Epilepsy & Behavior 71 (2017) 207–217

Contents lists available at ScienceDirect

Epilepsy & Behavior

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

Pharmacological and neuroethological study of the acute and chronic effects of lamotrigine in the genetic audiogenic seizure hamster (GASH:Sal)

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ARTICLE INFO

Article history:
Revised 3 November 2015
Accepted 6 November 2015
Available online 12 February 2016

Keywords:
Neuroethology
Antiepileptic drugs
Pharmacokinetics
Lamotrigine
Audiogenic seizures

ABSTRACT

The present study aimed to investigate the behavioral and anticonvulsant effects of lamotrigine (LTG) on the genetic audiogenic seizure hamster (GASH:Sal), an animal model of audiogenic seizure that is in the validation process. To evaluate the efficiency of acute and chronic treatments with LTG, GASH:Sals were treated with LTG either acutely via intraperitoneal injection (5–20 mg/kg) or chronically via oral administration (20–25 mg/kg/day). Their behavior was assessed via neuroethological analysis, and the anticonvulsant effect of LTG was evaluated based on the appearance and the severity of seizures. The results showed that acute administration of LTG exerts an anticonvulsant effect at the lowest dose tested (5 mg/kg) and that chronic oral LTG treatment exerts an anticonvulsant effect at a dose of 20–25 mg/kg/day. Furthermore, LTG treatment induced a low rate of secondary adverse effects.

This article is part of a Special Issue entitled "Genetic and Reflex Epilepsies, Audiogenic Seizures and Strains: From Experimental Models to the Clinic".

1. Introduction

Epilepsy is a complex neurological pathology with a high incidence rate and a significant number of negative consequences [1]. Animal models of epilepsy are essential for studying epileptic disorders, as they provide valuable insights into epilepsy development and its underlying mechanisms. Therefore, animal studies may provide novel approaches to test new pharmacological treatments. The main goals of using epileptic and seizure models with respect to antiepileptic drugs (AEDs) include the following: to discover new AEDs, to characterize the anticonvulsant effects of new AEDs, to evaluate the efficiency of chronic treatment with new AEDs, and to estimate the effective blood concentration of new AEDs in phase I clinical trials [2].

Epilepsy involves a variety of pathological conditions associated with its origin. Thus, it is reasonable to consider epilepsy as a disorder of complex systems. One of the newest approaches to the study of epilepsy is the application of neuroethology [1]. Neuroethology combines ethology [3], neurobiology, neurophysiology, and comparative behavioral analysis [4] to help explain how sensory information can structure and influence motion. Neuroethology is based on the principle that the evaluation of behavioral sequences is more consistent than the evaluation of isolated events [5]. Neuroethological studies have been performed in epilepsy models such as rat models of acute and chronic audiogenic seizures [6], models of systemically and intrahippocampally administered pilocarpine-induced seizures [5,7], and synapsin knockout mice [8]. Additional studies have been conducted on patients with temporal lobe [4,9] and frontal lobe [10] epilepsy.

Animal models of seizures and epilepsy have played a crucial role in developing our understanding of the physiological and behavioral disturbances associated with human epilepsy. Many preclinical trials of AEDs are conducted using models of seizures (e.g., maximum electroshock or pentylenetetrazole [11,12]) rather than models of epilepsy. As a consequence, these anticonvulsant effects of AEDs are not observed in a brain with an inherent dysfunction that renders it susceptible to epilepsy (genetic models and kindling models), since this dysfunction could affect drug efficacy and toxicity [13]. Epilepsy models are extensively used to understand the processes that lead to epilepsy development by facilitating the identification of the underlying

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http://dx.doi.org/10.1016/j.yebeh.2015.11.005
1525-5050/

cellular and molecular mechanisms, thus providing potential targets for AED development [14]. As such, there is an imperative need to study the response to these drugs from the molecular to the physiological level. Additionally, pharmacological studies of chronic AED treatment using epilepsy models are important because they help to characterize the anticonvulsant potential and spectrum of activity of the examined AED [2].

The genetic audiogenic seizure hamster (GASH:Sal), an inbred strain developed at the University of Salamanca, exhibits audiogenic seizures (AGs) [15–18] and is considered as a model of generalized tonic–clonic seizures [19–21]. The activation of reflex seizures via specific sensory stimulation, such as acoustic stimulation, of genetically susceptible animals may be suitable for the study of the cellular and electrical mechanisms underlying epilepsy that is partially attributable to a genetic component [2,22]. We consider the GASH:Sal as a reliable animal model of epilepsy because electroencephalographic recordings [16] show an evident progressive profile during seizures that is similar to the pattern of human generalized tonic–clonic seizures and to that of other animal models of epilepsy, such as the profile of spontaneous seizures in the Noda epileptic rat [23] and of tonic–clonic seizures present in the genetically epilepsy-prone rat model (GEPR-9) [24] and the Wistar Audiogenic Rat (WAR) strain model [6,25].

As a part of the validation process of the GASH:Sal strain, we designed the present pharmacological and neuroethological study of lamotrigine (LTG; 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine). This AED, which has been commercialized in the last 20 years [2,26,27], is indicated as a monotherapy or as an adjunctive therapy against partial and generalized tonic–clonic seizures. Lamotrigine is an antiepileptic drug chemically unrelated to other AEDs, and this distinction may explain its wide clinical spectrum for the treatment of not only epilepsy (including a wide variety of seizure types and epileptic syndromes [28,29]), but also bipolar disorder, neurological pain, and clinical depression [30–35]. Lamotrigine appears to act by inhibiting the release of glutamate from presynaptic membranes, primarily by inhibiting voltage-dependent sodium currents to block high-frequency repetitive spike firing, which is believed to occur during the spread of seizure activity, without affecting ongoing physiological neural activity [34,36]. However, in contrast to other sodium channel inhibitors, LTG acts on voltage-activated calcium channels (both N- and P-/Q-/R-type channels) by reducing the release of glutamate in response to paroxysmal neuronal firing [28,29,37–40]. Additionally, LTG is thought to block excitatory neurotransmitters by acting on N-methyl-D-aspartate (NMDA) receptors and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [41,42]. Furthermore, the characterization of the pharmacological activities of LTG requires the assessment of the drug concentration at its neuronal sites of action.

The goals of this study were to characterize the anticonvulsant efficacy of LTG in the GASH:Sal model and to characterize the effects of acute and chronic LTG administration on preictal and ictal behavior via neuroethological analysis [6,18]. We also determined the plasma and brain concentration profiles of LTG after the administration of a single intraperitoneal dose or multiple oral doses.

2. Materials and methods

2.1. Animals

Two-month-old male GASH:Sals were obtained from the Experimental Animal Service of the University of Salamanca (USAL, Spain) and were housed at the Institute of Neuroscience of Castilla and León (INCYL) animal housing unit for the duration of the experiments. The animals were provided with free access to food and water. The experimental animals were handled and cared according to the Guidelines of the European Communities Council Directive (2010/63/UE) and the current Spanish legislation for the use and care of laboratory animals (RD 53/2013, BOE 8/02/2013), under the supervision of the corresponding Institutional Animal Care and Use Committee. All efforts were made to avoid unnecessary animal suffering and to reduce the number of animals used.

