Original Paper

Ca_v2.3 (R-Type) Calcium Channels are Critical for Mediating Anticonvulsive and Neuroprotective Properties of Lamotrigine *In Vivo*

Maxine Dibué-Adjei^{a,b,c} Marcel A. Kamp^{a,c} Serdar Alpdogan^a
Etienne E. Tevoufouet^a Wolfram F. Neiss^d Jürgen Hescheler^a Toni Schneider^a

^aInstitute for Neurophysiology, ^bCenter for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, ^cDepartment of Neurosurgery, University Hospital, Heinrich-Heine-University, Düsseldorf, Düsseldorf, ^dInstitute for Anatomy I, University of Cologne, Cologne Germany

Key Words

Lamotrigine • R-type • Ca_v2.3 • Antiepileptic drugs • Toxicity • Quantitative electroencephalography

Abstract

Background/Aims: Lamotrigine (LTG) is a popular modern antiepileptic drug (AED), however, its mechanism of action has yet to be fully understood, as it is known to modulate many members of several ion channel families. In heterologous systems, LTG inhibits Ca_v2.3 (R-type) calcium currents, which contribute to kainic-acid- (KA) induced epilepsy *in vivo*. To gain insight into the role of R-type currents in LTG drug action *in vivo*, we compared the effects of LTG to topiramate and lacosamide in Ca_v2.3-deficient mice and controls on KA-induced seizures.

Methods: Behavioral seizure rating and quantitative electrocorticography were performed after injection of 20 mg/kg [and 30 mg/kg] KA. One hour before KA injection, mice were pretreated with either 30 mg/kg LTG, 50 mg/kg topiramate (TPM) or 30 mg/kg lacosamide (LSM). **Results:** Ablation of Ca_v2.3 reduced total seizure scores by 28.6% (p=0.0012) and pretreatment with LTG reduced seizure activity of control mice by 23.2% (p=0.02). In Ca_v2.3-deficient mice LTG pretreatment increased seizure activity by 22.1% (p=0.018) and increased the percentage of degenerated CA1 pyramidal neurons (p=0.02). All three tested AEDs reduced seizure activity in control mice, however only the non-calcium channel modulating AED, LSM had an anticonvulsive effect in Ca_v2.3-deficient mice. Furthermore LTG altered electrocorticographic parameters differently in the two genotypes, decreasing relative power of ictal spikes in control mice compared to Ca_v2.3-deficient mice. **Conclusion:** These findings give first *in vivo* evidence for an essential role for Ca_v2.3 in LTG pharmacology and shed light on a paradoxical effect of LTG in their absence. Furthermore, LTG appears to promote ictal activity in Ca_v2.3-deficient mice resulting in increased neurotoxicity in the CA1 region. This paradoxical mechanism, possibly reflecting rebound hyperexcitation of pyramidal CA1 neurons after increased inhibition, may be key in understanding LTG-induced seizure aggravation, observed in clinical practice.

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Introduction

Today lamotrigine (LTG) is among the most prescribed antiepileptic drugs worldwide. LTG is also FDA approved for treatment of bipolar disorder and has additionally become a popular off-label drug for treatment of other neurologic and psychiatric pathologies like borderline personality disorder. This diverse therapeutic capacity of LTG probably reflects the unspecificity of the drug, which is known to inhibit several different calcium, potassium and sodium currents [1]. LTG is thought to mediate its anticonvulsant and neuroprotective effects *in vivo* predominantly by inhibiting voltage-dependent sodium currents and the subsequent glutamate release, however recent evidence suggests that, in mice inhibition of $\text{Ca}_v2.3$ channels could play an important role in the mechanism of action of LTG during experimentally induced epilepsy. It has been demonstrated that LTG as well as another modern antiepileptic drug topiramate (TPM) inhibit R-type currents in heterologous systems and brain slices [2, 3]. Furthermore, $\text{Ca}_v2.3$ -deficient ($\text{Ca}_v2.3\text{-KO}$) mice display seizure resistance and reduced hippocampal neurotoxicity after kainic acid (KA) injection [4]. Parenteral administration of kainic acid is a well-established method of modeling temporal lobe epilepsy, causing seizures of the hippocampus and temporal lobe and degeneration of hippocampal pyramidal neurons [5, 6]. Using the kainic acid model of temporal lobe epilepsy, we investigated the effect of LTG next to TPM and lacosamide (LSM) in $\text{Ca}_v2.3\text{-KO}$ and control. The new AED lacosamide (LSM), which enhances slow inactivation of voltage-gated sodium channels [7], was used as a positive control, as it has been shown not to effect calcium or potassium currents [8, 9]. Gaining insight into the role of $\text{Ca}_v2.3$ calcium channels in antiepileptic pharmacotherapy may allow identification of new antiepileptic mechanisms and therefore of novel potential drug targets, offering hope for drug refractory epilepsy.

