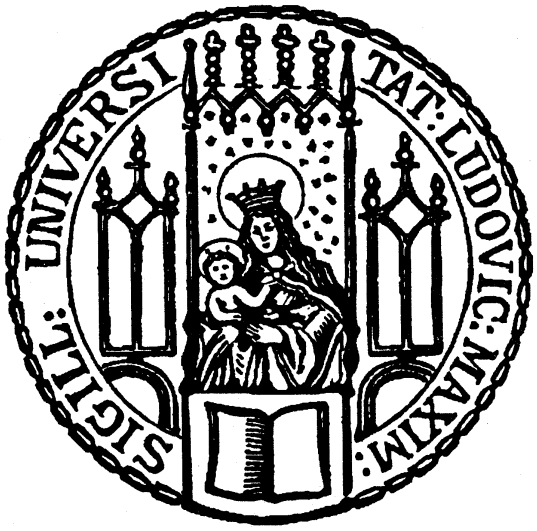
Pharmacological and genetic modulation of the endocannabinoid system: Evaluation of preventive strategies in the amygdala kindling model of temporal lobe epilepsy

Dr. med. vet. Eva-Lotta von Rüden,
née Schneider



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LMU Munich

Submitted by Dr. med. vet. Eva-Lotta von Rüden,

née Schneider

from Hansestadt Lübeck, Germany

First reviewer/supervisor: Prof. Dr. Heidrun Potschka

Second reviewer: Dr. Daniela Vogt-Weisenhorn

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To my family.

ABSTRACT

Epilepsy is one of the most common chronic neurological diseases worldwide and the prevention of epileptogenesis is so far unmet. A major challenge in epilepsy research is the development of new therapeutic approaches for patients with therapy-resistant epilepsies, for epilepsy prevention and for disease modification. The endocannabinoid system serves as a retrograde negative feedback mechanism and one of its key functions is regulating neuronal activity within the central nervous system. Thus, the endocannabinoid system can be considered a putative target for central nervous system diseases including epilepsies.

The purpose of this thesis was to evaluate the impact of the endocannabinoid and endovanilloid systems on both epileptogenesis and ictogenesis. Therefore, I modulated the systems pharmacologically and genetically and analyzed the impact on the generation of a hyperexcitable neuronal network as well as on ictogenesis in the kindling model of temporal lobe epilepsy. In addition, the impact of seizures on associated cellular alterations, like CB1-receptor (CB1R) expression and neurogenesis, was evaluated.

I established that the endocannabinoid system affects seizure and afterdischarge duration dependent on the neuronal subpopulation being modulated. Genetic deletion of CB1Rs from GABAergic forebrain neurons caused shorter seizure duration. Deletion of CB1R from principal neurons of the forebrain and pharmacological antagonism with rimonabant (5 mg/kg) resulted in the opposite effect. Along with these findings, the CB1R density was increased in mice with recurrent induced seizures. However, neither genetic knockout nor pharmacological antagonism had any impact on the development of generalized seizures. In contrast to genetic deletion or pharmacological blockade of CB1Rs, modulation of transient receptor potential vanilloid receptor 1 (TRPV1) neither genetically nor pharmacologically with SB366791 (1 mg/kg) had an effect on the duration of behavioral or electrographic seizure activity.

Pharmacological blockade of the 2-arachidonoylglycerol degrading enzyme, monoacylglycerol lipase (MAGL) with JZL184 (8 mg/kg), delayed the development of generalized seizures and decreased seizure and afterdischarge durations whereas in fully-kindled mice JZL184 (4, 8 and 16 mg/kg) had no relevant effects on associated seizure parameters. In addition, I confirmed by the use of conditional CB1R knockout mice that these effects are CB1R mediated.

In conclusion, my findings support the concept that the endocannabinoid system may be a therapeutic target for decreasing seizure duration and that it is involved in terminating seizures as an endogenous mechanism. Moreover, targeting MAGL may be a promising strategy for an antiepileptogenic approach. Respective strategies are of particular interest for the management of long-lasting refractory status epilepticus and cluster seizures as well as for the prevention of the development of symptomatic epilepsies after an initial insult.

TABLE OF CONTENTS

ABBREVIATIONS

ACAE	Arachidonyl-2'-chloroethylamide
2-AG	2-Arachidonylglycerol
AED	Antiepileptic drug
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
CB1R	Cannabinoid receptor type 1
CB2R	Cannabinoid receptor type 2
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
EEG	Electroencephalogram
FAAH	Fatty acid amide hydrolase
FGF-2	Fibroblast growth factor 2
GABA	Gamma aminobutyric acid
IBE	International Bureau for Epilepsy
IL	Interleukin
ILAE	International League against Epilepsy
KO	Knockout
MAGL	Monoacylglycerol lipase
Δ9-THC	Δ9-Tetrahydrocannabinol
SE	Status epilepticus
TLE	Temporal lobe epilepsy
TRPV1	Transient receptor potential vanilloid type 1 channel
VR1	Vanilloid receptor
WT	Wildtype

INTRODUCTION

1. Epilepsy

1.1. Definitions and classification

The disease *epilepsy* is a multifactorial disorder associated with complex and various different brain pathologies, e.g., among others, tumors, infection, stroke or traumatic brain injury (Froscher and Neher 1994; Fisher et al. 2005).

According to the working definition of the International League against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), epilepsies are characterized by recurrent and unprovoked seizures, which have their origin in the central nervous system (Fisher et al. 2005; Fisher et al. 2014). The common clinical symptom is the epileptic seizure, which is based on the transient occurrence of abnormal, paroxysmal changes in the electrical neuronal activity of the brain (Fisher et al. 2005; Fisher et al. 2014).

