

Mechanisms of the Ca<sub>v</sub>2.3 Calcium Channel's Role in Epileptogenesis and Antiepileptic  
Pharmacotherapy

Inaugural Dissertation

zur

Erlangung des Doktorgrades

Dr. nat. med.

der Medizinischen Fakultät

und

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln



vorgelegt von

Maxine Dibué-Adjei

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For Mom, Dad and Jason

*“If the brain were simple enough for us to understand it, we would be too simple to understand it.” — Ken Hill*

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## II. SUMMARY

**RATIONALE:** The Ca<sub>v</sub>2.3 (R-type) voltage-gated calcium channel represents the most enigmatic of all voltage-gated calcium channels due to its pharmacoresistance, mixed characteristics of high voltage-activated and low voltage-activated calcium channels and relatively low expression levels. Lamotrigine (LTG) is a modern antiepileptic drug however, its mechanism of action has yet to be fully understood, as it is known to modulate several ion channels and other targets. In heterologous systems, LTG inhibits Ca<sub>v</sub>2.3 (R-type) calcium currents, which contribute to kainic-acid (KA)–induced epilepsy *in vivo*. LTG has been suggested to increase the risk of sudden unexpected death in epilepsy (SUDEP), in which cardiac and respiratory mechanisms are proposed to be involved. In addition to the higher risk of SUDEP during sleep, epileptic patients are at higher risk of seizures during sleep, especially during slow wave sleep (SWS). The bidirectional relationship between sleep and epilepsy has long been acknowledged, however it remains far from understood.

**AIM:** The goal of the present project was to perform an in depth investigation of the role of R-type signaling in the epileptic brain and heart, by analyzing its contribution to experimental epilepsy, antiepileptic pharmacotherapy and sleep.

**METHODS:** In the first study we compared the effects of LTG to two other AEDs (Topiramate and Lacosamide) in Ca<sub>v</sub>2.3-deficient mice and controls on KA-induced seizures. Behavioral seizure rating and quantitative electrocorticography were performed after KA induced epilepsy, as well as immunohistochemistry and western blot analysis of Ca<sub>v</sub>2.3 expression in the brain. In the second study we investigated cardiac parameters during KA-induced epilepsy and LTG treatment in awake and sleeping C57Bl6 mice. Continuous electrocardiograms and electrocorticograms were collected telemetrically from freely moving mice, and time- and frequency-domain analysis performed on the electrocardiograms. In the third study, we analyzed sleep architecture in Ca<sub>v</sub>2.3-deficient and control mice also using radiotelemetric electrocorticography and electromyography during spontaneous and urethane-induced sleep.

**RESULTS:** LTG treatment displayed no antiepileptic potency in Ca<sub>v</sub>2.3-deficient mice, but contrarily significantly aggravated seizures and increased neurodegeneration in the

CA1 region of the hippocampus as well as increasing ultra-high frequency oscillations (ripples) known to be associated with seizure generation. This effect was specific to LTG in  $Ca_v2.3$ -deficient mice, as the two other AEDs tested - one with and one without  $Ca_v2.3$  inhibiting capacity- did not aggravate seizures. In our second study we found LTG to alter autonomous nervous control of the heart during SWS after induction of chronic epilepsy promoting sympatho-vagal imbalance. Furthermore, we found LTG to increase the squared-coefficient of variation of the heart rate during SWS, but not during wakefulness. Our third study was able to demonstrate, that ablation of  $Ca_v2.3$  robustly impacts sleep architecture, producing deficits in the amount and depth of SWS. Interestingly, although  $Ca_v2.3$  mice sleep less and display shorter SWS phases, they do not compensate for this deficit by increasing sleep depth, pointing to disturbances in sleep homeostasis.

**DISCUSSION:** We provide first *in vivo* evidence for a crucial role of R-type signaling in LTG pharmacology and shed light on a paradoxical effect of LTG in the absence of  $Ca_v2.3$ . LTG appears to promote ictal activity in  $Ca_v2.3$ -deficient mice by increasing high frequency components of seizures, resulting in increased neurotoxicity in the CA1. This paradoxical mechanism, possibly reflecting rebound hyperexcitation may be key in understanding LTG-induced seizure aggravation observed in patients. Furthermore, we find  $Ca_v2.3$  to be a critical mediator of sleep homeostasis, potentially representing a pivotal link between sleep and epilepsy.  $Ca_v2.3$  has been shown to be crucial for bursting in the reticular thalamus, which underlies delta-rhythm during SWS and generation of spike-and-wave discharges, the hallmark of absence epilepsy. Therefore, seizure resistance and SWS impairment of  $Ca_v2.3$ -deficient mice may be symptomatic of impairment of bursting in the thalamus and therefore of the generation and maintenance of highly synchronized slow rhythms. Remarkably, we found LTG to only affect autonomous control of the epileptic heart during SWS, possibly indicating a mechanism by which LTG could increase the risk for SUDEP. LTG-induced increased sympathetic tone during SWS, may also reflect impaired SWS, found in LTG-treated patients and in our  $Ca_v2.3$ -deficient mice.

**CONCLUSION:** Because  $Ca_v2.3$ -deficient mice display a subtle phenotype as opposed to an obvious one, because of the expression of  $Ca_v2.3$  in rhythmically active tissue and

because of  $Ca_v2.3$ 's unique electrophysiological properties, it is conceivable that a general function of R-type currents is the “fine tuning” of oscillatory networks. One may assume that a loss of “fine-tuning” in  $Ca_v2.3$ KO mice is only minimally noticeable under physiological conditions, but becomes evident in certain pathological conditions exerting a strain on an oscillatory network such as during experimentally induced epilepsy. This may explain how R-type signaling is crucial for sustaining physiological rhythmic activity of an entire network despite relatively low expression levels.

### III. ZUSAMMENFASSUNG

**HINTERGRUND:** Der  $Ca_v2.3$  (R-Typ) spannungsgesteuerte Kalziumkanal ist der enigmathischste aller Kalziumkanäle aufgrund von seiner Pharmakoresistenz, Charakteristika zwischen hoch- und niedrig- spannungsgesteuerten Kalziumkanälen und relativ niedrigen Expression. Lamotrigin (LTG) ist ein beliebtes modernes Antiepileptikum (AED), jedoch sind seine Wirkmechanismen noch unklar, da LTG viele Ionenkanäle und andere Zielstrukturen moduliert. Im heterologen System, inhibiert LTG R-Typ Ströme, die *in vivo* zu Kainat (KA)-induzierter Epilepsie beitragen. Es wurde gezeigt, dass LTG das Risiko für plötzlich auftretenden, ungeklärten Tod bei Epilepsie (SUDEP) erhöht, ein Phänomen, bei dem kardiale und respiratorische Mechanismen impliziert sind. Außerdem ist das Risiko für epileptische Anfälle bei Patienten höher im Schlaf, besonders im Langsame-Wellen-Schlaf (SWS). Die bidirektionale Beziehung zwischen Schlaf und Epilepsie ist schon lange bekannt, jedoch dem vollen Verständnis noch fern.

**ZIELE:** Ziel des gegenwärtigen Projekts, war es eine tiefgründige Analyse der Rolle des R-Typ Kalziumkanals in dem epileptischen Hirn und Herz durchzuführen, indem seine Mitwirkung bei experimenteller Epilepsie, antiepileptischer Pharmakotherapie und Schlaf analysiert wird.

**METHODEN:** In der ersten Studie wurden die Effekte von LTG und zwei anderen AEDs (Topiramamat und Lacosamid) auf  $Ca_v2.3$ -defizienten und Kontrollmäusen bei KA-induzierter Epilepsie untersucht. Neben telemetrischer Erfassung von Elektrokortikogrammen, wurde der Schweregrad der Anfälle quantifiziert. Immunohistochemie und Westernblot Analyse von  $Ca_v2.3$  Expression im Hirn wurden auch ausgeführt. In der zweiten Studie wurden kardiale Parameter nach KA-induzierter Epilepsie und anschließender LTG-Behandlung in wachen und schlafenden C57Bl6 Mäusen untersucht. Langzeit Elektrokardiogramme und Elektrokortikogramme wurden telemetrisch von sich-frei bewegenden Mäusen erhoben und anschließend Zeit- und Frequenzdomänen ausgewertet. In der dritten Studie wurde spontanes und Urethan-

induziertes Schlafverhalten in  $Ca_v2.3$ -defizienten und Kontrollmäusen mittels telemetrische Elektrokortikographie und Elektromyographie untersucht.

**ERGEBNISSE:** In  $Ca_v2.3$ -defizienten Mäusen zeigt LTG keine antiepileptische Potenz, sondern verstärkt epileptische Anfälle und Neurodegeneration in der CA1 Region des Hippokampus und verstärkt ultra-hochfrequente Oszillationen (Ripples), von denen gezeigt wurde, dass sie mit der Entstehung von iktaler Aktivität assoziiert sind. Dieser Effekt war spezifisch für LTG in  $Ca_v2.3$ -defizienten Mäusen und entstand nicht bei der Behandlung von  $Ca_v2.3$ -defizienten Mäusen oder Kontrollmäusen mit den anderen beiden getesteten AEDs. In der zweiten Studie konnte gezeigt werden, dass bei chronisch epileptischen Mäusen, LTG die autonom-nervöse Kontrolle des Herzes während SWS beeinflusst, indem sympathische Kontrolle der Herzaktivität verstärkt wird. Zudem fanden wir eine Zunahme des Variationskoeffizienten im Quadrat im SWS, jedoch nur bei SWS und nicht bei Wachheit. Unsere dritte Studie konnte zeigen, dass die Ablation von  $Ca_v2.3$  durch verkürzte SWS Phasen und reduzierte SWS Tiefe, robuste Veränderungen des Schlafverhaltens verursacht. Obwohl  $Ca_v2.3$ -defiziente Mäuse weniger Schlafen und kürzere SWS Phasen zeigen, kompensieren sie dieses nicht durch Vertiefung des SWS, was auf eine Beeinträchtigung von homöostatischen Mechanismen deutet.

**DISKUSSION:** Wir zeigen erste *in vivo* Belege für eine entscheidende Rolle von R-Typ Kalziumkanälen in dem Wirkmechanismus von LTG und decken einen paradoxen Effekt von LTG in der Abwesenheit von  $Ca_v2.3$  auf. LTG scheint iktale Aktivität in  $Ca_v2.3$ -defizienten Mäusen zu fördern, indem es ultra-hochfrequente Komponente von Anfällen verstärkt, was erhöhte Degeneration von CA1 Neurone verursacht. Dieser paradoxe Mechanismus, der womöglich reaktive Hyperexzitation reflektiert, könnte ausschlaggebend für das Verständnis von anfallsverstärkenden Effekten von LTG sein, die bei epileptischen Patienten auftreten.  $Ca_v2.3$  zeigt sich als bedeutender Vermittler der Schlafhomöostase, und könnte somit ein entscheidender Knotenpunkt zwischen Epilepsie und Schlaf darstellen.  $Ca_v2.3$  spielt eine entscheidende Rolle beim Bursting im Thalamus, welches dem Delta-Rhythmus unterliegt aber auch der Generation von Spike-and-Wave Discharges (SWD). Demzufolge könnte die Resistenz gegenüber Chemokunvulsiva und gestörtes SWS der  $Ca_v2.3$ -defizienten Mäuse symptomatisch für gestörtes Bursting im

Thalamus, und somit für die Generation und Aufrechterhaltung von hoch-synchronisierten langsamen Rhythmen sein. Bemerkenswerterweise, verschiebt LTG nur im SWS die autonom-nervöse Kontrolle des Herzes in sympathischer Richtung, was ein potentieller Mechanismus hinter dem erhöhten Risiko für SUDEP im Schlaf sein könnte. LTG-induzierter erhöhter Sympathikotonus im SWS könnte auch beeinträchtigtes SWS widerspiegeln, ein Phänomen, das auch in LTG-behandelten Patienten auftritt.

**KONKLUSION:** Weil  $Ca_v2.3$ -defiziente Mäuse einen subtilen statt einen eindeutigen Phänotyp zeigen, weil  $Ca_v2.3$  eine einzigartige Kombination elektrophysiologischer Eigenschaften besitzt und weil  $Ca_v2.3$  in verschiedenen rhythmisch-aktiven Geweben exprimiert wird, ist es annehmbar, dass eine Hauptfunktion dieses Kanals in der „Feinjustierung“ von oszillierenden Netzwerken besteht. Es ist möglich, dass ein Verlust von „Feinjustierung“ unter physiologischen Bedingungen kaum auffällt, jedoch unter pathophysiologischen Bedingungen bzw. wenn das Netzwerk stark belastet wird, große Auswirkungen hat wie z.B. bei der Epilepsie. Das würde erklären, wie R-Typ Kanäle, trotz relativ niedriger Expression, ausschlaggebend für die Generation und Aufrechterhaltung von rhythmischer Aktivität in ganzen Netzwerken sein können.

# 1. INTRODUCTION

## 1.1 Epilepsy

### 1.1.1 A Concise History of Epilepsy

Epilepsy has been known to man for over 3000 years. The earliest written account of an epilepsy disorder is an Akkadian text, the Sakikku, written around 1067-1046 BC (Kinnier Wilson and Reynolds, 1990), describing the “falling disease”. Although Hippocrates already identified epilepsy as a disorder of the brain in the 4<sup>th</sup> century BC, the belief that epilepsy was of supernatural origin, for example due to possession by ghosts, demons, ancestors or even gods persisted into the 18<sup>th</sup> century in many cultures.

The concept of epilepsy as a disorder of the brain slowly began to gain acceptance in the late 17<sup>th</sup> century and by the early 19<sup>th</sup> century it was thought that epilepsy was caused by vascular dysfunction. Robert Bentley Todd was the first to recognize electrical dysfunction as the underlying cause of epilepsy refuting the hypothesis of vascular congestion. In the Lumleian Lectures delivered at the Royal College of Physicians in London in 1849, Todd describes animal experiments, in which he found excitation of certain brain regions by “stimulus of galvanism” to cause convulsions (Todd, 2005). The International League Against Epilepsy (ILAE) was founded by a group of physicians in 1909 in Budapest, which greatly influenced the advancement of the understanding of epilepsy. However, it was only until the discovery of the electroencephalogram (EEG) in 1929 by Hans Berger (Berger, 1929), that the electrical basis of epilepsy could be fully confirmed. By this time, phenobarbital, the first antiepileptic drug (AED) (after potassium bromide, which has a very low therapeutic index) had been available for 17 years, offering a first non-toxic method of seizure control, but at a high price, as phenobarbital, a barbiturate is extremely sedative and becomes hypnotic at higher doses. Interestingly, despite the availability of over 30 FDA approved AEDs today, phenobarbital remains the most commonly used AED in the world.

### **1.1.2 Epilepsy Today**

Epilepsy is defined as the propensity for a person to suffer recurrent unprovoked seizures (WHO, 2005). The prominent British neurologist Gordon Morgan Holmes (1876-1965) defined a seizure as “a sudden, involuntary, time-limited alteration in behavior, motor activity, autonomic function, consciousness, or sensation, accompanied by an abnormal electrical discharge in the brain”.

Today, epilepsy is one of the most prevalent serious neurologic disorders, affecting over 50 million people worldwide (WHO, 2005). The mean number of people with epilepsy per 1000 is higher in low-income countries than in high income countries (9.55 vs 7.99), with 80-90% of people with epilepsy in most developing countries not receiving any treatment at all (WHO, 2005). Incidence rates of epilepsy are also higher in developing countries, which is thought to be due to higher rates of brain trauma, HIV, parasitic infection (particularly neurocysticercosis), perinatal morbidity and consanguinity (WHO, 2005). However, despite many other known circumstances that can cause epilepsy such as stroke, fever, tumors, infection or substance abuse, two thirds of epilepsy cases are idiopathic or cryptogenic (WHO, 2012). According to the ILAEs guidelines for classification, epilepsy, may be classified by etiology (1) and type of seizures (2):

- (1): symptomatic: identifiable etiology
  - cryptogenic: presumed symptomatic but unknown etiology
  - idiopathic: presumed genetic but unknown etiology
- (2): partial: focal onset
  - generalized: initial involvement of both hemispheres
  - unclassified: unknown onset

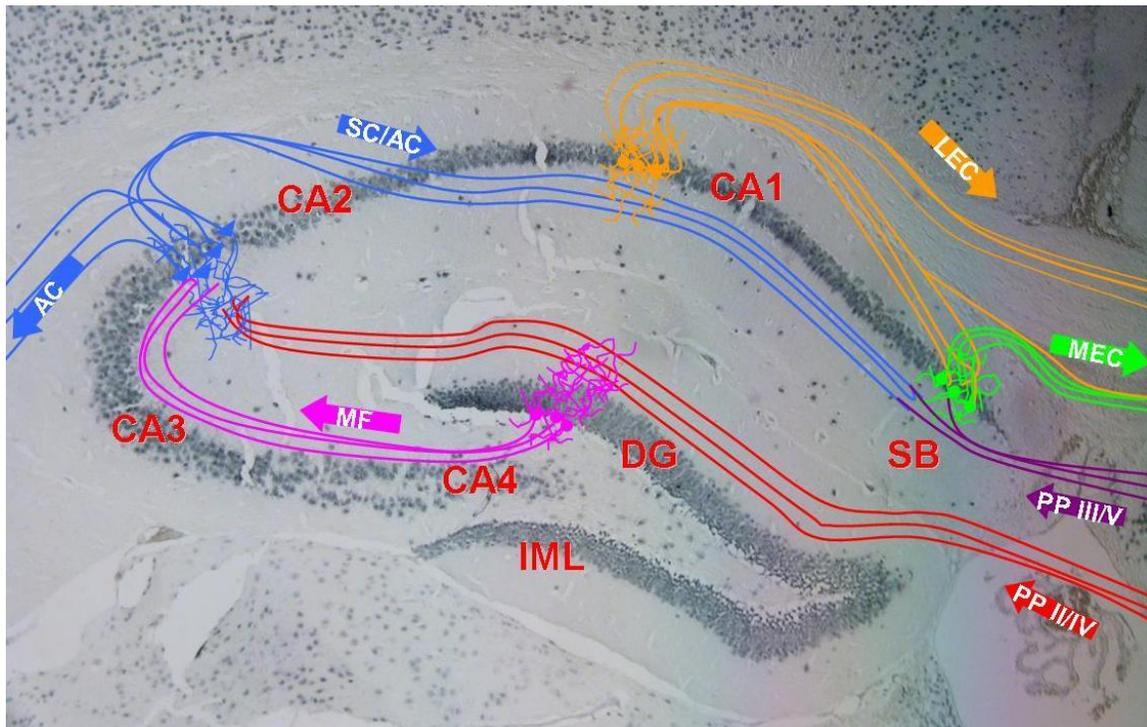
The ILAE recognizes 32 different epilepsy syndromes (Engel, 2006) with mesial temporal lobe epilepsy (MTLE) representing one of the most common epilepsy syndromes.

### **1.1.3 Mesial Temporal Lobe Epilepsy**

MTLE, the most common type of temporal lobe epilepsy (TLE) is characterized by seizures originating in the mesial temporal lobe i.e. in the hippocampus and parahippocampal gyrus (Margerison and Corsellis, 1966) (See Figure 1 for connectivity

of the mesial temporal lobe). In MTLE aura and automatisms lasting 1-2 minutes, accompanied by autonomic or psychic symptoms precede complex partial seizures, which can evolve into generalized seizures or status epilepticus. If left untreated, neurologic and psychiatric health of MTLE sufferers deteriorates rapidly: seizures occur more frequently and with greater severity causing always greater damage to the brain and manifestation of psychiatric disease such as depression or personality changes. Compared to the general population, morbidity and mortality are increased in people with MTLE, due to accidents occurring from sudden loss of consciousness accompanying seizures. However, high mortality also results from sudden unexpected death in epilepsy (SUDEP). Patients with therapy refractory MTLE, have a risk of sudden death that is 50 times greater than that in the general population, but are also at risk of dying during status epilepticus (SE). EEG is the primary tool used in diagnosis of MTLE. Interictal spikes and sharp waves (interictal epileptiform discharges (IEDs)), periodic-lateralized epileptiform discharges (PLEDs), lateralized focal or regional polymorphic delta activity (“slowing”) and a build-up of lateralized rhythmic 5-10 Hz sharp activity during seizures are characteristic for MTLE (Javidan, 2012). Generally, MTLE is considered to originate from lesions from febrile convulsions, status epilepticus (SE), mechanical trauma or cerebral infection during childhood, which cause spontaneous seizures after a 5-10 year latency period (Engel, 1993). Hippocampal sclerosis (HS), detectable in a structural MRI using a fluid-attenuated inversion recovery sequence (FLAIR), is considered by many to be the hallmark of MTLE; however one must note that HS does not occur in 30% of MTLE patients, who are therefore diagnosed with paradoxical temporal lobe epilepsy (PTLE). HS describes “scarring” of the hippocampus involving degeneration of neurons and gliosis in the cornu ammonis (Babb and Brown, 1986) as well as pathological sprouting of mossy fibers (MF) in the inner molecular layer of the dentate gyrus (DG) (Sutula et al., 1989). It is presumed that pathological remodeling of circuitry due to aberrant MF sprouts, forming synapses with spines of predominantly excitatory granule cells represents recurrent excitatory circuitry, which may underlie seizure generation (Lothman et al., 1992; Buckmaster et al., 2002). Seizures in MTLE are also referred to as “limbic” seizures, as the Papez circuit (=subiculum → fornix → mammillary bodies → mammillothalamic tract → anterior thalamic nucleus → cingulum → entorhinal cortex

→ (via perforant path) hippocampus), which is part of the limbic system, may be involved in seizure generation. It has been suggested that pathologic hyperexcitation from the hippocampus is transmitted to other limbic areas via the Papez circuit and then returns to the hippocampus as amplified input resulting in generalized seizures.



**Figure 1** Schematic of connectivity of the hippocampus superimposed on a NeuN-stained coronal paraffin section of mouse brain at approximately -1.5 mm Bregma. The hippocampus forms a uni-directional network receiving most input from the entorhinal cortex (EC) and projecting back to the EC. The different layers of the EC project to the dentate gyrus (DG) and CA3 pyramidal layer via the perforant path (PP). The CA3 also receives input from the DG via the mossy fibers (MF) and projects to the ipsilateral CA1 via the Schaffer Collateral Pathway (SC) and to the contralateral CA1 via the Associational Commissural Pathway (AC). The main output of the hippocampus is from the CA1 to the lateral EC (LEC) and from the subiculum (SB) to the medial EC (MEC).

### 1.1.4 Treatment of Mesial Temporal Lobe Epilepsy

MTLE is the most common type of epilepsy referred for epilepsy surgery, making it the most medically refractory type of epilepsy (Wass et al., 1996; Engel, 2001). Approximately half of MTLE patients achieve complete seizure control with antiepileptic pharmacotherapy, with lamotrigine (LTG) and carbamazepine (CBZ) often being the AEDs of choice, to which another AED can be added if necessary. A small group of

MTLE patients achieve seizure freedom by combination of three or more AEDs, however in many cases, MTLE is susceptible to AED (mono-) therapy in childhood and adolescence and becomes pharmaco-resistant with age. For intractable MTLE, anteromedial temporal resection (AMTR) is the treatment of choice, achieving seizure control in 60-90% of patients (Engel, 1996). Despite the high success rate, this surgical procedure comes at a high price, often causing anterograde amnesia and verbal memory deficits. Vagal nerve stimulation is a less invasive surgical procedure that is sometimes recommended before AMTR, however success rates are drastically lower.

