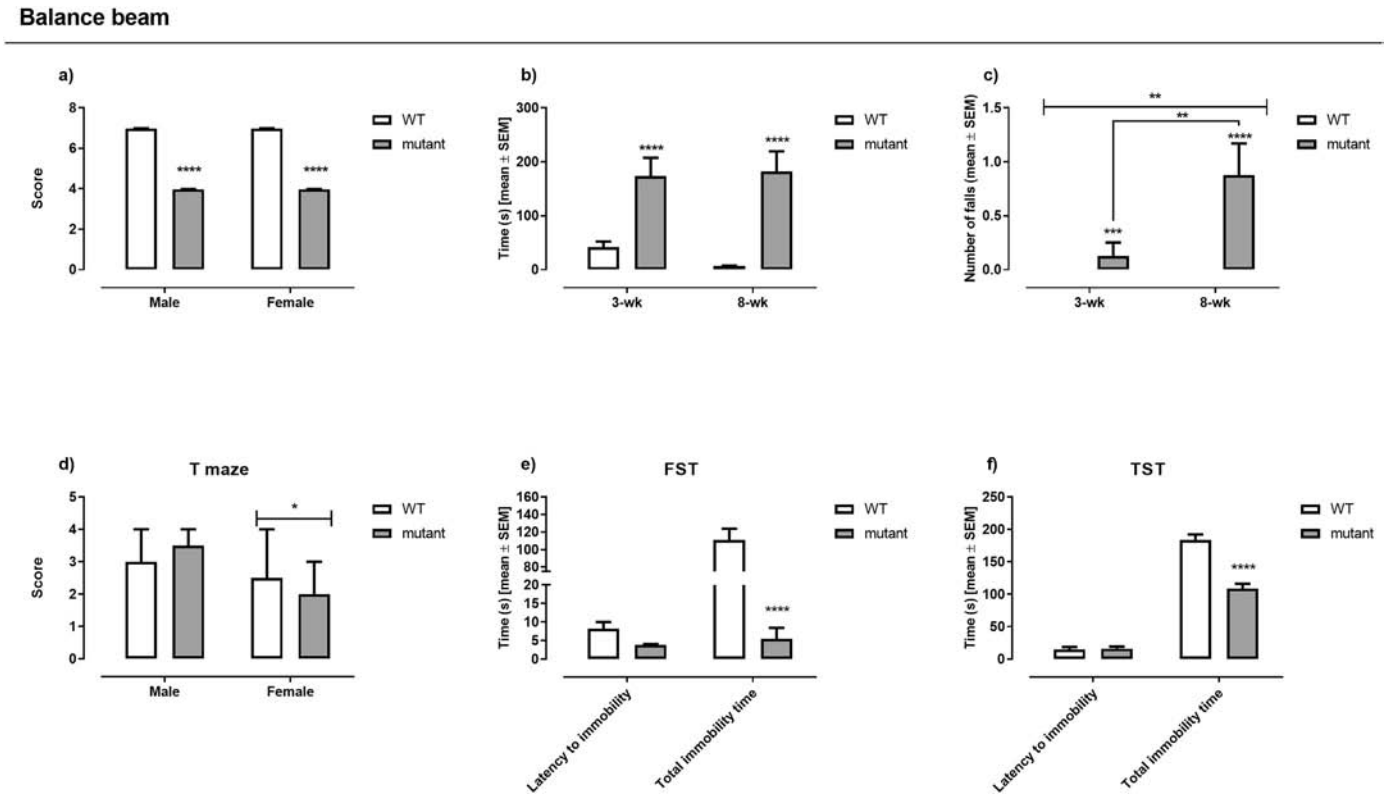


**Fig. 4.** Balance beam test: (a) scores (n = 16 mutants and 16 WT); (b and c) time taken to cross the beam and number of falls (n = 8 mutants and 11 WT); (d) T-maze alternation test (n = 20 mutants and 20 WT); (e and f) latency to the first immobility and total immobility time in the FST and TST in mutant (n = 8) and WT (n = 8) mice. Data are presented as the means ± SEM or medians and the interquartile range. Two-way ANOVA followed by Sidak's multicomparison test was employed to evaluate strain and sex differences.

and Gabra1 gene expression, as well as of glycine, GABA, glutamate, aspartate, NOR, 5-HT and 5HIAA content, and VMA/NOR ratio. Then, taken all the alterations together, *tremor* mice may be a useful tool for the study of epilepsy.