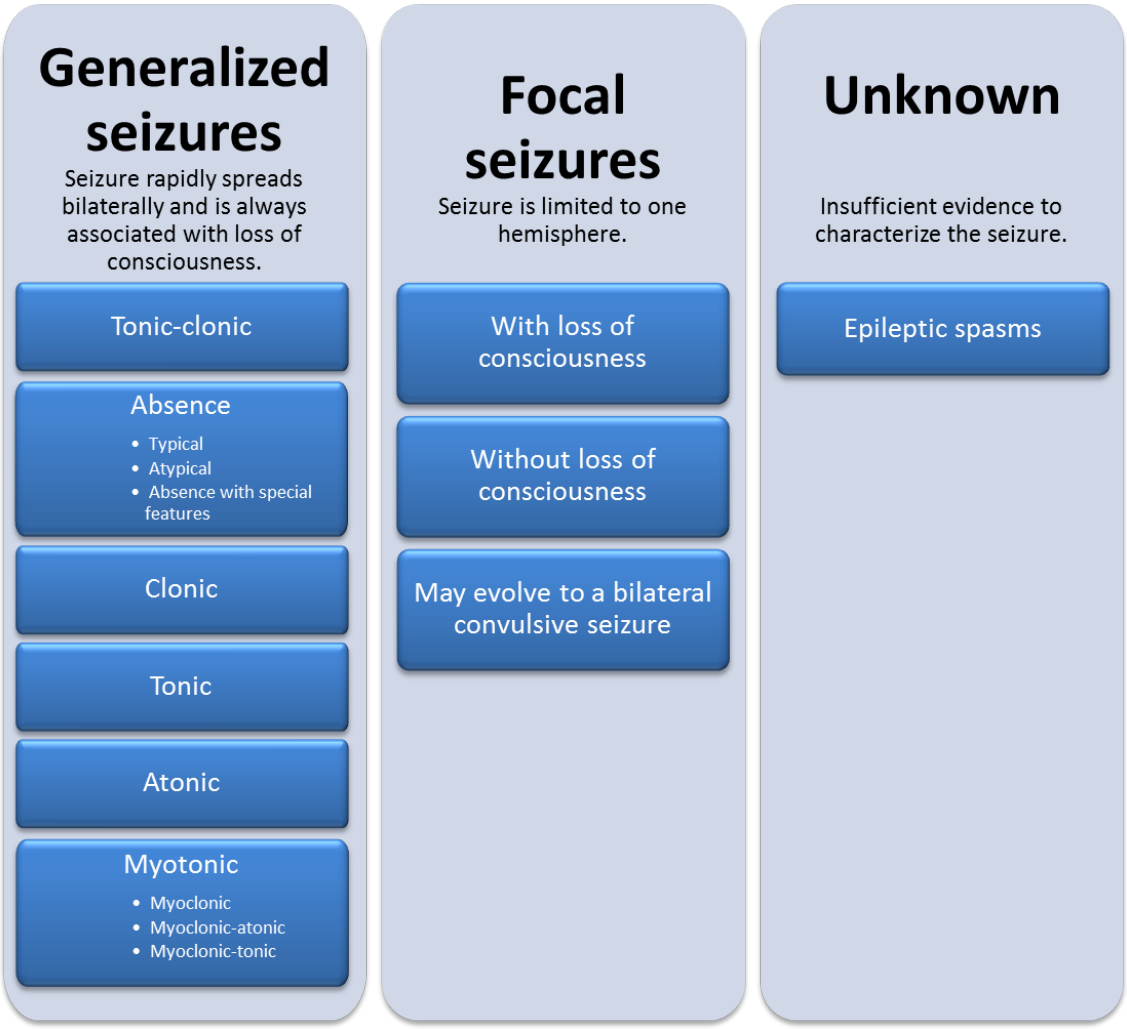
Approximately 50 million people suffer from epilepsy worldwide (World Health Organization 2006), whereof at least 40 million live in developing countries (World Health Organization 2006) and an estimated 6 million live in Europe (Cross 2011). Simply put, epilepsy is one of the most prevalent chronic neurological diseases.

For a successful treatment regime, seizure characterization followed by a thorough diagnosis of the epileptic syndrome and the underlying cause is indispensable. The framework for an accurate diagnosis is provided by a classification system of the ILAE. The first proposal for seizure and epilepsy classification was made in 1960 and was updated in 1981 and 1989 (Dreifuss et al. 1981; Classification and Epilepsy 1989). Advances in neuroimaging, genomic technologies and new concepts in molecular biology have revealed that the classification concepts of 1981 and 1989 are outdated and have been refined recently. The approach presented in the 2010 ILAE Commission report represents a major step forward, but is still under revision (Berg et al. 2010; Berg and Millichap 2013). The concept for the classification of epilepsies and seizures is based on the mode and age of seizure onset, the underlying cause (etiology), EEG features as well as provoking or triggering factors (Berg et al. 2010).

Etiologically, epilepsies are classified as symptomatic, idiopathic and cryptogenic epilepsies (Kwan and Brodie 2006). Since 2010, the ILAE recommends replacing this terminology with the following terms: structural or metabolic epilepsies, genetic epilepsies and epilepsies of unknown origin (Berg et al. 2010). *Structural-metabolic* (formerly “symptomatic”) epilepsies are caused by a structural or metabolic disorder of the brain. The underlying cause of g*enetic* (formerly “idiopathic”) epilepsies is a genetic defect that contributes to epilepsy with seizures as the main symptom. If the cause is unknown and might be genetic, structural or metabolic the epilepsy is classified as *unknown* (formerly “cryptogenic”; Engel 2001; Berg et al. 2010).

Seizures are classified into *generalized seizures* and *focal seizures*. The generalized seizure originates at some place and rapidly spreads to the other hemisphere associated with loss of consciousness. Generalized seizures are further classified into tonic-clonic, absence, clonic, tonic, atonic, or myoclonic seizures (Berg et al. 2010). If the seizure activity continues over a long period without full recovery of the patient between single seizures, it is called status epilepticus (Fisher et al. 2005).
In contrast, focal seizures are limited to one hemisphere and are accompanied either with or without loss of consciousness. Focal seizures may secondarily generalize and spread to the entire brain (Fisher et al. 2005). **Figure 1** gives an overview of the clinical seizure classification.

Figure 1.