### **1.1.5 The Kainic-Acid Model of Temporal Lobe Epilepsy**

2-carboxy-4 (1-methylethenyl)-3-pyrrolidiacetic acid or “kainic-acid” (KA) is a non-degradable cyclic analog of glutamate, which causes hyperexcitation by agonism of kainate-class ionotropic glutamate receptors (Wang et al., 2005). Rodent models using systemic or local injections of KA are among the most popular models of epilepsy and neurodegeneration. MTLE is easily modeled by systemic injection of KA, which induces automatisms, partial seizures which can evolve into generalized seizures and progressive sclerosis of the hippocampus leading to recurrent spontaneous seizures (Sharma et al., 2007). KA, administered by intraventricular injection, was first used to experimentally model MTLE in rats in 1978 and was found to selectively destroy hippocampal pyramidal cells (Nadler et al., 1978). Since then numerous KA models have probed different protocols (several low doses vs one high dose) and administration routes from intrahippocampal injection to intravenous injection to intraperitoneal injection. Because systemic administration of KA still causes specific degeneration of hippocampal pyramidal cells, does not require surgery but is easily executed as an intraperitoneal (i.p) or subcutaneous (s.c.) injection, it is preferred by many researchers. Rodent KA models closely mimic phenomena observed in MTLE patients. KA causes necrosis and apoptosis of pyramidal cells in the CA1 and CA3 subfields of the hippocampus and MF sprouting in the inner molecular layer (IML) of the DG (Tauck and Nadler, 1985; Okazaki et al., 1995) as well as changes in expression of AMPA-receptor subunits GluR1 and GluR2 (Sommer et al., 2001). Neurodegeneration is also detectable in associated limbic and neocortical structures (Ben-Ari et al., 1979; Pollard et al., 1994). Astrogliosis, microgliosis

and neurogenesis in the hippocampus have also been confirmed by TUNEL, MHC I and II and BrdU immunohistochemistry, respectively. All in all, histopathologic alterations of the rodent hippocampus after systemic KA injection are very similar to histopathology of surgically resected hippocampal tissue from MTLE patients. Mouse models of human pathology offer several advantages, most notably genetic homogeneity and availability of transgenic animals, but they can be technically challenging due to the small size of mice. The same evolution of seizures found in MTLE patients i.e. aura → automatisms → partial seizure → generalized seizure → status epilepticus can analogously be observed in mice after systemic KA injection as: immobility (“freezing”) → automatisms → myoclonic jerking → tonic-clonic seizure → status epilepticus. When faced with the task of quantifying behavioral severity of seizures, seizure rating scales adapted for mice such as one used by Morrison and colleagues allow for reproducible and accurate assessment of seizure intensity (Morrison et al., 1996).

## **1.2 The Ca<sub>v</sub>2.3 (R-type) Voltage-Gated Calcium Channel**

### **1.2.1. Structure of Voltage-gated Calcium Channels**

In vertebrates, calcium is one of the most tightly regulated physiological variables. The intracellular calcium concentration must be kept within precise limits in order to maintain physiological signal transduction, during which calcium ions are crucial as charge carriers and also as second messengers, neurotransmitter release, contraction of myocytes, hormone release and several other processes (Burgen, 1968;Rubin, 1970;Ashley, 1971;Rasmussen et al., 1976;Rasmussen and Barrett, 1984).

Voltage-gated calcium channels (VGCCs) are heterooligomeric complexes of up to four subunits ( $\alpha_1$ ,  $\alpha_2\delta$ ,  $\beta$ , and sometimes  $\gamma$ ), however they are primarily characterized by their pore-forming  $\alpha_1$  subunit (212-250 kDa), which harbours the voltage-sensing machinery and the drug/toxin-binding sites and for which ten different encoding genes in the human genome are known (Catterall, 2011;Catterall, 2000). The  $\alpha_1$  subunit consists of four homologous repeats (I–IV) containing six transmembrane  $\alpha$ -helical segments each (S1–S6)(Lacinova, 2005). The linker between segments S5 and S6 of each domain, which

loops back from the extracellular side of the cell membrane (pore loop or P-region), forms the inner pore surface, determining ion conductance and selectivity (Guy and Conti, 1990). With their positively charged residues (arginine or lysine), S4 segments act as the main voltage-sensors, carrying gating charges through the membrane upon opening or closing. It remains a matter of debate how exactly the S4 segments move at gating, and movement as a helical screw or a sweeping paddle have been proposed (Guy and Seetharamulu, 1986; Jiang et al., 2003; Tombola et al., 2006). Negative residues of segments S2 and S3 are thought to create the electric field necessary for movement of S4 (Tombola et al., 2006).

In addition to phosphorylation sites for protein kinases such as PKA, PKC and CAMKII, the  $\alpha_1$  subunit also harbours the so-called alpha subunit interaction domain (AID), an 18 amino-acid-long motif in the I-II linker representing the site of interaction with the  $\beta$  subunit (Pragnell et al., 1994). Not only does the  $\beta$  subunit mediate trafficking of the  $\alpha_1$  subunit to the plasma membrane, partly by masking an endoplasmic reticulum retention signal in the  $\alpha_1$  subunit (Bichet et al., 2000), its association with the  $\alpha_1$  subunit specifically modulates biophysical properties of the channel (Sokolov et al., 2000; Dolphin, 2003). Co-expression with some  $\alpha_2\delta$  subunits such as  $\alpha_2\delta$ -1 enhances membrane trafficking of  $\alpha_1$  subunits and can increase current amplitude and activation and inactivation kinetics as well as induce a hyperpolarizing shift in the voltage dependence of activation (Felix et al., 1997; Gao et al., 2000). Initially,  $\gamma$  subunits were considered to be restricted to skeletal muscle VGCCs, however later studies could prove the existence of a neuronal  $\gamma$  subunit (Letts et al., 1998). In contrast to  $\alpha_2\delta$  and  $\beta$  subunits,  $\gamma$  subunits do not appear to influence surface expression of the channels, instead they appear to only alter biophysical properties of the channel for example:  $\gamma_1$  and  $\gamma_2$  subunits can exert an inhibitory effect on some calcium currents and can modulate activation and inactivation kinetics (Kang et al., 2001; Rousset et al., 2001).

### **1.2.2. Classification of Voltage-gated Calcium Channels**

In accordance with newer nomenclature used for naming sodium and potassium channels, VGCCs are named by the chemical symbol of the permeating ion i.e. (“Ca”), followed by

the physiological regulator (“v” for voltage) in subscript, followed by the number corresponding to the  $\alpha 1$  subunit gene family (1-3), followed by the subunit. Traditionally, VGCCs are grouped into low- and high-voltage activated calcium channels (LVACCs and HVACCs), according to the membrane potential at which the channels activate (Catterall, 2000;Lacinova, 2005;Zamponi et al., 2010). VGCCs can be further grouped into L, N, P/Q, R and T subtypes with L standing for long-lasting current, comprising the dihydropyridine-sensitive channels  $Ca_v1.1$  through  $Ca_v1.4$ . The LVACCs  $Ca_v3.1$ - $Ca_v3.3$  are referred to as “T-type” channels, standing for tiny, transient current, whereas the remaining HVACCs i.e.  $Ca_v2.1$ - $Ca_v2.3$  (P/Q-type, N-type and E/R-type) are often referred to as “non-L-type” channels. Non-L-type HVACCs are insensitive to dihydropyridines but can be inhibited by specific spider and marine snail toxins ( $Ca_v2.1$  by  $\omega$ -agatoxin IVA,  $Ca_v2.2$  by  $\omega$ -conotoxin GVIA and  $Ca_v2.3$  by SNX-482)(Catterall et al., 2005). After blockade of L-type, T-type,  $Ca_v2.1$  and  $Ca_v2.2$  channels, the remaining or resistant current was referred to as R-type current and was later found to be carried by  $Ca_v2.3$  channels (Randall and Tsien, 1995;Zhang et al., 1993). Characterization of this channel proved difficult until the discovery of its fairly specific inhibitor SNX-482 in 1998 (Newcomb et al., 1998), which remains the only specific modulator of  $Ca_v2.3$  to date. However, although not specific,  $Ca_v2.3$  channels are highly sensitive to blockade by  $Ni^{2+}$  and potentially other divalent heavy metal cations (Zamponi et al., 1996;Kang et al., 2006).

### **1.2.3 The $Ca_v2.3$ (R-type) Voltage-Gated Calcium Channel and its Physiological Functions**

The  $Ca_v2.3$  (R-type) voltage-gated calcium channel represents the most enigmatic of all voltage-gated calcium channels due to its pharmacoresistance and to its mixed characteristics of HVA and LVA calcium channels. Its eponymous attribute of pharmacologic inertness initially made in depth investigation of the channel difficult, however the identification of the tarantula toxin SNX-482 as a fairly specific inhibitor of  $Ca_v2.3$  in the nanomolar range has enabled insights into the channels properties.

Ca<sub>v</sub>2.3 splice variants are expressed in several regions of the central nervous system as well as in the heart and endocrine tissues. In the brain high expression levels are found in the hippocampus, cerebellum, neocortex and reticular thalamus (Soong et al., 1993). Ca<sub>v</sub>2.3 triggers the release of several neurotransmitters such as dopamine in the substantia nigra (Bergquist and Nissbrandt, 2003) and contributes to fast glutamatergic transmission (Gasparini et al., 2001) in the hippocampus, where it is also involved in long term potentiation at the mossy fiber – CA3 synapses. By these mechanisms, Ca<sub>v</sub>2.3 is involved in basic processes related to learning and memory formation (Kubota et al., 2001; Isomura et al., 2002; Breustedt et al., 2003; Dietrich et al., 2003). R-type currents are available at resting potential and contribute to after-depolarization, and therefore to the initiation of burst firing in CA1 hippocampal neurons (Metz et al., 2005).

Moreover, R-type currents have been shown to be involved in the secretion of several different hormones. Ca<sub>v</sub>2.3KO mice display disturbances in glucose-induced insulin release (Pereverzev et al., 2002; Jing et al., 2005), glucose-mediated glucagon suppression (Pereverzev et al., 2005), and most importantly in glucose-mediated somatostatin-release (Zhang et al., 2007). SNX-482 sensitive R-type currents have been shown to mediate the release of gonadotropin-releasing hormone (Watanabe et al., 2004) and of oxytocin (Wang et al., 1999; Ortiz-Miranda et al., 2005).

Whether Ca<sub>v</sub>2.3 is functionally expressed in cardiomyocytes is controversial: although Ca<sub>v</sub>2.3 transcripts have been amplified from microscopically identified myocytes (Lu et al., 2004; Weiergräber et al., 2005), Ca<sub>v</sub>2.3 protein have yet to be reliably detected in murine cardiomyocytes. Cardiac arrhythmia and impairment of autonomic cardiac control are displayed by Ca<sub>v</sub>2.3KO mice, suggesting that in pacemaker cells and in autonomic nerve endings R-type currents may be crucial for cardiac rhythmicity (Galetin et al., 2013).

Because Ca<sub>v</sub>2.3KO mice display a subtle phenotype as opposed to an obvious one, and because of the expression of Ca<sub>v</sub>2.3 in rhythmically active tissue, it is conceivable that a general function of R-type currents is “fine tuning” of oscillatory networks. The distinct biophysical properties of Ca<sub>v</sub>2.3 bestow upon the channel the unique capacity to elicit a rapid calcium current at relatively low i.e. close to resting potentials, enabling modulatory effects on oscillatory activity such as on the after-depolarization, necessary

for initiation of burst firing in CA1 hippocampal neurons. One may assume that a loss of “fine-tuning” in Ca<sub>v</sub>2.3KO mice is only minimally noticeable during normal conditions, but becomes evident in certain pathological conditions exerting a strain on an oscillatory network such as during experimentally induced epilepsy.

### **1.3 Ca<sub>v</sub>2.3 in Epilepsy and Antiepileptic Pharmacotherapy**

#### **1.3.1 Ca<sub>v</sub>2.3 VGCCs in Epilepsy**

In some epilepsy syndromes aberrant function or pathologic expression patterns of neuronal VGCCs is a major contributor to hyperexcitability and therefore to epileptogenesis (Turnbull et al., 2005). For example reticular thalamic (RT) neurons of the generalized absence epilepsy rat of Strasbourg (GAERS) display 55% larger LVA calcium currents than controls and also display a 16% increase in mRNA of the LVACC Ca<sub>v</sub>3.2 (Talley et al., 2000;tsakiridou et al., 1995), corresponding to data from human studies identifying CACNA1H, the gene coding for Ca<sub>v</sub>3.2’s functional subunit, as an epilepsy susceptibility gene (Chen et al., 2003). Findings in mice reveal that pilocarpine induced status epilepticus (SE) causes a selective increase in Ca<sub>v</sub>3.2 mRNA and protein in the CA1 region of the hippocampus (Becker et al., 2008). Furthermore, Ca<sub>v</sub>3.2KO mice were protected from hippocampal sclerosis found in CA1 and CA3 regions of control mice 10 days after SE.

Interestingly, GAERS also exhibits changes in Ca<sub>v</sub>2.3 expression: Ca<sub>v</sub>2.3 transcripts have been found to be significantly reduced in the cerebellum and brain stem (De Borman et al., 1999;Lakaye et al., 2002), which projects to rostral reticular thalamic nucleus (rRTn), a key region in the control of thalamic oscillation and bursting and therefore in absence epilepsy. Interestingly, whole-cell current clamp measurements revealed that pharmacologic inhibition or genetic ablation of the Ca<sub>v</sub>2.3 calcium channel not only strongly reduces (and in some cases also almost eliminates) rhythmic bursting, but also reduces the amplitude of the slow AHP following the initial low threshold burst (Zaman et al., 2011). The same study also demonstrates that for bursting, LVACCs recruit Ca<sub>v</sub>2.3 channels to generate depolarizations, providing preliminary evidence for a synergistic method of action of Ca<sub>v</sub>2.3 and Ca<sub>v</sub>3.2 in absence epilepsy. In the genetically epilepsy-

prone rat (GEPR), a model of acoustically evoked seizures (audiogenic epilepsy), Ca<sub>v</sub>2.3 protein and R-type currents have been shown to be increased in the inferior colliculus (IC) compared to controls thereby contributing to hyperexcitability (N'Gouemo and Morad, 2003;N'Gouemo et al., 2010). In the CA1 region of the hippocampus, where R-type currents, have been shown to be increased by stimulation of muscarinic acetylcholine receptors (Meza et al., 1999;Bannister et al., 2004;Tai et al., 2006), increased R-type currents after cholinergic stimulation enhance plateau potentials possibly promoting epileptiform discharges (Williams and Kauer, 1997;Kuzmiski et al., 2005).

### **1.3.2 Experiments with Ca<sub>v</sub>2.3KO Mice Experimental Models of Epilepsy**

A 2006 study investigating the effects of two different chemoconvulsants in Ca<sub>v</sub>2.3KO mice found that while there was no difference in 4-aminopyridine susceptibility between Ca<sub>v</sub>2.3KO and control mice, Ca<sub>v</sub>2.3KO mice were less susceptible to seizures induced by pentylenetetrazol (PTZ), a compound that impairs GABA-mediated inhibitory neurotransmission (Weiergräber et al., 2006). In this study, lethality of Ca<sub>v</sub>2.3KO mice after 80mg/kg s.c. PTZ was significantly lower, as was the latency, duration and frequency of tonic-clonic-seizures. In a later study, the same authors found that in the KA-model of TLE, Ca<sub>v</sub>2.3KO mice displayed strong seizure resistance as well as protection from degeneration of CA3 pyramidal neurons (Weiergräber et al., 2007). 50% of control mice reportedly died as a result of 30 mg/kg i.p. KA and those surviving exhibited a loss of 90% of CA3 pyramidal neurons 7 days later. Contrastingly not one single Ca<sub>v</sub>2.3 mouse died as a result of 30 mg/kg KA with these mice displaying a loss of only 10% of CA3 pyramidal neurons. Interestingly however, although a similar tendency for seizure severity in NMDA induced seizures was found, the authors failed to identify differences in EEG ictal activity between both genotypes, possibly due to the chosen dose of KA, which is quite high.

### **1.3.3 Ca<sub>v</sub>2.3 as a Target of Antiepileptic Drugs**

Ion channels are popular targets for many AEDs, however Ca<sub>v</sub>2.3 is not considered a classical target. Lamotrigine (LTG) was approved by the FDA for the treatment of partial

seizures in 1994, showing favorable pharmacokinetics, improved tolerability and lower potential for drug interactions compared to several older antiepileptic drugs (AEDs). In addition to its approval for maintenance treatment of bipolar I disorder, off-label prescription of LTG is becoming increasingly popular, as it shows therapeutic effects on several neurologic and neuropsychiatric diseases of completely diverse etiologies such as borderline syndrome or cocaine dependence. LTG's wide therapeutic applicability reflects the "unspecificity" of the drug which has shown to modulate several different sodium, calcium and potassium currents (Beck and Yaari, 2012). Anticonvulsive properties of LTG were initially mainly attributed to its capacity to inhibit transient and persistent sodium currents, selectively prolonging slow inactivation thereby suppressing the release of excitatory amino acids (Brodie, 1996; Xie et al., 1995). Over the following years more than 15 other targets of LTG could be identified including several voltage- and ligand-gated cation channels and neurotransmitter receptors and transporters (see Table 1). One of the newly found mechanisms of LTG was potentiation of the outward cation current through hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Poolos et al., 2002), which are localized on CA1 dendrites, where they are proposed to modulate dendritic integration of excitatory input (Magee, 1998; Magee, 1999).

Furthermore, LTG as well as its derivative sipatrigine have been shown to inhibit R-type currents carried by  $Ca_v2.3$  voltage-gated calcium channels in heterologous systems (Hainsworth et al., 2003). In this study, R-type currents from human  $\alpha 1E$  subunits with  $\beta 3$  subunits stably transfected in HEK 293 cells were inhibited by 10  $\mu M$  of LTG, a concentration within the estimated range of therapeutic brain concentrations of 4–40  $\mu M$  (Leach et al., 1995).

Topiramate (TPM) another broad spectrum anticonvulsant was FDA approved two years after LTG in 1996 and like LTG enjoys a wide-spectrum of use from treatment of Lennox-Gastaut Syndrome and bipolar disorder to migraine. In addition to its known mechanisms involving sodium channel blockade, TPM was later found to be an inhibitor of R-type currents at concentration within the estimated range of therapeutic brain concentrations ( $IC_{50} = 50.9 \mu M$ ) (Kuzmiski et al., 2005). In transiently transfected tsA-201 cells, application of 100  $\mu M$  TPM causes a negative shift in the voltage dependence

of steady-state inactivation of Ca<sub>v</sub>2.3. Because the authors also found that R-type inhibition by TPM in CA1 neurons causes a reduction in the Ca<sup>2+</sup> influx, necessary for plateau potential activation, they hypothesize that TPM directly inhibits R-type calcium spike generation by increasing steady-state inactivation of Ca<sub>v</sub>2.3 calcium channels at resting potentials.

**Table 1** Known molecular targets of lamotrigine.

<i>Target / Mechanism</i>	<i>System / Organism</i>	<i>Author</i>
Inhibition of Na <sup>+</sup> current: negative shift of the steady-state inactivation curve, delay of recovery from inactivation,	mouse primary spinal cord neurons; rat primary hippocampal pyramidal neurons; rat cerebellar granule cells; rat primary pyramidal cortical neurons;	<i>Cheung et al 1992 Epilepsy Res</i> <i>Xie et al 1995 Pflugers Arch</i> <i>Zona et al 1997 Epilepsia</i> ; <i>Stefani et al 1997 Exp Neurol</i>
Inhibition of N-type HVACC (Ca <sub>v</sub> 2.2)	rat primary pyramidal cortical neurons; rat amygdalar neurons	<i>Stefani et al 1996 Eur J Pharmacol</i> ; <i>Wang et al 1996 Neuroreport</i>
Inhibition of P-type HVACC (Ca <sub>v</sub> 2.1)	rat primary pyramidal cortical neurons	<i>Stefani et al 1996 Eur J Pharmacol</i>
Inhibition of R-type HVACC (Ca <sub>v</sub> 2.3)	transfected HEK-293 cells	<i>Hainsworth et al 2003 Eur J Pharmacol</i>
Weak inhibition of LVACC through alpha1G subunits (Ca <sub>v</sub> 3.1)	transfected HEK-293 cells	<i>Hainsworth et al 2003 Eur J Pharmacol</i>
Enhancement of K <sup>+</sup> current	rat CA1 pyramidal cells; rat primary cortical neurons and slices	<i>Grunze et al 1998 Brain Res</i> ; <i>Zona et al 2002 Epilepsia</i>
Inhibition of 5-HT-, noradrenaline- and dopamine uptake	human platelets and rat brain synaptosomes	<i>Sotham et al 1998 Eur J Pharmacol</i>
Inhibition of TRPM7 (csNSC)	mouse hippocampal neurons	<i>Xiong et al 2001 J Neurophysiol</i>
Increase of HCN current	rat hippocampal neurons	<i>Poolos et al 2002 Nat Neurosci</i>
Increase of gene expression of GABA <sub>A</sub> R beta3 subunit	primary rat hippocampal neurons	<i>Wang et al 2002 Neuropsychopharmacology</i>
Inhibition of inwardly-rectifying K <sup>+</sup> current	transfected HEK-293 cells	<i>Danielsson et al 2005 Epilepsy Res</i>

Inhibition of monoamine oxidases (MAO) A and B	in vitro assay	<i>Southam et al 2005 Eur J Pharmacol</i>
Phosphorylation of GluR1, enhancing surface expression of GluR1 and GluR2	embryonic rat primary hippocampal neurons	<i>Du et al 2007 Neuropsychopharmacology</i>
Open channel block of nicotinic acetylcholine receptor (nAChR)	transfected CHO-K1/A5 cells	<i>Vallés et al 2007 Neuroreport</i>
Transition from the resting (closed) state to the desensitized state (nAChR)	transfected CHO-K1/A5 cells	<i>Vallés et al 2008 Biochim Biophys Acta</i>
Inhibition of TRESK K <sup>+</sup> channels (KCNK18)	primary rat DRG neurons and transfected COS-7 cells	<i>Kang et al 2008 Biochem Biophys Res Commun</i>
Inhibition of postsynaptic AMPAR	rat primary dentate gyrus granule cells	<i>Lee et al 2008 Epilepsia</i>
Downregulation of COX-2 mRNA and protein	rat frontal cortex	<i>Lee et al 2008 Neurochem Res</i>

## 1.4 Cardiac Phenomena during Epilepsy

### 1.4.1 Cardiac Phenomena in Epileptic Patients

In epileptic patients, seizures have been shown to be preceded and accompanied by ECG changes, fueling the discussion of brain to heart interactions, which may be of great relevance in the context of sudden-unexpected death in epilepsy (SUDEP). Ictal tachycardia is detectable in almost all patients of different age groups (Jansen et al., 2013). Several studies of different epilepsy syndromes have described the occurrence of sympathovagal imbalance (increase of sympathetic and decrease of parasympathetic control of heart rhythm)(Brotherstone and McLellan, 2012;Lotufo et al., 2012;Meghana et al., 2012;Ponnusamy et al., 2012), which is known to contribute to mortality and morbidity in cardiovascular disease (Mortara et al., 1997;Schwartz et al., 1988). Furthermore, pre-ictal tachycardia has been observed in children and adults with generalized seizures (Schernthaner et al., 1999;Jansen et al., 2013), in adults with refractory epilepsy (Zijlmans et al., 2002) and in children with refractory TLE (Mayer et al., 2004).

### **1.4.2 Effects of AEDs on Cardiac Function**

Conflicting data has resulted in an ongoing debate about how AEDs affect cardiac function in epileptic patients, and the clinical relevance thereof. Moreover, mechanisms underlying SUDEP, proposed to be mainly of cardiac and respiratory origin, remain unknown, further complicating the debate. One study found AEDs to ameliorate sympathovagal imbalance (Hallioglu et al., 2008), another found AEDs to reduce ECG power and heart rate variability (HRV) (Lossius et al., 2007), which may predispose epileptic patients to cardiac arrhythmia, a potential contributor to SUDEP. A recently performed meta-analysis identified a trend of increased low frequency power (LF) in patients taking AEDs, (Lotufo et al., 2012), possibly representing a cardiac risk.