The prevailing phenotype of the spontaneous mutant *tremor*, which enables distinguishing them from WT mice after three weeks old, is a constant tremor while moving even when in home cage; WT mice moved quickly in the cage while mutant mice were stalled and showed ataxia and full-body tremor when stimulated on that purpose. With aging, tremor phenotype intensifies and motor coordination decreases, while generalized clonic audiogenic seizures are observed. Initial observations showed that both sexes were fertile; females, however, presented inferior fertility compared to WT mice. Heterozygous animals were indistinguishable from Swiss mice.

Postnatal physical and reflexology development evaluation have shown no differences between WT and mutant mice from birth to three weeks of age, demonstrating that mutants have a normal development until three weeks of age, as suggested by the initial observations about mutant phenotype. Besides postnatal development evaluation, WT and mutant mice went through OFT at three and eight

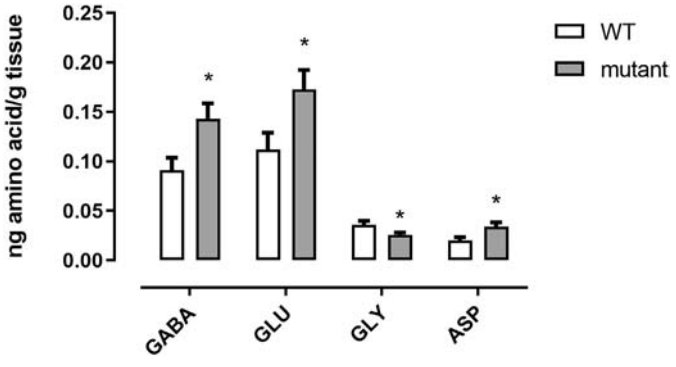


**Fig. 5.** Neurotransmitter amino acid tissue contents (ng/g wet weight tissue) in hippocampus of WT and mutant male mice. Data are presented as the means ± SEM. Unpaired *t*-test with Welch's correction was employed to evaluate strain differences. GABA (gamma-aminobutyric acid), GLU (glutamate), GLY (glycine), and ASP (aspartate).

**Table 2**
Neurotransmitter monoamine tissue contents (ng/g wet weight tissue) and turnover rates in hippocampus of WT and mutant male mice. Data are presented as the means ± SEM.

| | Group | |
|---|---|---|
| Monoamines and metabolites | WT | mutant |
| DA | 27.47 ± 9.17 (n = 9) | 23.77 ± 4.43 (n = 9) |
| DOPAC | 10.22 ± 0.94 (n = 9) | 11.36 ± 1.29 (n = 9) |
| HVA | 45.98 ± 6.72 (n = 9) | 69.54 ± 10.95 (n = 10) |
| (DOPAC + HVA)/DA ratio | 4.30 ± 1.40 (n = 9) | 3.64 ± 0.58 (n = 9) |
| NOR | 479.10 ± 27.14 (n = 10) | 608.93 ± 18.51* (n = 10) |
| VMA | 241.57 ± 18.79 (n = 10) | 260.49 ± 13.24 (n = 10) |
| VMA/NOR ratio | 0.50 ± 0.02 (n = 10) | 0.42 ± 0.01* (n = 10) |
| 5HT | 551.09 ± 38.93 (n = 10) | 727.51 ± 44.78* (n = 10) |
| 5HIAA | 363.51 ± 24.38 (n = 10) | 545.68 ± 44.92* (n = 10) |
| 5HIAA/5HT ratio | 0.66 ± 0.03 (n = 10) | 0.74 ± 0.04 (n = 10) |

DA (dopamine), DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid), NOR (noradrenalin), VMA (vanillylmandelic acid), 5HT (5-hydroxytryptamine), and 5HIAA (5-hydroxyindole acetic acid).