Overview of the clinical seizure classification *(modified after Berg et al. (2010)).*

Until now, the main goal of epilepsy treatment has been to control the clinical symptoms and to reach seizure freedom by suppression of recurrent seizures. Usually, patients are treated with antiepileptic or anticonvulsant drugs (AEDs). The choice of medication should consider several factors, like the AED's mechanism of action, drug interactions, the side-effect profile, and should include the epilepsy classification concept. The first choice should be an AED that is expected to be well-tolerated with

few side effects. Classical side effects caused by AEDs are dizziness, imbalance, nausea, and diplopia. Although the new AEDs have minimal side effects and have only a small potential for drug interaction, little improvement in AED efficacy has been made since phenobarbital was launched in 1912. In up to 25-30% of patients, epileptic seizures cannot be controlled by AED treatment. These patients are considered to be drug resistant (Kwan and Brodie 2006). Moreover, *cure* cannot be achieved with AEDs used in clinical practice (Loscher 2002b). In addition, pharmacological treatment does not prevent the development or progression of epilepsy (Loscher and Brandt 2010). Surgical resection of the epileptic focus is an alternative treatment strategy but is rather applied to patients with drug resistant epilepsy or to patients with severe side effects due to AED treatment.

Among the epilepsy syndrome, temporal lobe epilepsy (TLE) is the most common form and is frequently very difficult to treat (Engel et al. 1998). TLE is considered to belong to the group of *structural-metabolic* epilepsies, although there are known forms of TLE with an underlying genetic cause (Berkovic et al. 1996; Vadlamudi et al. 2003; Salzmann and Malafosse 2012). Typically, TLE is an acquired epilepsy syndrome in which seizures originate in the temporal lobe triggered by a precipitating factor, such as traumatic brain injury, febrile seizure, stroke, encephalitis, perinatal hypoxia, or even a tumor. The precipitating factor is called the *initial insult* (Stefan et al. 2006). The initial insult initiates pathophysiological alterations in the hippocampus and parahippocampal regions leading to the formation of a proconvulsant neural network and finally to chronic epilepsy. This process is called *epileptogenesis* and is described below in more detail.

1.2. Epileptogenesis and seizure-associated alterations

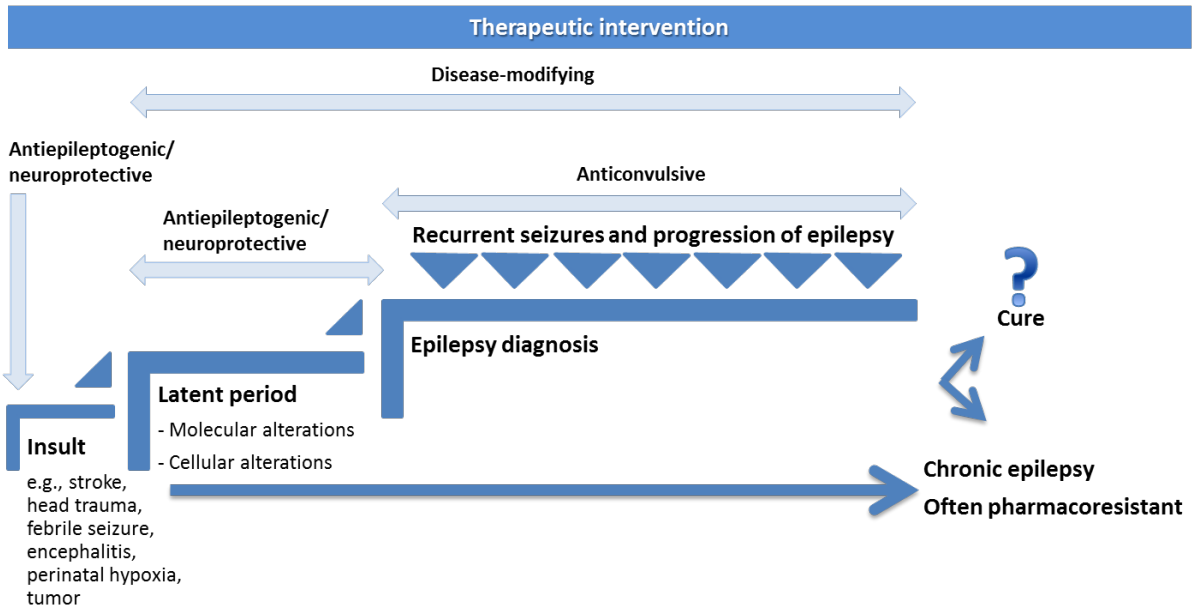
Epileptogenesis is a dynamic process and is associated with the development of acquired epilepsies. Multiple pathophysiological alterations at the molecular, cellular and circuit level lead towards an imbalance of neuronal inhibition and excitation and to the manifestation of chronic epilepsy (Ziburkus et al. 2006; Ziburkus et al. 2013). However, the exact pathophysiology underlying the development of TLE remains still unknown.

Classically, epileptogenesis is a three-step process: first, the *initial insult*; second, the *latent* or *silent period*; and third, chronic epilepsy with spontaneous and recurrent seizures. The concept of epileptogenesis is illustrated in **figure 2**.

The *initial insult*, e.g., traumatic brain injury, febrile seizure, stroke, encephalitis, perinatal hypoxia, or a tumor, is the trigger, which sets the pathophysiological process of epileptogenesis in motion.

The *latent or silent period* is defined as the time from a brain insult to the first clinical seizure (Pitkanen and Lukasiuk 2011). In this period no clinical seizures occur. However, data from animal experiments declare that non-convulsive electroencephalographic seizures occur before the first visible convulsive seizure (Bertram and Cornett 1993; Bertram and Cornett 1994; Williams et al. 2009). These studies point towards the possibility that in humans subclinical unrecognized seizures may predate the first clinical event. In humans the asymptotic period can range from a few weeks to some years (Annegers et al. 1998; Tsai et al. 2009).

Figure 2.