In this regard LTG is of special interest, as it modulates several targets which are involved in cardiac pacemaking such as hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which contribute to pacemaking in the sinoatrial and atrioventricular node and Purkinje fibers in the ventricle (DiFrancesco, 1993;DiFrancesco, 1995;Poolos et al., 2002) and the delayed rectifier potassium current, which is crucial for cardiac repolarization and therefore plays a critical role in maintenance of cardiac rhythm (Danielsson et al., 2005). Furthermore,  $Ca_v2.3$  VGCCs, which contribute to cardiac autonomous control and to intrinsic rhythm propagation (Galetin T. et al., 2012) are also inhibited by LTG (Hainsworth et al., 2003), representing another potential arrhythmogenic mechanism. Indeed, based on their own isolated observations, some physicians have voiced concern about unwanted and potentially dangerous cardiac side effects of LTG, especially prolongation of the QT-interval, a risk factor of arrhythmia and sudden cardiac death. This prompted an investigation by GlaxoSmithKline, manufacturer of the initial LTG product Lamictal®, which did not observe QT prolongation or related safety issues (Dixon et al., 2008). However, LTG was found to significantly prolong the PR interval (Matsuo et al., 1993;Dixon et al., 2011). For this reason, and because LTG has been controversially found to increase the risk of SUDEP (Hesdorffer et al., 2011;Aurlien et al., 2012), clarification whether LTG has potentially arrhythmogenic effects on the epileptic heart is of great interest.

## 1.5 Epilepsy and Sleep

### 1.5.1 Physiological Sleep

In physiological sleep, two main states can be distinguished: rapid-eye-movement (REM) sleep and non-rapid-eye-movement (NREM) sleep, which is also referred to as slow-wave-sleep (SWS) (Rechtschaffen and Kales, 1968; Iber et al., 2007). Sleep architecture is one of the rare things many vertebrates have in common: the same cyclic pattern of REM and SWS can be recorded in mammals, birds and monotremes. During the night, SWS and REM sleep phases alternate cyclically with sleep initiating with SWS stage 1 progressing through SWS stages 2 and 3, finally reaching the deepest sleep phase SWS4 (which in newer studies is grouped together with stage 3), before entering REM sleep. This cycle is repeated every 90 to 120 minutes throughout the night, with later cycles lasting slightly longer than earlier ones (Guilleminault and Kreutzer, 2003).

EEG was the first and remains the most important tool in characterizing sleep. Upon falling asleep i.e. transitioning into the first sleep stage (S1) the EEG slows down with alpha waves (8-12 Hz) increasingly becoming substituted by theta waves (4-7 Hz) (Iber et al., 2007). S2 is characterized by sleep spindles, which are visible in the EEG burst of 12-14 Hz oscillations and K-complexes, which are brief negative high-voltage peaks followed by a slow positive complex and a final negative peak (Silber et al., 2007). Slow delta waves (0.5-4 Hz) reflect synchronization of periods of neuronal depolarization or high firing (up-phase) followed by periods of hyperpolarization (down phase) within large areas of the cortex in S3 and globally in S4 (Rechtschaffen and Kales, 1968; Iber et al., 2007; Silber et al., 2007). Sleep-depth is measured as SWS intensity by quantifying delta power by Fast Fourier Transform of the delta band of the EEG. REM sleep is also commonly referred to as “paradoxical sleep”, as like wakefulness it is characterized by a desynchronized EEG, however hippocampal theta rhythms are also present and can be measured depending on the quality of the EEG recording. REM sleep can be further subdivided into intermittent episodes of “phasic REM sleep”, during which muscle

twitches and rapid-eye-movements occur, and persistent “tonic REM sleep”, which is characterized by muscle atonia.

### **1.5.2 Regulation of Sleep**

Duration, timing and depth of sleep are regulated by two interacting factors: a homeostatic factor, regulating depth and duration of sleep, within a circadian rhythm, which in turn is the factor that determines the timing of sleep (Borbely, 1995;Borbely and Achermann, 1999). While SWS activity is not strongly influenced by circadian factors, sleep-depth and therefore delta power as well as the propensity to sleep (=“sleepiness”) are both proportional to the duration of prior wakefulness (Feinberg et al., 1985). Sleep homeostasis thus is dependent on the interval since the last sleep episode and the depth of this sleep. Circadian regulation is demonstrated by the maintenance of a 24 hour rhythm in sleep propensity even in the absence of temporal clues. Circadian rhythm is less dependent on sleep history but instead is generated by an intrinsic pacemaker that regulates several physiological processes and variables such as core body temperature, and production and secretion of hormones such as cortisol and melatonin. The circadian rhythm of human sleep propensity is approximately the inverse of the core body temperature rhythm: maximal sleep propensity and the highest continuity of sleep occur in proximity to the minimum temperature (Dijk and Czeisler, 1993).

### **1.5.3 The Relationship between Sleep and Epilepsy**

The bidirectional relationship of sleep and epilepsy has been acknowledged for a long time; however underlying interdependent mechanisms have yet to be fully understood despite decades of experimental and clinical research. Not only does epilepsy impact sleep (epileptic patients display a variety of sleep disturbances) but sleep is known to impact epilepsy, the simplest example being the pro-convulsive effect of sleep deprivation. In most human epilepsy syndromes seizures and inter-ictal epileptiform discharges (IED) are precipitated during SWS and relatively inhibited by REM sleep (Bazil and Walczak, 1997;Malow et al., 1998;Kumar and Raju, 2001). The relationship between spike-and-wave discharges (SWD), the hallmark of absence epilepsy, and SWS has been well established (Shouse et al., 1996) and is known to contribute to electrical

status epilepticus in sleep (ESES) (Nickels and Wirrell, 2008). Continuous spike-and-wave activity during slow sleep (CSWS) are classified by the International League Against Epilepsy (ILAE) as an epilepsy syndrome but also represent an EEG pattern occurring in other epilepsy syndromes such as Landau-Kleffner syndrome (Veggiotti et al., 1999). Based on the available data, the concept of physiological brain oscillations being “hijacked” and used as a template from which paroxysmal waveforms are generated has gained wide acceptance (Beenhakker and Huguenard, 2009). Indeed transition from cortical slow oscillations (CSO) to SWD has been demonstrated in epileptic patients and animals (Steriade and Amzica, 1994; Steriade and Amzica, 2003; Tucker et al., 2009).

## **1.6 Experimental Goals**

The goal of the present project was to perform an in depth investigation of the role of R-type signaling in the epileptic brain and heart, by analyzing its contribution to antiepileptic pharmacotherapy and sleep. To date a third of epileptic patients suffer from pharmacoresistant epilepsy, with no hope for pharmacological seizure control and the constant fear of cognitive deterioration and sudden death. Therefore identification of novel pharmacological targets is of urgent importance.

## 2. RESULTS

### 2.1 Ca<sub>v</sub>2.3 (R-type) calcium channels are critical for mediating anticonvulsive and neuroprotective properties of lamotrigine in vivo

**Maxine Dibué**, Marcel A. Kamp, Serdar Alpdogan, Etienne E. Tevoufouet, Wolfram F. Neiss, Jürgen Hescheler, Toni Schneider

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<sub>v</sub>2.3 (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 two other AEDs in Ca<sub>v</sub>2.3-deficient mice and controls on KA-induced seizures. 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 30 mg/kg LTG, 50 mg/kg topiramate (TPM) or 30 mg/kg lacosamide (LSM).

Ablation of Ca<sub>v</sub>2.3 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<sub>v</sub>2.3-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 AEDs reduced seizure activity in control mice, however only the non-calcium channel modulating AED, LSM had an anticonvulsive effect in Ca<sub>v</sub>2.3-deficient mice. Furthermore LTG altered electrocorticographic parameters differently in the two genotypes, decreasing relative power of ictal spikes in control mice but increasing relative power of high frequency fast ripple discharges during seizures in Ca<sub>v</sub>2.3-deficient mice.

These findings give first in vivo evidence for an essential role for Ca<sub>v</sub>2.3 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 by increasing high frequency components of seizures resulting in increased neurotoxicity in the CA1. 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.

### **Own Contribution to Publication I**

I independently conceptualized and designed this study after establishing the hypothesis, that LTG would lack antiepileptic potency in  $Ca_v2.3$ KO mice with my principal investigator Prof. Toni Schneider. I performed all data analysis on my own, as well as writing the manuscript and creating all images. I performed surgery on the mice, initially under supervision of Dr. Kamp. I isolated membrane fractions from mouse brains with the assistance of Serdar Alpdogan. I performed immunohistochemistry on paraffin sections, western blot of membrane fractions, electrocorticography and behavioral seizure analysis without assistance. Epilepsy induced by kainic acid injection and all pharmacological treatment (antiepileptic and analgesic post-operative care) was carried out by me. I also handled the submission and review process of the manuscript.

**All images were created by Maxine Dibué depicting data collected by Maxine Dibué.**

### **Contribution of Co-authors to Publication I**

As a neurosurgeon, Dr. Kamp initially supervised me during mouse surgery, and aided in the optimization of the procedure. Serdar Alpdogan, at that time a Bachelor's student assisted me during isolation of membrane fractions. Etienne E. Tevoufouet was involved with establishing the novel method of membrane fraction isolation our lab. Prof. Wolfram Neiss prepared paraffin sections of brains from mice used in the other experiments. Prof. Hescheler provided the facilities for experimentation and Prof. Schneider, as principal investigator supervised and chaperoned the project, making adjustments to experimental procedures, aiding in interpreting data and proof-reading the manuscript.

## **2.2 Cardiac Phenomena During Kainic-acid Induced Epilepsy and Lamotrigine Antiepileptic Therapy**

**Maxine Dibué**, Marcel A. Kamp, Felix Neumaier, Hans-Jakob Steiger, Daniel Hänggi, Jürgen Hescheler, Toni Schneider.

Pathologic ECG events are known to accompany seizures and to persist in several chronic epilepsy syndromes. The contribution of antiepileptic drugs (AEDs) to these events and the implications in the etiology of sudden-unexpected death in epilepsy (SUDEP) continue to be a matter of debate. We therefore investigated cardiac parameters during kainic-acid (KA) induced experimental epilepsy and antiepileptic treatment with lamotrigine (LTG). Epilepsy was induced in seven C57Bl/6 mice by injections of KA (20 mg/kg) on days 1 and 5, which produced severe acute seizures and spontaneous seizures 10 days later. Treatment with LTG (30 mg/kg) was initiated on day 11 and repeated on day 12. Continuous ECGs and ECoGs were collected telemetrically from freely moving mice. Mice displayed pre-ictal but not ictal tachycardia. The squared coefficient of variation (SCV) of R—R intervals was significantly elevated 30 s before and during seizures compared to control conditions. LTG produced a significant reversible increase in SCV and LF/HF ratio during slow-wave sleep (SWS), potentially indicative of sympatho-vagal imbalance during this state of vigilance, in which epileptic patients are known to be particularly vulnerable to SUDEP. The KA model used in this study permits the investigation of cardiac phenomena during epilepsy, as it features many effects found in human epileptic patients. Increased LF/HF, a known risk factor for cardiac disease, which is often found in epileptic patients, was observed as a side-effect of LTG treatment during SWS, suggesting that LTG may promote imbalance of the autonomous nervous system in epileptic mice.

### **Own Contribution to Publication II**

I formulated the hypothesis for this project, based on observations I had made in previous experiments and designed the study accordingly. After optimizing the surgical procedure with Dr. Kamp and Prof. Hänggi, I performed surgery on mice. Epilepsy induced by kainic acid injection and all pharmacological treatment (antiepileptic and analgesic post-operative care) was carried out by me. I independently acquired all data, analyzed all data, wrote the manuscript and created all images. I also handled the submission and review process of the manuscript.

**All images were created by Maxine Dibué depicting data collected by Maxine Dibué.**

### **Contribution of Co-Authors to Publication II**

As neurosurgeons, Dr. Kamp and Prof. Hänggi aided in the optimization of the surgical procedure. Felix Neumaier was crucial in overcoming technical difficulties concerning signal quality of both simultaneously recorded biopotentials (ECG and ECoG). Prof. Hescheler and Prof. Steiger provided the facilities for experimentation and Prof. Schneider, as principal investigator supervised and chaperoned the project, proof-reading the manuscript.

## 2.3 Ca<sub>v</sub>2.3 E-/R-type voltage-gated calcium channels modulate sleep in mice

Angela Münch\*, **Maxine Dibué\***, Jürgen Hescheler, Toni Schneider

\*contributed equally

Mammalian sleep is characterized by cycles of REM and non-REM (NREM), i.e. slow-wave sleep (SWS) phases. The major neuroanatomical basis of SWS is the thalamocortical circuitry, which operates in different functional modes to determine the state of vigilance. At high vigilance, the tonic mode predominates; stages of low vigilance and SWS are characterized by rebound burst firing. Electrophysiologically, rebound bursting depends on low-threshold Ca<sup>2+</sup> spikes and T-type Ca<sup>2+</sup> channels have been shown to modulate SWS. We recently demonstrated that Ca<sub>v</sub>2.3 R-type Ca<sup>2+</sup> channels are capable of modulating absence seizures, a pathophysiological aberration of the thalamocortical oscillations related to SWS. We thus analyzed sleep architecture in control and Ca<sub>v</sub>2.3(-/-) mice using implantable electroencephalography (EEG)/electromyography (EMG) radiotelemetry during spontaneous and urethane-induced sleep. The results demonstrate significantly reduced total sleep time and impairment of SWS generation in Ca<sub>v</sub>2.3(-/-) mice, which affects global sleep architecture (i.e. the ratio of REM to NREM). Furthermore, the relative  $\delta$  power is significantly reduced in Ca<sub>v</sub>2.3(-/-) mice during NREM sleep although these mice display longer prior wakefulness, possibly indicating disturbances in sleep homeostasis. This observation is supported by recordings following urethane administration. This is the first study to shed light on the fundamental role of Ca<sub>v</sub>2.3 channels in rodent sleep physiology.

### **Own Contribution to Publication III**

I performed frequency domain analysis for this study and wrote the manuscript with the exception of the results section of the time domain analysis, which was written by Prof. Schneider. I created image 4 and handled the review process of the manuscript.

#### **Figure 4: Spectral analysis of normal sleep from control and $Cav2.3$ -deficient mice**

This image was created by **Maxine Dibué** depicting results from the spectral analysis performed by **Maxine Dibué**.

### **Contribution of Co-Authors to Publication III**

Angela Münch performed surgery on the mice, recorded electrocorticograms and created images 2 and 3. Next to designing, supervising and chaperoning the project Prof. Schneider created images 1 and 5. Prof. Hescheler provided the facilities for experimentation

## **3. DISCUSSION**

### **3.1 Results from the three presented studies in summary:**

1. R-Type calcium channels are critical in epileptogenesis.
2. R-Type calcium channels are critical in mediating anticonvulsive and neuroprotective effects of LTG.
3. R-type calcium channels are critical in maintenance of SWS
4. LTG promotes sympathovagal imbalance during SWS of epileptic mice
5. In the absence of R-type calcium channels LTG enhances ultra-high frequency oscillations, promoting neurodegeneration in the CA1 region of the hippocampus.

### **3.2 Ca<sub>v</sub>2.3 in Oscillatory Networks**

#### **3.2.1 R-type Currents in Thalamo-Cortical and Hippocampal Rhythmicity**

Viewing the above results in summary, the importance of R-type signaling in the generation and maintenance of rhythmic activity such as in the delta rhythm of slow wave sleep or in repetitive spiking in the delta-theta range during experimentally induced epilepsy becomes evident. Spike-and-wave discharges (SWDs), the hallmark of absence epilepsy reflect pathological hyperoscillations generated by bidirectional thalamo-cortical circuitry. Accordingly, Zaman et al recently demonstrated in brain slices that Ca<sub>v</sub>2.3 channels are critical for oscillatory burst discharges in the reticular thalamus (RT) and that Ca<sub>v</sub>2.3KO mice display decreased sensitivity to  $\gamma$ -butyrolactone-induced absence epilepsy (Zaman et al., 2011). This study revealed that oscillatory burst discharges and the slow after-hyperpolarization (AHP) were both significantly reduced in RT neurons in brain slices from Ca<sub>v</sub>2.3KO mice. Contrarily, Weiergräber et al found increased susceptibility of Ca<sub>v</sub>2.3KO mice to  $\gamma$ -butyrolactone-induced absence epilepsy: in Ca<sub>v</sub>2.3KO mice the latency to SWDs and the number of SWD episodes increased, however contrarily the duration of SWD episodes was reduced (Weiergräber et al., 2008). However, Zaman et al point out that their data may not be directly comparable to that of Weiergräber et al due to their use of monopolar EEGs instead of the bipolar subtraction

method used by Weiergräber et al. Zaman et al found SWDs in bipolar recordings to be 10-fold smaller than in monopolar recordings, possibly due to cancellation of the hemispherically symmetrical synchronous SWDs characteristic of absence seizures. Nevertheless, both studies report altered ictal activity of  $Ca_v2.3$ KO mice after systemic administration of  $\gamma$ -butyrolactone and in addition, the direct role of R-type currents in oscillatory bursting of RT neurons has been elucidated.

RT neurons are reciprocally connected to thalamocortical (TC) neurons, which they hyperpolarize by GABA release upon bursting. The hyperpolarized membrane potential deinactivates low-voltage gated calcium channels enabling regenerative calcium spikes promoting bursting of TC neurons, which in turn causes excitation of RT neurons, initiating the next cycle of bursting. Rebound burst firing of RT neurons is known to occur during cortical slow-wave activity, proposed to serve as a motif from which ictal activity can evolve (Beenhakker and Huguenard, 2009). It is therefore conceivable that, genetic ablation or modulation of  $Ca_v2.3$  by LTG significantly alters bursting of RT neurons and post-inhibitory rebound firing of TC neurons, possibly disturbing slow-wave sleep and preventing the generation of SWDs.

Similar mechanisms are conceivable in the hippocampus, where  $Ca_v2.3$  also contributes to AHP and thereby to bursting (Metz et al., 2005) and is suggested to mediate atropine-sensitive theta oscillations (Muller et al., 2012). In hippocampal CA1 neurons during carbachol-induced epilepsy, R-type currents are enhanced, promoting  $Ca^{2+}$  spikes and thus plateau potentials, which share characteristics with ictal depolarizations (Kuzmiski et al., 2005; Tai et al., 2006). Here inverse effects of LTG in  $Ca_v2.3$ KO mice may reflect post-inhibitory rebound firing of CA1 pyramidal neurons after stimulation of HCN channels, a paradoxical phenomenon observed after increased inhibition via HCN currents as a reaction to experimentally induced seizures (Chen et al., 2001). As the neuroprotection and seizure resistance of  $Ca_v2.3$ KO mice, may involve increased synaptic inhibition due to robustly reduced calcium influx into hippocampal neurons, rebound excitation of CA1 pyramidal neurons may be increased to a greater degree in  $Ca_v2.3$ KO mice when HCN currents are stimulated by LTG.

### **3.2.2 R-type Signaling as a Mediator of Sleep Homeostasis –an Intersection Point between Sleep and Epilepsy?**

It is remarkable that the genetic ablation of a calcium channel that is expressed at relatively low levels is sufficient to disturb sleep depth but also sleep homeostasis itself, considering all the neurotransmitter systems and brain structures involved in sleep and its homeostasis.  $Ca_v2.3$ KO mice sleep less and display less SWS than controls. If homeostatic mechanisms were intact,  $Ca_v2.3$ KO mice should compensate for lack of SWS or sleep in general by increasing delta-power i.e. the depth of sleep which they do not. However, one may also argue inversely i.e. that reduced delta-power, that  $Ca_v2.3$ KO display would be compensated by increased sleep time if homeostatic mechanism were intact. In either way, one may hypothesize that this points to impaired generation and maintenance of highly synchronized delta-activity due to lack of R-type signaling, which ultimately undermines homeostatic mechanisms. Delta activity reflects highly synchronized switching of cortical cells between a hyperpolarized and a depolarized state. Delta rhythm occurs in both isolated thalamic and cortical slices, however is more regular *in vivo* and therefore thought to be cortically generated but also thalamically driven. The hyperpolarization required for delta-initiation is induced by corticothalamic volleys, pointing to GABA-ergic RT neurons in facilitation and synchronization of delta rhythm (Steriade et al., 1991). Thalamically generated delta rhythm is the result of interplay between T-type and HCN currents. During SWS, hyperpolarized TC neurons initiate slow activation of HCN currents, which depolarize the cell triggering rebound bursting, mediated by LVACCs, which were de-inactivated by the hyperpolarization (McCormick and Pape, 1990;Llinas and Steriade, 2006). Both channels inactivate during the burst, promoting hyperpolarization, which in turn initiates the next cycle of bursting. As post-inhibitory rebound activation of T-type currents was found to recruit R-type currents mediating the burst response, this may be the point at which R-type ablation impairs delta-activity. This impairment however, may be at the root of the seizure resistance of  $Ca_v2.3$ KO mice: impairment of the thalamic “bursting apparatus” may

hamper SWS but may prevent the generation of SWDs, lending weight to the widely accepted hypothesis that SWDs “mutate” from slow oscillations. Although speculative, one may hypothesize that the impairment of SWS by LTG found in epileptic patients (Foldvary et al., 2001;Placidi et al., 2000b;Placidi et al., 2000a) corresponds to inhibition of R-type currents, as it mirrors the findings from Cav2.3KO mice. Reduction of SWS time and intensity may represent a key antiepileptic strategy of LTG, as seizures are precipitated by SWS and inhibited by REM sleep, (Bazil and Walczak, 1997;Malow et al., 1998;Kumar and Raju, 2001), which we found to be significantly increased in Cav2.3KO mice.

### **3.2.3 Ca<sub>v</sub>2.3, a “Fine Tuner” of Oscillatory Activity?**

Intrinsic properties of Ca<sub>v</sub>2.3 may enable this channel to exert a specialized pacemaking function. This may also be reflected by its’ expression in oscillating tissues such as the heart, RT, hippocampus and endocrine pancreas. A calcium channel capable of eliciting a rapid calcium influx, due to fast activation and deactivation kinetics at an activation potential much lower than all other HVACCs and much higher than LVACCs is unique and may represent a key element in certain physiological rhythms such as bursting in RT neurons. The role of T-type channels in bursting of RT neurons has been studied in great detail and it appears that due to their slow kinetics T-type VGCCs are more suitable for sustaining slow pacemaker activity such as post-inhibitory rebound firing, whereas R-type VGCCs can convey a more rapid and transient influx of calcium (Randall and Tsien, 1997). In RT neurons post-inhibitory rebound activation of T-type VGCCs recruits R-type VGCCs which then mediate the burst response (Zaman et al., 2011). Furthermore, activation of R-type currents also promotes the calcium-dependent slow AHP in RT neurons, which increases burst firing and is crucial for intrinsic rhythmic discharge within RT neurons reinforcing synaptic network activity (Paz and Huguenard, 2012). Data from Cav2.3KO mice, which display reduced rebound bursts, reduced post-burst AHPs as well as a severely impaired ability of the neuron to discharge oscillatory bursts (Zaman et al., 2011) depicts how R-type signaling is crucial for sustaining physiological rhythmic activity of an entire network despite relatively low expression levels.

### 3.3 Ca<sub>v</sub>2.3 as a Key Player in Lamotrigine Pharmacology

The investigation of Ca<sub>v</sub>2.3's eligibility as an antiepileptic target is complicated by the lack of specific modulators that can be employed in *in vivo* models. SNX-482, the only Ca<sub>v</sub>2.3 specific inhibitor is a small peptide which is very costly due to its recombinant generation and which would have to be applied intra-theccally, rendering its use inefficient in animal models. Our investigation of LTG as a potent but unspecific Ca<sub>v</sub>2.3 inhibitor in Ca<sub>v</sub>2.3KO mice and controls reveals the importance of Ca<sub>v</sub>2.3 inhibition in the antiepileptic and neuroprotective mechanisms of this unspecific drug. It is therefore conceivable that R-type inhibition alone could represent a novel and effective antiepileptic and neuroprotective strategy, with lesser unwanted side-effects.