2.2. Acoustic stimuli

Auditory stimulation was performed inside an acrylic cylinder (height: 50 cm, diameter: 37 cm). The sound of shaking keys was recorded using a high-pass filter (>500 Hz, Bruel & Kjaer #4134 microphone and Bruel & Kjaer #2619 preamplifier), digitized above 4 kHz, and reproduced by a computer coupled to an amplifier (FONESTAR MA-25T, Revilla de Camargo, Spain) and a speaker (Beyma T2010, Valencia, Spain) placed on the upper side of the cage. The final sound was a semirandom sound of 0–18 kHz with an intensity of 115–120 dB.

2.3. Drugs and reagents

Lamotrigine was provided by GlaxoSmithKline (UK). The stock solution was made by first dissolving the LTG in propylene glycol (Merck, Madrid, Spain) with vortexing and then adding an equal volume of distilled water, producing a final 50:50 (v:v) solution. For blood extraction, the animals were anesthetized using isoflurane (IsoFlo®, Esteve Veterinaria, Abbot, Barcelona, Spain). Chloramphenicol (Sigma-Aldrich, Madrid, Spain) was used as an internal standard (IS) for HPLC/MS analysis of the concentration of LTG in the brain and in blood. For HPLC, the mobile phase consisted of the following: A: water containing 0.1% formic acid and B: acetonitrile. Both reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Pharmacological study of LTG

2.4.1. Brain and blood extraction and sample preparation

The right hemispheres of two animals receiving acute or chronic LTG treatment were collected in Falcon tubes after decapitation following CO_2 euthanasia. Each sample was frozen in liquid nitrogen and stored until use. Each sample was weighed, homogenized in 2 ml of 0.1 M perchloric acid using a Polytron® homogenizer, and then centrifuged at 13,000 *g* for 15 min. The supernatant was collected, placed in a new tube, and supplemented with the internal standard (at a known concentration), 50 mg of alumina, 100 μl of 5 mM sodium metabisulfite, and 800 μl of 10 mM Tris/1 mM EDTA solution, pH 8. After vortexing and agitating for 10 min, the mixture was centrifuged at 11,000 *g* for 5 min. The 20 μl of the supernatant (equivalent to approximately 5.4 mg of tissue) were analyzed via HPLC/MS.

Blood was extracted from the cranial cava vein according to the procedure described by Picazo et al. [43] under anesthesia (induction: 4% isoflurane and 1 l/min O_2, maintenance: 3% isoflurane and 0.4 l/min O_2). At each time point, 100 μl of blood was extracted, and the animal was rehydrated with 150 μl of 0.9% saline. We performed serum extraction as described by Barrera-Bailón et al. [18]. After air drying, the samples were suspended in 20 μl of 20% methanol in dH_2O containing 10 μg/ml of the IS.

2.4.2. Drug concentrations in blood and the brain

The drug concentrations in blood and the brain were determined via HPLC/MS at the Mass Spectrophotometer General Service Lab of USAL. The equipment used for the analysis of the samples was an Agilent 1100 HPLC (Agilent Technologies, Santa Clara CA, USA) for the HPLC assay and an Agilent trap XCT mass spectrometer (Agilent Technologies, Santa Clara CA, USA) for the MS assay. The mobile phase was delivered at a flow rate of 0.2 ml/min to the X-Bridge C-18 column to elute LTG using an isocratic method consisting of 75% of A and 25% of B for 15 min. For MS, pseudomolecular ion transition was monitored from 304 to fragmentation at 258, and for LTG and IS from 305 to 196 [44,45].

2.4.3. Determination of the time and concentration of the maximal drug level in blood

To determine the timing of the maximal levels of the drug, we measured the drug levels in blood after the intraperitoneal administration of 20 mg/kg LTG. After drug injection, 150 μl of blood were extracted from five animals at different time points (15, 30, 45, 60, 90, 180, 240, 360, 480, 600, and 720 min).

2.5. Treatments

2.5.1. Anticonvulsant drug effect: acute treatment

The doses for acute treatment with LTG (5, 10, 15, and 20 mg/kg) were chosen based on their efficacy in suppressing seizures in other experimental models of epilepsy [46–48]. Six animals received one of the specified doses of LTG 60 min before sound stimulus exposure. In the first week, the animals received vehicle (saline 0.9%, 0.001 ml/g body weight), and in the following four weeks, the drug was administered once per week. One week after the last dose, vehicle was administered, followed by the application of a final stimulus. A second group of animals was included as a control group, which received saline instead of the drug preceding all five stimuli. The median effective dose (ED50) for the treatment was calculated according to the method of Litchfield and Wilcoxon [49] using PASW Statistics 18 (IBM).

2.5.2. Anticonvulsant drug effect: chronic treatment

Lamotrigine was administered to five animals twice a day for 30 days. The starting dose was determined from previous assays (not shown) and was based on the parenteral ED50 and the oral bioavailability of LTG in other species. The dose was increased according to the response of the animals to the drug according to its efficiency to abolish seizure activity and its toxic effects. The doses of LTG used were 20 mg/kg/day for the first and second weeks and 25 mg/kg/day for the last two weeks. The AED was administered into the mouth with a pipette according to the weight of each animal, which was determined every three days. The drugs were dissolved in honey diluted in distilled water to a convenient stock concentration. Prior to starting the treatment, all animals were stimulated once to observe seizure activity without any influence of the AED. Then, once per week, a stimulus was applied approximately two hours after the morning drug administration to monitor the effect of the drug. A second control group of animals was included in order to account for any kindling effects. Animals were manipulated and stimulated according to the protocol used with the LTG group; dissolved honey was administered instead of AED.

Table 1
Severity index (SI) [6] using the behavioral descriptions and categorized severity index (cSI) transformed into discreet variables for statistical purposes [50].

SI	Seizure behaviors	cSI
0.00	No seizures	0
0.11	One wild running	1
0.23	One wild running (running plus jumping plus atonic fall)	2
0.38	Two wild runnings	3
0.61	Tonic convulsion (opisthotonus)	4
0.85	Tonic seizures plus generalized clonic convulsions	5
0.90	Head ventral flexion plus cSI5	6
0.95	Forelimb extension plus cSI6[a]	7
1.00	Hindlimb extension plus cSI6[a]	8

[a] Categories which are generally followed by hindlimb clonic convulsions (CCV_2).

2.6. Neuroethological study

2.6.1. Stimulus observation

The stimuli consisted of three phases of behavioral observation: 1-minute presound exposure, one minute of sound exposure or until the onset of opisthotonus, and 3-minute postsound exposure in the case of seizure occurrence or 1-minute postsound exposure in the case of no seizure occurrence. All stimuli were video-recorded for analysis (Fig. 1).

2.6.2. Behavioral indexes and neuroethological analysis

Three behavioral evaluations were used. The severity index (cSI) (Table 1) was used to determine the severity of the seizures [5,50,51].