Epileptogenesis of TLE and therapeutic intervention points. *The stepwise process of epileptogenesis includes the initial insult, the latency period and the occurrence of recurrent seizures often associated with progression leading to pharmacoresistant epilepsy. The overall treatment goal is cure with the reversal of the pathophysiological alterations. The light blue arrows indicate possible therapeutic intervention points (modified from Löscher et al. (2008) and Pitkänen and Lukasiuk (2011)).*

During the latent period, molecular signaling pathways convert, e.g., immediate-early gene expression and the expression of ion channel and neurotransmitter receptors occur (Loscher et al. 2008; Pitkanen and Lukasiuk 2011). Furthermore, altered circuit-level mechanisms, like synaptic reorganization, dendritic plasticity, axonal sprouting and subsequently the formation of new excitatory synaptic connections (the so-called mossy fiber sprouting), are described (Babb et al. 1991; Peng et al. 2013; Singh et al. 2013). In addition, changes on the cellular level, e.g. neurodegeneration and neurogenesis, are hallmarks of epileptogenesis (Parent et al. 1997; Sutula et al. 2003; Pekcec and Potschka 2007; Scharfman and Gray 2007; Licko et al. 2013). Neuronal death, especially of GABAergic interneurons, consequently results in decreased GABA inhibition (Toth et al. 2010; Huusko et al. 2013). On the other hand, dentate gyrus mossy cells die as well (Danzer et al. 2010; Zellinger et al. 2011a). This causes reactive gliosis along with cell dispersion and generation of new dentate granule cells in ectopic locations (Scharfman et al. 2000; Scharfman et al. 2003; Cameron et al. 2011). All this is accompanied by inflammatory processes (Vezzani et al. 2011) and damage of the blood-brain-barrier (Tomkins et al. 2007; van Vliet et al. 2007). Some of these changes may be compensatory and reparative, but others are actively involved in the pathogenesis of epilepsy. Some alterations may contribute to the latent period and also continue beyond the diagnosis of epilepsy, and are further involved in the progressive nature of some epilepsies like TLE (Pitkanen and Lukasiuk 2009).

The prevention of epileptogenesis is still an unmet challenge. This raises the question of when the right time point for therapeutic intervention is and which are the best targets to aim for. **Figure 2** summarizes possible intervention points during epileptogenesis.

The diversity of pathophysiological alterations during epileptogenesis suggests that the whole process is multifactorial and that the targets can vary over time. Relevant intervention time points of antiepileptogenesis are not only the prevention or delay of epilepsy, but also strategies to modify seizure frequency and seizure severity. Moreover, because epilepsy can be associated with psychiatric comorbidities, e.g., memory impairment or anxiety disorders (see chapter 1.3.), comorbidity modification should also be taken into account. Considering the complex pathophysiology of epilepsy, polytherapy might be more successful than monotherapy.

1.3. Epilepsy-associated psychiatric comorbidities

Many people suffering from epilepsy do not only have to cope with the disease itself, but also with co-morbidities that tremendously affect their daily activities and quality of life. A comorbidity is defined as *"a condition that occurs in association with another (i.e., epilepsy) at frequencies that are significantly greater than those observed in the appropriate control group"* (Brooks-Kayal et al. 2013). The comorbidity may be a cause of epilepsy, a consequence or even a separate condition. Among others, cognitive impairment, depression, anxiety disorders, attention deficit, autism, suicidality, psychosis and migraine can be associated with epilepsy. Very often, epilepsy associated with mood disorders is accompanied by poor seizure control and drug resistance (Hesdorffer et al. 2006). For example, TLE is both the most common form of drug-resistant epilepsy and also the most common form associated with comorbidities, such as depression and cognitive impairments (Kanner 2006a; Hoppe et al. 2007). Evidence suggests that the structural damage, like pyramidal cell loss in CA1 and CA3, gliosis, axonal sprouting and aberrant migration of granule cells in the hippocampus, is progressive (Yilmazer-Hanke et al. 2000). These changes in hippocampal neuronal networks may explain the progressive cognitive impairments and problems in memory consolidation (Hermann et al. 2006). This may explain why an estimated large number of children (25-40 %) with epilepsy have impaired intellectual function and poor educational outcomes (Berg et al. 2004; Sogawa et al. 2010).

In addition, there is growing evidence from animal models that epilepsy and behavioral abnormalities may share the same mechanisms of underlying neuropathology (Groticke et al. 2007, 2008; Takechi et al. 2011; Yutsudo et al. 2013). Serotonin receptor dysfunction, for example, is discussed as one of the main pathological mechanisms of depression (Yang et al. 2012). The same system may also be involved in lowering seizure thresholds of epilepsy patients (Choi et al. 2010; Gholipour et al. 2010). Since defects in the serotonin system are linked to both conditions, this could be an obvious explanation for a shared pathology of depression and epilepsy (Jobe 2003; Jobe and Browning 2005; Kanner 2006b). Combined animal models of the diseases may offer helpful tools to find possible targets for new treatment strategies in the complex relationship between epileptogenesis, neurodegeneration and behavioral and cognitive disturbances.

1.4. Antiepileptogenic and disease-modifying strategies

The defining hallmark of epilepsy is recurrent spontaneous seizures. Unfortunately, the pathophysiological mechanisms leading to the hyperexcitable condition are poorly understood. Moreover, it is unlikely that only one central pathophysiologic mechanism underlies all different types of epilepsy. During the last decade research focused on different pharmacological strategies to modify pathophysiological molecular alterations with the overall goal of mitigating, preventing or even curing epilepsy. Molecular studies on neurodegeneration, neuronal plasticity and neurogenesis revealed that a combination of them contributes to the development of hyperexcitability and spontaneous seizures (Pitkanen and Lukasiuk 2011).

One of the first ideas for antiepileptogenic strategies is the prolonged administration of conventional AEDs, e.g.,, diazepam, phenobarbital, phenytoin, valproate or carbamazepine after an initial insult has occurred. Many clinical trials and experimental studies have been carried out with the aim of preventing epilepsy, but none of them was successful (Temkin 2001; Loscher 2012). At least some AEDs, like carbamazepine, diazepam, levetiracetam, phenytoin, pregabalin, lacosamide or topiramate, mitigate neurodegeneration and over the long term they ease memory impairments (Capella and Lemos 2002; Andre et al. 2003; Frisch et al. 2007; Zhou et al. 2007; Cunha et al. 2009; Licko et al. 2013). These studies indicate that prevention of neurodegeneration alone is not sufficient as an antiepileptogenic treatment strategy.

Recently, neurotrophic factors, in particular brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF-2), have been suggested to be promising candidates for the prevention of recurrent seizures. Many of the cellular mechanisms altered in epilepsy are regulated by neurotrophic factors in the healthy brain. Indeed, some research groups applied a combination of BDNF and FGF-2 in different animal models and achieved reduced mossy fiber sprouting, neuroinflammation and less spontaneous seizures (Paradiso et al. 2009; Paradiso et al. 2011; Liu et al. 2013). On the contrary, others found that long lasting overexpression of BDNF instead promotes epileptogenesis (Heinrich et al. 2011). Nevertheless, the molecular mechanisms that control the synthesis of neurotrophic factors play a role in epilepsy and may be an attractive target for AED development.