The paradoxical effects caused by LTG in Ca<sub>v</sub>2.3KO mice found in our study may potentially represent the first experimental description of paradoxical effects of LTG found in epileptic patients. In severe myoclonic childhood epilepsy, there is a frequent aggravating effect (clinical and EEG) of LTG at therapeutic doses (Guerrini et al., 1998;Genton, 2000). Another study reports, pro-ictogenic effects of LTG in adults: patients with idiopathic generalized epilepsies treated with LTG experienced exacerbation or de novo appearance of myoclonic jerks (Crespel et al., 2005). In Unverricht-Lundborg Disease, a progressive myoclonic epilepsy with tonic-clonic seizures, LTG had an aggravating effect in three out of five patients and had no therapeutic effect in the other two patients (Genton et al., 2006). Furthermore, aggravation of absence seizures by LTG leading to absence status epilepticus (Hasan et al., 2006) and seizure deterioration, appearance of a new seizure type, and transient cognitive impairment in idiopathic rolandic epilepsy at a low LTG dose after slow titration have been reported (Cerminara et al., 2004). As the occurrence of paradoxical effects of LTG does not appear to be restricted to specific epilepsy disorders or to a certain age group, identification of underlying mechanisms remains difficult. However paradoxical effects of LTG may be symptomatic of misbalance of the various mechanisms of the drug.

Furthermore, LTG also offers another valuable insight into the relationship between sleep and epilepsy in regard to cardiac mechanisms, which next to respiratory mechanisms are proposed to underlie SUDEP. Only during slow-wave sleep i.e generalized delta-activity does LTG affect autonomous control of the heart. One may hypothesize that inhibition of “fine-tuning” of delta rhythm, which is highly synchronized throughout the cortex, has downstream effects possibly affecting autonomous nervous control of cardiac activity. The identified increase in sympathetic activity during slow wave sleep, reflected by an increase in LF/HF ratio, a known risk factor in heart disease may represent a possible mechanism by which LTG could increase the risk for SUDEP as found by (Hesdorffer et al., 2011;Aurlien et al., 2012). LTG-induced increased sympathetic activity during SWS may also reflect impaired SWS, found in LTG-treated patients, as parasympathetic tone is known to dominate during SWS, the opposite applying to REM sleep (Tobaldini et al., 2013).

### **3.4 Concluding Remarks**

For an unlucky few, therapy resistant epilepsy is a frightening debilitating and life-threatening burden, bringing great sadness and distress to sufferers and their families. Here, it is evident that the classic targets of AEDs such as GABA receptors, sodium channels and T-type calcium channels are not suitable, rendering the search for new targets vital. A growing body of evidence implicates the “pharmacoresistant” R-type calcium channel to be involved in epileptogenesis, antiepileptic pharmacotherapy and mechanisms associated with epilepsy such as SWS. The fact that  $Ca_v2.3$ KO mice display impaired delta rhythm generation and reduced seizure susceptibility may reflect an essential link between slow-wave sleep and epilepsy and the role of R-type signaling therein. It is therefore conceivable that pharmacological inhibition of R-type currents has the potential to prevent seizures developing from delta waves during slow-wave sleep, a state during which epileptic patients are at higher risk of seizures and SUDEP. Development of specific  $Ca_v2.3$  inhibitors, which can be administered orally and

intravenously, could represent a new frontier in epilepsy therapy, possibly bringing hope to the millions of sufferers of therapy refractory epilepsy disorders.

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# Curriculum Vitae

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Cardiac phenomena during kainic-acid induced epilepsy and lamotrigine antiepileptic therapy. *Epilepsy Res.* 2014 May;108(4):666-74.
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APLP1 and Rab5A interact with the II-III loop of the voltage-gated Ca-channel Ca(v)2.3 and modulate its internalization differently. *Cell Physiol Biochem*. 2011 Dec;28(4):603-12.

## **Erklärung:**

Ich versichere, dass ich die mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten, und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. rer. nat. Toni Schneider betreut worden.

### Publikationen:

Cav 2.3 (R-type) calcium channels are critical for mediating anticonvulsive and neuroprotective properties of lamotrigine in vivo.

Dibué M, Kamp MA, Alpdogan S, Tevoufouet EE, Neiss WF, Hescheler J, Schneider T.  
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Cardiac phenomena during kainic-acid induced epilepsy and lamotrigine antiepileptic therapy.

Dibué M, Kamp MA, Neumaier F, Steiger HJ, Hänggi D, Hescheler J, Schneider T.

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Cav2.3 E-/R-type voltage-gated calcium channels modulate sleep in mice.

Münch A, Dibué M, Hescheler J, Schneider T.

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Maxine Dibué-Adjei

## FULL-LENGTH ORIGINAL RESEARCH

# Ca<sub>v</sub>2.3 (R-type) calcium channels are critical for mediating anticonvulsive and neuroprotective properties of lamotrigine in vivo

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### SUMMARY

**Purpose:** 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<sub>v</sub>2.3 (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 two other AEDs in Ca<sub>v</sub>2.3-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 30 mg/kg LTG, 50 mg/kg topiramate (TPM), or 30 mg/kg lacosamide (LSM).

**Key Findings:** Ablation of Ca<sub>v</sub>2.3 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<sub>v</sub>2.3-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 AEDs reduced seizure activity in control mice; however, only the non-calcium channel modulating AED, LSM, had an anticonvulsive effect in Ca<sub>v</sub>2.3-deficient mice. Furthermore, LTG altered electrocorticographic parameters differently in the two genotypes: decreasing relative power of ictal spikes in control mice but increasing relative power of high frequency fast ripple discharges during seizures in Ca<sub>v</sub>2.3-deficient mice.

**Significance:** These findings provided the first in vivo evidence for an essential role for Ca<sub>v</sub>2.3 in LTG pharmacology and shed light on a paradoxical effect of LTG in their absence. Furthermore, LTG appears to promote ictal activity in Ca<sub>v</sub>2.3-deficient mice by increasing high frequency components of seizures, resulting in increased neurotoxicity in the CA1. 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.

**KEY WORDS:** Lamotrigine, R-type, Ca<sub>v</sub>2.3, Antiepileptic drugs, Toxicity, Quantitative electroencephalography.

Today lamotrigine (LTG) is among the most prescribed antiepileptic drugs (AEDs) worldwide. In addition, LTG is approved by the U.S. Food and Drug Administration (FDA) for treatment of bipolar disorder and has become a popular off-label drug for treatment of other neurologic and psychiatric conditions such as borderline personality disorder. This diverse therapeutic capacity of LTG probably reflects the nonspecificity of the drug, which is known to inhibit several different calcium, potassium, and sodium currents

(Beck & Yaari, 2012). 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 Ca<sub>v</sub>2.3 channels could play an important role in the mechanism of action of LTG during experimentally induced epilepsy. It has been demonstrated that LTG and another modern AED, topiramate (TPM), inhibit R-type currents in heterologous systems and brain slices (Hainsworth et al., 2003; Kuzmiski et al., 2005). Furthermore, Ca<sub>v</sub>2.3-deficient (Ca<sub>v</sub>2.3-KO) mice display seizure resistance and reduced hippocampal neurotoxicity after kainic acid (KA) injection (Weiergräber et al., 2007). Parenteral administration of KA is a well-established method of modeling temporal lobe epilepsy, causing

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seizures in the hippocampus and temporal lobe and degeneration of hippocampal pyramidal neurons (Tremblay & Ben Ari, 1984; Sperk et al., 1985). Using the KA model of temporal lobe epilepsy, we investigated the effect of LTG next to TPM and lacosamide (LSM) in Ca<sub>v</sub>2.3-KO and controls. The new AED lacosamide (LSM), which enhances slow inactivation of voltage-gated sodium channels (Errington et al., 2008), was used as a positive control, as it has been shown not to effect calcium or potassium currents (Errington et al., 2006; Wang & Khanna, 2011). Gaining insight into the role of Ca<sub>v</sub>2.3 calcium channels in antiepileptic pharmacotherapy may allow identification of new antiepileptic mechanisms and therefore of novel potential drug targets, offering hope for patients with drug refractory epilepsy.

## MATERIALS AND METHODS

### Animals

Ca<sub>v</sub>2.3-KO and control mice are separate mouse lines derived from heterozygous parents (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 (Pereverzev et al., 2002; Weiergräber et al., 2006). Mice were kept at 20°C in polycarbonate cages under a 12 h 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 a.m., saline or AEDs 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 intraperitoneally into male mice of both genotypes (Ca<sub>v</sub>2.3-KO and Ca<sub>v</sub>2.3+/+) within the age range of 20–25 weeks. One hour later 20 mg/kg KA (n = 40) or 30 mg/kg KA (n = 46) (Sigma Aldrich) dissolved in saline was injected intraperitoneally. Immediately after administering KA, mice were set in separate single cages and filmed for the next 24 h.

### Behavioral seizure analysis

The initial 2 h after the application of KA was evaluated using an adapted version of Morrison's Seizure Rating Scale (Morrison et al., 1996) (see Data S1 for further information):

- 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 2 h period the highest seizure score was noted for every 5-min interval. Interval scores were added for total seizure scores. Total seizure scores of pretreated groups were compared to those of the untreated group of the same genotype and differences were expressed as relative changes in total seizure score from the untreated group.

### Radiotelemetric electrocorticographic recording of seizures

Radiotelemetric electrocorticography (ECoG) of KA-induced seizures was recorded on LTG pretreated and untreated animals of both genotypes (n = 4 per group). Animals were anesthetized with 100 mg/kg body weight (BW) ketamine hydrochloride (Ketanest, Parke-Davis/Pfizer, Berlin, Germany) and 10 mg/kg BW xylazine hydrochloride (RompunR 2%; Bayer Vital, Leverkusen, Germany). TL11M2-F20-EET transmitters (Datascience International, Lexington, MA, U.S.A.) were implanted subcutaneously and burr holes were 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, Kent, United Kingdom). Animals were allowed 7 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. ECoG studies were obtained at a sampling rate of 1,000 Hz without cutoff from freely moving animals in their cages, which were placed on the telemetry receiver platforms.

### ECoG analysis

NEUROSCORE 2.1.0 (Datascience International) 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 (16–24 Hz), Gamma (30–80 Hz), Ripples (80–200 Hz), and Fast Ripples (200–500 Hz). An automated seizure detection protocol was written to quantify ictal activity. The protocol recognizes waveforms shorter than 200 msec that are between 2.5- and 25-fold the baseline amplitude as spikes. Spikes occurring in intervals between 30 and 1,500 msec are recognized as belonging to a spike train, which must be at least 300 msec 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 the following equation:  $(LF - HF)/(LF + HF)$ .

### Histology and immunohistochemistry

Seven days after injection of 30 mg/kg, KA brains were extracted and kept in 30% sucrose for 24 h prior to freezing them in methyl-butane. Brains were sliced 10  $\mu$ m thick in a cryotome (CM3050S; Leica Microsystems, Wetzlar, Germany), and then fixed in 4% formaldehyde and Nissl stained according to standard protocol. Brains for immunohistochemistry were kept in 4% paraformaldehyde for 24 h and—using a slow regimen 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, U.S.A.). 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 [Wisniewski, 2009]). Fifty micrograms of membrane protein per sample were separated by electrophoresis on an sodium dodecyl sulfate polyacrylamide gel and then blotted onto a polyvinylidene fluoride membrane. The  $Ca_v2.3$  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  $\alpha 1E$  subunit (for further information see [Pereverzev et al., 1998]), ECL-Anti-Rabbit IgG and ECL detection system (GE Healthcare, Buckinghamshire, United Kingdom). Because the expression of the reference protein synaptophysin (SYN) has been shown to be unaffected by hyperexcitation (Chen et al., 2001a; Wierschke et al., 2010),  $Ca_v2.3$  bands were quantified by normalizing them to SYN, which was detected using anti-SYN antibody from mouse (Antibodies-online, Atlanta, GA, U.S.A.) and ECL-Anti-Mouse IgG (GE Healthcare).  $Ca_v2.3$  protein was quantified manually using IMAGEJ 1.46 (NIH) and automatically using GELSCAN 6.0 (BioSciTec, Frankfurt, Germany).

### Statistical analysis

Seizure scores and relative spectral power were assessed using the Shapiro-Wilk test of normality and found to be mostly nonnormally distributed. Therefore, the nonparametric Mann-Whitney test was used to determine significance of

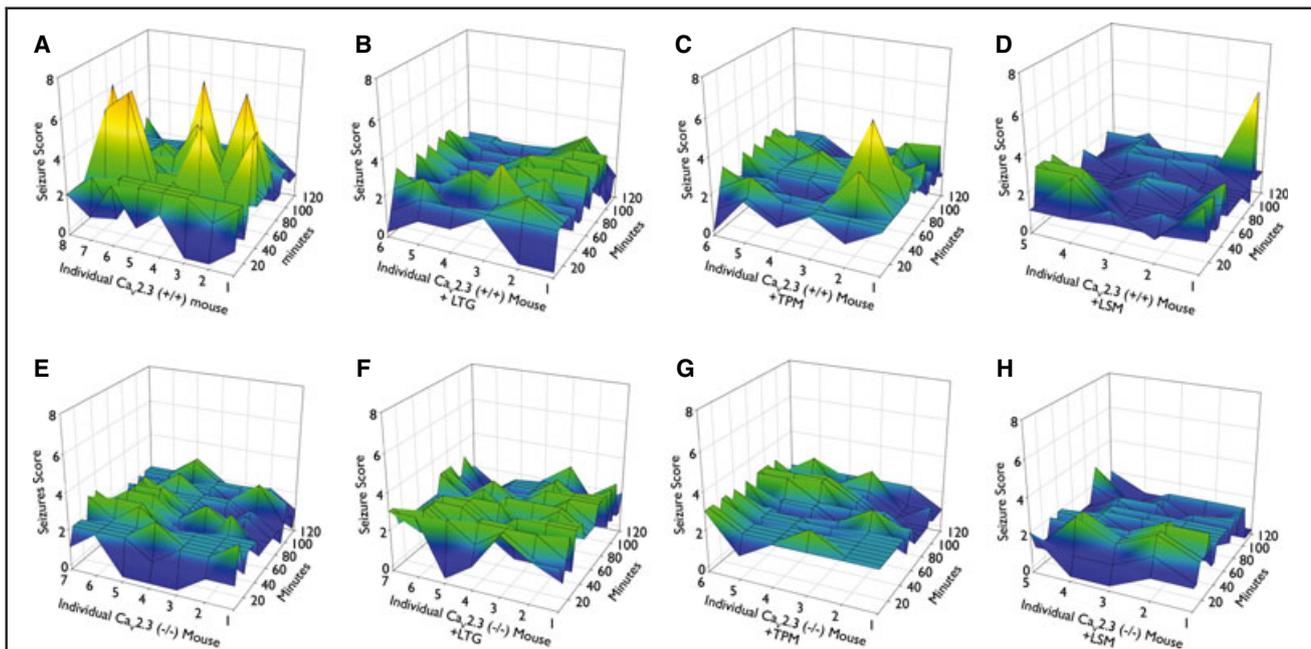
seizure scores. Relative power values were log transformed ( $\log(x/[1 - x])$ ) to obtain a more Gaussian distribution and were then subjected to analysis of variance (ANOVA) (Gasser et al., 1982). Statistical significance of frequencies of the seizure stages was determined using Fisher's exact probability test.  $p$ -Values of 0.05 and below were considered statistically significant.

## RESULTS

### Behavioral seizure analysis

After injection of 20 mg/kg KA in all groups, normal explorative behavior ceased within 10 min 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 of eight control mice experienced tonic-clonic seizures (Fig. 1A), whereas  $Ca_v2.3$ -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  $57.8 \pm 2.6$  to  $41.4 \pm 3.7$ ,  $p = 0.0012$ ;  $U = 2.5$ ) (Fig. 2A). In control animals, LTG prevented tonic-clonic seizures (Fig. 1B) and reduced total seizure scores by 23.2%, from  $57.8 \pm 2.6$  to  $44.3 \pm 3.6$  ( $p = 0.02$ ;  $U = 6.5$ ). TPM did not prevent tonic-clonic seizures in all control mice (Fig. 1C) but reduced total seizure scores by 21%, from  $57.8 \pm 2.6$  to  $45.6 \pm 3.8$  ( $p = 0.029$ ;  $U = 5$ ). LSM was most effective in reducing seizure scores in control mice, eliciting a reduction of the total seizure score of 42.2%, from  $57.8 \pm 2.6$  to  $33.4 \pm 2.5$  ( $p = 0.0016$ ;  $U = 0$ ) (Fig. 1D). TPM had no significant effect on total seizure scores in  $Ca_v2.3$ -KO mice, whereas LTG significantly increased total seizure scores by 22.1%, from  $41.4 \pm 3.7$  to  $50.6 \pm 1.5$  ( $p = 0.018$ ;  $U = 6.5$ ) and the frequency of the convulsive stage 3 (Fig. 1F and Table S1) in  $Ca_v2.3$ -KO mice. Both LTG and TPM were effective in reducing total seizure scores of control mice but were ineffective in doing the same in  $Ca_v2.3$ -KO mice. LSM was the only AED of the three that reduced seizure scores in  $Ca_v2.3$ -KO mice, doing so by 19.4%, from  $41.4 \pm 3.7$  to  $33.4 \pm 0.6$  ( $p = 0.048$ ;  $U = 5$ ) (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  $Ca_v2.3$ -KO mice (Table S1A). TPM did not alter the frequencies of occurrence of the seizure stages in  $Ca_v2.3$ -KO mice.

We retested the effect of LTG in  $Ca_v2.3$ KO and control mice at 30 mg/kg KA (Fig. 2B), a dosage at which  $Ca_v2.3$ 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  $Ca_v2.3$ KO mice and to further investigate the convulsive effect of LTG in  $Ca_v2.3$ KO mice observed at 20 mg/kg. At 30 mg/kg KA, LTG pretreatment reduced



**Figure 1.**

Individual seizure scores of mice at 20 mg/kg kainic acid. Seizure scores are plotted for each mouse during the course of the 2 h video-monitoring period. (A–D) Seizure scores for control mice without (A) and after pretreatment with LGT (B), TPM (C), or LSM (D). (E–H) Seizure scores for Ca<sub>v</sub>2.3KO without (E) and after pretreatment with LGT (F), TPM (G), or LSM (H).

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total seizure scores of control mice by 30% ( $p = 0.0079$ ;  $U = 0$ ) and total seizure scores of LGT-treated Ca<sub>v</sub>2.3-KO mice were 33% ( $p = 0.015$ ;  $U = 1.5$ ), higher than those of LGT-treated control mice ( $69 \pm 6.4$  compared to  $51.6 \pm 1$ ). An increase (not significant) of total seizure scores of 15.8% ( $69 \pm 6.4$  compared to  $59.6 \pm 4.1$ ) was observed in LGT-treated Ca<sub>v</sub>2.3-KO mice compared to Ca<sub>v</sub>2.3-KO mice without pretreatment, which is in line with the significant increase of total seizure scores caused by LGT in Ca<sub>v</sub>2.3-KO mice observed at 20 mg/kg KA. At both KA concentrations, LGT increased the frequency of stage 3 (Tables S1A,B) in Ca<sub>v</sub>2.3-KO mice (but not in controls), which contributes to the increased total seizure scores of LGT-treated Ca<sub>v</sub>2.3-KO mice compared to untreated Ca<sub>v</sub>2.3-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 (Fig. 3) CA2, CA3, and dentate gyrus (DG) regions of the hippocampus. Ca<sub>v</sub>2.3-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. (2007). Both stains revealed that LGT-treated

Ca<sub>v</sub>2.3-KO mice displayed significantly increased degeneration of pyramidal CA1 neurons compared to untreated Ca<sub>v</sub>2.3-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. Furthermore, in control mice, LGT significantly reduced neurodegeneration in the CA1, CA3, and DG. Ca<sub>v</sub>2.3-KO and LGT-treated control mice displayed similar degrees of degeneration in all evaluated regions except the CA2.

### Expression of Ca<sub>v</sub>2.3 protein

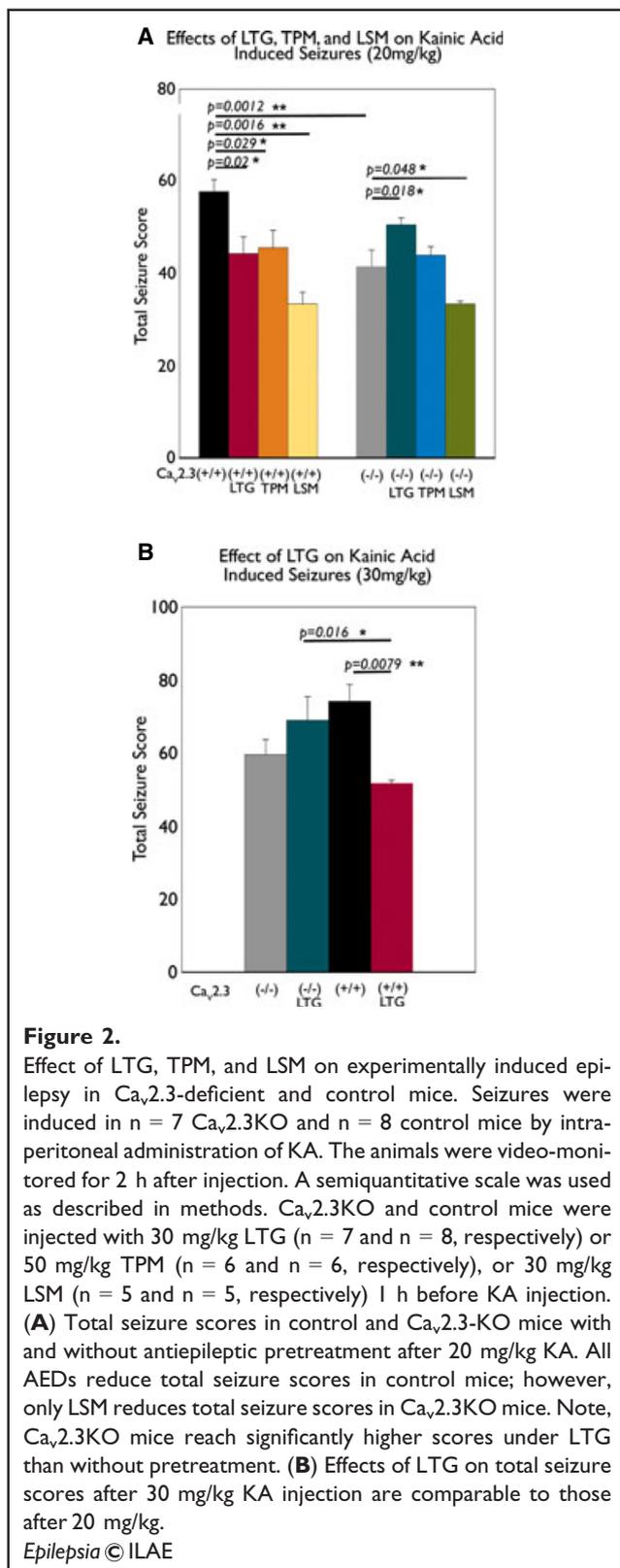
Both manual and automated quantification of western blotted Ca<sub>v</sub>2.3 bands by normalization to SYN revealed no significant differences in Ca<sub>v</sub>2.3 protein expression between KA- and saline-injected groups (Fig. 4).

### Electrocorticography studies

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 LGT in control condition

Spectral analysis of the recorded ECoG studies revealed significant differences between Ca<sub>v</sub>2.3-KO and control mice and between the effects of LGT in both genotypes in control recordings and after injection of 20 mg/kg KA. In



control conditions, Ca<sub>v</sub>2.3-KO mice displayed significantly reduced relative delta power compared to control mice ( $29 \pm 1.7\%$  vs.  $22.1 \pm 2.0\%$  [ $p = 0.037$ ]) (Fig. S1A). LTG

treatment increased relative beta power in control mice (from  $4.6 \pm 0.48\%$  to  $7.5 \pm 1\%$  [ $p = 0.037$ ]) (Fig. S1B), but not in Ca<sub>v</sub>2.3-KO mice in which LTG reduced relative alpha power from  $16 \pm 1.5\%$  to  $11.1 \pm 0.9\%$  ( $p = 0.034$ ) (Fig. S1C).