Materials and Methods

Animals

$\text{Ca}_v2.3\text{-KO}$ (= $\text{Cav}2.3\text{-[-/-]}$) and $\text{Ca}_v2.3$ -competent mice (= $\text{Ca}_v2.3\text{[+/+]}$ -mice, called here control mice), represent separate mouse lines derived from heterozygous parents with a mixed C57Bl/6x129SvJ background (fourth backcrossing into C57Bl/6). Homozygous littermates are regularly interbred with each other and back-bred into C57Bl/6 (for further information on knock out generation see [10, 11]. Mice were kept at 20°C in makrolon type II cages under a 12h light-dark cycle (7:00 a.m./p.m.) with food and water *ad libitum*. All animal experiments were in line with the European Communities Council Directive for the care and use of laboratory animals and were approved by the local institutional committee on animal care.

Antiepileptic Pretreatment and Seizure Induction

Between 9:00 and 10:30 in the morning saline or antiepileptic drugs LTG (30 mg/kg), TPM (50 mg/kg) (both Sigma Aldrich, Crailsheim Germany) or LSM (30 mg/kg) (UCB Pharma SA, Brussels, Belgium) dissolved in saline were injected intra-peritoneally into male mice of both genotypes ($\text{Ca}_v2.3\text{-KO}$ and $\text{Ca}_v2.3\text{[+/+]}$) within the age range of 20 to 25 weeks. Effective concentrations of the antiepileptic drugs were taken from the literature. One hour later 20 mg/kg KA (n= 40) or 30 mg/kg KA (n=46) (Sigma Aldrich, Crailsheim Germany) dissolved in saline was injected intra-peritoneally. Immediately after administering KA, mice were set in separate single cages and filmed for the next 24 hours.

Behavioral Seizure Analysis

The initial 2 hours after the application of KA were evaluated using an adapted version of Morrison's Seizure Rating Scale [12]:

Stage 0: normal behavior

Stage 1: immobility

Stage 2: facial clonus, head bobbing/nodding, automatisms

Stage 3: limb clonus, jerking of the torso

Stage 4: rearing

Stage 5: falling

Stage 6: tonic clonic seizure

Stage 7: tonic clonic seizure with jumping

Stage 8: tonic clonic seizure causing death

For a two hour period the highest seizure score was noted for every five-minute interval. Interval scores were added for total seizure scores. Total seizure scores of pretreated groups were compared to those the untreated group of the same genotype and differences expressed as relative changes in total seizure score from the untreated group.

Radio Telemetric Electrographic Recording of Seizures

Radio telemetric electrocorticograms (ECoG) of KA induced seizures were recorded on LTG pretreated and untreated animals of both genotypes (n=4 per group). Animals were anesthetized with 100 mg/kg BW ketaminhydrochloride (Ketanest, Parke-Davis/Pfizer, Germany) and 10 mg/kg BW xylazinehydrochloride (RompunR2% BayerVital, Leverkusen, Germany). TL11M2-F20-EET transmitters (Datascience International, Lexington, USA) were implanted subcutaneously and burr holes drilled over the somatosensory cortex (-1 mm and 3 mm lateral from bregma) and cerebellum (-6.3 mm and 1 mm lateral from bregma), leaving the dura intact. Electrodes were inserted and fixed into position with glass ionomer cement (Kent DentalR, Kent Express, UK). Animals were allowed seven days to recover from surgery (all made full recovery) and were then recorded before (control condition) and after injection of 20 mg/kg KA i.p. ECoGs were obtained at a sampling rate of 500 Hz without cut-off from freely moving animals in their cages, which were placed on the telemetry receiver platforms.

ECoG Analysis

Neuroscore 2.1.0 (Datascience International, Lexington, USA) was used to calculate absolute and relative power of frequency bands (Fast Fourier Transform based using a Hamming window) in the first hour after KA injection (totally and fractioned into 5 min intervals). The frequency spectrum was defined as follows: Delta (0.5-4 Hz), Theta (4-8 Hz), Alpha (8-12 Hz), Sigma (12-16 Hz), Beta (16-24Hz) and Gamma (30-80 Hz). Ripples (80-200 Hz) and Fast Ripples (200-250 Hz) could not exactly be determined because of the limitation of the bandwidth of the transmitters in use (for discussion see: [13, 14]). An automated seizure detection protocol was written to quantify ictal activity. The protocol recognizes waveforms shorter than 200 ms in length that are between 2.5- and 25-fold the baseline amplitude as spikes. Spikes occurring in intervals between 30 and 1500 are recognized as belonging to a spike train which must be at least 300 ms long and contain a minimum of four spikes. No ictal events were detected in the control condition (before KA injection). The Z-Ratio reflecting the ratio between low and high frequency power (LF (0.5-8 Hz) and HF (8-20 Hz) respectively) was calculated using following equation: $(LF-HF)/(LF+HF)$.