* $p < .05$, unpaired *t*-test with Welch's correction was employed to evaluate strain differences.

weeks of age, in which general activity was analyzed. Mutants showed higher grooming frequency than WT mice at 3 weeks of age. On the other hand, grooming frequency was reduced in mutants with 8 weeks of age, denoting ongoing motor impairment. Additionally, decreasing in rearing frequency, shorter distance traveled and lower average speed in mutants with three and eight weeks of age compared to WT mice occurred as a result of motor disability caused by the mutation.

Concomitantly to phenotypic characterization of mutant mice, genetic mapping with microsatellite markers was performed in an attempt to identify the mutated gene. A linkage was identified on chromosome 14, between 33.21 and 38.21 cM, making Egr3 the main candidate gene; Egr3-deficient mice have severe ataxia, resting tremors, scoliosis, and ptosis caused by lack of muscle spindles, which are skeletal muscle sensory organs involved in the proprioception [29]. However, this phenotype is quite different of our mutants, which presented movement tremors and audiogenic seizures, and do not show scoliosis or ptosis.

The Egr3, a member of the Egr transcription factor family that includes four proteins (Egr 1, 2, 3, and 4), is responsible for transcription factors of zinc-finger family and may have multiple roles in epileptogenesis [30,31]; Egr3 overexpression was related to susceptibility of audiogenic seizures in the WAR rats and GASH:Sal hamsters, likewise Egr3 expression was increased in the hippocampus of patients and animal models of temporal lobe epilepsy [8]. Induction of Egr family transcription factors occurs after pilocarpine-induced status epilepticus in mice, showing increased mRNA and protein levels of Egr3 in the hippocampus [32]. In the pilocarpine-induced rodent model, Egr3 is involved in the modulation of GABAergic receptors in dentate granule cells of the hippocampus, resulting in decreased expression of GABAA receptor alpha 1 subunit and increased alpha 4 subunit expression [30].

Corroborating with the literature, our results demonstrated overexpression of Egr3 mRNA in the hippocampus of mutants that was 3.7-fold higher when compared to WT mice. Data distribution (Fig. 1) showed that Egr3 expression range was 6.65 in mutant mice. As naive mice (i.e., do not submit to acoustic stimuli) were used for gene expression assessment, this finding suggests that spontaneous seizures could occur in mutants, ranging between animals.

As a general view, even though no mutation has been found in the Egr3 DNA sequences analyzed, the reports above reinforce that an impairment of the Egr3 gene function, which is located on the interval mapped for the mutation, could explain the mechanism involved in seizures and tremor observed in our mutant strain.

Literatures have described both TST and FST as tools to evaluate depression-like responses in mice [23,24]. However, recent publications have interpreted immobility time in the FST as a way to express passive coping behavior that might be determined by genetic factors [33,34]. Our results showed reduced immobility period in mutant compared to WT mice in the TST and FST, which can be understood as lack of coping behavior to an acute stressor of mutant animals; however, these data require further investigation. Corroborating with our findings, Egr3-deficient mice showed hyperactivity facing a new environment, and they failed to habituate to repeated acoustic stimulus that might indicate a lack of behavioral adaptation to an acute stressor [35]. However, tail suspension test and forced swim test showed lack of behavioral adaptation to an acute stressor in mutants.