### 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 (Fig. S2A,C), however, to a greater degree in control mice. Of interest, in control mice, LTG pretreatment prevented the KA-induced shift in spectral distribution (Fig. S2B), whereas in LTG-pretreated Ca<sub>v</sub>2.3-KO mice, KA injection caused a significant reduction of alpha power (Fig. S2D).

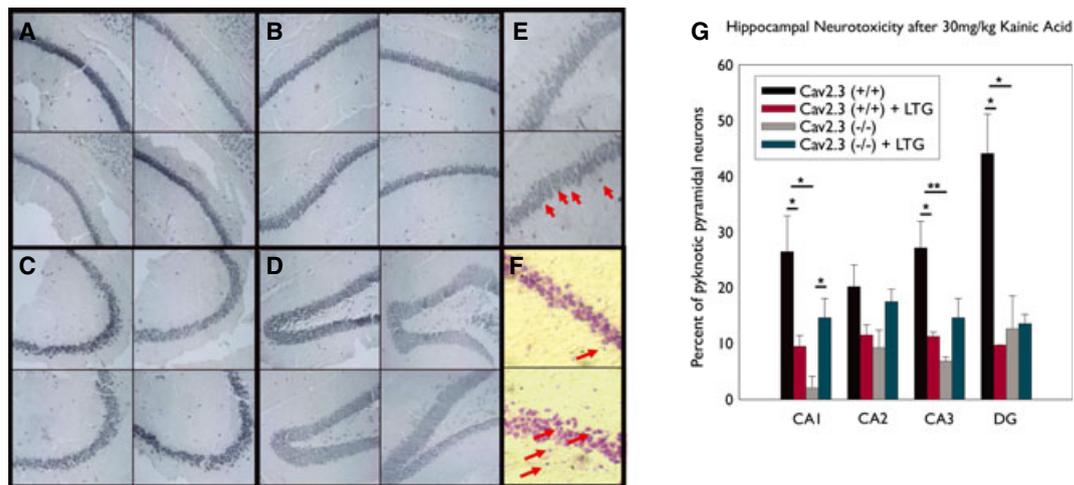
### Effect of LTG on KA-induced seizures

Both genotypes displayed different spectral distribution after KA injection (Fig. 7), with Ca<sub>v</sub>2.3-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 (Weiergräber et al., 2007) 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 toward sigma and beta frequencies ( $5.4 \pm 0.9\%$  vs.  $3.2 \pm 0.1\%$  [ $p = 0.007$ ] and  $6.5 \pm 1\%$  versus  $3.5 \pm 0.3\%$  [ $p = 0.008$ ], respectively) away from delta–theta frequencies and thus less ictal activity in this frequency range. In contrast, LTG pretreatment of Ca<sub>v</sub>2.3-KO mice did not significantly alter spectral distribution when the complete recording period was analyzed.

### Analysis of maximal seizure activity

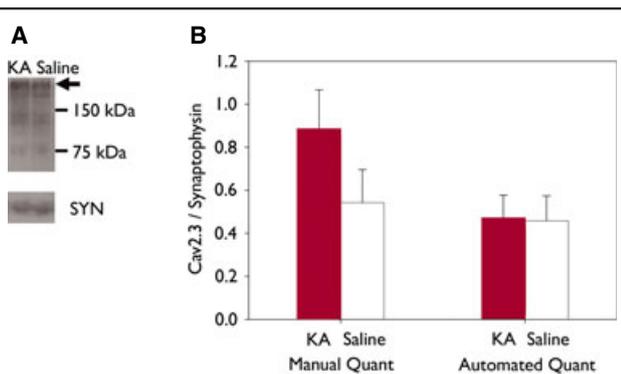
Using the automated spike detection protocol, the longest spike train, that is, maximal seizure activity, was identified and analyzed in further detail in order to gain more detailed insight into the effect of LTG on ictal activity in both genotypes. Although analysis of the parameters latency to first 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 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.  $24.9 \pm 2.6\%$  [ $p = 0.03$ ]) (Fig. 8B). This effect of LTG on maximal seizure activity did not occur in Ca<sub>v</sub>2.3-KO mice.



**Figure 3.**

Neurotoxicity after 30 mg/kg KA. NeuN-stained paraffin sections of the CA1 (A), CA2 (B), CA3 (C), and DG (D) regions of the hippocampus at 20 $\times$  magnification from control mice (top left of each quadrant), LTG pretreated control mice (bottom left), Ca<sub>v</sub>2.3KO mice (top right), and LTG pretreated Ca<sub>v</sub>2.3KO mice (bottom right). Higher (40 $\times$ ) magnification of NeuN- (E) and Nissl- (F) stained sections showing the CA1 region. Arrows indicate pyknotic pyramidal neurons, that is, pyramidal neurons with condensed nuclear material indicating apoptosis. (G) Evaluation of the percentage of pyknotic neurons in regions of the hippocampus.

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**Figure 4.**

Ca<sub>v</sub>2.3 protein expression in the hippocampus. (A) Western blot of control mice hippocampal membrane fractions. (B) Student's *t*-test of manual (using IMAGEJ 1.46) and automated (using GELSCAN 6.0) quantification of Ca<sub>v</sub>2.3 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.

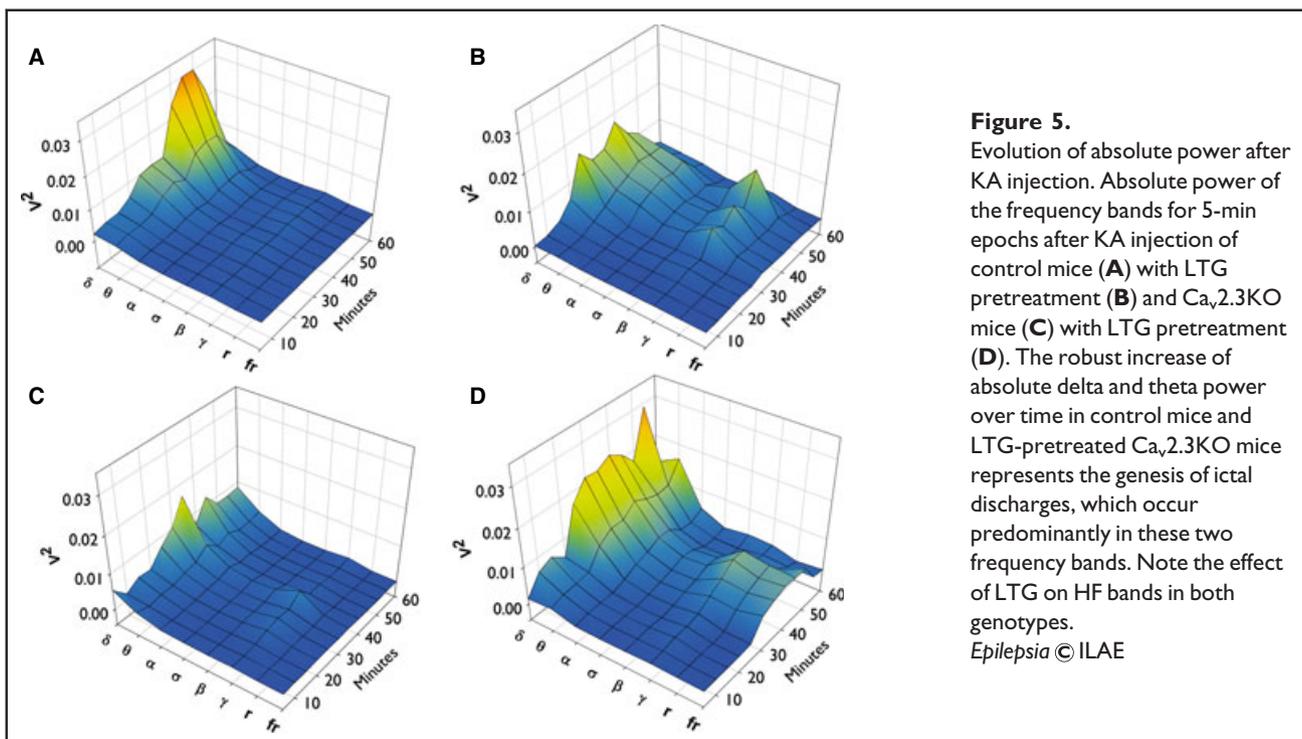
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Of interest, LTG-pretreated Ca<sub>v</sub>2.3-KO mice displayed significantly increased relative fast ripple power compared to untreated Ca<sub>v</sub>2.3-KO mice ( $1.2 \pm 0.4\%$  vs.  $0.036 \pm 0.001\%$  [ $p = 0.003$ ]), possibly underlying the pro-ictogenic effect of LTG observed in behavioral seizure analysis (Fig. 8C). Correspondingly, in control mice but not in Ca<sub>v</sub>2.3-KO mice, LTG significantly reduced the Z-ratio of

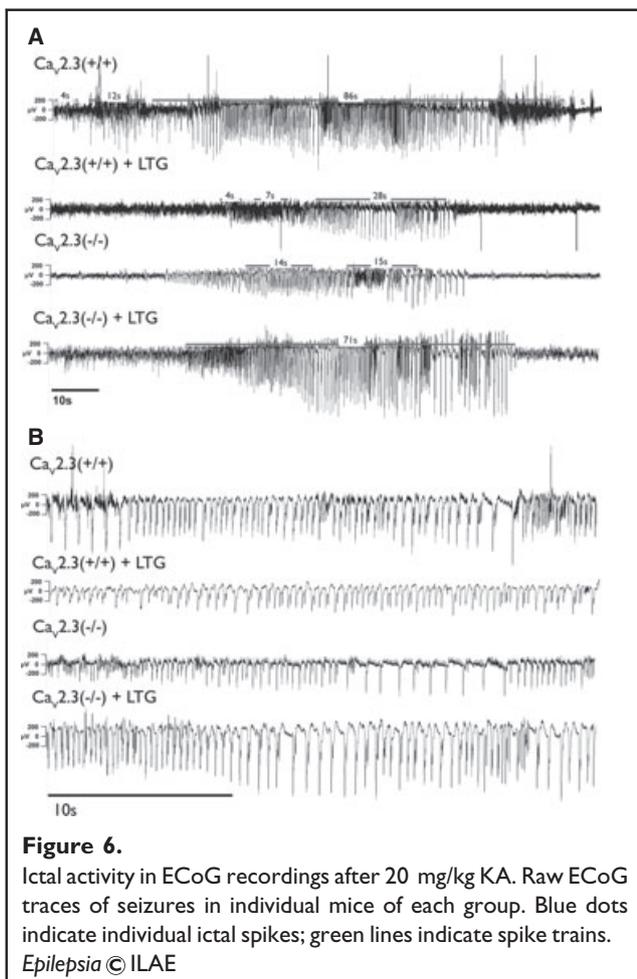
maximal seizure activity from 0.51 to 0.07 ( $p = 0.04$ ), indicating an increase of high frequency power and thus a shift away from spiking in the delta–theta range.

## DISCUSSION

In this study we show that the Ca<sub>v</sub>2.3 calcium channel is critical in mediating the anticonvulsant properties of LTG in the KA model of epilepsy and that LTG elicits pro-ictogenic effects in mice lacking the Ca<sub>v</sub>2.3 calcium channel. Neither LTG nor TPM, which have been shown to inhibit R-type currents in heterologous systems, could reduce seizure scores in Ca<sub>v</sub>2.3-KO mice, indicating the importance of Ca<sub>v</sub>2.3 inhibition in mediation of their anticonvulsive effects. In contrast, LSM, which has no calcium channel modulating properties, was the only AED of the three tested that could reduce seizure scores in Ca<sub>v</sub>2.3-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 Ca<sub>v</sub>2.3 is ablated. Furthermore, this study reveals a convulsive and neurotoxic effect of LTG in the absence of Ca<sub>v</sub>2.3 calcium channels. Of interest, toxicity of LTG was located in the CA1 region of the hippocampus, where LTG is known to be most neuroprotective (Leach et al., 1991; Crumrine et al., 1997; Englund et al., 2011). Therefore, it is assumable that the underlying neuroprotective mechanisms may include inhibition of signaling through Ca<sub>v</sub>2.3, which we found not to be upregulated after KA injection. The fact that the convulsive effect of LTG is more specifically related to the



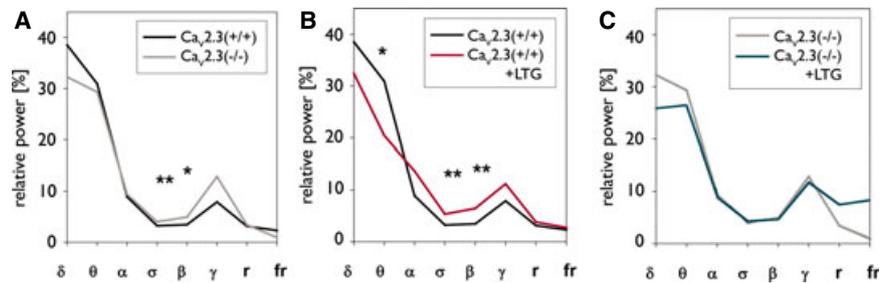
**Figure 5.** Evolution of absolute power after KA injection. Absolute power of the frequency bands for 5-min epochs after KA injection of control mice (A) with LTG pretreatment (B) and  $Ca_v2.3$ KO mice (C) with LTG pretreatment (D). The robust increase of absolute delta and theta power over time in control mice and LTG-pretreated  $Ca_v2.3$ 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. *Epilepsia* © ILAE



**Figure 6.** Ictal activity in ECoG recordings after 20 mg/kg KA. Raw ECoG traces of seizures in individual mice of each group. Blue dots indicate individual ictal spikes; green lines indicate spike trains. *Epilepsia* © ILAE

CA1 region, must lead to a novel interpretation of its mechanism of action. Underlying this finding could be postinhibitory 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 (Chen et al., 2001b). LTG has been shown to enhance HCN currents in CA1 pyramidal neurons, conveying an inhibitory effect (Poolos et al., 2002). However, due to the capacity of HCN channels to activate at hyperpolarized potentials and slow deactivation kinetics, increased synaptic inhibition, a condition predictable in  $Ca_v2.3$ 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 was identified after injection of 30 mg/kg KA in hippocampi of  $Ca_v2.3$ -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).

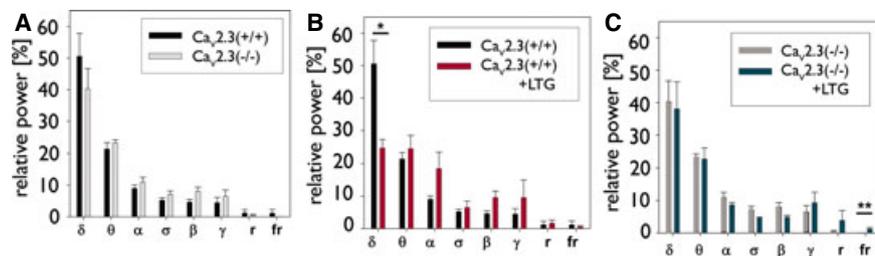
Furthermore, in this study, telemetrically recorded ECoG revealed that LTG cannot attenuate ictal discharges in  $Ca_v2.3$ KO mice as it does in control mice, but instead increases ultra-high frequency components of ictal activity, which are known to be associated with generation of epileptic activity in humans and in animals (Allamand et al., 1997; Traub et al., 2001; Bragin et al., 2004). Clinically, this 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



**Figure 7.**

Spectral distribution after KA injection. Relative power of the frequency bands after KA injection averaged from 10-s epochs of 60-min recording time. **(A)** Ca<sub>v</sub>2.3KO versus control mice. **(B)** Control versus LTG-pretreated control mice. **(C)** Ca<sub>v</sub>2.3KO versus LTG pretreated Ca<sub>v</sub>2.3KO mice. Note the similar effect of ablation of Ca<sub>v</sub>2.3 and LTG pretreatment (of control mice).

*Epilepsia* © ILAE



**Figure 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-s epochs. **(A)** Ca<sub>v</sub>2.3KO versus control mice. **(B)** Control versus LTG-pretreated control mice. **(C)** Ca<sub>v</sub>2.3KO versus LTG-pretreated Ca<sub>v</sub>2.3KO mice. LTG increases fast ripples and fails to reduce spiking in the delta theta range in Ca<sub>v</sub>2.3KO mice as it does in control mice.

*Epilepsia* © ILAE

several (nonsedative) 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 frequent aggravating effect of LTG at therapeutic doses (Guerrini et al., 1998; Genton, 2000). Another study reports that adults with idiopathic generalized epilepsies treated with LTG experienced exacerbation or de novo appearance of myoclonic jerks (Crespel et al., 2005). 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 Ca<sub>v</sub>2.3 in human patients with epilepsy. Although gain-of-function mutations in the CACNA1H gene encoding for the low-voltage activated (T-type) calcium channel Ca<sub>v</sub>3.2 have been identified in patients with hereditary forms of absence epilepsy (Liang et al., 2006), no variants of Ca<sub>v</sub>2.3 have been identified to date 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 (N'Gouemo et al., 2010). Whether expression of Ca<sub>v</sub>2.3 is altered in the hippocampus of human patients with epilepsy 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, investigation of genetic variants of CACNA1E in patients with epilepsy who experience a worsening of symptoms with LTG could produce valuable insights.

Because LTG is not able to prevent or attenuate ictal activity in the absence of Ca<sub>v</sub>2.3 calcium channels, one must assume that its anticonvulsive properties are not based primarily 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 such as bipolar depression, borderline disorder, and anxiety disorder, and to contribute to a better outcome in animal models of stroke and subarachnoid hemorrhage.

## ACKNOWLEDGMENTS

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## DISCLOSURE

None of the authors has any conflict of interest 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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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Seizure stages.

**Figure S1.** Spectral distribution in control condition.

**Figure S2.** Comparison of spectral distribution pre- and post-KA injection.

**Table S1.** Frequency of seizure stages.



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# Cardiac phenomena during kainic-acid induced epilepsy and lamotrigine antiepileptic therapy



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Kainic-acid;  
SUDEP;  
Sleep;  
Electrocorticography;  
Electrocardiography

## Summary

**Rationale:** Pathologic ECG events are known to accompany seizures and to persist in several chronic epilepsy syndromes. The contribution of antiepileptic drugs (AEDs) to these events and the implications in the etiology of sudden-unexpected death in epilepsy (SUDEP) continue to be a matter of debate. We therefore investigated cardiac parameters during kainic-acid (KA) induced experimental epilepsy and antiepileptic treatment with lamotrigine (LTG).

**Methods:** Epilepsy was induced in seven C57Bl/6 mice by injections of KA (20 mg/kg) on days 1 and 5, which produced severe acute seizures and spontaneous seizures 10 days later. Treatment with LTG (30 mg/kg) was initiated on day 11 and repeated on day 12. Continuous ECGs and ECoGs were collected telemetrically from freely moving mice.

**Results:** Mice displayed pre-ictal but not ictal tachycardia. The squared coefficient of variation (SCV) of R–R intervals was significantly elevated 30 s before and during seizures compared to control conditions. LTG produced a significant reversible increase in SCV and LF/HF ratio during slow-wave sleep (SWS), potentially indicative of sympatho-vagal imbalance during this state of vigilance, in which epileptic patients are known to be particularly vulnerable to SUDEP.

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*Significance:* The KA model used in this study permits the investigation of cardiac phenomena during epilepsy, as it features many effects found in human epileptic patients. Increased LF/HF, a known risk factor for cardiac disease, which is often found in epileptic patients, was observed as a side-effect of LTG treatment during SWS, suggesting that LTG may promote imbalance of the autonomous nervous system in epileptic mice.

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## Introduction

Kainic-acid (KA) is a non-degradable analog of glutamate, which causes excitotoxicity by agonism of kainate-class ionotropic glutamate receptors (Wang et al., 2005). Animal models involving systemic or local injection of KA are among the most popular models of epilepsy and neurodegeneration, with over 1500 articles published on the subject in the last 10 years<sup>1</sup>. Systemic injection of KA is commonly used to model mesial temporal lobe epilepsy (MTLE), as it produces generalized seizures and progressive sclerosis of the hippocampus, which in turn -like in MTLE patients- leads to recurrent spontaneous seizures (Sharma et al., 2007). Mouse models of human pathology offer several advantages, most notably genetic homogeneity and availability of transgenic animals, but they can be technically challenging due to the small size of mice.

In epileptic patients, seizures have been shown to be preceded and accompanied by ECG changes, fueling the discussion of brain to heart interactions, which may be of great relevance in the context of sudden-unexpected death in epilepsy (SUDEP). Ictal tachycardia is detectable in almost all patients of different age groups (Jansen et al., 2013). Several studies of different epilepsy syndromes have described the occurrence of sympathovagal imbalance (increase of sympathetic and decrease of parasympathetic control of heart rhythm) (Brotherstone and McLellan, 2012; Lotufo et al., 2012; Meghana et al., 2012; Ponnusamy et al., 2012), which is known to contribute to mortality and morbidity in cardiovascular disease (Mortara et al., 1997; Schwartz et al., 1988). Furthermore, pre-ictal tachycardia has been observed in children and adults with generalized seizures (Jansen et al., 2013; Scherthner et al., 1999), in adults with refractory epilepsy (Zijlmans et al., 2002) and in children with refractory TLE (Mayer et al., 2004). How and whether antiepileptic drugs (AEDs) affect cardiac function of epileptic patients is a matter of debate, as data is conflicting and non-conclusive. One study found AEDs to ameliorate sympathovagal imbalance (Hallioglu et al., 2008), whereas another found AEDs to reduce ECG power and heart rate variability (HRV) (Lossius et al., 2007), possibly predisposing patients to cardiac arrhythmia which may be an important contributor to SUDEP. A recent meta-analysis of 39

studies found a trend of increased low frequency power (LF) in patients taking AEDs, presumably reflecting increased sympathetic tone (Lotufo et al., 2012), possibly posing a cardiac risk. Of AEDs potentially affecting cardiac function, the modern broad spectrum AED lamotrigine (LTG) is of special interest. LTG, FDA approved for treatment of partial seizures in 1994, and later for maintenance treatment of bipolar I disorder, enjoys great popularity and is employed in treatment of several epilepsy syndromes and [also as an off-label drug] of several neuropsychiatric diseases. LTG's wide therapeutic applicability reflects the multi-target nature of the drug which has been shown to modulate several different sodium, calcium and potassium currents (Beck and Yaari, 2012). In particular, LTG has been demonstrated to inhibit the delayed rectifier potassium current, which is crucial for cardiac repolarization and therefore plays a critical role in maintenance of cardiac rhythm (Danielsson et al., 2005). Ca<sub>v</sub>2.3 (R-type) voltage-gated calcium channels, which contribute to cardiac autonomous control and to intrinsic rhythm propagation (Galetin et al., 2012) are also inhibited by LTG (Hainsworth et al., 2003), representing another potential arrhythmogenic mechanism. Prolongation of the QT-interval, a risk factor of arrhythmia and sudden cardiac death, was an initial concern in regard to LTG treatment, however, some studies could dismiss this concern (Saetre et al., 2009) and a study by GlaxoSmithKline, manufacturer of the initial lamotrigine product Lamictal® found no QT prolongation or related safety concerns (Dixon et al., 2008). Interestingly however, prolonged PR interval due to LTG treatment has been reported (Dixon et al., 2011; Matsuo et al., 1993). Clarification whether LTG has potentially arrhythmogenic effects on the epileptic heart is of great importance, also because it has been reported that LTG increases the risk of SUDEP (Aurlien et al., 2012; Hesdorffer et al., 2011), although findings are controversial. Therefore exact characterization of cardiac phenomena in the murine KA model of epilepsy and investigation of cardiac effects of LTG in this model are of great importance.

## Materials and methods

### Animals

Seven male C57Bl/6 mice between 18 and 22 weeks of age were used in this study. Mice were kept at 20–22 °C in makrolon type II cages under a 12 h 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

<sup>1</sup> PubMed search of the words "kainic" and "acid" occurring in combination with the words "epilepsy", "seizure", "excitotoxicity", "neurodegeneration", "hyperexcitation" in the title or abstract of articles published between October 2003 and October 2013.

and were approved by the local institutional committee on animal care.