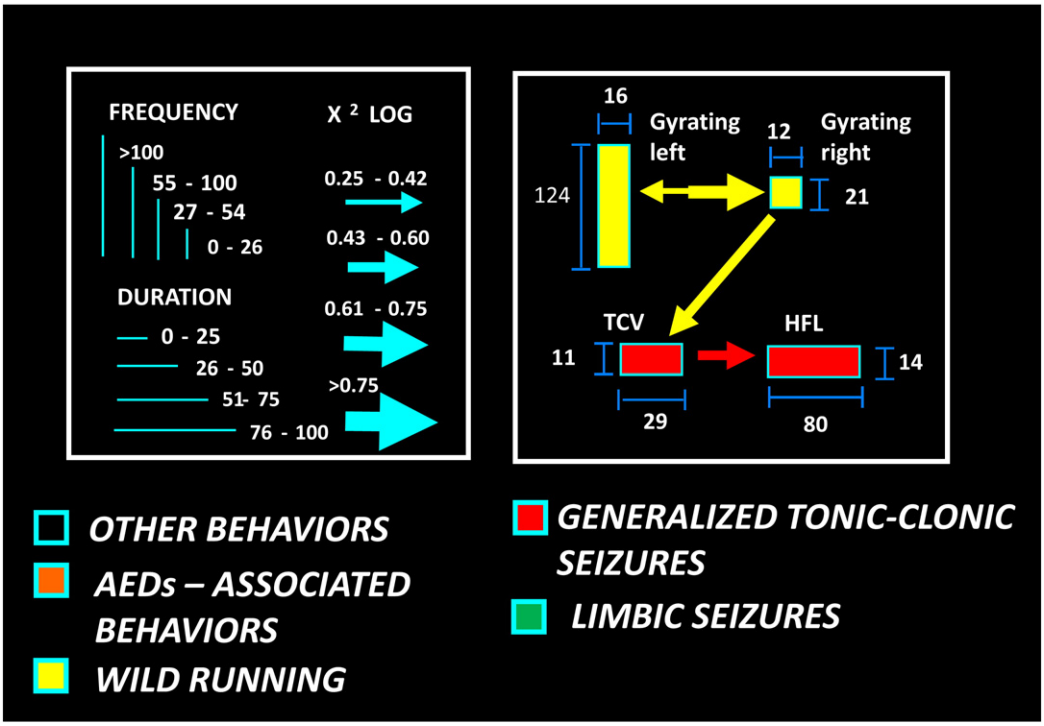

Fig. 1. Flow-charts illustrating the graphical and statistical aspects of the observed behaviors. The frequency and time spent performing each behavior are proportional to the height and width of the rectangle, respectively. The arrow width and direction indicate the statistical intensity and preference association between two items.
Adapted from Garcia-Cairasco et al. [6] and Barrera-Bailon et al. [18].

Table 2
Ataxia index (AI) scores according to Lösher and Hönack [52], taken from Rosetti et al. [50].

Score	Behaviors
1	Slight ataxia in hind leg (tottering of hind quarters), no decrease in abdominal muscle tone
2	More pronounced ataxia with dragging of hind legs and slight decrease of muscle tone
3	Further increase in ataxia and more pronounced dragging of hind legs and decrease in muscle tone
4	Marked ataxia, animals lose balance during forward locomotion, total loss of abdominal muscle tone
5	Very marked ataxia with frequent loss of balance during locomotion, loss of abdominal muscle tone

Table 3
Sedation index (SdI) scores according to Lösher and Hönack [52], taken from Rosetti et al. [50].

Score	Behaviors
1	Slightly reduced forward locomotion
2	Reduced locomotion with rest periods in between (partly with closed eyes)
3	Reduced locomotion with more frequent rest periods
4	No forward locomotion, animal sits quietly with closed eyes

The ataxia (Table 2) and sedation (Table 3) indexes were used to measure the toxic effects of LTG on normal behavior [50,52].

After each animal was scored using the cSI, we divided the animals into two groups for further analysis. Those with a cSI greater than or equal to 2 (SI = 0.23) were considered as animals that experienced a seizure, and those with a cSI less than 2 were considered as animals in which seizure activity was blocked.

The behavioral sequences observed during the different stimuli were assessed using neuroethological methods. Every behavior presented in a given time window was recorded second by second according to a dictionary of behavioral items (Table 4) described by Garcia-Cairasco [6]. Once the data were obtained, the ETHOMATIC [5,6,18] program for statistical analysis was used to analyze the data. This program displays the mean frequency and mean duration of each behavioral item in the given observation window. The program also performs statistical analysis to reveal the significant associations between pairs of behavioral items and to calculate X^2 values. Flowcharts representing all of the statistically significant data were constructed using Microsoft PowerPoint 2011 (see Fig. 1 for flowchart calibration).

Table 4
Behavioral dictionary.

Acronym	Behavior	Acronym	Behavior
AF	Atonic fall	GRH	Grooming of head
ATF[a]	Ataxic fall	GRHL	Grooming of head, left
BE	Blinking eyes	GRL	Grooming of body, left
BRL	Barrel rolling, left	GRN	Grooming of neck
BRR	Barrel rolling, right	GRR	Grooming of body, right
CCV1	Clonic convulsions (forelegs)	HFL	Head ventral flexions
CCV2	Clonic convulsions (hind legs)	HP1	Forelimb extensions
CCVg	Clonic convulsions (generalized)	HP2	Hindlimb extensions
CVL1	Clonic convulsions (forelegs—left)	IM	Immobility
CVL2	Clonic convulsions (hind legs—left)	JP	Jumping
DYS	Dyspnea	LI	Licking
ER	Erect posture	LIC	Licking of claws
EXC	Excretion of feces and urine	MT	Masticatory movements
STR	Straighten	PIM	Postictal immobility
FR	Freezing posture	RU	Running
GL	Gyrating, left	SN	Sniffing
GN	Gnawing	TCP	Tachypnea
GR	Gyrating, right	WA	Walking
GRB	Grooming	WDS	Wet dog shaking
GRF	Grooming of face	NOD	Nodding
GRG	Grooming of genitals	TCV	Tonic–clonic convultions
STA	Startle		

[a] Observation: The behavioral item ataxic fall (ATF) was created to show the presence of the toxic effect of drugs on motor behavior.

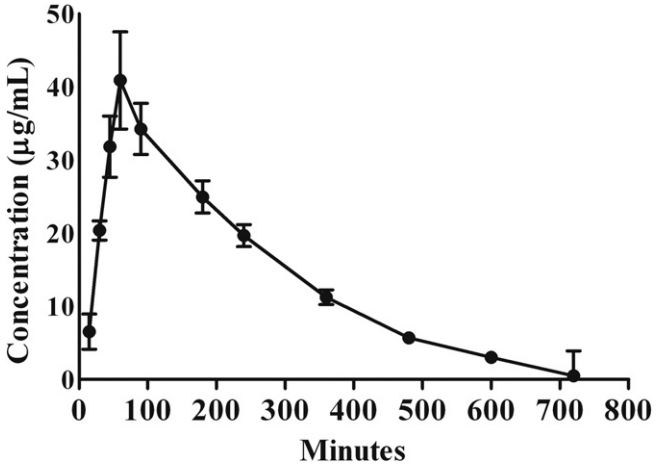

Fig. 2. Serum concentration levels in different extraction times after intraperitoneal injection of 20 mg/kg of LTG. Each point is the mean (+/− S.E.M.) of 5 animals.