Histology and Immunohistochemistry

Seven days after injection of 30 mg/kg KA brains from both genotypes were extracted and kept in 30% sucrose for 24 hours prior to freezing them in methyl-butane. Brains were sliced 10µm thick in a cryotome (CM3050S, Leica Microsystems, Wetzlar Germany), then fixed in 4% formaldehyde and Nissl stained according to standard protocol. Brains for immunohistochemistry were kept in 4% paraformaldehyde for 24 hours and – using a slow regime of manual changes over 12 days – embedded in paraffin. Ten micron sections were cut with slim Feather blades for low compression (cutting angle 25 degrees) on a motor-driven rotary microtome (Reichert-Jung 1140 Autocut, Leica Microsystems, Nussloch, Germany) and mounted on silanized glass slides. Sections were deparaffinized and rehydrated before incubation with anti-neuron specific nuclear protein (anti- NeuN) antibody from mouse (GeneTex Asia Ltd, Hsinchu City, Taiwan) and detection thereof using VECTOR® M.O.M Peroxidase Immunodetection Kit (Vector Laboratories Inc, Burlingame CA, USA). Using the cell counter tool of NIH IMAGEJ software (<http://rsbweb.nih.gov/ij/>), hippocampal neurons were counted and the percentage of pyknotic neurons calculated.

Protein Isolation, Western Blot Analysis and Protein Quantification

Twenty-four hours after 30 mg/kg KA (or saline) injection membrane proteins were isolated from control mouse (n = 10) hippocampi using a high-salt high-pH extraction method (for further information see [15]). 50 µg of membrane protein per sample were separated by electrophoresis on an SDS gel and then

blotted onto a PVDF membrane. The $\text{Ca}_v2.3$ calcium channel was detected using a self-generated antibody (rabbit) directed against AA 256-272 in the loop IS5 to pore region of the human $\alpha1E$ subunit (for further information see [16]), ECL™-Anti-Rabbit IgG and ECL™ detection system (GE Healthcare, Buckinghamshire, UK). As the expression of the reference protein synaptophysin (SYN) has been shown to be unaffected by hyperexcitation [17, 18], $\text{Ca}_v2.3$ bands were quantified by normalizing them to SYN, which was detected using anti-SYN antibody from mouse (Antibodies-online, Atlanta GA, USA) and ECL™-Anti-Mouse IgG and (GE Healthcare, Buckinghamshire, UK). $\text{Ca}_v2.3$ protein was quantified manually using ImageJ 1.46 (NIH) and automatically using Gelscan 6.0 (BioSciTec, Frankfurt, Germany).

Statistical Analysis

Normal distribution of seizure scores and relative spectral power was assessed using the Shapiro-Wilk test of normality, and were found to be mostly non-normally distributed. Therefore, the nonparametrical Mann-Whitney test was used to determine significance of seizure scores. Relative power values were log transformed ($\log(x/(1-x))$) to obtain a more Gaussian distribution and were then subjected to ANOVA [19]. Statistical significance of frequencies of the seizure stages was determined using Fisher's exact probability test. P-values of 0.05 and below were considered significant.