Another discussed strategy to prevent the progression of neurodegenerative conditions is the application of small molecules that resemble naturally occurring body peptides with protective potentials (Skaper 2008). These peptides lack unwanted side effects, e.g., bone marrow–stimulating properties of erythropoietin, but keep beneficial properties, e.g., neuroprotective, neurotrophic, and antiinflammatory properties. They are called mimetic peptides, because they resemble endogenous proteins. Over the past six years, our group was able to promote cell differentiation and neuroprotection and attenuate spatial learning deficits in epileptic animals by the administration of different mimetic peptides (Seeger et al. 2011; Zellinger et al. 2011a; Zellinger et al. 2011b; Russmann et al. 2013). Even if the number of animals exhibiting spontaneous recurrent seizures was not affected (Seeger et al. 2011), cellular and cognitive changes following SE are modified over the long term.

These results are encouraging and might suggest mimetic peptides as another molecular strategy with disease-modifying properties.

Another reasonable strategy is to target inflammatory processes. In recent years, evidence has accumulated suggesting that the initial brain insult promotes inflammation, which is thought to be a trigger for the onset and progression of epilepsy (Vezzani et al. 2011). *In vitro* and *in vivo* findings suggest that proinflammatory cytokines like interleukin (IL)-1β, cyclooxygenase-2 and complement factors of the innate immune system are up-regulated at all three stages of epileptogenesis (Xiong et al. 2003; Balosso et al. 2008; Oliveira et al. 2008; Ravizza et al. 2008a). This prompted the hypothesis that "*brain inflammation, in addition to its established contribution to ictogenesis, may play a role in the development of the epileptogenic process"* (Vezzani et al. 2011). Indeed, modulatory strategies of the IL1-β system, e.g., by pharmacological inhibition of the Interleukin Converting Enzyme, blocked kindling acquisition and prevented seizures after a washout period (Ravizza et al. 2008b). Moreover, a combinatory approach of pharmacological inhibition of the Interleukin Converting Enzyme and the human recombinant IL1-β receptor antagonist in the post-Status-Epilepticus-model provides neuroprotection, but did not affect the onset of epilepsy, frequency and duration of seizures (Noe et al. 2013).

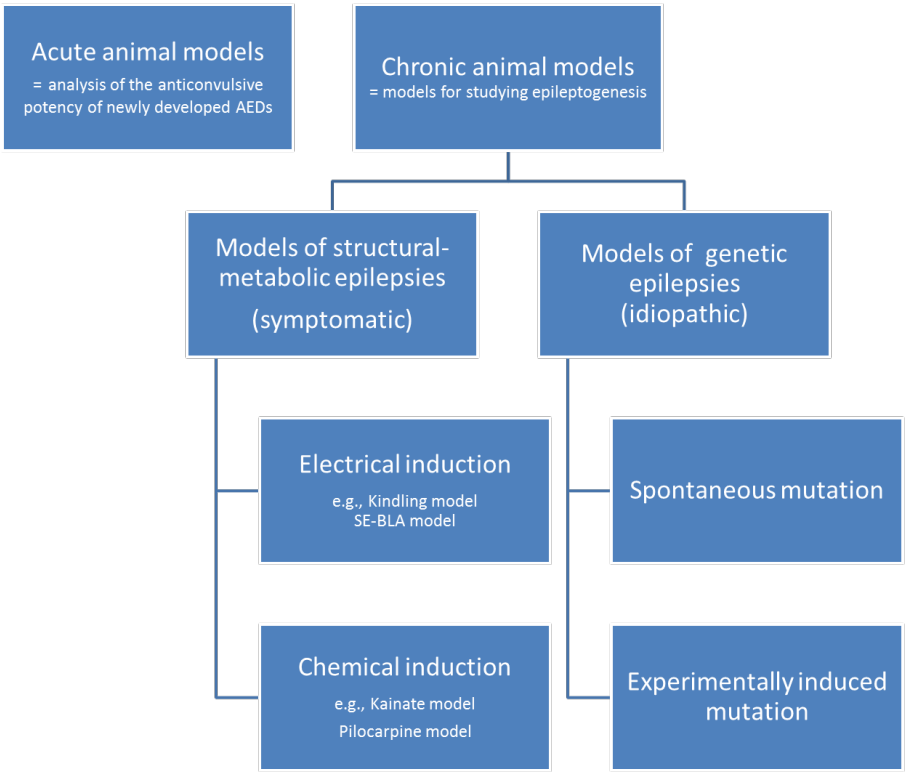
In summary, all strategies aim to modulate the molecular or cellular reorganization caused by the initial brain insult. So far, all efforts have been unsuccessful or at least did not lead to prevention or cure of epilepsy but only influenced the acute process of ictogenesis (Pitkanen 2002; Loscher and Brandt 2010). An expert group proposes that antiepileptogenesis treatment should completely prevent seizures (= ultimate goal), prolong the latent period (= partial prevention), or modify seizure frequency, duration and severity (= disease modification; Kobow et al. 2012). A combination of different strategies and a mixture of different drugs applied at different intervention time points may help to identify the optimum "drug-cocktail" and the time window of therapeutic intervention.

1.5. Animal models of epileptogenesis

Animal models of TLE are fundamental to the study of epileptogenesis. In general, an excellent animal model of a disease postulates three basic demands: constructive validity, face validity and predictive validity. Both the clinical symptoms (= face validity) and the pathogenesis (= constructive validity) in the model should resemble the human disease in many key aspects. Concerning the clinical efficacy of drugs in human medicine, animal models should allow the prediction of the therapeutic outcomes of future treatments (= predictive validity). A translational approach from *bench to bedside* can only be made with robust animal models.

Epileptogenesis can be studied in many types of animal models (**Figure 3**). Basically, *chronic* animal models are distinguished from *acute* animal models. Chronic models are usually applied to study epileptogenesis whereas the acute seizure models are required for testing the anticonvulsant potency of newly developed antiepileptic drugs.