2.6.3. Statistical analysis

All statistical analyses were performed using PASW Statistics 18 (IBM) software. For comparisons between different AED doses, we used the nonparametric Friedman test with Conover post hoc comparisons [53]. To estimate the statistical significance of potential differential growth between the control and LTG groups, ANOVA test for repetitive measurements was used.

3. Results

3.1. Pharmacology of LTG in GASH:Sal

3.1.1. Blood and brain LTG levels

The results obtained from the blood LTG concentration analysis (Fig. 2) show that the GASH:Sal displayed the peak serum LTG concentration (41.05 ± 6.65 μg/ml) at 60 min postinjection of a single dose of 20 mg/kg; subsequently, the LTG levels decreased slowly for the following 12 h, resulting in LTG levels of 0.53 ± 1 μg/ml. The brain LTG concentration determined 60 min after intraperitoneal injection was 0.82 ± 0.25 μg/ml. For chronic treatment, the blood levels of LTG (Fig. 3) were maintained between 4 and 5 μg/ml during the first week

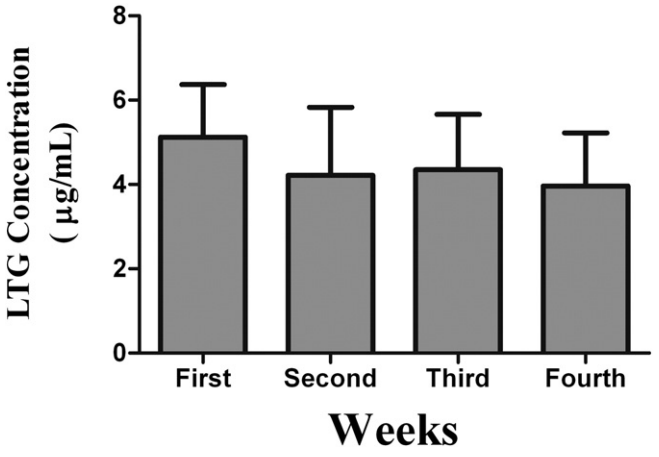

Fig. 3. Serum concentration levels during chronic treatment with LTG. Each point is the mean (+/− S.E.M.) of 6 animals.

of oral administration. These levels gradually decreased in the fourth week (3.96 ± 1.25 μg/ml), after which the dose was increased from 20 to 25 mg/kg/day. Lamotrigine was not detected in the brain after oral administration, possibly because of the lower detection limit of the HPLC technique (0.1 μg/ml).

3.2. Acute LTG treatment

3.2.1. Anticonvulsant effects

Exploratory behaviors in LTG-treated animals during the prestimulus window were decreased in frequency and associations when compared with the saline-treated animals. A clear disappearance of the cluster of grooming behaviors was noticed in the LTG treatment group. Lamotrigine was evaluated for its anticonvulsant efficacy based on its ED50, the dose that completely blocks seizures in 50% of animals stimulated after intraperitoneal injection of 5, 10, 15, or 20 μg/ml LTG. The lowest dose of LTG that produced 50% seizure blockade was 5 mg/kg, demonstrating the anticonvulsant properties of LTG at the lowest dose tested (Figs. 4 and 5). Furthermore, those animals that experienced seizures exhibited a reduction in the maximal seizure severity score (cSI) from 8 to 6 (Fig. 5). Although there were still wild running behaviors (yellow cluster) and back-arching tonus (opisthotonus), there was a clear lack of forelimb (HP_1) and hindlimb (HP_2) hyperextensions and an increase in the duration of postictal immobility (PIM, blue open symbols).

No seizures were observed in the animals receiving an LTG dose of 10 mg/kg or greater. In the three observation windows, only exploratory behavior was observed. Furthermore, as the LTG dose increased, the sniffing component of the exploratory cluster congruently increased in duration, and grooming behavior reappeared (GRF and LIC) at 20 mg/kg dose. Immobility was prolonged at the two highest doses. The interaction between behaviors also increased. At the two highest doses, especially at 15 mg/kg and 20 mg/kg, drug toxicity was