Results

Behavioral Seizure Analysis

After injection of 20 mg/kg KA in all groups normal explorative behavior ceased within ten minutes and mice "froze" exhibiting a rigid posture and staring into space (immobility stage i.e. stage 1). In this stage mice only reacted scarcely to their environment (i.e. when nudged) if at all. Six out of eight control mice experienced tonic clonic seizures (Fig. 1A), whereas $\text{Ca}_v2.3$ -KO mice did not develop tonic-clonic seizures or enter seizure stages higher than stage 3 (Fig. 1E), displaying a reduction of total seizure scores of 28.6% compared to control mice (from 57.8 ± 2.6 to 41.4 ± 3.7 , $p = 0.0012$; $U = 2.5$) (Fig. 2A). In control animals LTG prevented tonic-clonic seizures (Fig. 1B) and reduced total seizure scores by 23.2%, from 57.8 ± 2.6 to 44.3 ± 3.6 ($p = 0.02$; $U = 6.5$). TPM did not prevent tonic-clonic seizures in all control mice (Fig. 1C) but reduced total seizure scores by 21%, from 57.8 ± 2.6 to 45.6 ± 3.8 ($p = 0.029$; $U = 5$). LSM was most effective in reducing seizure scores in control mice, eliciting a reduction of the total seizure score of 42.2%, from 57.8 ± 2.6 to 33.4 ± 2.5 ($p = 0.0016$; $U = 0$) (Fig. 1D). TPM had no significant effect on total seizure scores in $\text{Ca}_v2.3$ -KO mice, whereas LTG significantly increased total seizure scores by 15.8%, from 41.4 ± 3.7 to 50.6 ± 1.5 ($p = 0.018$; $U = 6.5$) and the frequency of the convulsive stage 3 in $\text{Ca}_v2.3$ -KO mice. Both LTG and TPM were effective in reducing total seizure scores of control mice but were ineffective in doing the same in $\text{Ca}_v2.3$ -KO mice. LSM was the only AED of the three that reduced seizure scores in $\text{Ca}_v2.3$ -KO mice, doing so by 19.4%, from 41.4 ± 3.7 to 33.4 ± 0.6 ($p = 0.048$; $U = 5$) (Fig. 2A). No animals died as a result of 20 mg/kg kainic acid injection. In control mice, all three AEDs significantly increased the frequency of stage 1, the lowest pathologic seizure stage, whereas LTG had the opposite effect in $\text{Ca}_v2.3$ -KO mice. TPM did not alter the frequencies of occurrence of the seizure stages in $\text{Ca}_v2.3$ -KO mice.

We retested the effect of LTG in seven $\text{Ca}_v2.3$ -KO and eight control mice at 30 mg/kg KA (Fig. 2B), a dosage at which $\text{Ca}_v2.3$ -KO mice develop tonic clonic seizures and exhibit similar seizure activity as control animals at 20 mg/kg KA, to determine whether LTG can prevent tonic clonic seizures in $\text{Ca}_v2.3$ -KO mice and to further investigate the convulsive effect of LTG in $\text{Ca}_v2.3$ -KO mice observed at 20mg/kg. At 30 mg/kg KA, LTG pretreatment reduced total seizure scores of control mice by 30% ($p = 0.0079$; $U = 0$) and total seizure scores of LTG-treated $\text{Ca}_v2.3$ -KO mice were 33% ($p = 0.015$; $U = 1.5$) higher than those of LTG-treated control mice (69 ± 6.4 compared to 51.6 ± 1). An increase (not significant) of total seizure scores of 15.8% (69 ± 6.4 compared to 59.6 ± 4.1) was observed in LTG-treated $\text{Ca}_v2.3$ -KO mice compared to $\text{Ca}_v2.3$ -KO mice without pretreatment, which is in line with the significant

Fig. 1. Individual seizure scores of mice at 20 mg/kg kainate. Seizure scores are plotted for each mouse during the course of the 2 hour video-monitoring period. A. – D.: Seizure scores for control mice without (A, n = 8) and after pretreatment with 30 mg/kg LTG (B, n = 6), 50 mg/kg TPM (C, n = 6) or 30 mg/kg LSM (D, n = 5). E. – H.: Seizure scores for $\text{Ca}_v2.3\text{KO}$ without (E, n = 7) and after pretreatment with 30 mg/kg LTG (F, n = 7), 50 mg/kg TPM (G, n = 6) or 30 mg/kg LSM (H, n = 5).

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increase of total seizure scores caused by LTG in $\text{Ca}_v2.3\text{-KO}$ mice observed at 20 mg/kg KA. At both kainic acid concentrations LTG increased the frequency of stage 3 in $\text{Ca}_v2.3\text{KO}$ mice, (but not in controls), which contributes to the increased total seizure scores of LTG-treated $\text{Ca}_v2.3\text{-KO}$ mice compared to untreated $\text{Ca}_v2.3\text{-KO}$ mice.