In the balance beam test, mutant mouse presented a characteristic posture, by grabbing the beam with its hind limbs and rolling tail around the beam to avoid falling. To walk across the beam, the mouse supports the abdomen on it. In spite of the difficulty to cross the beam, mutants were able to do it, showing that their severe motor impairment did not interfere with environment exploration (Supplementary Material – S4 and S5, Videos 3 and 4). In addition, a longer period was taken to cross the bar by mutants compared to WT, and mutants showed the highest number of falls at eight weeks of age. Additionally, results from T-maze alternation test showed that mutants presented a prominent motor deficiency but did not show spatial memory alterations when compared to WT mice. Different from our data, Egr3-deficient mice performed fewer sequential arm entries compared with controls, in a Y-maze spontaneous alternation task, suggesting immediate memory impairment [35].

Often clonic seizures can occur in mutant mice in response to acoustic and/or environmental stimuli, but the mechanism involved in these seizures has not been fully acknowledged yet. Our results showed increased content of GABA, glutamate and aspartate in the hippocampus of mutants. Very similar results were described for spontaneous recurrent seizures on the pilocarpine-induced model in rats [36] and after a single acoustic stimulation in audiogenic seizure-prone Rb1 mice [5]. Increased concentration levels of glutamate and GABA in the extracellular matrix might influence on epilepsy development and evolution, possibly because of the activation of extrasynaptic receptors [37]. Impairment of GABAergic neurotransmission has been described as a main neurochemical mechanism involved in audiogenic seizures in different animal models of epilepsy. Low levels of GABA were detected in the hippocampus of Il-6 KO, Rb1 (clonic–tonic seizure-prone) and Rb2 (clonic seizure-prone) mice, susceptible to audiogenic seizures. Seizure-prone BALB/c mice showed reduced GABA concentrations in the striatum, and DBA/2 mice presented fewer GABA biding sites in whole brain when compared to seizure-resistant mice [6,28].

Diazepam administration prior to acoustic stimuli inhibited seizures in mutant mice, probably due to its action on facilitating GABAergic transmission and reducing extracellular hippocampal glutamate levels. It has been demonstrated that activation of GABAA receptors, by binding on regulatory sites, was the main mechanism of diazepam to prevent pilocarpine-induced seizures [38], since diazepam interaction with these sites increases frequency of chlorine channels opening and enhances neuronal chloride-ion influx, resulting in postsynaptic membrane hyperpolarization and reduced neuronal excitability. In previous studies, diazepam had demonstrated protective effects against the tonic, clonic, and wild running phase of audiogenic seizures in DBA/2J mice [39].

Audiogenic seizures are triggered in the brainstem neural network involved in sound processing, mainly the inferior colliculus [27]. The hippocampus seems to be included in the modulation of that network, considering that impairment of the hippocampal integrity or function are associated with the development of audiogenic seizures susceptibility in previously normal animals [40–42]. Additionally, rodents genetically prone to audiogenic seizures show alterations in the hippocampal function [6,28,43–47]. Imbalance between excitatory and inhibitory activity, which is regulated by neurotransmitters, results on increased seizure frequency [48]. Disparity of neurotransmitters in the hippocampus concerned with hypoactivity of GABA and hyperactivity of glutamate has been reported in generalized epilepsy [49]. Therefore, studying neurochemical changes in CNS is essential for a better understanding of seizure development and occurrence.

Increased extracellular glutamate is an important biochemical marker of animal and human epileptic nervous tissues and provokes neurotoxicity and seizures [50]; GABA presents inhibitory effects on hippocampal excitability and seizure activity, and rising levels of this amino acid during seizures probably leads to inhibition of the following seizure. Raised levels of glutamate might be related to seizure initiation process while the following rising level of GABA contributes to end the process [37].

Raised aspartate concentration levels on mutants' hippocampus may have occurred because of raised levels of glutamate, considering that studies showed both are synchronously released and are related to excitatory synaptic activity on hippocampus [51]. Alike, increased aspartate levels were demonstrated in the hippocampus and brainstem of Il-6 KO seizure-prone mice [6] and inferior colliculus of Rb1 mice [5].