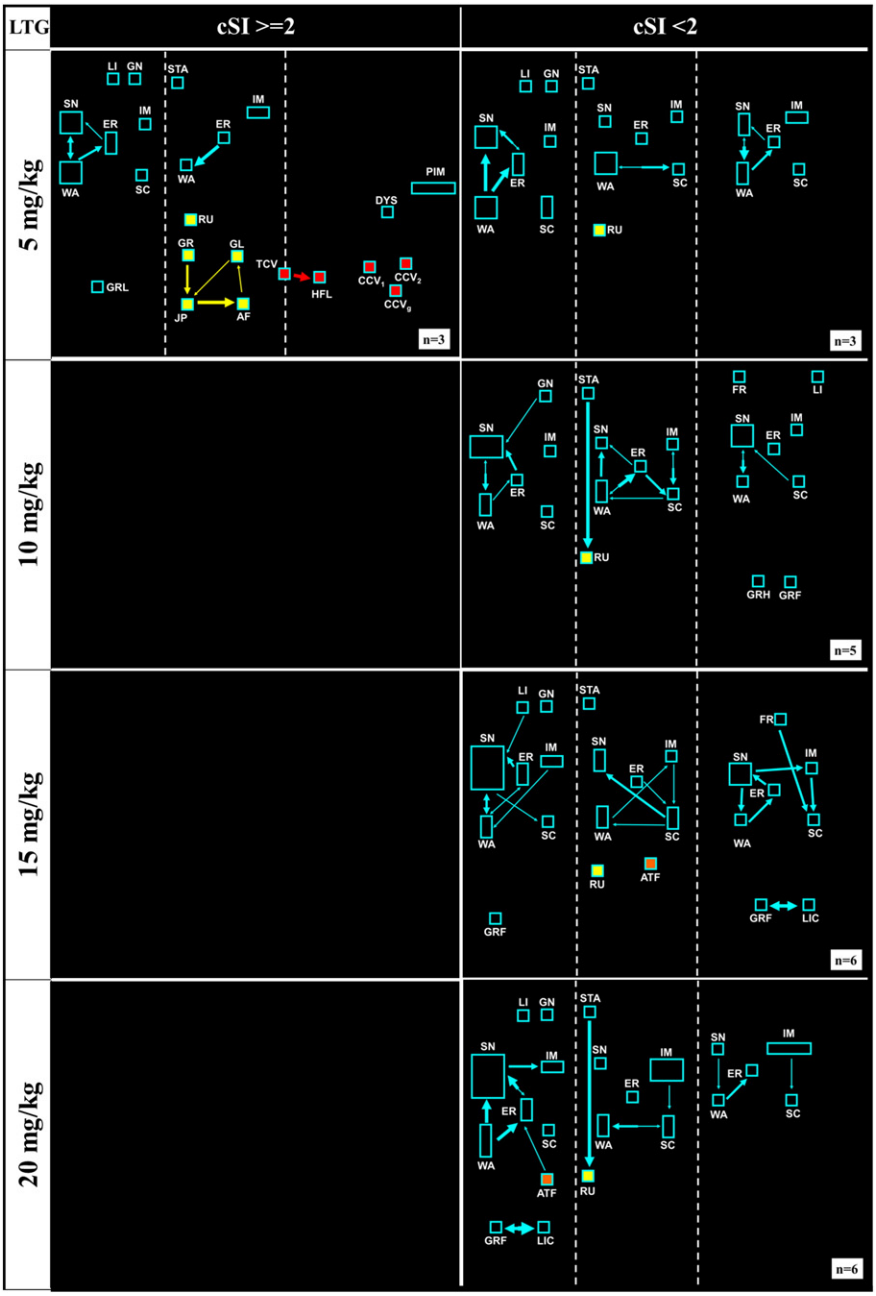

Fig. 4. Flowchart of the sequences of behaviors of the GASH:Sal injected with different doses of LTG.

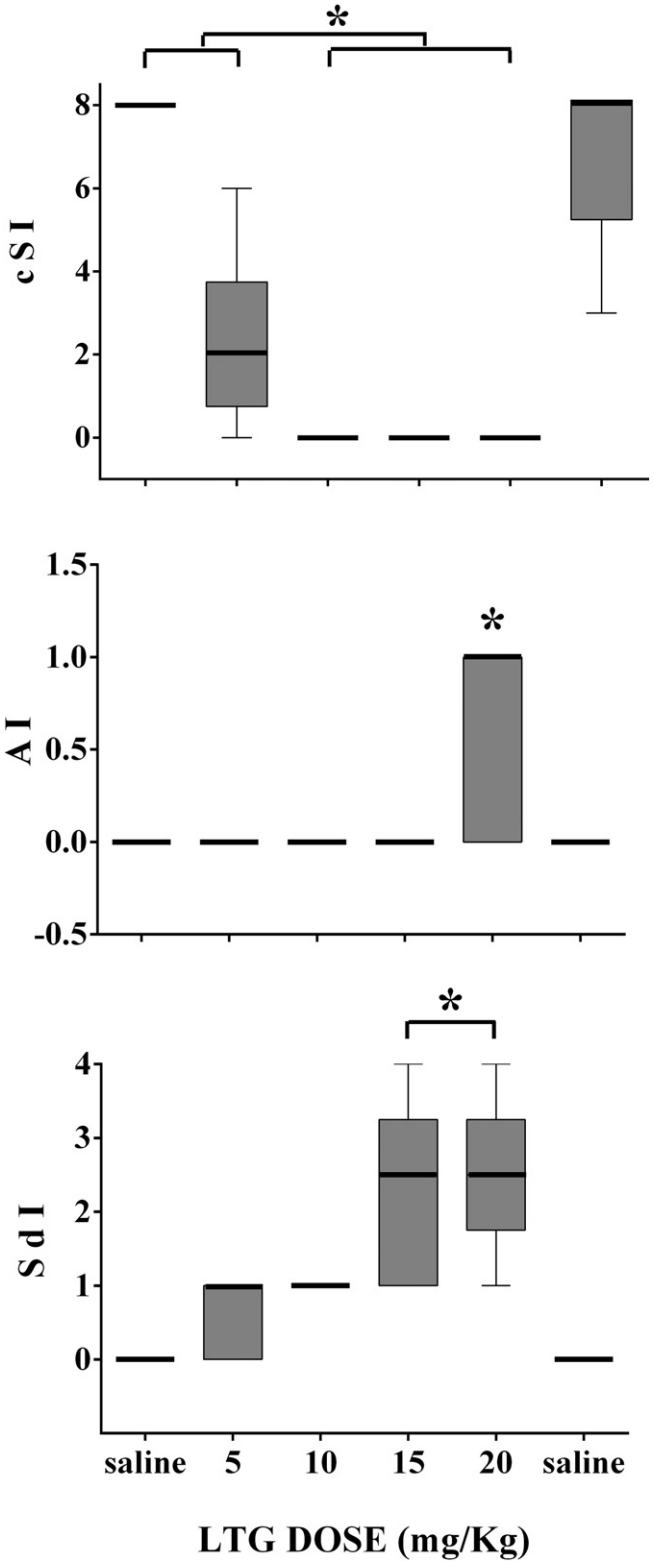

Fig. 5. Box plots showing the categorized severity index (cSI), the ataxic index (AI), and the sedation index (SdI) on the different doses of LTG in acute treatment. Nonparametric Friedman test with Conover post hoc comparisons was used to calculate significant differences between the indexes on the different acute doses.

increased, as reflected by the appearance of ataxic falls (ATF, yellow symbol) and by increased ataxic (Fig. 5, middle panel) and sedation (Fig. 5, bottom panel) index scores. No differences in seizure behaviors were observed between the preacute and postacute saline treatment groups.

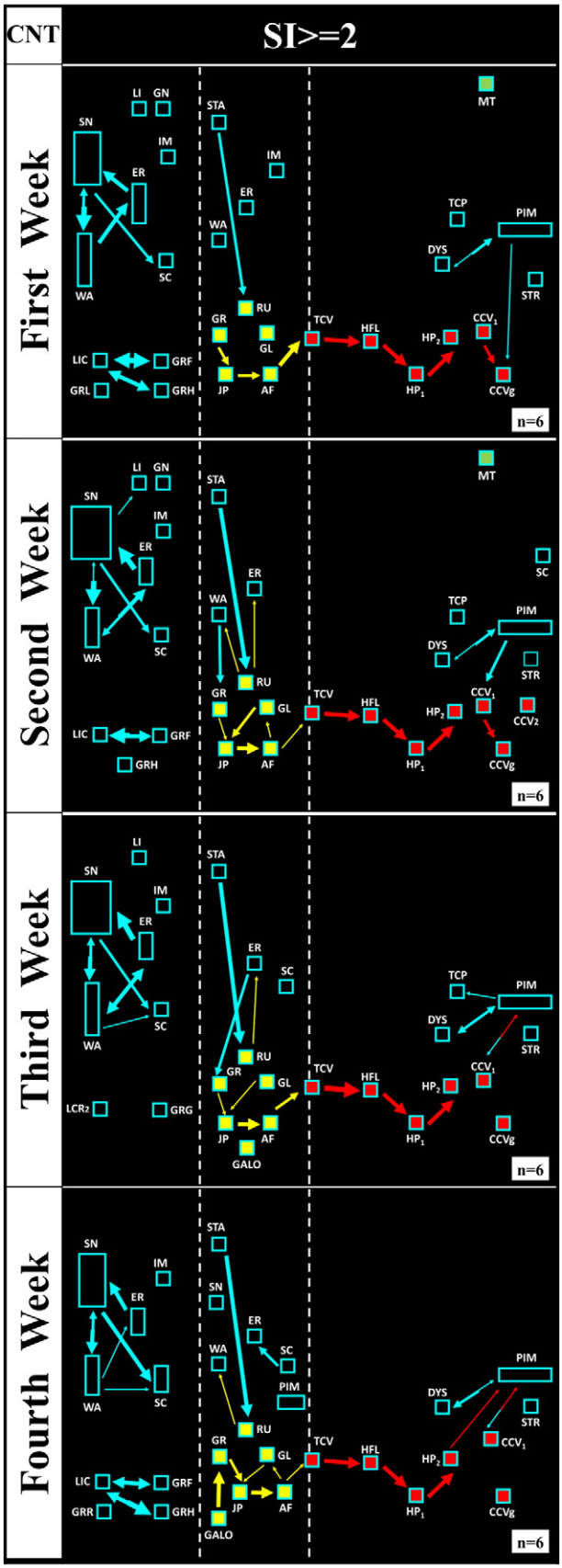

Fig. 6. Flowchart of the behavioral sequences of the GASH:Sal control animals (n = 6) during the chronic treatment. All animals had observed seizures (cSI = >2) during the length of the experiment. A. first week, B. second week, C. third week, and D. fourth week.