Histology and Immunohistochemistry

NeuN and Nissl stained brain sections of mice from 30 mg/kg groups (n=4 per group) were evaluated by determining the percentage of pyknotic to healthy pyramidal neurons in the CA1 CA2, CA3 and dentate gyrus (DG) regions of the hippocampus (Fig. 3). $\text{Ca}_v2.3\text{-KO}$ mice were found to display significantly less pyknotic pyramidal neurons than control mice in the CA1 and CA3 regions of the hippocampus (CA1 $4.14\% \pm 2.07$ compared to $26.5\% \pm 6.41$; CA3 $6.89\% \pm 0.75$ compared to $27.17\% \pm 4.75$) which is in line with findings from Weiergräber et al [4]. Both stains revealed that LTG-treated $\text{Ca}_v2.3\text{-KO}$ mice displayed significantly increased degeneration of pyramidal CA1 neurons compared to untreated $\text{Ca}_v2.3\text{KO}$ mice (NeuN $14.65\% \pm 3.45$ compared to $4.14\% \pm 2.07$, $p=0.048$; Nissl $20.6\% \pm 2.6$ compared to $11\% \pm 2$, $p=0.02$), although a similar trend is visible in the other three regions.

Fig. 2. Effect of LTG, TPM and LSM on experimentally induced epilepsy in $\text{Ca}_v2.3$ -deficient and control mice. Seizures were induced in $n=7$ $\text{Ca}_v2.3\text{KO}$ and $n=8$ control mice by i.p. administration of KA. The animals were video-monitored for 2 h after injection. A semi-quantitative scale was used as described in methods. $\text{Ca}_v2.3\text{KO}$ and control mice were injected with 30 mg/kg LTG ($n=7$ and $n=8$ respectively) or 50 mg/kg TPM ($n=6$ and $n=6$ respectively) or 30 mg/kg LSM ($n=5$ and $n=5$ respectively) one hour before KA injection. A.: Total seizure scores in control and $\text{Ca}_v2.3\text{-KO}$ mice with and without antiepileptic pretreatment after 20 mg/kg KA. All AEDs reduce total seizure scores in control mice, however only LSM reduces total seizure scores in $\text{Ca}_v2.3\text{KO}$ mice. Note, $\text{Ca}_v2.3\text{KO}$ mice reach significantly higher scores under LTG than without pretreatment. B.: Effects of LTG on total seizure scores after 30 mg/kg KA injection are comparable to those after 20 mg/kg. The error bars represent SEM values in all Figs.

Furthermore, in control mice LTG significantly reduces neurodegeneration in the CA1, CA3 and DG. $\text{Ca}_v2.3\text{KO}$ and LTG-treated control mice displayed similar degrees of degeneration in all evaluated regions except the CA2.

Expression of $\text{Ca}_v2.3$ Protein

Both manual and automated quantification of western blotted $\text{Ca}_v2.3$ bands by normalization to SYN revealed no significant differences in $\text{Ca}_v2.3$ protein expression between KA and saline injected groups (Fig. 4).

ECoGs

Relative power was used in the evaluation and statistical testing due to better inter-individual comparability, however absolute power was also computed and is shown in Fig 5.

Effect of LTG in Control Condition

Spectral analysis of the recorded ECoGs revealed significant differences between $\text{Ca}_v2.3\text{-KO}$ and control mice and between the effects of LTG in both genotypes in control recordings and after injection of 20 mg/kg KA. In control conditions, $\text{Ca}_v2.3\text{-KO}$ mice displayed significantly reduced relative delta power compared to control mice ($29\% \pm 1.7$ vs $22.1\% \pm 2.0$ ($p=0.037$)). LTG treatment increased relative beta power in control mice (from $4.6\% \pm 0.48$ to $7.5\% \pm 1$ ($p=0.037$)), but not in $\text{Ca}_v2.3\text{-KO}$ mice in which LTG reduced relative alpha power from $16\% \pm 1.5$ to $11.1\% \pm 0.9$ ($p=0.034$).

Effect of KA Compared to Control Condition

KA injection elicited spikes, sharp waves and spike trains in all four groups with ictal activity predominantly occurring within the delta-theta range (Fig. 6). Accordingly, KA injection significantly increased relative delta power in both genotypes, however to a greater degree in control mice. Interestingly, in control mice, LTG pretreatment prevented the KA-induced shift in spectral distribution, whereas in LTG-pretreated $\text{Ca}_v2.3\text{-KO}$ mice KA injection caused a significant reduction of alpha power.

Fig. 3. Neurotoxicity after 30mg/kg KA. NeuN stained paraffin sections of the CA1 (A), CA2 (B), CA3 (C) and DG (D) regions of the hippocampus at 20x magnification from control mice (top left of each quadrant), LTG pretreated control mice (bottom left), Cav2.3KO mice (top right) and LTG pretreated Cav2.3KO mice (bottom right) (n = 4 for each group). Higher (40x) magnification of NeuN (E) and Nissl (F) stained sections showing the CA1 region. Arrows indicate pyknotic pyramidal neurons i.e. pyramidal neurons with condensed nuclear material indicating apoptosis. (G) Evaluation of the percentage of pyknotic neurons in regions of the hippocampus.