Figure 3.



Schematic representation of chronic animal models in epilepsy research. *Acute (seizure) models can be distinguished from chronic models. Chronic animal models are not only used to study epilepsy but also to study epileptogenesis. Chronic models are subdivided into models of structural-metabolic and genetic epilepsies. SE – status epilepticus; BLA – basolateral amygdala.*

To investigate the underlying mechanisms of epileptogenesis, animal models with either induced or spontaneously occurring seizures are available, including kindling, post-Status-epilepticus-models, traumatic brain injury and stroke models, models of febrile seizures, genetic rodent models of generalized epilepsy and even naturally occurring epilepsy in dogs (Pitkanen et al. 2007; Potschka 2012; Potschka et al. 2013). In electrical and chemical animal models, seizures are triggered by electrical or chemical stimulation of the brain. In these models the seizures occur only associated with the stimuli. In contrast, in animal models with a strong initial electrical or chemical insult the animals develop spontaneous and recurrent seizures after a latency period. Both model types reflect the clinical situation of patients with symptomatic epilepsies.

The animal model used in this study is the kindling model of TLE. The model is explained in more detail below.

The kindling model of temporal lobe epilepsy

Kindling is an animal model and seizures contribute to the progression of a hyperexcitable neuronal network. The generation of repeated, brief, focal seizures caused by electrical or chemical stimulation leads to permanently enhanced sensitivity to these stimuli with enhanced seizure duration and seizure severity, and ultimately to chronic epilepsy.

The kindling model was developed by Goddard and colleagues in 1967 and has been utilized in epilepsy research for almost fifty years (Goddard 1967). The verb “to kindle” literally means to enflame or to excite. This refers to repeated electrical stimulations inducing focal and later generalized seizures. These seizures promote or “kindle” the generation of a hyperexcitable neuronal network with long lasting cellular and molecular alterations. In the beginning of kindling only a small neural circuit is involved, visible as short focal seizures with eye blinking, facial clonus and chewing. Subsequently, additional circuits are recruited and the animals exhibit generalized seizures. After some stimulations seizure severity and seizure duration reach a plateau and the animal is supposed to be fully-kindled (McNamara 1984). This process of hyperexcitable neuronal network formation is compared to the process of epileptogenesis because the kindling model has many parallels with TLE in humans. Seizure duration increases and severity becomes more severe also in humans with recurrent seizures (Goddard et al. 1969; Racine 1972). Furthermore, EEG features in kindled mice resemble those in humans. High frequency spiking is followed by a progression to clonic bursts before a terminal suppression of the EEG appears (Bertram 2007). In addition, molecular and cellular alterations in the temporal lobe of kindled animals are comparable to the alterations in human TLE. Furthermore, kindling increases hippocampal neurogenesis (Parent et al. 1998; Scott et al. 1998) in correlation with seizure frequency (Jafari et al. 2012) and enhances synaptogenesis (Li et al. 2002). Moreover, in the kindling model neurodegeneration (von Bohlen und Halbach et al. 2004) and the generation of mossy fiber sprouting (Armitage et al. 1998), one of the hallmarks of TLE (Isokawa et al. 1993), was observed. These findings strongly support the idea of using the kindling model as a model to study epileptogenesis. However, the term “epileptogenesis” has to be used with great caution in this context. Epileptogenesis is defined as leading to recurrent and spontaneous seizures. In the kindling model only prolonged electrical stimulations over months may lead to spontaneous seizures (Pinel and Rovner 1978; Michalakis et al. 1998; Sayin et al. 2003; Brandt et al. 2004). Spontaneous seizures in kindled animals have been difficult to replicate, thus questioning the model as an appropriate model for epileptogenesis.

Nevertheless, repeated electrical stimuli with the sum of cellular and molecular alterations perfectly mimic the generation of a hyperexcitable neuronal network. Moreover, acute effects on seizure severity and duration can be easily examined in testing new pharmacological compounds. In addition, an excellent predictive validity for detecting clinically relevant treatment effects has been demonstrated (Loscher 2002a). Therefore, the kindling model was chosen to investigate the impact of the endocannabinoid and the endovanilloid systems on epileptogenesis and to test acute and chronic effects of cannabinoid type 1 receptor (CB1R) agonists and antagonists.

2. The endocannabinoid system

2.1. Physiology of the endocannabinoid system

For millennia humankind has known about psychoactive and medicinal effects of the marijuana plant (Russo 2007). In medieval Arabian medicine many of the currently known indications have already been described (Zuardi 2006). Lastly, after the chemical structure of Δ9-Tetrahydrocannabinol (THC) has been identified as the main active substance of the marijuana plant, detailed knowledge of the cannabinoid system and its effects could be obtained over the recent decades. Essentially, the endocannabinoid system consists of endogenous ligands (endocannabinoids) and cannabinoid receptors and enzymes, which coordinate endocannabinoid synthesis, release and degradation.

In 1988 two different cannabinoid receptor types were identified in the rat brain (Devane et al. 1988): The cannabinoid receptor 1 (CB1R) and 2 (CB2R). The CB1R is localized in various tissues in the periphery and in the central nervous system, whereas the CB2R is almost exclusively expressed on immune cells and only to a limited extent in the central nervous system (Munro et al. 1993; Galiegue et al. 1995). The vast majority of the currently known effects of cannabinoids in the central nervous system are CB1R mediated and CB2R independent. Therefore, CB2Rs will not be covered any further here.

The CB1R is a seven transmembrane G-protein coupled receptor, which is predominantly expressed on presynaptic termini of inhibitory as well as of excitatory synapses (Katona et al. 1999; Misner and Sullivan 1999). Among others, activation of the receptor and the metabotropic signal cascade inhibits the calcium influx by N- and P/Q- voltage gated calcium channels, the activity of adenylate cyclase and increases the open probability of different potassium channels (Twitchell et al. 1997; McAllister and Glass 2002). As a consequence of activation, cells, especially neurons, change their functional characteristics and neurotransmitter release is decreased (Freund et al. 2003).