3.3. Chronic LTG treatment

Chronic LTG administration induced an anticonvulsant effect. From the first week of treatment, LTG showed an anticonvulsant effect in 50% of the animals. The animals affected by seizures (cSI > 2) displayed all phase components of the seizures (Fig. 7), which means the presence of wild running behaviors (yellow symbols), followed by tonic–clonic seizures (red symbols) and postictal immobility (PIM, yellow open symbol). However, in successive weeks, the interactions among tonic–clonic convulsive behaviors became weaker, whereas the associations among wild running behaviors (yellow cluster) increased. The associations among behaviors in the prestimulus window were affected by LTG treatment. Specifically, the sniffing item of the exploratory cluster (open blue symbols) increased in duration, and in the first two weeks, grooming behaviors were more obvious and increased in frequency.

In the third week, with an increase in the LTG doses from 20 to 25 mg/kg, fewer animals (33%) were affected by seizures, and a maximum cSI value of 3 (Fig. 8) was recorded. In the final week of LTG administration, seizures were abolished in 83% of the animals. From the first to the third week, the animals lacking seizures displayed mainly exploratory behaviors; in the prestimulus observation window, sniffing and walking were the most affected behaviors. In the stimulus observation window, the only seizure-related behavior was running, and the remaining time of the observation window consisted of exploratory

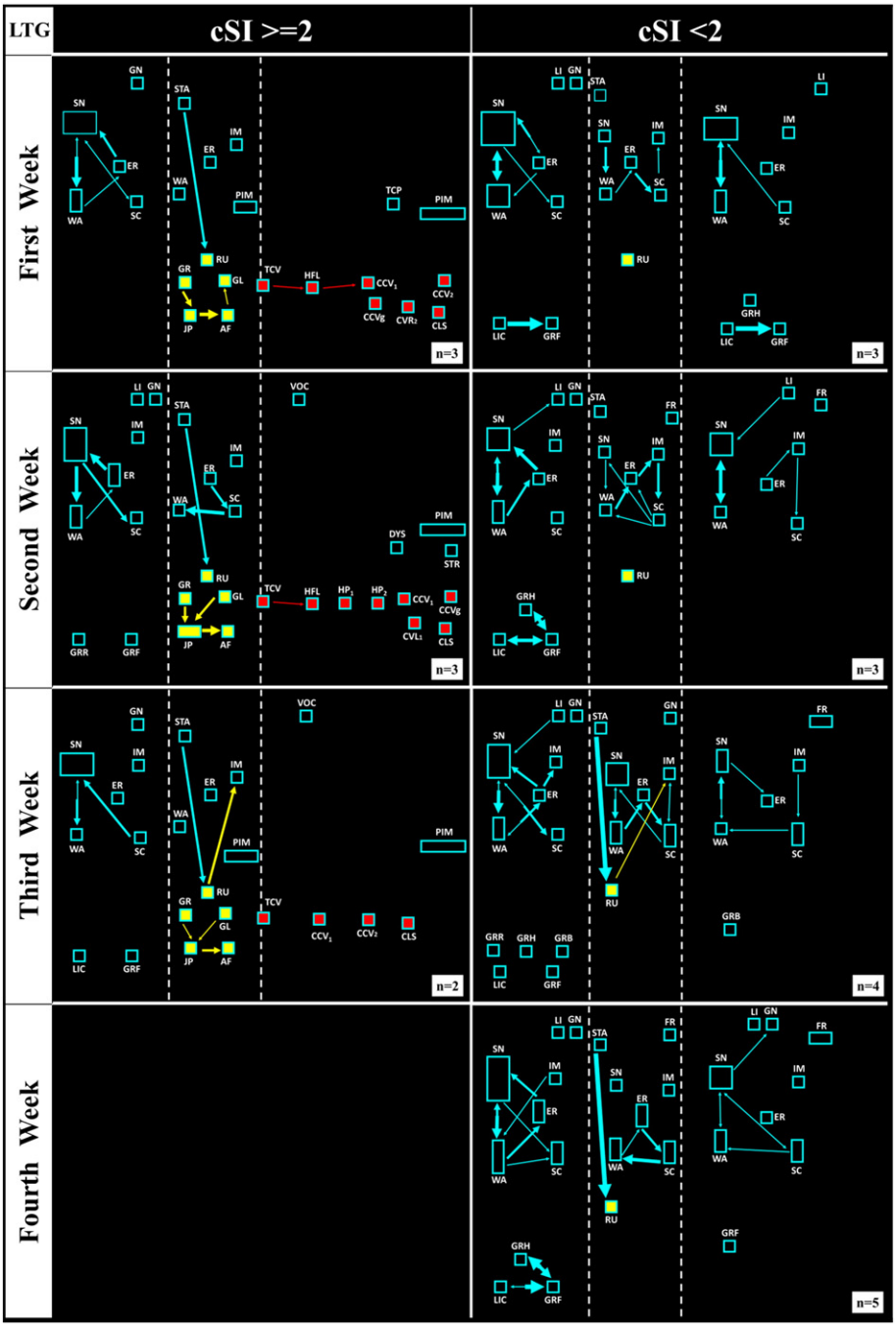

Fig. 7. Flowchart of the behavioral sequences of the GASH:Sal treated with LTG. 20 mg/kg/day for the 1st and 2nd weeks and 25 mg/kg/day for the 3rd and 4th weeks; (n = 6).