Effect of LTG on KA-induced Seizures

Both genotypes displayed different spectral distribution after KA injection (Fig. 7), with Cav2.3-KO mice exhibiting significantly increased relative sigma

and beta power compared to control mice ($4\% \pm 0.2$ vs $3.2\% \pm 0.1$ ($p = 0.009$) and $4.9\% \pm 0.4$ vs $3.5\% \pm 0.3$ ($p = 0.024$) respectively), reflecting less ictal activity in the delta theta range and thus the reduced seizure susceptibility found by other authors [4] and observed in behavioral analysis in this study.

Similarly LTG-pretreated control mice exhibited reduced relative theta power compared to untreated control mice ($31\% \pm 1.9$ vs $20.4\% \pm 2.7$ ($p = 0.04$)) and therefore a distinct shift in spectral distribution towards sigma and beta frequencies ($5.4\% \pm 0.9$ vs $3.2\% \pm 0.1$ ($p = 0.007$) and $6.5\% \pm 1$ vs $3.5\% \pm 0.3$ ($p = 0.008$) respectively) away from delta-theta frequencies and thus less ictal activity in this frequency range. In contrast, LTG pretreatment of Cav2.3-KO mice did not significantly alter spectral distribution when the complete recording period was analyzed.

Analysis of Maximal Seizure Activity

Using the automated spike detection protocol, the longest spike train i.e. maximal seizure activity was identified and analyzed in further detail in order to gain more detailed insight into the effect of LTG on ictal activity in both genotypes. Although analysis of the parameters latency to first spike, longest spike train, spikes per second and average spike interval revealed trends corresponding to the rest of the data, results did not reach statistical

Fig. 4. $\text{Ca}_v2.3$ Protein expression in the hippocampus. A.: Western blot of control mice hippocampal membrane fractions (n = 3 blots). B.: Student's t-test of manual (using ImageJ 1.46) and automated (using Gelsacn 6.0) quantification of $\text{Ca}_v2.3$ protein by normalization to synaptophysin expression revealed no significant difference between KA and saline injected animals. Although manual quantification produced a greater difference between the groups, standard error was also much increased.

Fig. 5. Evolution of absolute power after KA injection. Absolute power of the frequency bands for five minute epochs after KA injection of control mice (A, n = 8) with LTG pretreatment (B, n = 6) and $\text{Ca}_v2.3\text{KO}$ mice (C, n = 7) with LTG-pretreatment (D, n = 7). The robust increase of absolute delta and theta power over time in

control mice and LTG-pretreated $\text{Ca}_v2.3\text{KO}$ mice represents the genesis of ictal discharges, which occur predominantly in these two frequency bands. Note the effect of LTG on HF bands in both genotypes.

significance, as inter-individual spiking patterns proved to be highly variable within the groups. However, analysis of maximal seizure activity revealed a robust reduction of relative delta power in LTG pretreated control mice compared to those without pretreatment ($51\% \pm 7.2$ vs 24.9 ± 2.6 ($p=0.03$)) (Fig. 8B). This effect of LTG on maximal seizure activity does not occur in $\text{Ca}_v2.3\text{-KO}$ mice (Fig. 8C).

Interestingly, LTG pretreated $\text{Ca}_v2.3\text{-KO}$ mice displayed significantly increased relative fast ripple power compared to untreated $\text{Ca}_v2.3\text{-KO}$ mice ($1.2\% \pm 0.4$ vs $0.036\% \pm 0.001$ ($p=0.003$)), which is difficult to interpret, because of the limitations of the transmitter's sampling rate used [20]. Therefore, it only could be speculated that the extracted difference between both genotypes at the elevated frequency band may possibly underlying the pro-ictogenic

Fig. 6. Ictal activity in electrocorticographic recordings after 20mg/kg KA. Raw EcoG traces of seizures in individual mice of each group. Blue dots indicate individual ictal spikes, green lines indicate spike trains.

effect of LTG observed in behavioral seizure analysis (Fig. 8C). Correspondingly, in control mice but not in $Ca_v2.3$ -KO mice, LTG significantly reduced the Z-ratio of maximal seizure activity from 0.51 to 0.07 ($p = 0.04$), indicating an increase of high frequency power and thus a shift away from spiking in the delta-theta range.