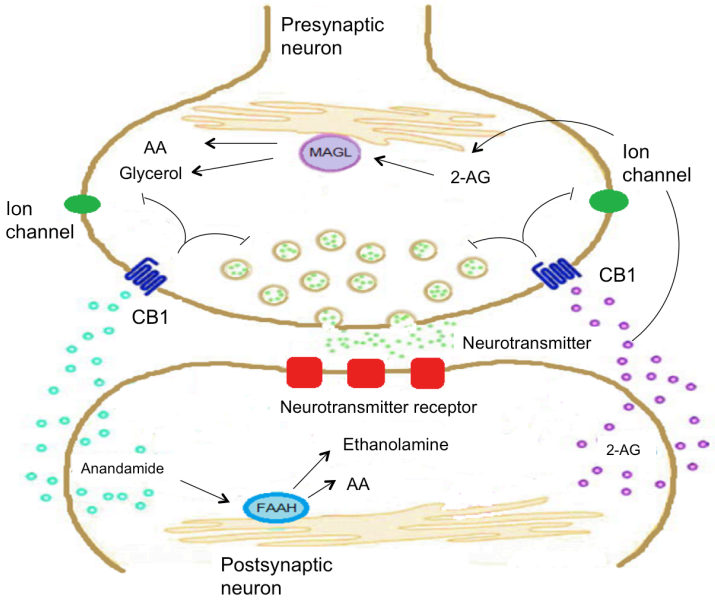
The hydrophobic properties of THC suggested that the endogenous ligand of the cannabinoid system must be a lipid molecule. In 1992, the arachidonic acid derivative arachidonylethanolamide (anandamide) was isolated from pig brains and identified as an endogenous ligand (Devane et al. 1992). Anandamide (after ananda, the Sanskrit word for bliss) is synthesized from N-arachidonyl phosphatidyl ethanolamide by elimination of phosphorous acid. Three years later a second endocannabinoid, 2-Arachidonoylglycerol (2-AG), was discovered (Mechoulam et al. 1995). 2-AG is an arachidonic ester (Mechoulam et al. 1995), which arises from a different precursor molecule than anandamide.

The synthesis of the endogenous ligands is initiated by presynaptic axonal depolarization, which causes an activity dependent Ca^{2+} influx (Di Marzo et al. 2004). Once the endocannabinoids are released into the synaptic cleft, they travel backwards to activate CB1Rs on presynaptic nerve terminals. To regulate the feedback loop, specific uptake transporters and degrading enzymes of neurons terminate the physiological effects of the ligands in the synaptic cleft (Beltramo et al. 1997;

Bisogno et al. 1997). 2-AG is hydrolyzed by monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol (Dinh et al. 2002), whereas anandamide is degraded by the enzyme fatty acid amidhydrolase (FAAH) into arachidonic acid and ethanolamide (Cravatt et al. 1996; Hillard and Campbell 1997). Up to now, anandamide and 2-AG are the two most commonly studied endocannabinoids (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995; Di Marzo and De Petrocellis 2012).

In simple terms, the endocannabinoid system is a retrograde feedback mechanism that is activated on demand. The overall effect of CB1R activation is a reduced inhibitory or excitatory neurotransmission. **Figure 4** schematically summarizes the key features of the feedback loop.

Figure 4.



Schematic representation of the endocannabinoid system's retrograde signaling. *Neuronal depolarization causes neurotransmitter release (e.g., glutamate or GABA). Neurotransmitter receptors on the postsynaptic neuron are activated, leading to an increase of intracellular Ca^{2+}-ions and induction of the on-demand endocannabinoid biosynthesis. Endocannabinoids like anandamide and 2-AG diffuse across the synaptic cleft to presynaptic CB1R. Once activated, CB1Rs regulate ion channels to decrease neuronal activity. Termination of endocannabinoid activity is achieved by hydrolyzing enzymes. Anandamide is degraded by fatty acid amide hydrolase (FAAH) in the postsynaptic neuron, and 2-AG by monoacylglycerol lipase (MAGL) in the presynaptic neuron (modified from Ahn et al. (2008)).*

2.2. Genetic modulation of the endocannabinoid system

Excessive glutamate release is the pathophysiological key factor leading to the generation of epileptic seizures (ictogenesis). Therefore, it is mandatory to understand the mechanisms of excitotoxic glutamate release and related neuronal circuits for the development of new therapeutic strategies. Given the fact that the endocannabinoid system strongly participates in neuroprotection (Abood et al. 2001; Marsicano et al. 2003; Khaspekov et al. 2004) and that it regulates neurotransmitter release on

demand (Freund et al. 2003), it is crucial to identify in detail the neuronal subtypes, which are controlled by the endocannabinoid system.

Genetic techniques are the most powerful tools to answer these questions. In this context gene targeting is a technique to investigate the function of a particular gene and its product (Thomas and Capecchi, 1987). However, this technique may also have some disadvantages: increased embryonic lethality and compensatory effects during development may preclude gene analysis in adult mice. In addition, pleiotropic effects in several organs and tissues may lead to a complex interpretation of the phenotype. For these circumstances the method of choice is to switch off a gene in a specific tissue at any given time, thus creating so called *conditional knockout mice*. Today the Cre / loxP technology is well-established. This technique allows the production of conditional somatic mutagenesis by the enzyme *Cre-recombinase* ("causes recombination", 38 kDa). This enzyme is originally synthesized by P1 bacteriophage (Austin et al. 1981). It efficiently catalyzes the intramolecular recombination of the *loxP sites* (meaning “locus of crossing over [x] of P1 recombinase”; 34 bp long). If both loxP sites are orientated in 5’ -3’ – direction in a cis-localization, the Cre enzyme catalyzes the cleavage and rejoins the DNA, thus excising the intervening piece of DNA. If the Cre enzyme is controlled by a specific promotor, it is cell- or tissue-specific expressed (Abremski and Gottesman 1981; Sternberg and Hamilton 1981; Sauer and Henderson 1988). Thus, the Cre / loxP technology enables researchers to study the tissue-specific function of a gene product.

The generation of conditional knockout mice is a stepwise process. By gene targeting an essential part of the gene of interest is flanked with two Cre-recognition sequences, the loxP sites. Alleles like this are called *floxed* alleles (meaning “flanked by loxP sites”). If these floxed animals are also transgenic for a cell-specifically expressed Cre-recombinase, the gene of interest will be displayed and knocked out here (Tsien et al. 1996) but will preserve its function in every other cell or tissue.