### Radio telemetric electrocorticographic and electrocardiographic recording

Radio telemetric electrocorticograms (ECoG) and electrocardiograms (ECG) were recorded from a total of 7 mice. Animals were anesthetized with 100 mg/kg BW ketaminhydrochloride (Ketanest, Parke-Davis/Pfizer, Germany) and 10 mg/kg BW xylazinehydrochloride (RompunR 2% BayerVital, Leverkusen, Germany). TL11M2-F20-EET transmitters (Datascience International, Lexington, USA) were implanted subcutaneously over the left hindlimb and burr holes drilled over the somatosensory cortex (−1 mm and 3 mm lateral from bregma) for the positive lead and cerebellum (−6.3 mm and 1 mm lateral from bregma) for the negative lead, leaving the dura intact. Electrodes were inserted and fixed into position with glass ionomer cement (Kent DentalR, Kent Express, UK). Subcutaneous ECG leads were positioned laterally on either side of the animals' torso. Animals were allowed seven days to recover from surgery (all made a full recovery) before initiating the experimental conditions. ECoGs and ECGs, were recorded by Dataquest™ A.R.T.™ 3.1 software (DSI) at a sampling rate of 1000 Hz without cut-off or filtering from freely moving animals in their cages (1 mouse per cage), which were placed on the telemetry receiver platforms located in the same room in which the animals' cages were kept previously. Locomotion was also recorded at a sampling rate of 250 Hz.

### Chronic epilepsy and antiepileptic therapy protocol

In order to reduce inter-individual variability of overall seizure severity and to enhance the occurrence of spontaneous seizures, a double kainic acid injection protocol was employed. For further information of repetitive injection protocols see [Dudek et al. \(2005\)](#). The experimental protocol to produce a chronic epileptic condition characterized by spontaneous seizures involved 2 injections of KA: on days 1 and 5 20 mg/kg KA (Sigma Aldrich, Crailsheim Germany) dissolved in saline was injected intra-peritoneally between 9:00 and 10:30 in the morning. 30 mg/kg LTG KA (Sigma Aldrich, Crailsheim Germany) was injected in the same manner on days 11 and 12. 24 h recordings were carried out on days 1, 3, 5, 10, 11, 12 and 13. Before the initial KA injection, recording was carried out for an hour to establish the control condition.

### ECoG analysis

NEUROSCORE 2.1.0 (Datascience International) was used to analyze recordings. An automated seizure detection protocol was written to be able to quantify ictal activity. The protocol recognizes waveforms shorter than 200 ms that are between 2.5- and 25-fold the baseline amplitude as spikes. Spikes occurring in intervals between 30 and 1500 ms are recognized as belonging to a spike train, which must be at least 300 ms long and contain a minimum of four spikes. Seizure with motor signs (SMS) were defined as spike-trains lasting longer than 10 s during which locomotion took place, whereas non-convulsive seizures (NCS) were defined as a

cluster of spike-trains occurring with inter-train intervals of less than 3 s lasting longer than 10 s during which no locomotion took place.

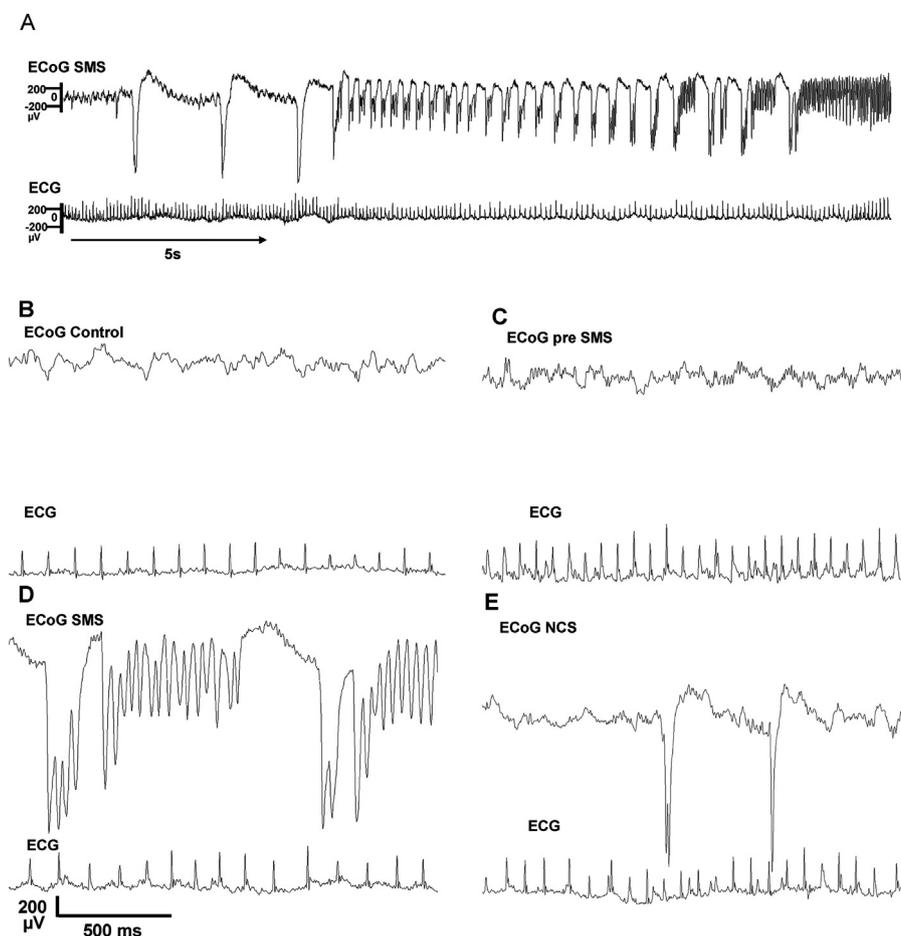
In a previous study, in which we collected ECoGs and video-monitored mice after injection of 20 mg/kg (Dibué et al., 2013), we observed increased locomotion, recorded by TL11M2-F20-EET transmitters to reflect motor components of seizures. Generalized seizures began with myoclonic jerks in the forelimbs, which then expanded to the whole body, increased in frequency and then became increasingly tonic when the seizure had progressed for longer than 10 s. Underlying repetitive myoclonic jerking were spikes, polyspikes and spike-and-wave discharges occurring in the delta and theta ranges (0.4–8). Tonic components were reflected by the progression of spiking to higher frequencies in the sigma and beta range (10–25 Hz) (see [Fig. 1A](#)). Digital filtering also revealed bursts of ultra high frequency oscillations (ripples and fast-ripples) during SMS. During the episodes of spiking without locomotion, that we termed NCS, mice appeared to “freeze”, exhibiting a rigid posture and display impaired consciousness, as they do not reacting to any stimuli (for details on murine KA-induced epilepsy and seizure severity rating see [Dibué et al. \(2013\)](#)). SWS was detected by Fast Fourier Transformation (FFT) (Hamming window with 50% overlap) of the ECoG signal: periods in which the ratio of delta power (0.5–4 Hz) to total power exceeded 0.4 (i.e. dominant delta band) and in which there was no locomotion were defined as SWS periods. Wakefulness was defined as periods in which neither SWS nor rapid-eye-movement (REM) sleep (defined by a dominant theta band (4–8 Hz) and rise in theta/delta ratio) occurred, excluding periods with maximal locomotion.

### ECG analysis

Frequency domain analysis of the ECG was performed in order to calculate LF/HF ratios during the various experimental conditions. The R-peak of the ECG QRS complex was detected automatically and used to calculate heart rate and R–R intervals. The coefficient of variation (CV) was calculated from R–R intervals. For each animal, data from five independent 1 min SWS periods (for each of the different experimental conditions) were combined and used in the analysis. For the wake periods (control, pre-SMS, SMS, post-SMS, NCS, LTG (measured on day12) and withdrawal from LTG) data from three independent 30 s periods were combined and analyzed. If SMS lasted less than 30 s, data from shorter SMS periods was combined to obtain the same amount of data points..

### Statistical analysis

GraphPad Prism 4 (GraphPad Software, Inc, La Jolla, CA, USA) was used for statistical analysis. Coefficients of variation were below 30% and were therefore squared and subjected to the *F*-test for comparison of equality of variance according to ([Lewontin, 1966](#)). Heart-rate and LF/HF ratios were assessed for significant differences using students' paired samples *t*-test. Data are presented as the mean ± SEM based on *n*, the number of independent recordings in the corresponding condition.



**Figure 1** Sample ECoG and ECG traces of control and ictal conditions. Example of progression of a seizure with motor signs (SMS), which begins with spike-and-wave discharges, which increase in frequency and then become accompanied by high frequency ictal discharges (A). Control ECoG and ECG (B). Heart rate is increased before SMS (C) but not during the seizure itself (D). Variation of R–R intervals however, is increased before and during SMS and also during non-convulsive seizures (NCS), which is shown in (E).

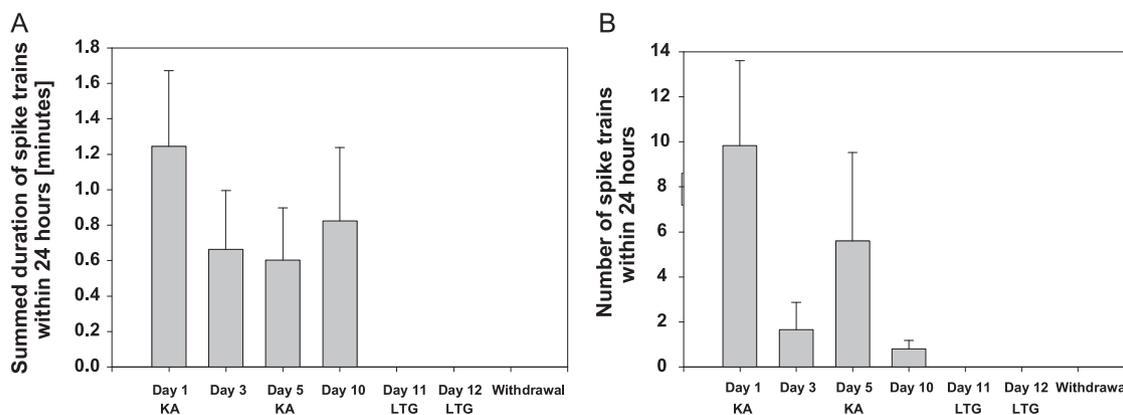
## Results

### Kainic acid-induced model of chronic epilepsy

The first KA injection elicited severe seizures in all animals persisting for about 2–3 h. Seizures were characterized by several ictal waveforms like spike-and-wave discharges, spikes and poly-spike complexes (Fig. 1). One animal died nearly two hours after the first KA injection. The second KA injection on day 5 elicited seizures that were slightly less severe than those observed on day 1. Spontaneous spike trains occurred on days 3 and 10. No spike trains occurred on the days LTG was injected or on the day of LTG withdrawal, however inter-ictal spikes during sleep and wakefulness were observed on these days. No spike trains were detected in the control condition (i.e. prior to KA injection), however in 3/7 animals, sleep spindles and other high amplitude waveforms during slow wave sleep (SWS) caused false positive detection of single spikes. Therefore, each SWS period used for analysis was manually evaluated for false positive spikes, to allow distinction from seizures occurring during SWS.

### Time domain ECG analysis

FFT of the ECG signal during wakefulness under the control condition produced a periodogram with two peaks (Fig. 3). Accordingly, for frequency domain analysis, the low frequency ECG band (LF) was defined as 0.1–1 Hz and the high frequency band (HF) as 1–5 Hz. Evaluation of heart rate during the different experimental conditions revealed the occurrence of pre-ictal tachycardia. In the 30 s preceding SMSs, heart rate increased from  $387 \pm 23$  bpm to  $436 \pm 23$  bpm ( $p = 0.0007$ ), whereas during SMSs, heart rate was significantly lower than in pre-ictal periods ( $388 \pm 27$  bpm vs  $436 \pm 23$  bpm  $p = 0.0038$ ) (Fig. 4A). LTG treatment did not affect heart rate, however it did show effects on the squared coefficient of variation (SCV) of R–R intervals. The SCV exhibited a highly significant increase in the pre-ictal period as well as during SMS ( $259 \pm 136$  and  $292 \pm 187$ ) compared to the control condition and to the post-ictal period ( $91 \pm 16$  and  $97 \pm 15$ )  $p < 0.001$  for all comparisons (Fig. 4B). A small but significant increase in SCV was also seen during NCSs compared to the control condition ( $145 \pm 49$  vs  $91 \pm 16$   $p = 0.0152$ ). Interestingly, on the second day of LTG treatment (day 12), the SCV was



**Figure 2** Kainic acid-induced model of TLE. Plotted are the average summed duration of spike trains occurring in 24 h (A). Number of spike trains occurring in 24 h recording periods. KA injections on days 1 and 5 elicit severe seizures with motor signs and non-convulsive seizures. Spontaneous seizures occur on days 3 and 10, but not on days on which lamotrigine was injected, or upon withdrawal (B). Interestingly, although less individual spike trains occur on days 3 and 10 compared to the days on which KA was injected (1,5) the overall duration of ictal activity is comparable on days 1,3,5 and 10.

significantly elevated compared to the control condition ( $187 \pm 82$  vs  $91 \pm 16$   $p=0.0015$ ). This LTG-induced elevation also occurred during SWS compared to control SWS ( $216 \pm 93$  vs  $88 \pm 28$   $p=0.0129$ ) and was found to be reversible, as the SCV returned to baseline at withdrawal from LTG ( $216 \pm 93$  to  $95 \pm 25$   $p=0.0322$ ) (Fig. 4C).

#### Frequency domain ECG analysis

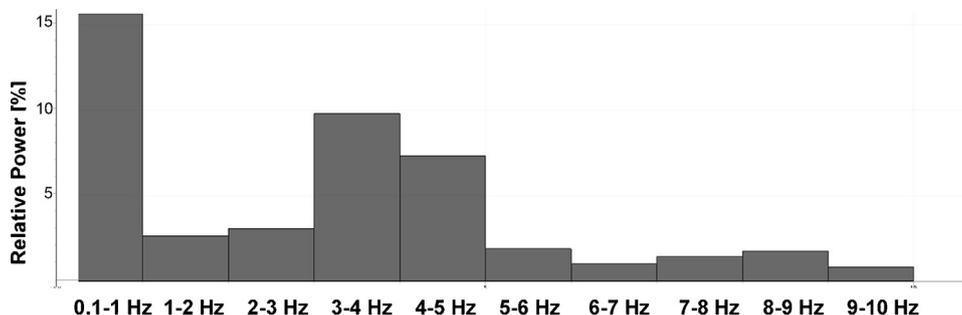
Analysis of the LF/HF ratio as a measure of sympatho-vagal balance in the autonomic nervous system (ANS) did not reveal sympatho-vagal imbalance during wakefulness after KA injection. Although a trend for higher ratios after KA injection, especially during SMS and NCS was observed, it did not reach significance, as inter-individual differences were quite large. However, LTG treatment significantly increased the LF/HF ratio during SWS compared to control SWS ( $3 \pm 0.28$  vs  $1.5 \pm 0.33$   $p=0.0167$ ). This effect was mostly reversed by termination of LTG treatment with the LF/HF ratio returning to values closer to baseline (from  $3 \pm 0.28$  to  $2.1 \pm 0.17$   $p=0.0418$ ), potentially indicating a sympathomimetic or parasympatholytic effect of LTG on the epileptic murine heart during slow wave sleep (Fig. 5).

#### Death case

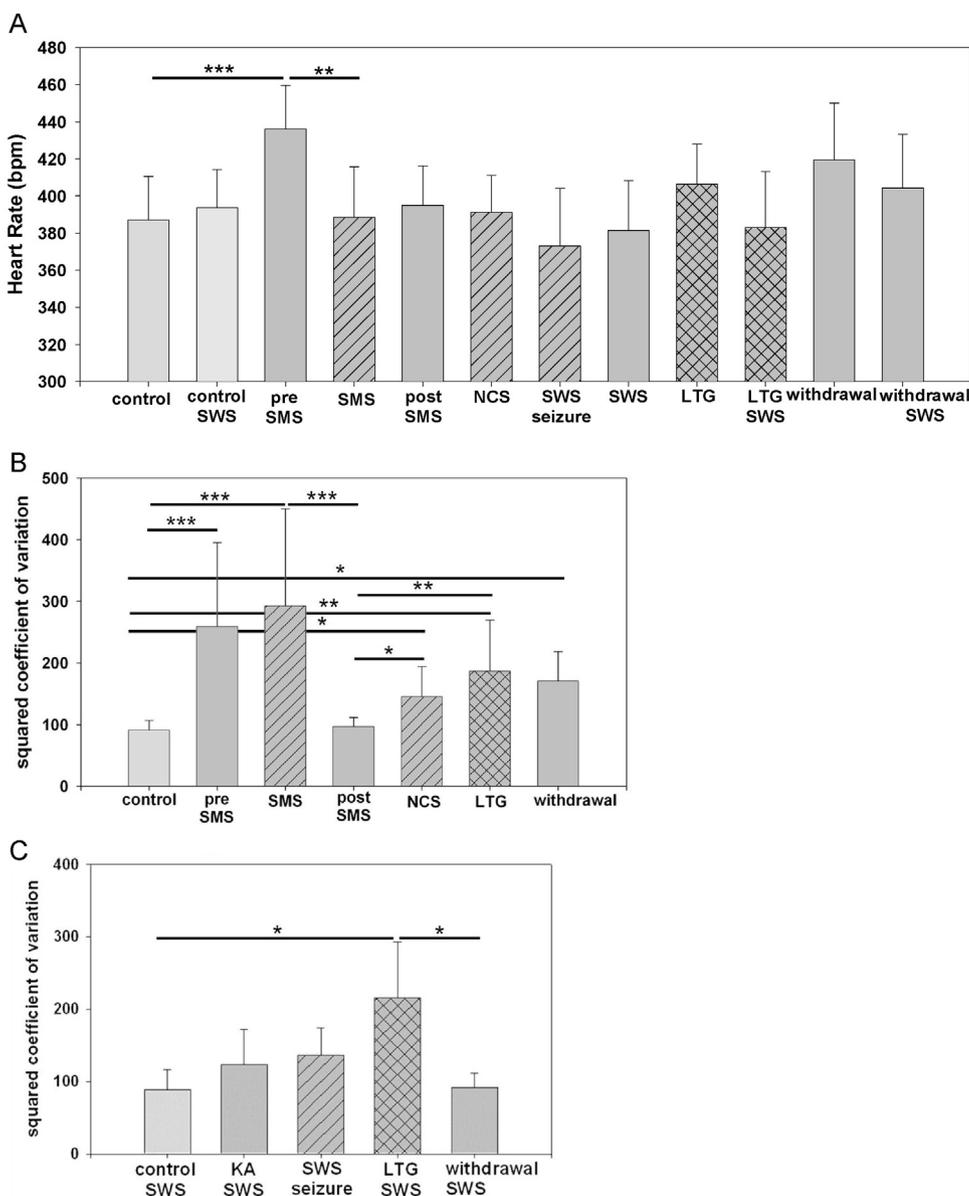
One mouse died 110 min after the first KA injection. The mouse did not display more severe seizure activity than other mice and did not die due to acute SMSs as the last SMS occurred 24 min before death (Fig. 6A and B). However, single high amplitude spikes with inter-spike intervals of 10–20 s did occur peri-mortally (about 10 min before death). In the last 7 min before death, respiration took on to a gasping pattern, with gasps occurring every 10–15 s. The EcoG flatlined for 3 min before cardiac arrest occurred. In the frequency domain of the ECG, about 30 min after KA injection LF power, which was previously dominant, decreased and the ECG shifted to a pattern dominated by HF components, which persisted for 30 min. 60 min after KA injection total ECG power finally dropped and remained low until death occurred 50 min later (Fig. 6C).

#### Discussion

In this study we identified several cardiac phenomena associated with KA-induced epilepsy in mice. Like in some human epilepsy disorders, tachycardia preceded SMSs, however



**Figure 3** FFT periodogram of the ECG signal under control wake condition revealed two peaks between 0.1 and 10 Hz. Therefore “low frequency power” = LF was defined as 0.1–1 Hz and “high frequency power” = HF was defined as 1–5 Hz. The LF/HF ratio represents sympatho-vagal balance with high values reflecting high sympathetic tone and low values parasympathetic tone.

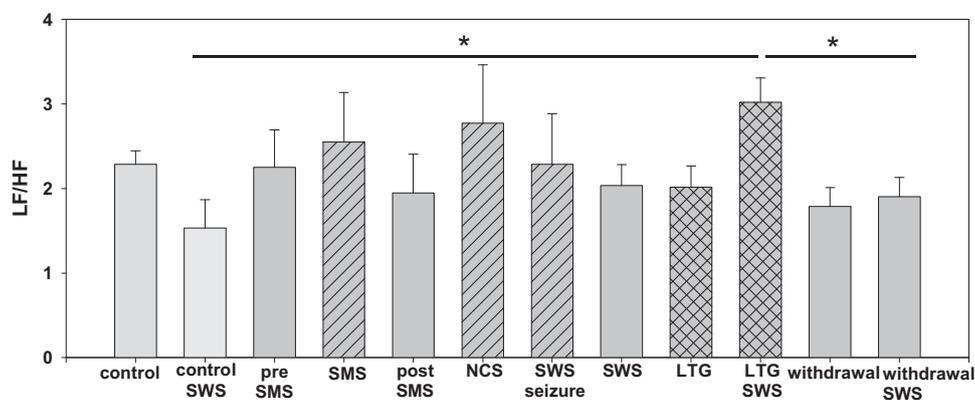


**Figure 4** Paired *t*-test revealed that heart rate significantly increased in the 30s preceding seizures with motor signs (SMS), but not during the SMS itself ( $p = 0.0038$ ) (A). LTG treatment did not affect heart rate in any state of vigilance. The squared coefficient of variation (SCV) increased significantly in the 30s preceding SMS and during SMS. Non-convulsive seizures were also characterized by increased SCV. Lamotrigine significantly increased SCV compared to control conditions (B). This increase in SVC due to lamotrigine was also observed during slow wave sleep and was found to be reversible at withdrawal (C).