In contrast to results described by Cavalheiro et al. [36] during chronic seizures in the pilocarpine-induced model, glycine levels were decreased in mutants' hippocampus. Low glycine levels in different brain areas were associated with increased susceptibility of audiogenic seizures in DBA/2J, Il-6 KO, seizure-prone BALB/c, and Rb1 mice [6,52,53]. Glycine can present opposite effects on hippocampus development, depending on a pre- and postsynaptic neurons balance activation [54]. Also, Chen et al. [55] demonstrated that low glycine concentration in hippocampus has proconvulsant effects while high concentrations seem to attenuate recurrent epileptic discharges. Mutants presented raised levels of 5-HT and the corresponding metabolite 5HIAA in the hippocampus; however, the neurotransmitter activity was similar to WT mice. In agreement with our findings, other authors described higher baseline contents of serotonin and its metabolite in the temporal cortex, hippocampus, and medulla of Krushinsky–Molodkina (KM) rats than in control Wistar rats [7], and in the hippocampus of Egr3-deficient mice [56]. Contrary to glutamate and GABA, extracellular increasing of 5-HT is not directly related to seizure activity in the hippocampus [37]. According to the authors, increased levels of 5-HT after pilocarpine and picrotoxin injections might result from muscarinic receptors activation or GABAA receptors blockage on serotonergic nerve endings in hippocampus.

In addition, serotonin is associated with physiological functions in the CNS, such as motor control. This monoamine produces a complex modulatory control over neurotransmission in the hippocampus, frontal cortex, and cerebellum, modulating excitatory effects by glutamate and inhibitory ones by GABA [57]. Endogenous or exogenous 5-HT exerts an anticonvulsant effect, because of raised GABAergic responses from hippocampus neurons, mediated by 5-HT1 receptors activation. Still, high concentrations of 5-HT in extracellular matrix led to more intense seizures, and has been associated with significant increased levels of extracellular glutamate, suggesting a modulatory effect of glutamate on monoamine anticonvulsant activity [48]. In sudden unexpected death in epilepsy, 5-HT abnormal activity is involved in the pathogenesis of seizure-induced respiratory arrest in DBA mice genetically susceptible to audiogenic seizures [58].

Noradrenalin plays an important role in the limbic system preventing the damage induced by status epilepticus [59]. The extracellular concentration of NOR was increased in the hippocampus, but its activity was decreased in the mutant compared to WT mice. The anticonvulsant effect of NOR has been described in the literature, and their levels are reduced after seizures [48,59]. Low levels of NOR in the brain of GEPR-9 rats susceptible to audiogenic seizure were associated with the severity of epileptic phenotype [59]. The administration of NOR at low concentrations in rats hippocampal CA3 region induced a proconvulsant effect, while high concentrations had an anticonvulsant activity [49]. On the other hand, either low or high levels of NOR may also have proconvulsant effects, which might be mediated by alpha2-adrenergic receptors. However, the mechanism of action by which NOR may achieve its pro- or anticonvulsant effects remains unclear [48,59].

## 5. Conclusions

In this work, data demonstrated that audiogenic seizures of *tremor* mice are associated with progressive motor impairment, evidenced by reduction of rearing and grooming frequency and increased number of falls in balance beam crosses at 8 weeks old, as well as to hippocampal alterations of the Egr3 and Gabra1 gene expression and amino acid and monoamine content. In addition, our mutant could be useful for study of neurotransmission pathways as modulators of epilepsy. Further studies are required to better characterize behavior, electroencephalography, and brain pathology of the *tremor* mice, which could be a new mouse model to better understand the pathogenesis of epilepsies occurring with generalized clonic seizures.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yebeh.2020.106945.

## Declaration of competing interest

We have no conflicts of interest to declare. All authors declare no competing interests.

## Acknowledgments

This manuscript is based upon work supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under grant number 2017/21103-3, and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior — Brasil (CAPES) — Finance Code 001. We thank the Core Facility for Scientific Research – University of Sao Paulo (CEFAP-USP/GENIAL). The authors would like to thank too Mr. Mauro de Mattos who found out the mutant in our mice colony, and Mrs. Luciana Bandini responsible for animal care and husbandry.

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