Discussion

In this study we show that the $Ca_v2.3$ calcium channel is critical in mediating the anticonvulsant properties of LTG in the kainic acid model of epilepsy. Different from a pure C57Bl/6 genetic background [21], our mice develop limbic seizures connected with neuronal cell death, both, in the CA3 and CA1 region. The percentage of pyknotic to healthy pyramidal neurons was largest reduced by LTG in the CA1 and the DG region of control mice (Fig. 3G).

In mice lacking the $Ca_v2.3$ calcium channel LTG-pretreatment results in increased seizure severity in this acute kainic-acid model as well as delayed increased pyknosis of pyramidal neurons in the CA1 region of the hippocampus. Whether this cell loss increases the frequency of spontaneous seizures beyond the first hours of KA intoxication was not assessed in this study but would be of interest as reorganization specifically of the CA1 region is suggested to be a critical mechanism in ictogenesis in both animal epilepsy models and in temporal lobe epilepsy patients [22].

Why pretreatment with LTG a powerful broad-spectrum anti-epileptic agent has ictogenic effects in animals lacking the $Ca_v2.3$ calcium channel is not clear. It is well known that LTG can aggravate seizures in certain epilepsies and especially in Dravet Syndrome, which is commonly associated with a mutation of the SCN1A gene coding for the $Na_v1.1$ calcium channel [23], however epilepsy phenotypes in humans associated with CACNA1E mutations have yet to be reported more than once [24]. Further investigations will be necessary to elucidate why $Ca_v2.3$ signaling is critical for LTG's anti-epileptic effects in the acute kainic acid model of epilepsy.

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Fig. 7. Spectral distribution after KA injection. Relative power of the frequency bands after KA injection averaged from 10-second epochs of 60 minutes recording time. For the individual groups, the same number of animals were used as described in Fig. 1. and Fig. 2. A.: $\text{Ca}_v2.3\text{KO}$ vs control mice. B.: Control vs LTG-pretreated control mice. C: $\text{Ca}_v2.3\text{KO}$ vs LTG pre-treated $\text{Ca}_v2.3\text{KO}$ mice. Note the similar effect of ablation of $\text{Ca}_v2.3$ and LTG-pretreatment [of control mice].

Fig. 8. Spectral distribution during maximal seizure activity. Relative power of the frequency bands during the longest spike train identified by the seizure detection protocol averaged from 10-second epochs. For the individual groups, the same number of animals were used as described in Fig. 1. and Fig. 2. A.: $\text{Ca}_v2.3\text{KO}$ vs control mice. B.: Control vs LTG-pretreated control mice. C: $\text{Ca}_v2.3\text{KO}$ vs LTG pre-treated $\text{Ca}_v2.3\text{KO}$ mice. LTG increases fast ripples and fails to reduce spiking in the delta theta range in $\text{Ca}_v2.3\text{KO}$ mice as it does in control mice.

Furthermore, in these animals the total seizure score was significantly increased after KA-induced seizures at 20 mg/kg (Fig. 2A). The EEG recordings led us suggest rather an increased seizure severity than an increase in spontaneous seizures (Fig. 6). The consequences of acute LTG overdoses may be compared with the situation in $\text{Ca}_v2.3$ -deficient mice and were summarized recently characterized mostly by mild or no toxicity [25]. But some cases with very high serum LTG levels (> 25 mg/L) accidentally caused in a child led to severe and polymorphic neurological symptoms acutely [26]. In an adult man after a suicidal attempt LTG overdose reveals severe neurological and cardiac symptoms [27].

In our mouse model, neither LTG nor TPM, which have been shown to inhibit R-type currents in heterologous systems, could reduce seizure scores in $\text{Ca}_v2.3\text{-KO}$ mice, indicating the importance of $\text{Ca}_v2.3$ inhibition in mediation of their anticonvulsive effects. Contrastingly, LSM which has no calcium channel modulating properties was the only AED of the three tested, which could reduce seizure scores in $\text{Ca}_v2.3\text{-KO}$ mice. It should be taken into account that, in control mice neither LTG nor TPM was capable of reducing seizure scores beyond the degree that is reached when the $\text{Ca}_v2.3$ is ablated. Furthermore, this study reveals a convulsive and neurotoxic effect of LTG in the absence of $\text{Ca}_v2.3$ calcium