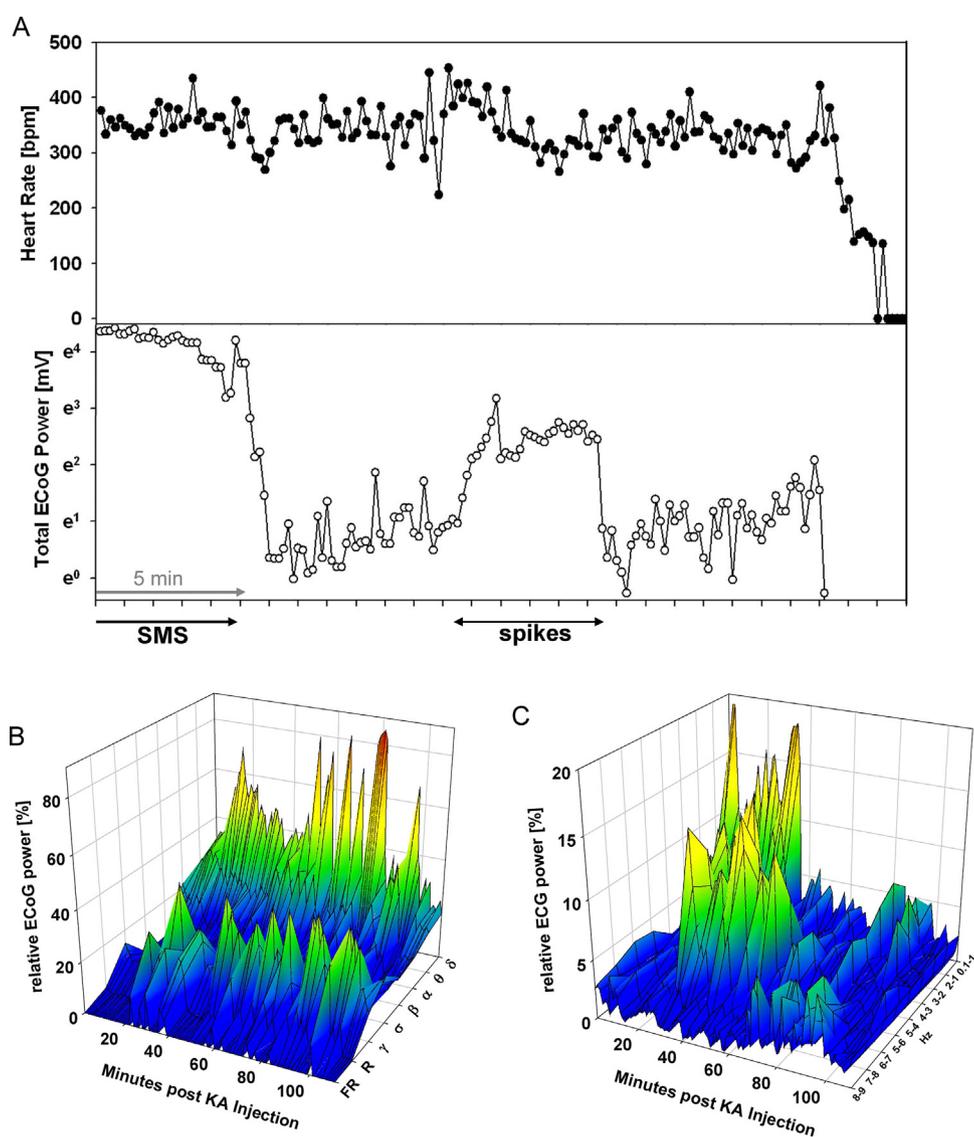
ictal tachycardia was not observed. Furthermore, pre-ictal and ictal increases in the coefficient of variation may reflect the potential for peri-ictal cardiac arrhythmia. LTG treatment produced a reversible increase of the coefficient of variation, which was most prominent during SWS. Furthermore, during SWS LTG reversibly increased the LF/HF ratio (Fig. 7), presumably reflecting dominating sympathetic tone, a known risk factor for cardiovascular disease. Interestingly, effects of LTG on sleep architecture have been reported before. Three previous studies have found LTG to reduce SWS time, stage shifts and number of arousals and increase REM sleep time in epileptic patients (Foldvary et al., 2001; Placidi et al., 2000a, 2000b). This may be an antiepileptic mechanism of LTG, as seizures and inter-ictal

epileptiform discharges (IED) are precipitated during SWS and relatively inhibited by REM sleep in most human epilepsy syndromes (Bazil and Walczak, 1997; Kumar and Raju, 2001; Malow et al., 1998). However, because sympathetic tone is known to be predominant during REM sleep, the opposite applying to SWS sleep (Tobaldini et al., 2013), sympathetic tone during sleep may be generally enhanced by LTG, possibly also affecting SWS. Whether this poses a potential cardiac risk, must be investigated in further studies, as well as possible underlying mechanisms.

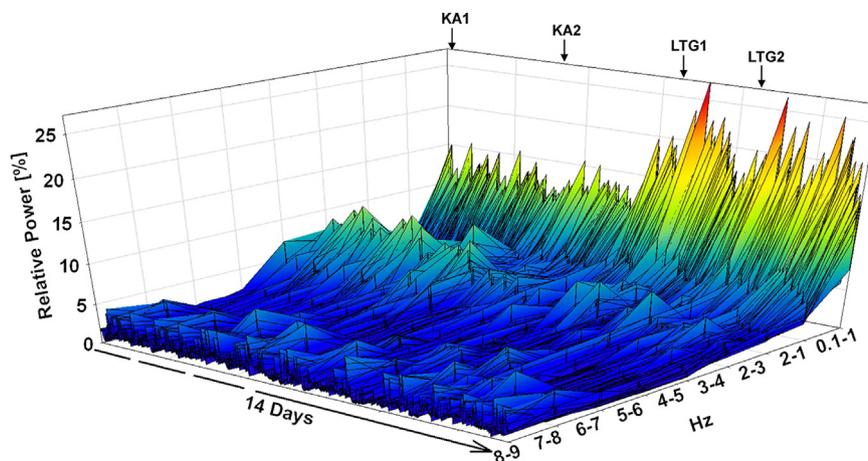
Furthermore, SUDEP appears to occur more commonly during sleep, although mechanisms remain unclear (Asadi-Pooya and Sperling, 2009; Nobili et al., 2011). Several studies have observed autonomic changes in epileptic



**Figure 5** LF/HF ratios, reflecting autonomous nervous system balance during the different experimental conditions. Lamotrigine significantly increased LF/HF during slow wave sleep (SWS) compared to control SWS ( $3 \pm 0.28$  vs  $1.5 \pm 0.33$   $p=0.0167$ ), indicating increased sympathetic tone during SWS. This increase is reversible as LF/HF ratios are no longer elevated upon withdrawal SWS.



**Figure 6** Heart rate and total ECoG power of the 30 min before death of the only mouse that died. Heart rate drastically dropped when the ECoG flatlined but continued to beat regularly for another 3 min (A). ECoG (B) and ECG (C) spectra of the mouse that died nearly 2 h after the first KA injection. Note the clear shift of LF dominance to HF dominance of the ECG around 30 min.



**Figure 7** ECG spectrum of one mouse during the full experimental period sampled from 10 min epochs. A trend toward gradual elevation of LF power and reduction of HF power is visible until LF power surges after each LTG injection.

patients during sleep, finding decreased heart rate variability during night-time, suggesting that nocturnal sleep may be more vulnerable to impaired autonomic control (Ferri et al., 2002; Persson et al., 2007; Ronkainen et al., 2005). A growing body of evidence lends weight to the hypothesis of extreme autonomic imbalance in SUDEP victims and it has been suggested that mechanisms similar to those involved in sudden cardiac death, such as reduced vagal tone during sleep and a sudden increase of catecholamines upon awakening are involved (Nei et al., 2004). Nevertheless, the exact identification of risk factors for SUDEP may be key in understanding potentially reciprocal mechanisms behind brain-heart interactions during epilepsy and antiepileptic pharmacotherapy. We conclude that this model permits the investigation of cardiac phenomena during epilepsy, as it features many effects found in human epileptic patients.

## Disclosure

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# Ca<sub>v</sub>2.3 E-/R-type voltage-gated calcium channels modulate sleep in mice

## Introduction

Voltage-gated calcium channels (VGCCs) are key components in the pathogenesis of various forms of epilepsy [1]. Recently, we could demonstrate that Ca<sub>v</sub>2.3 E-/R-type Ca<sup>2+</sup> channels are important in modulating absence epilepsy [2], a pathophysiological aberration of the thalamocortical oscillations that are related to slow-wave sleep (SWS) [3, 4]. During the last decade, several reports have discussed the intimate relationship between sleep and epilepsy. Even Aristotle noted that “sleep is like epilepsy and epilepsy is like sleep” (cited in [5]). Epilepsy and sleep both represent states of the brain. However, while sleep is a normal physiological event, epileptic seizures are of pathophysiological origin. Critical comparison of both states has led to a more careful distinction between them [6], particularly in relation to the underlying mechanisms of the thalamocortical oscillations observed during electroencephalogram (EEG) recordings, for which some common features were initially discussed [6, 7]. Differential origins of sleep spindles and spike-and-wave discharges (SWDs) were identified [6], which led to the conclusion that an understanding of the underlying mechanisms involved—in which the thalamus and the thalamocortical network play a central role [9]—will require a detailed description of sleep-related ion channels, transporters [8] and transmitter receptors.

Shortly after the cloning of T-type VGCCs, their neuronal distribution was determined by *in situ* hybridization [10]. The transcripts of each of the three T-type Ca<sup>2+</sup> channels had a unique distribution

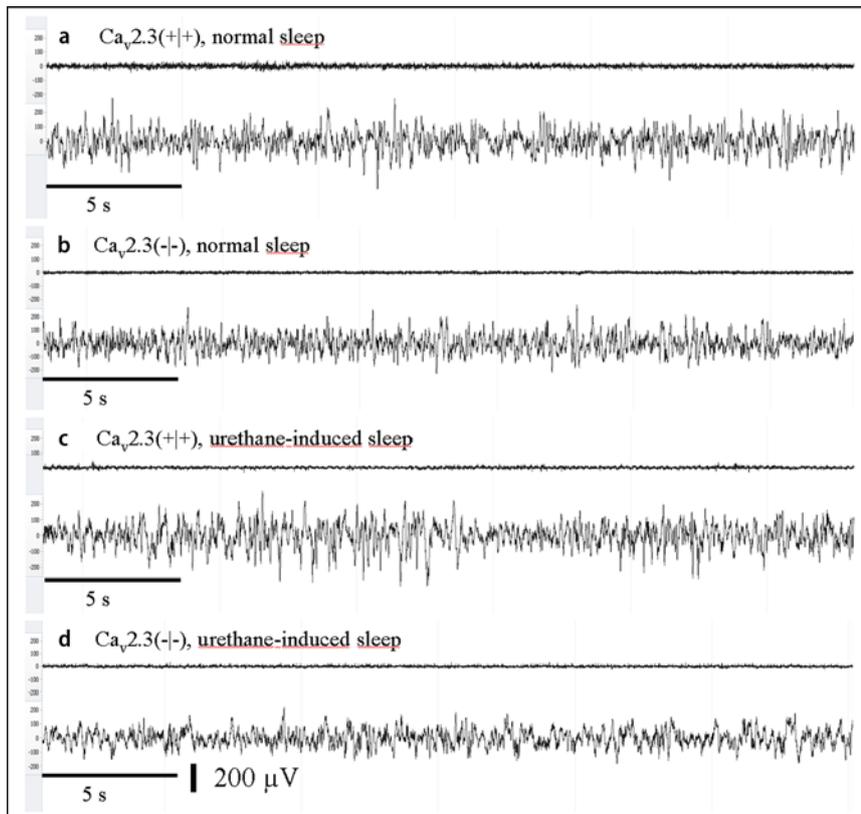
in the central nervous system, particularly in the thalamus and the thalamic reticular nucleus (nRt), and their patterns of expression were largely complementary. As T-type Ca<sup>2+</sup> channels affect afterpotential properties and excitability in neurons [11], their distribution was also determined in the Genetic Absence Epilepsy Rats from Strasbourg (GAERS). In addition to expression of Ca<sub>v</sub>3.1 (α1G), Ca<sub>v</sub>3.2 (α1H) and Ca<sub>v</sub>3.3 (α1I), transcripts for Ca<sub>v</sub>2.3 (α1E) were also analyzed [12]. Compared to non-epileptic control rats, juvenile GAERS rats display higher amplitude T-type Ca<sup>2+</sup> currents in neurons of the nRt. Transcripts of Ca<sub>v</sub>2.3, which should be considered a mid-voltage-gated Ca<sup>2+</sup> channel, did not differ between the two animal groups. This led to the conclusion that Ca<sub>v</sub>2.3 may not contribute to the pathological thalamocortical oscillations characteristically found in GAERS rats. However, in another study using quantitative reverse transcription (RT)-PCR and *in situ* hybridization, reduced Ca<sub>v</sub>2.3 levels were found in the cerebellum and brain stem of GAERS rats compared to non-epileptic animals [13]. No difference was found in younger animals lacking the GAERS epileptic phenotype and no difference was recorded for Ca<sub>v</sub>3.1 transcripts.

As important as T-type Ca<sup>2+</sup> channels are in epileptogenesis, they are also crucial for the regulation of sleep and wakefulness. T-type Ca<sup>2+</sup> channels contribute substantially to the oscillatory output of the thalamus region [14, 15, 16, 17, 18]. Although it is well known that all three Ca<sub>v</sub>3 T-type Ca<sup>2+</sup> channels are complementarily expressed in the thalamocortical system, their respective distinct con-

tributions to sleep have yet to be fully described. In the nRt, the Ca<sub>v</sub>3.3 T-type Ca<sup>2+</sup> channel is expressed abundantly, similarly to Ca<sub>v</sub>2.3 E-/R-type channels. Consequently, inactivation of Ca<sub>v</sub>3.3 causes a disturbance in the synchronized thalamic network oscillations underlying sleep-spindle waves. These were markedly weakened because of a reduced inhibition of the thalamocortical neurons by the nRt cells, confirming the central role of Ca<sub>v</sub>3.3 T-type channels in rhythmogenesis of the sleep-spindle generator [19].

Although it was believed that high-voltage-activated Ca<sup>2+</sup> channels may not be involved in seizure processes in rat models of absence epilepsy, recent evidence from Ca<sub>v</sub>2.3-deficient mice suggests their involvement in the murine model [20]. Recordings from tissue slices revealed that neurons of the reticular thalamus (RT) display oscillatory discharges, which are believed to be critical for thalamocortical network oscillations related to absence epilepsy. In brain slices from Ca<sub>v</sub>2.3-deficient mice, injection of hyperpolarizing currents initiated a low-threshold burst of spikes in RT neurons. However, subsequent oscillatory burst discharges were severely suppressed and accompanied by significantly reduced slow afterhyperpolarization (AHP), suggesting that not only T-type Ca<sup>2+</sup> channels, but also mid-voltage-gated Ca<sub>v</sub>2.3 E-/R-type channels contribute to oscillatory burst discharges in RT neurons. Ca<sub>v</sub>2.3 (highly expressed in the nRt) may cooperate with the T-type Ca<sup>2+</sup> channels expressed in the same or different neurons

A. Münch and M. Dibué contributed equally to this work.



**Fig. 1** ▲ Example 30 s radiotelemetric EEG recordings from control (panel **a** and **c**) and  $Ca_v2.3$ -deficient mice (panel **b** and **d**). Example traces were selected from recordings during typical slow-wave sleep (SWS) periods. During normal sleep, EMG (top) and EEG traces (bottom) were collected during the light period, when  $Ca_v2.3$ -competent control mice (**a**) and  $Ca_v2.3$ -deficient mice (**b**) had started to sleep. During urethane-induced sleep, EMG (top) and EEG traces (bottom) were collected from  $Ca_v2.3$ -competent control mice (**c**) and for  $Ca_v2.3$ -deficient mice (**d**) for 1 h after injection

of the thalamocortical loop, which is part of a common neuronal network for sleep spindles and SWDs [21].

Since the thalamocortical network is connected to absence epilepsy [2, 22] and sleep [9, 23], the present study was undertaken to elucidate the role of the  $Ca_v2.3$  VGCC in rodent sleep. Sleep architecture was investigated in control and  $Ca_v2.3(-/-)$  mice using implantable EEG/electromyography (EMG) radiotelemetry in a spontaneous 24 h sleep paradigm and pharmacologically (urethane)-induced sleep.

## Materials and methods

### Chemicals

All chemicals were analytical grade. Urethane (>99% purity) was obtained from Sigma-Aldrich (Steinheim, Germany) and dissolved in 0.9% (w/v) NaCl solution.

### Study animals

The *cacnale* gene encoding  $Ca_v2.3$  was targeted by homologous recombination in E14.1 embryonic stem (ES) cells. A loxP-flanked neomycin cassette was inserted into the NsiI site of intron 2 and a third loxP site was inserted downstream of the HindIII site. The cyclic DNA-producing recombinase (Cre-recombinase) was transiently expressed in correctly targeted ES cells, from which the Ta1E1E8 clone was identified by Southern blotting of its genomic DNA. From those ES cells surviving on puromycin, cells were selected that had lost their neomycin cassette (type II deletion) to generate a recombined *cacnale* gene in which exon 2 was flanked by two loxP sites [24]. Exon 2 corresponds to nucleotides 269–375 of the murine  $Ca_v2.3$  subunit (GB L29346).

LoxP-flanked exon 2 ES cells were injected into C57Bl/6 blastocysts. Resulting male chimeras were bred to C57Bl/6

females and the  $Ca_v2.3^{fl/+}$  genotype of agouti-colored offspring was determined by Southern blot analysis. The *cacnale* gene was ablated in vivo by mating  $Ca_v2.3^{fl/+}$  and Cre-deleter mice expressing Cre-recombinase under the constitutive control of the cytomegalovirus promoter. The  $Ca_v2.3$  null mutant was backcrossed into C57Bl/6 as previously described [25].  $Ca_v2.3(+/+)$  control animals and  $Ca_v2.3(-/-)$  mice were generated from  $Ca_v2.3(+/-)$  littermates by inbreeding two parallel mouse lines with identical genetic backgrounds.  $Ca_v2.3$ -deficient and control mice of both genders were used in this study. Mice were housed in Makrolon type II cages and maintained on a conventional light/dark cycle with food and water available ad libitum.

The mean body weight of the six mice used from each genotype was  $32.5 \pm 1.0$  g for control mice and  $32.7 \pm 2.2$  g for  $Ca_v2.3$ -deficient animals. Mice from both genotypes were age-matched at the day of implantation (age was  $161 \pm 5$  days for controls and  $164 \pm 10$  days for  $Ca_v2.3$ -deficient mice). Mice were used for EEG recordings after 10–12 days of implantation.

All animal experimentation was approved by the local institutional committee on animal care. All efforts were made to minimize animal suffering and to use the minimum number of animals required to produce reliable scientific data.

### Telemetric EEG recordings

The telemetry system, anesthesia, implantation procedure and postoperative treatment have been described in detail previously [26]. The positive electrode of the F20-EET transmitter (DSI, St. Paul, MN, USA) was implanted over the primary somatosensory cortex (–1 mm caudally, –3 mm laterally from bregma) and the negative electrode over the cerebellum (–6.3 mm caudally, –1 mm laterally from bregma). Electrodes from the second channel of the transmitter were implanted into the trapezius muscle. Mice were allowed 10–12 days to recover from surgery before radiotelemetric EEGs and EMGs [26] were recorded from six freely moving  $Ca_v2.3$ -deficient and six control mice over a 24 h period to permit recording of spontaneous sleep. Thereafter, pharma-

cologically-induced sleep was invoked by systemic intraperitoneal (i.p.) administration of 800 mg urethane/kg body weight, which was followed up by EEG/EMG recording for 24 h. Because urethane develops its maximum effect within 30 min after injection, the first 60 min after injection were selected for the evaluation of sleep stage. During induced sleep, mice were placed on a heating plate to maintain proper body temperature.

Dataquest™ A.R.T.™ 3.1 software (DSI) was used to record EEGs, which were sampled at 1000 Hz without filtering. Neuroscore 2.1.0 (DSI) was used to calculate the absolute and relative power of frequency bands (fast Fourier transform based using a Hamming window). The frequency spectrum was defined as follows:  $\delta$ : 0.5–4 Hz,  $\theta$ : 4–8 Hz,  $\alpha$ : 8–12 Hz,  $\beta$ : 16–24 Hz. The rodent sleep scoring analysis protocol assigned a vigilance stage to each epoch based on EEG, EMG and activity data. Accordingly, the sleep stages were grouped into: “active wake”, “quiet wake”, “paradoxical sleep” (PS), “slow-wave sleep 1” (SWS1) and “slow-wave sleep 2” (SWS2). “Total sleep time” is the time from sleep onset until the last wake episode, excluding wakefulness occurring in that interval. “Wake time after sleep onset” (WASO) is the summed wake time occurring within the sleep period. “Sleep onset” is the duration from start of scoring until the first occurrence of sleep stages (PS or SWS). “PS onset” is the duration from start of scoring until the first occurrence of PS. “SWS onset” is the duration from start of scoring until the first occurrence of SWS.

The active wake stage was scored when muscle tone was high and movements of the mice were between low and high. PS was recognized by a dominant  $\theta$  power band and an increase in the  $\theta/\delta$  ratio. A dominant  $\delta$  component is significant for SWS (i.e. deep sleep) and when the ratio of  $\delta$  power to total power exceeds the threshold of 0.35, SWS2 is awarded, otherwise SWS1. The EMG threshold was lowered from 20 to 10  $\mu$ V to ensure a more sensitive detection of movements. In four randomly picked mice (two of each genotype), the accuracy of the automatic sleep scoring protocol was assessed by manually determining REM and NREM sleep according to classic EEG definitions and evaluating

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**Ca<sub>v</sub>2.3 E-/R-type voltage-gated calcium channels modulate sleep in mice**

**Abstract**

Mammalian sleep is characterized by cycles of REM and non-REM (NREM), i.e. slow-wave sleep (SWS) phases. The major neuroanatomical basis of SWS is the thalamocortical circuitry, which operates in different functional modes to determine the state of vigilance. At high vigilance, the tonic mode predominates; stages of low vigilance and SWS are characterized by rebound burst firing. Electrophysiologically, rebound bursting depends on low-threshold Ca<sup>2+</sup> spikes and T-type Ca<sup>2+</sup> channels have been shown to modulate SWS. We recently demonstrated that Ca<sub>v</sub>2.3 R-type Ca<sup>2+</sup> channels are capable of modulating absence seizures, a pathophysiological aberration of the thalamocortical oscillations related to SWS. We thus analyzed sleep architecture in control and Ca<sub>v</sub>2.3(–/–) mice using implantable electroencephalography (EEG)/electromyography (EMG) radiotelemetry

during spontaneous and urethane-induced sleep. The results demonstrate significantly reduced total sleep time and impairment of SWS generation in Ca<sub>v</sub>2.3(–/–) mice, which affects global sleep architecture (i.e. the ratio of REM to NREM). Furthermore, the relative  $\delta$  power is significantly reduced in Ca<sub>v</sub>2.3(–/–) mice during NREM sleep although these mice display longer prior wakefulness, possibly indicating disturbances in sleep homeostasis. This observation is supported by recordings following urethane administration. This is the first study to shed light on the fundamental role of Ca<sub>v</sub>2.3 channels in rodent sleep physiology.

**Keywords**

Telemetry · Electroencephalography · Slow-wave sleep · REM sleep · Ion channel

**Modulation des Schlafs durch spannungsgesteuerte Ca<sub>v</sub>2.3-E- und Ca<sub>v</sub>2.3-R-Typ-Kalziumkanäle in Mäusen**

**Zusammenfassung**

Säugerschlag ist von zyklischen REM- und Non-REM-Stadien [Langsame-Wellen-Schlaf, „slow-wave sleep“ (SWS)] gekennzeichnet. Die wichtigste neuroanatomische Grundlage für den SWS bildet der thalamokortikale Schaltkreis, der in zwei verschiedenen funktionalen Modi arbeitet, die die Vigilanz bestimmen: im tonischen und im Reboundburst-Modus. Bei hoher Aufmerksamkeit dominiert der tonische Modus, wohingegen schwache Aufmerksamkeit und langsamer Wellenschlaf von „rebound bursts“ geprägt sind. Elektrophysiologisch hängen „rebound bursts“ von niederschweligen Ca<sup>2+</sup>-Spikes ab. Dementsprechend wurde gezeigt, dass T-Typ-Kalziumkanäle SWS modulieren. Kürzlich zeigten wir, dass Ca<sub>v</sub>2.3-E- bzw. Ca<sub>v</sub>2.3-R-Typ-Kalziumkanäle für die Modulation von Absenzen verantwortlich sind. Diese beinhalten eine pathophysiologische Störung der thalamokortikalen Oszillation, die mit dem SWS verwandt ist. Deswegen untersuchten wir die Schlafarchitektur von Ca<sub>v</sub>2.3-kompetenten und Ca<sub>v</sub>2.3-

defizienten Mäusen wofür wir die implantierbare Elektroenzephalographie(EEG)/Elektromyographie(EMG)-Radiotelemetrie während des natürlichen 24-h-Schlafs und des mit Urethan pharmakologisch induzierten Schlafs einsetzten. Unsere Ergebnisse zeigen im Vergleich mit Kontrollmäusen, dass die generelle Inaktivierung der Ca<sub>v</sub>2.3-Kanäle in einer Mauslinie die Gesamtschlafzeit signifikant reduziert, die Generierung von SWS in Ca<sub>v</sub>2.3-defizienten Mäusen beeinflusst und in die globale Schlafarchitektur eingreift, d. h., das Verhältnis von REM und SWS verändert. Interessanterweise wird diese Beobachtung auch durch die Aufzeichnungen bei der darauf folgenden Gabe von Urethan gestützt. Dies ist die erste Studie, die eine fundamentale Rolle des Ca<sub>v</sub>2.3-Kanals in der Schlafphysiologie der Nager aufzeigt.