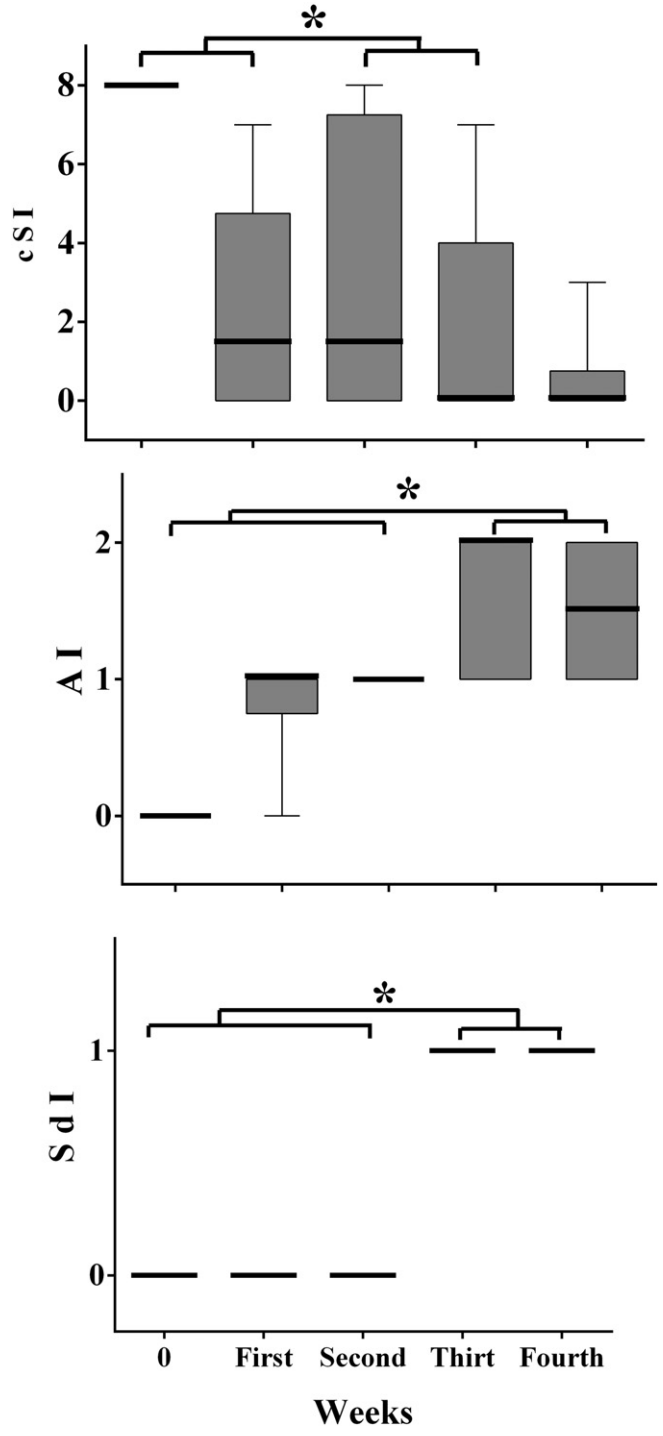

Fig. 8. Box plots showing the categorized severity index (cSI), the ataxic index (AI), and the sedation index (SdI) in chronic treatment. Nonparametric Friedman test with Conover post hoc comparisons was used to calculate significant differences between the indexes found during the consecutive weeks of treatment.

behavior. The correlation coefficient between behaviors was highest at the third week. Additionally, a freezing posture (FR, blue symbol) appeared during the stimulus window and extended into the postsound stimulus period. In the third and fourth weeks, the ataxia index value increased to two, and sedation behavior appeared at a low level (Fig. 8). In contrast, the prestimulus behavior of the treatment group resembled that of the control group (Fig. 6) in terms of frequency, duration, and the interactions among the behavioral items.

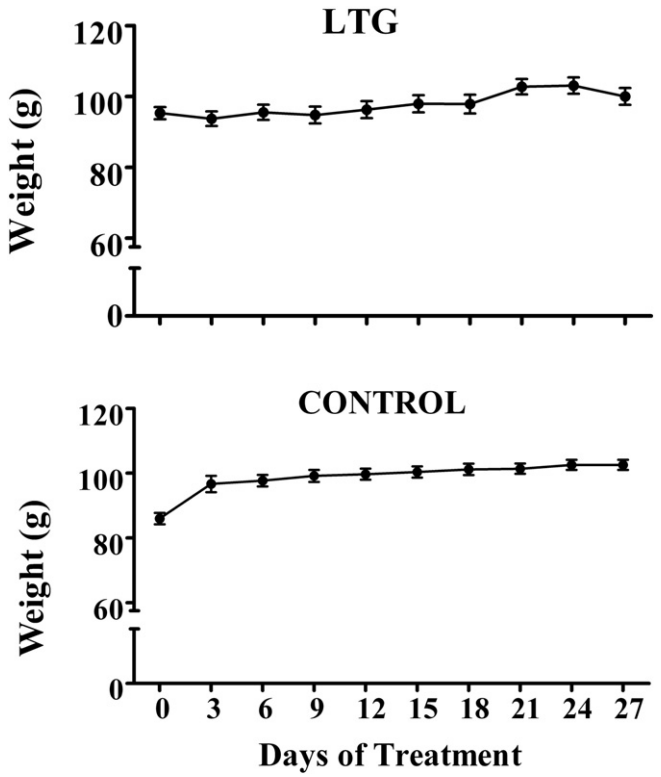

Fig. 9. Body weight mean values during chronic treatment with LTG and control group.

3.3.1. Effect on body weight

In regard to body weight (Fig. 9), chronic administration of LTG did not affect the natural growth of the animals compared with the control treatment. The average growth was 0.73 g/week for the LTG treatment group and 0.83 g/week for the control group, with no statistical significant difference (ANOVA test $p < 0.05$, d.f. $= 1$).

4. Discussion

Following our publication [18] with the evaluation of phenobarbital, valproic acid and levetiracetam in GASH:Sal, in the current study, we continued to validate the GASH:Sal as a model of epilepsy using LTG. Our results show that either acute or chronic LTG treatment exerts a strong anticonvulsant effect, displaying different characteristics depending on the route of administration. Following acute treatment (i.p. injection), 10–20 mg/kg LTG exerted an anticonvulsant effect in all animals tested. Alternatively, following chronic treatment (p.o. route), 25 mg/kg/day LTG exerted an anticonvulsant effect in 100% of the tested animals during the four weeks of treatment. For acute treatment, the exploratory items were most strongly affected. Specifically, a decrease in the duration and frequency of forward locomotion was observed. This behavior was replaced by more frequent sniffing and several periods of freezing. The latter increased proportionally to the LTG dose, and at 20 mg/kg LTG, freezing was the principal behavior associated with the sedation index. For chronic treatment, freezing also appeared, mainly in the sound and postsound stimulus observation windows, although the main relevant feature that we detected was the appearance of an increase in grooming behaviors over the weeks of LTG treatment.

The GASH:Sal strain has been suggested as a useful model for studies of AEDs using both acute and chronic administration protocols. Chronic studies of AEDs in experimental epilepsy models are needed because they help to characterize the anticonvulsant potential and spectrum of activity of the AEDs in the brain of subjects with a genetic predisposition

to epilepsy (e.g., genetic models), since the efficacy and toxicity of the drugs in genetic models may differ from those established in nongenetic models [2]. Chronic studies are scarce because they involve time-consuming experiments, require extensive researcher commitment, and present specific technical difficulties [28,54]. One of the biggest technical limitations is the priming of the animals to generate seizures, and more specifically, to generate seizures of a specific severity or intensity by either producing the lesion (mechanical or chemical) or selecting animals with a genetic predisposition for the studied pathology. The GASH:Sal has repeatedly been shown [15,16,18] to display reliable generalized tonic–clonic seizures of genetic origin, reaching maximal seizure severity at two months of age. Furthermore, seizure onset is simply triggered by a controlled acoustic stimulus and requires no kindling or other induction of seizure susceptibility. All of these features make this experimental model an excellent candidate for AED screening. Additional experiments with audiogenic kindling in GASH:Sal animals are in progress, in order to compare with data from other chronic protocols obtained with audiogenic seizure models.