channels. Interestingly, toxicity of LTG was located in the CA1 region of the hippocampus, where LTG is known to be most neuroprotective [28-30]. Therefore, it is assumable that the underlying neuroprotective mechanisms may include inhibition of signaling through $\text{Ca}_v2.3$, which we found not to be upregulated after KA injection. The fact that the convulsive effect of LTG is more specifically related to the CA1 region, must lead to a novel interpretation of its mechanism of action. Underlying this finding could be post-inhibitory rebound firing of CA1 pyramidal neurons promoted by HCN channels (hyperpolarization-activated cyclic nucleotide-gated channels), a paradoxical phenomenon observed as a reaction to increased inhibition after experimentally induced seizures [31]. LTG has been shown to enhance HCN currents in CA1 pyramidal neurons, conveying an inhibitory effect [32]. However due to HCN channels' capacity to activate at hyperpolarized potentials and slow deactivation kinetics, increased synaptic inhibition, a condition predictable in $\text{Ca}_v2.3\text{KO}$ mice, may cause rebound excitation of CA1 pyramidal neurons when HCN currents are stimulated by LTG. It should be noted that no compensatory upregulation of other cation channels, that may increase excitability were identified after injection of 30 mg/kg KA in hippocampi of Cav2.3-KO mice compared to control mice in a full transcriptome analysis that was performed in our laboratory prior to the present study (results not shown).

Furthermore, in this study telemetrically recorded ECoGs reveal that LTG cannot attenuate ictal discharges in $\text{Ca}_v2.3\text{KO}$ mice as it does in control mice. However, LTG may increase high frequency components of ictal activity, which are known to be associated with generation of epileptic activity in humans and in animals [33-35]. This result must be taken with precaution. The radiotransmitter used in the present study may cause aliasing with frequencies higher than 125 Hz. The nominal sampling rate was given by the company DSI as 50 Hz. Controlling the transmitted sine waves from a GW Instek AFG-2012 signal generator at 200 and 250 Hz revealed that the responses recorded by the TL11M2-F20-EET transmitter dropped severely in the transmitter output. Sine waves at 200 and 250 Hz, which were introduced into the system with an input amplitude of 1 to 3 mV yielded only 0.3 to 1.3 % of the incoming voltage-intensity for the mentioned frequency ranges. This low transfer rate was higher than the noise, as it increased significantly with increasing amplitude of the input signal. In conclusion, at the elevated frequencies, differences exist between both genotypes, but the exact frequency range must be confirmed with improved transmitter sampling rates.

Clinically, the LTG phenomenon observed in mice and in brain slices, may be represented by the capacity of LTG to aggravate seizures in certain epilepsy syndromes. Although toxic doses of several (non-sedative) AEDs can cause seizures, LTG has been reported to cause and aggravate seizures and seizure frequency at doses within its therapeutic range. In severe myoclonic childhood epilepsy, there is a very frequent aggravating effect of LTG at therapeutic doses [23, 36]. Another study reports, that adults suffering from idiopathic generalized epilepsies treated with LTG experienced exacerbation or de novo appearance of myoclonic jerks [37]. Whether this paradoxical effect of LTG in clinical practice reflects rebound hyperexcitation after increased inhibition, possibly due to antiepileptic polytherapy or intake of other drugs with an inhibitory effect on certain neuron types, must be investigated in further studies. It is notable that nothing is known about expression or genetic variants of Cav2.3 in human patients with epilepsy. Although gain of function mutations in the CACNA1H gene encoding for the low-voltage activated (T-type) calcium channel $\text{Ca}_v3.2$ have been identified in patients with hereditary forms of absence epilepsy [38], no variants of $\text{Ca}_v2.3$ have been identified in patients with epilepsy. However, increased R-type currents have been measured in the genetically epilepsy-prone rat (GEPR) suggesting that increased R-type signaling contributes to the genetic basis of the enhanced seizure susceptibility of GEPR [39]. Whether expression of $\text{Ca}_v2.3$ is altered in the hippocampus of human epileptic patients is a matter of great interest; however gaining access to resected hippocampal tissue can be difficult, and is a limiting factor for several epilepsy researchers. Nevertheless, investigating genetic variants of CACNA1E in epileptic patients that experience a worsening of symptoms under LTG, could produce valuable insights.

Conclusion

Because LTG is not able to prevent or attenuate ictal activity in the absence of $\text{Ca}_v2.3$ calcium channels, one must assume that its anticonvulsive properties are not primarily based on inhibition of sodium currents, but that R-type modulation plays a major role in mediating net anticonvulsive properties of LTG. A complex and multimodal mechanism of LTG is highly likely, also considering that LTG has been shown to attenuate several neuropsychiatric disorders like bipolar depression, borderline disorder and anxiety disorder and to contribute to a better outcome in animal models of stroke and subarachnoid hemorrhage.

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Disclosure Statement

Dr. Maxine Dibué-Adjei is an Employee of LivaNova PLC. None of the authors has anything other to disclose.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines

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