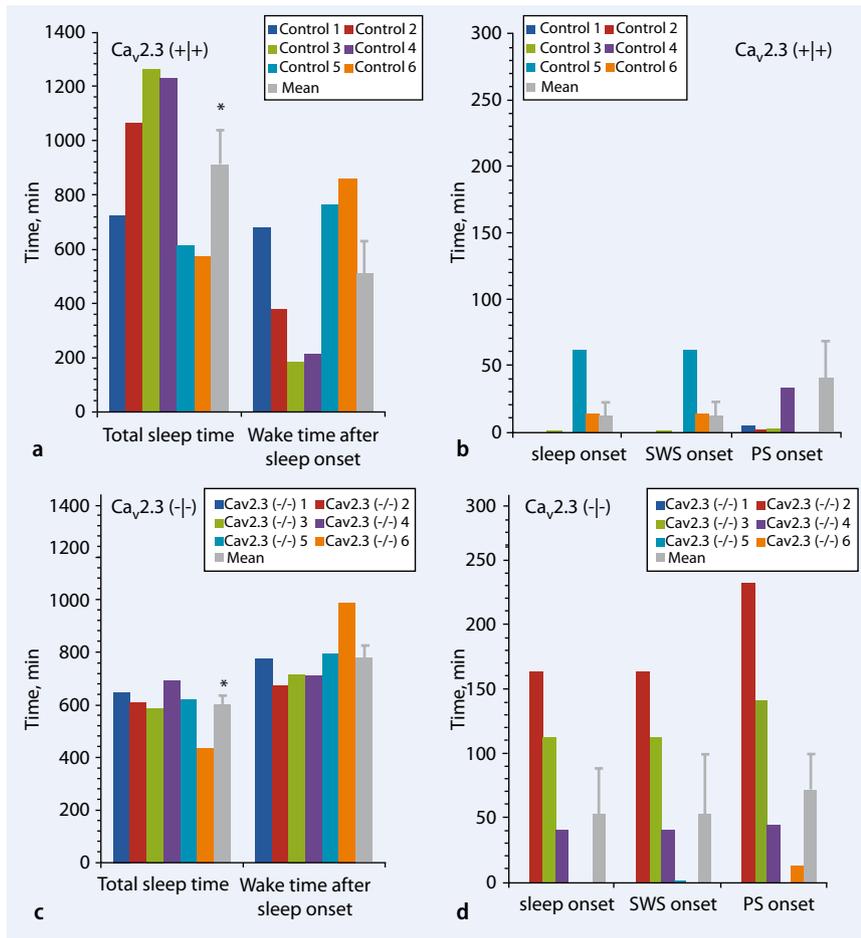
**Schlüsselwörter**

Telemetrie · Elektroenzephalographie · Langsame-Wellen-Schlaf · REM-Schlaf · Ionenkanal

the level of activity (locomotion) independently of EMG. In these evaluated cases, we found the automated protocol to be comparable to classic definitions of NREM and

REM sleep and therefore suitable for comparison of sleep parameters in this study.

Spectral analysis was performed on the first clearly distinguishable full sleep cy-



**Fig. 2** ▲ Time-based analysis of normal sleep–wake states as determined by EEG/EMG for control (**a**, **b**) and Ca<sub>v</sub>2.3-deficient mice (**c**, **d**). The primary signal used for scoring rodent sleep was the cortical EEG. Additional signals used were neck EMG,  $\theta$  EEG (located directly over the hippocampus) and activity score from the telemetry system. After 10–12 days postsurgical recovery, EEGs and EMGs were recorded from six freely moving Ca<sub>v</sub>2.3-deficient and six control mice for a 24 h period to enable recording of spontaneous sleep. Colored bars indicate time values from individual mice; white bars represent the mean  $\pm$  SEM. Total sleep time for Ca<sub>v</sub>2.3-competent mice was significantly longer than for Ca<sub>v</sub>2.3-deficient animals (\* in **a** and **c** and Fig. 5a). The data for sleep onset, slow-wave sleep (SWS) onset and paradoxical sleep (PS) onset presented in **b** and **d** are at a higher magnification. No difference was found between Ca<sub>v</sub>2.3-competent and -deficient mice for wake time after sleep onset, time to sleep onset, time to SWS onset or time to onset of PS (compare **a** with **c** and **b** with **d**)

cle of the 24 h recording of spontaneous sleep performed for each mouse. A sleep cycle was defined as an episode of all three sleep stages (SWS1, SWS2 and PS), bordered by at least 1 min of active wakefulness. The average relative power of the frequency bands  $\delta$ ,  $\theta$ ,  $\alpha$  and  $\beta$  was computed for the three sleep stages and compared between the two genotypes.

### Statistical analysis

Data are displayed as mean  $\pm$  SEM. The distribution of relative spectral power was assessed using the Shapiro–Wilk test of normality and found to be mostly non-

normally distributed. Therefore, relative power values were log transformed ( $\log(x/(1-x))$ ) to obtain a more Gaussian distribution, before being subjected to analysis of variance (ANOVA) [27]. Statistical comparison was performed for the data in **Fig. 4** and **Fig. 5** using the student t-test, with  $p < 0.05$  considered as significant (\*) and  $p < 0.01$  as highly significant (\*\*). For the parameters of total sleep time, WASO and active wake (**Fig. 1**, **2**, **3**, **4**), the denoted values were certified with the Levene's test for equality of variances and represent assumed variances. The Mann–Whitney U test verified the significances for the duration of SWS2, the SWS2–

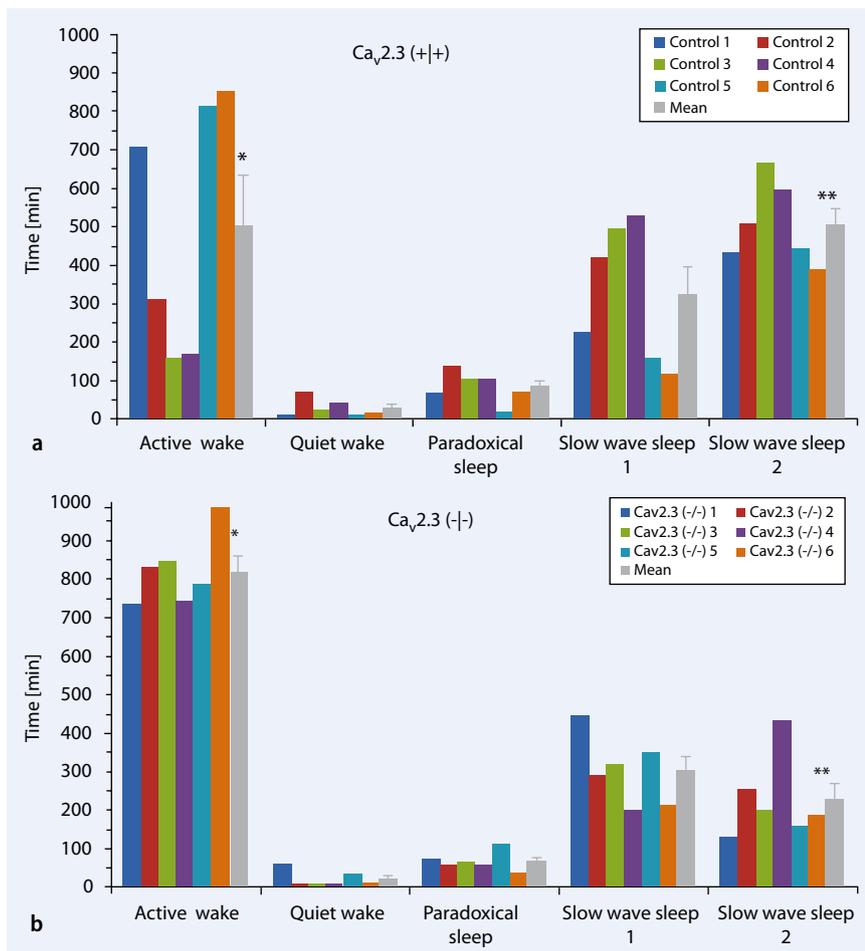
PS transition and the single longest active wake and SWS2 sleep stages.

## Results

### Analysis of duration and latencies of sleep stages during the 24 h spontaneous sleep

Telemetric EEG recordings of normal and urethane-induced sleep reveal no obvious differences between Ca<sub>v</sub>2.3-competent (**Fig. 1a** and **c**) and Ca<sub>v</sub>2.3-deficient mice (**Fig. 1b** and **d**) during short 30 s recordings from SWS periods. However, differences between the genotypes became evident when evaluating the total recording periods (24 h for normal sleep and 60 min for urethane-induced sleep).

During the 24 h of spontaneous sleep/wakefulness activity, WASO, sleep onset and PS onset were comparable between both genotypes (**Fig. 2**), however the total sleep time of control animals ( $910 \pm 127$  min) was significantly longer ( $p = 0.039$ ) than that of Ca<sub>v</sub>2.3(-/-) mice ( $597 \pm 36$  min, compare the mean values labeled by an asterisk in **Fig. 2a** and **c**). As depicted in **Fig. 3**, the duration of sleep stages quiet wake, PS and SWS1 did not differ between the two genotypes, however control animals exhibit significantly shorter ( $p = 0.043$ ) active wake periods ( $501 \pm 133$  min) compared to Ca<sub>v</sub>2.3-deficient mice ( $821 \pm 38$  min). SWS2 was significantly longer in control ( $503 \pm 43$  min, **Fig. 3a**) than Ca<sub>v</sub>2.3 knockout (KO) mice ( $227 \pm 44$  min,  $p = 0.001$  **Fig. 3b**). Correspondingly, Ca<sub>v</sub>2.3(-/-) mice exhibited the longest single active wake ( $p = 0.015$ ) and the shortest SWS2 stage ( $p = 0.002$ , **Fig. 3b**). Furthermore, the number of transitions from SWS2 to PS shows a significant difference between the genotypes (controls:  $16 \pm 5$  vs. Ca<sub>v</sub>2.3-KO:  $2 \pm 1$ ,  $p = 0.022$ ), while all other transitions were comparable between both genotypes. Noticeably, a high scatter was found in control animals for total sleep time, WASO and active wake (**Fig. 2** and **Fig. 3**). In conclusion, ablation of Ca<sub>v</sub>2.3 disturbs normal sleep behavior in adult male mice by shortening SWS2 and total sleep time, which is logically accompanied by an increase in active wake time in Ca<sub>v</sub>2.3-deficient mice.



**Fig. 3** ▲ Sleep/wake stages of normal sleep from control (a) and Ca<sub>v</sub>2.3-deficient (b) mice. Time-based values of active wake (muscle tone high; movements of mice low–high), quiet wake, paradoxical sleep, slow-wave sleep 1 and slow-wave sleep 2 sleep stages. Colored bars represent individual values for each of six mice; white bars represent the mean ± SEM. Duration of the active wake stage is longer in Ca<sub>v</sub>2.3-deficient mice than in controls (\*). Furthermore, slow-wave sleep 2 is significantly longer in Ca<sub>v</sub>2.3-competent mice compared to Ca<sub>v</sub>2.3-deficient animals (\*\*)

### Spectral analysis of spontaneous sleep

Spectral analysis of the first sleep cycle revealed significant differences in the spectral distributions of the two genotypes during all three sleep stages (■ Fig. 4a). During both SWS stages, Ca<sub>v</sub>2.3-KO mice display significantly less relative  $\delta$  power compared to controls, potentially representing reduced depth of NREM sleep (■ Fig. 4b, left and middle panels). During SWS and PS, Ca<sub>v</sub>2.3-KO mice display significantly increased relative  $\theta$  power compared to control mice, reflected by the decrease in other frequency bands (■ Fig. 4b). Interestingly, although Ca<sub>v</sub>2.3-KO mice appear to sleep less, they do not display increased  $\delta$  power that would reflect a greater intensity of SWS.

### Urethane-induced sleep

For the evaluation of urethane-induced sleep, the first 60 min after injection of urethane were selected, since urethane develops its maximum effect 30 min after injection. As shown in ■ Fig. 5, during urethane-induced, i.e. artificial sleep, total sleep time was significantly longer in control animals ( $p=0.034$ ). The duration of active wake is increased in Ca<sub>v</sub>2.3(-/-) mice (controls:  $7\pm 2$  min vs. Ca<sub>v</sub>2.3-KO:  $22\pm 6$  min;  $p=0.027$ ). Furthermore, a significant difference in the duration of PS was observed between both genotypes (controls:  $1.4\pm 1.5$  vs. Ca<sub>v</sub>2.3-KO:  $0.03\pm 0.08$  min;  $p=0.048$ ).

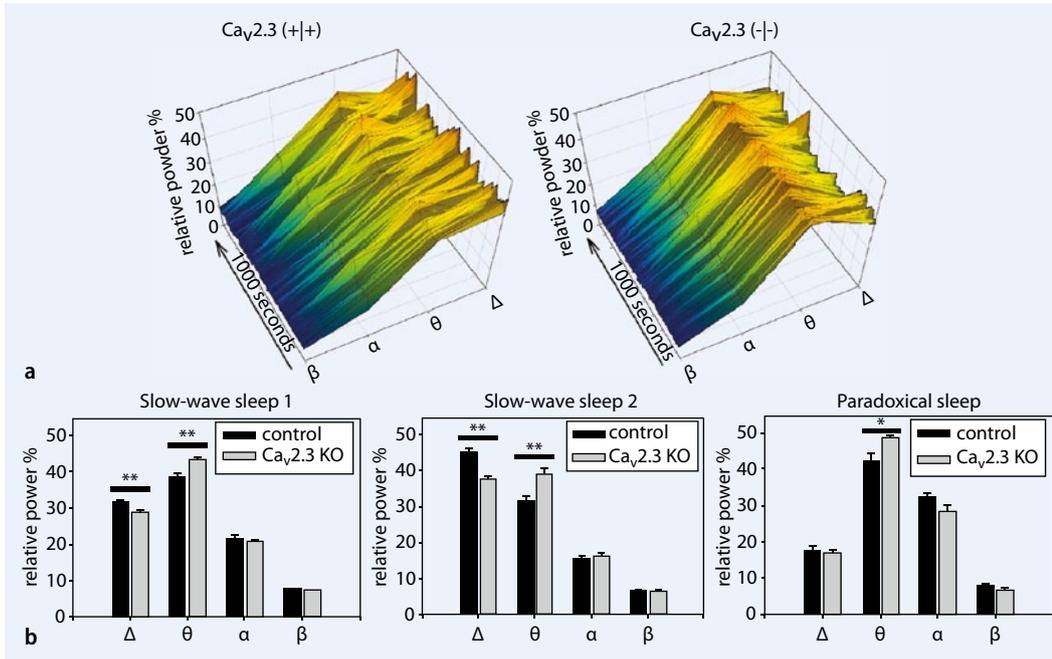
In contrast, no difference was found between the two genotypes in the duration of deep sleep (SWS2) (controls:

$45\pm 2$  min vs. Ca<sub>v</sub>2.3-KO  $35\pm 6$  min; student's t-test  $p=0.16$ ). SWS1 comparisons were also equal. The number of transitions from active wake to quiet wake was significantly increased in Ca<sub>v</sub>2.3-KO mice ( $p=0.022$ ). Furthermore, the maximum single active stage duration is also increased in control animals ( $p=0.008$ ). The Mann–Whitney U test verified the significances for the total sleep time, sleep onset, SWS onset, duration of active wake and PS and the active wake–quiet wake transition. Interestingly, the scatter is higher in the Ca<sub>v</sub>2.3(-/-) mice group, whereas controls exhibit homogeneous values (for all p-values: two-tailed t-test with  $n=6$  and significance level  $p<0.05$ ). The results for urethane-induced sleep are summarized and compared to normal sleep (■ Fig. 5).

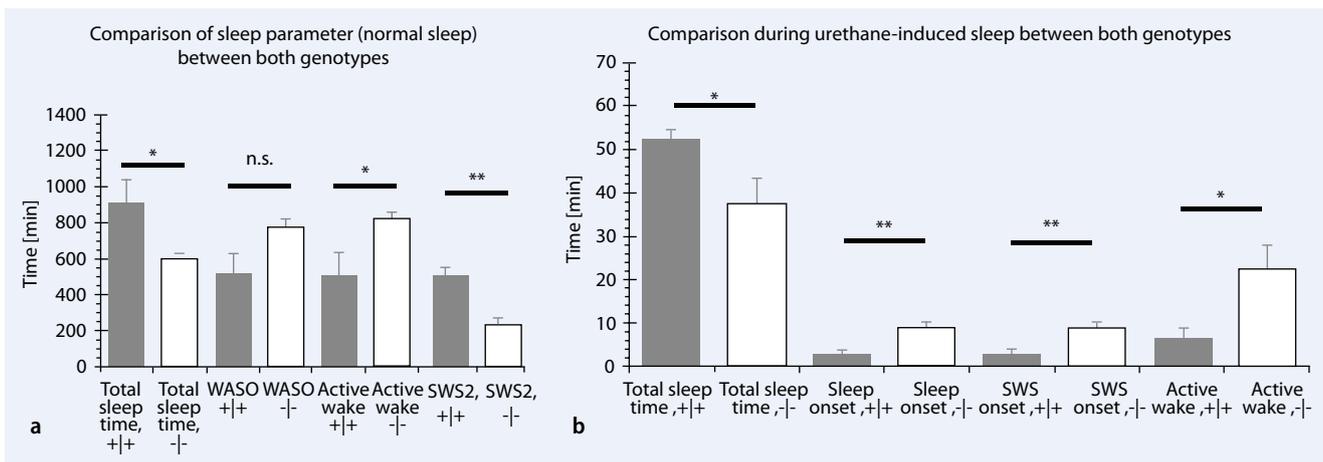
### Discussion

The observed differences in sleep architecture mainly affected the duration (■ Fig. 5) and intensity of deep NREM sleep (■ Fig. 4). The number of transitions from SWS2 to PS during 24 h, as well as the duration of SWS2 and PS implicate Ca<sub>v</sub>2.3 E-/R-type Ca<sup>2+</sup> channels as modulators of thalamocortical signal transduction. Duration, timing and depth of sleep are regulated by two interacting processes: a homeostatic factor (which regulates depth and duration of sleep), within a circadian rhythm (which is the factor that determines the timing of sleep) [28]. While slow-wave activity is mainly not influenced by circadian factors, the intensity of  $\delta$  waves in deep sleep stages and the propensity to sleep are both proportional to the duration of prior wakefulness [29, 30]. Sleep homeostasis is therefore dependent on the interval since the last sleep episode and the depth of this sleep. Remarkably, although Ca<sub>v</sub>2.3-KO mice sleep less and therefore endure longer periods of wakefulness, they do not exhibit deeper sleep, indicating disturbances in sleep homeostasis and therefore a role of Ca<sub>v</sub>2.3 calcium channels in sleep homeostasis.

As recently summarized for other regions and species [33], murine Ca<sub>v</sub>2.3 VGCCs are expressed in most basal ganglia regions, the thalamus, the hypothalamus, the amygdala, the hippocampus and the cortex [31, 32]. Two major splice vari-



**Fig. 4** ◀ Spectral analysis of normal sleep from control and  $Ca_v2.3$ -deficient mice. **a** During the first sleep cycle, spectral distribution of relative ECG power was compared for 100 epochs of 10 s duration. **b** Bar diagrams showing the averaged relative power for slow-wave sleep 1 (left), slow-wave sleep 2 (middle) and paradoxical sleep (right) from both genotypes.  $Ca_v2.3$ -deficient mice display decreased relative  $\delta$  power during non-REM sleep and increased  $\theta$  power during REM sleep. In the student t-test,  $p < 0.05$  was considered significant (\*) and  $p < 0.01$  (\*\*), highly significant



**Fig. 5** ▲ Summary of significant differences between the two genotypes for the time-based analysis of sleep/wake stages. For the overall comparison of sleep data, the automatic evaluation software NeuroScore (version 2.0.1., D.S.I.) was used. Thresholds were only changed for the EMG (from 20 to 10  $\mu V$ ). **a** Comparison for normal sleep. **b** Comparison for urethane-induced sleep. WASO wake time after sleep onset, SWS1 slow-wave sleep 1, SWS2 slow-wave sleep 2, \* statistically significant, \*\* highly significant, n.s. not statistically significant, grey bars control mice, white bars  $Ca_v2.3(-/-)$  mice

ants exist in the central nervous system [34]; one being unique to the cerebellum [35]. On the protein level,  $Ca_v2.3$  has been identified in the nRt region, the neocortex and the hippocampus [34]. Thus,  $Ca_v2.3$  is expressed in regions that are known to contribute to the modulation of the thalamocortical network.

Humans and animals spend a third of their lives asleep. However, we have yet to fully understand why sleep is necessary. Although it is evident that it is mainly the brain that requires sleep, the main function of sleep and the induction there-

of have yet to be completely elucidated. Sleep induction and the increasing depth of NREM sleep measured as slow oscillations are proposed to result from the inhibition of the thalamic relay nuclei mediated by the nRt, where  $Ca_v2.3$  is highly expressed [12]. A finely tuned interplay between GABAergic inhibitory postsynaptic potentials received from nRt neurons and two types of voltage-gated channels (T-type  $Ca^{2+}$  channels and hyperpolarization-activated cation channels) modulates the activity of thalamocortical projections [36]. Besides this interplay, additional changes

in conductance contribute to such oscillations as well [37, 38]. Recently, a T-type  $Ca^{2+}$  channel has been connected to the initiation of synchronized thalamic network oscillations underlying sleep spindle waves: deletion of  $Ca_v3.3$  markedly weakened (but did not abrogate) thalamic network oscillations suggesting a central role for  $Ca_v3.3$  VGCCs in the rhythmogenic properties of the sleep spindle generator [39]. One may postulate that  $Ca_v2.3$  could contribute to such a regulation, since both channels are expressed in the nRt region. Repetitive burst firing in nRt neurons was

also shown to be dependent on intracellular  $\text{Ca}^{2+}$  homeostasis, which is affected by several mechanisms and actions of transporters and ion channels [8, 40].

The use of  $\gamma$ -butyrolactone to induce absence epilepsy in two  $\text{Ca}_v2.3$ -deficient mouse models has led to opposite conclusions regarding the ability to induce absence epilepsy [2, 41]. In brain slices from  $\text{Ca}_v2.3$ -deficient mice, injection of hyperpolarizing current initiated low-threshold bursts of spikes in nRt neurons. However, subsequent oscillatory burst discharges were severely suppressed in  $\text{Ca}_v2.3$ -deficient mice, with a significantly reduced slow AHP [41].

In our own model of  $\text{Ca}_v2.3$ -deficient mice, we investigated absence-specific SWD susceptibility by systemic administration of  $\gamma$ -hydroxybutyrolactone followed by electrocorticographic radiotelemetric recordings, behavioral analysis and histomorphological characterization. Based on motoric studies, SWD and power-spectrum density (PSD) analysis, our results demonstrated that  $\text{Ca}_v2.3$ -deficient mice exhibit increased absence seizure susceptibility [2]. So far, the discrepancy between the different mouse knockout models is unclear. However thalamocortical rhythmicity is altered in both models as compared to the corresponding control mouse line.

Furthermore, spectral analysis revealed differences between both genotypes during REM (PS) sleep. Interestingly, SNX-482—a selective antagonist of  $\text{Ca}_v2.3$ —was found to attenuate carbachol inhibition of somatic spike-evoked calcium transients in the laterodorsal tegmentum (LDT), which projects to the REM-induction site of the brainstem [42]. Because the spike-evoked calcium influx dampens excitability in the LDT, it is conceivable that muscarinic control of  $\text{Ca}_v2.3$  regulates firing rate and responsiveness to excitatory inputs during states of high firing, like during REM sleep.

Future studies involving region-specific neuronal inactivation of  $\text{Ca}_v2.3$  may help to understand the interplay of ion channels, transporters and signaling receptors that is important for sleep homeostasis. In mice,  $\text{Ca}_v2.3$  evidently represents an important participant, as far as it can be concluded from its general ablation.

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**Conflict of interest.** On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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