In rodents, LTG is nearly completely absorbed. Its bioavailability was reported to be >90% [55,56], and the time required to reach its peak concentration in plasma following oral administration is 0.5–3 h [28,56]. The peak plasma concentrations of LTG in the GASH:Sal were observed approximately 60 min after parenteral drug administration, and LTG had an elimination half-life of 6 h, which is shorter than that recorded in guinea pigs (approximately 11.5 h) [57], albino rats (12 h) [58], and Sprague–Dawley rats (33.5 h) [28] but is comparable to the 120–240 min observed in CD-1 mice by Arban et al. [59]. According to Patsalos et al. [60], in patients with no comedication interaction, the half-life of LTG ranges from 15 to 35 h. We should take into account the possibility of more rapid elimination due to the low body weight of small animals and to various physiological and metabolic parameters, such as differences between the levels of metabolizing enzymes (CYPs) across species [61].

For acute treatment, the LTG doses used were in the range reported in the literature for rats and mice, 2–20 mg/kg [46,59,62], and in our model, the anticonvulsant effect of LTG was observed at 5 mg/kg (the lowest dose used) and at 10 mg/kg LTG, and 83% of the animals were seizure-free. High anticonvulsant potency of LTG may be partially due to its joint action on sodium and calcium voltage-activated channels; this action of LTG is shared with phenobarbital and valproic acid. A unique characteristic of LTG is its interaction with HCN ion channels. These channels are activated upon the hyperpolarization-driven cellular entry of sodium. Lamotrigine selectively reduces action potential firing from dendritic depolarization by activating HCN channels [42].

For chronic treatment, the blood levels of LTG in the first week were approximately 5 μg/ml, and with each subsequent week, the blood levels of LTG gradually decreased despite increasing the dose. Notwithstanding, from the second to the fourth week, the blood LTG concentration (approximately 4 μg/ml) was maintained in the therapeutic range, which should be 2.5–15 μg/ml according to Patsalos et al. [60]. Brant and Löscher [28] found that the effective chronic treatment dose for phenobarbital-resistant rats was 10 mg/kg i.p., resulting in higher blood LTG concentrations during treatment (between 13 and 17 μg/ml). During the chronic LTG treatment study, we used the oral route to administer the drug to minimize any interference when handling the animals, which could provoke seizures. Lamotrigine is documented to be well absorbed after oral administration, although the rate and the extent of its absorption may be dose-dependent [63]. Interestingly, the administration route plays a significant role in dose efficacy. The i.p. route produces an anticonvulsant action at low doses [57], whereas the oral route takes longer and requires higher doses to achieve the same action. This finding is in disagreement with the 98% absorption bioavailability observed in humans for oral LTG administration.

One common hurdle facing chronic studies with AEDs is that rodents (mice, rats, or hamsters) eliminate these drugs much more rapidly than humans [64]. The elimination rate may affect the response of GASH:Sal to chronic treatment. In contrast to chronic treatment, acute treatment with LTG evoked an anticonvulsant effect in 100% of the cases at doses between 10 and 20 mg/kg. This response was observed as soon as 60 min after i.p. administration, the time at which LTG reached a sufficient blood concentration to elicit a response (at 20 mg/kg, the blood LTG concentration was between 35 and 45 μg/ml). Note that the acoustic stimulus is presented far before the 3-hour half-life recorded for the highest dose. As such, it may be possible that the plasma levels of LTG are good indicators of the concentration of the drug at its sites of action. In summary, for chronic treatment, drug absorption for the oral route and the drug elimination rate affect the concentration of the drug in blood. The sound stimuli and the blood extraction were performed approximately two hours after LTG administration, and as analyzed, the blood LTG concentration was 4- to 5-fold less for p.o. administration than for i.p. administration. As the weeks passed, more animals exhibited an anticonvulsant response while maintaining the same blood LTG levels. These results suggest not only an immediate anticonvulsant effect, but also a progressive neuroprotective effect due to the continuous presence of LTG in the body. In another study [65], LTG displayed no effect on absence seizures in Long–Evans rats during the early phases of oral treatment, although after 2 weeks of treatment, LTG administration gradually reduced seizure manifestation, and this phenomenon became even more significant after 1 month. These phenomena are analogous to the actions of LTG in human patients for a period of 4–12 weeks [66–68]. Therefore, in the GASH:Sal, the oral route and chronic drug administration mimic the human use of LTG.

In our study, during acute treatment, no ataxia was generated, although sedation was clearly observed beginning at 15 mg/kg LTG. With chronic treatment, toxicity was observed to affect only forward locomotion, albeit in an insignificant manner. This low toxicity has been observed in other studies using rats [46,59,62], and this characteristic differentiates LTG from other AEDs. A common side effect of LTG is exanthema (in approximately 10% of patients) [29,69]. No rash was perceived in any of the animals during treatment, but an increase in grooming was noted throughout the treatment period and was more intense in those animals exhibiting an anticonvulsant response (Fig. 7). Nevertheless, further studies should be performed to determine whether LTG causes any skin alterations. Additionally, no effect on body weight was observed, implying that the administration of LTG did not affect food or water intake.

It is important to study the differences between acute and chronic treatment with AEDs in a genetic model of epilepsy. This study may help to explain that one of the discrepancies between animal models of epilepsy and human patients with epilepsy may be due to the different routes of drug administration (i.p. versus oral) and differences in the duration of treatment (acute versus chronic).

5. Conclusions

In our effort to validate the GASH:Sal as an animal model of epilepsy, we found that LTG exerts an anticonvulsant effect following both acute and chronic treatment, respectively, by abolishing generalized tonic–clonic seizures. Moreover, LTG shows the highest anticonvulsant potency upon acute treatment compared with other AEDs studied in the GASH:Sal; these results may be due to the wide range of action of LTG in different receptors, predominantly through sodium and calcium voltage-activated channels. Differences in the administration routes were also observed between acute and chronic treatments. Oral administration resulted in reduced relative blood LTG concentrations, even though continuous administration of LTG generated an anticonvulsant response with the potential for a still untested (at least in the GASH:Sal model) neuroprotective effect. Sedation appears at the highest acute doses of LTG, but no significant secondary effects were observed after chronic treatment.

Acknowledgments

This study was supported by the Spanish JCyL (#SA023A12-2), the University of Salamanca Research grant to Dr. Dolores E. López (2015) and the University of São Paulo (USP)/University of Salamanca (USAL) Program for the Promotion of the Bilateral Cooperation in the Field of Research (#2011.1.23386.1.3). We thank GlaxoSmithKline Research & Development Limited for providing lamotrigine.

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest, or expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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