Gene targeting strategy for conditional CB1R knockout

We used two different conditional CB1R knockout mouse lines generated by using the cre / loxP system (Marsicano et al. 2003; Monory et al. 2006).

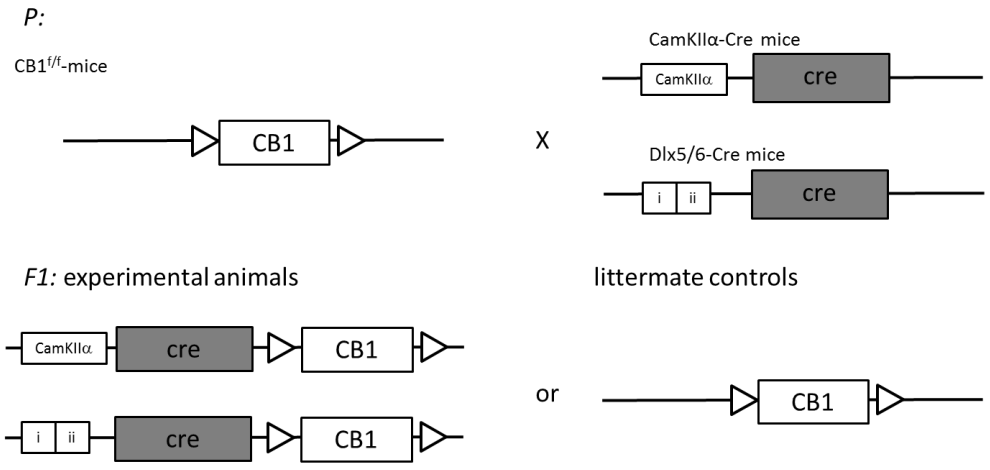
The conditional knockout mice were obtained using a stepwise breeding protocol. They were maintained on a predominant C57BL/6N background. The final breeding step (see **Figure 4**) was to cross CB1R floxed ($CB1^{f/f}$) mice (Marsicano et al. 2003) with the respective Cre-expressing (CamKIIα-Cre and Dlx5/6-Cre) mouse line (Marsicano et al. 2003; Monory et al. 2006).

For the first mouse line the regulatory sequence of the Cre enzyme is CamKIIα, a Ca^{2+}/calmodulin-dependent kinase IIα from a bacterial artificial chromosome (Casanova et al. 2001). The artificial as well as the natural regulatory sequence is highly expressed in the dentate gyrus, cornu ammonis, cortex, olfactory bulb and amygdala (Sola et al. 1999; Casanova et al. 2001). Lower levels of CamKIIα expression are detected in the striatum, the thalamus and the hypothalamus, whereas the expression is absent in the cerebellum and in the periphery (Sola et al. 1999; Casanova et al. 2001). Therefore, $CB1^{f/f,CamKII_{\alpha}}$ littermates lack CB1R in all principal, mainly glutamatergic, neurons of the

forebrain, but not in adjacent interneurons, in the cerebellum and in the periphery. These mice show an increased level of fear adaption comparable to the behavior of total CB1R knockout mice (Kamprath et al. 2009). In addition, the corticosterone release is enhanced when animals are subjected to acute stress (Steiner et al. 2008). Moreover, these animals are more susceptible to seizures caused by chemical noxa (Marsicano et al. 2003; Monory et al. 2006).

For the second mouse line the regulatory sequence of the Cre enzyme is the I56i and I56ii intergenic enhancer sequence of the zebrafish, which is very similar to the mouse's Dlx5/Dlx6 genes (Zerucha et al. 2000). This gene region was transfected into a plasmid containing the Cre-coding gene sequence and the excised transgene was injected into fertilized eggs from mice (Zerucha et al. 2000) to gain Dlx5/6-Cre mice. These mice were further crossed with $CB1^{f/f}$ mice (see **Figure 5**). $CB1^{f/f,Dlx5/6\text{-}Cre}$ mice lack expression of CB1Rs in all GABAergic neurons of the forebrain (particularly in the hippocampal formation), but are rescued in pyramidal neurons (Monory et al. 2006). These animals show increased exploratory behavior in the basal state (Lafenetre et al. 2009), but otherwise the phenotype is not altered (Monory et al. 2006).

Figure 5.



Breeding protocol of the two conditional CB1R-knockout mouse lines. *$CB1^{f/f}$-mice were generated by Marsiciano et al. (2003) and crossed with the respective Cre mouse lines to obtain experimental animals and littermate controls (Marsicano et al. 2003; Monory et al. 2006). Experimental animals express the Cre enzyme either in principal neurons of the forebrain (promotor: open box; CamKIIα) or in GABAergic neurons of the forebrain (promotor: open boxes; i/ii (=Dlx5/6)), and therefore lack CB1Rs in specific neuronal subpopulations. P, parental generation; F1, Filial generation; open triangles, loxP sites; open box 'CB1', CB1 open reading frame; grey box 'cre', open reading frame Cre recombinase.*

2.3. Pharmacological modulators of the endocannabinoid system

More than 40 years ago the most well-known cannabinoid Δ9-THC was isolated, synthesized and its psychoactive characteristics were demonstrated (Gaoni and Mechoulam 1964). Until now, a large number of cannabinoid analogues have been synthesized and characterized in animal models (Pertwee

2008, 2012; Starowicz and Di Marzo 2013). In 1991 the Sterling Research Group discovered a new class of aminoalkylindol analgesics acting as agonists at CB1R in the brain (Pacheco et al. 1991). The stereochemical structure of WIN55.212-2 has a high similarity to Δ9-THC, but the affinity and selectivity is higher for CB1R and CB2R (Pacheco et al. 1991; Compton et al. 1992). WIN55.212-2 is considered to be the prototype of the agonists, fulfilling the typical tetrad effect (analgesia, hypolocomotion, catalepsy and immobility) of cannabinoids. Today, a variety of chemical substances are available that affect endocannabinoid signaling either directly by interaction with the receptor (agonists and antagonists) or indirectly by modulating endocannabinoid levels and thus the receptor output (indirect agonists and indirect antagonists). Because of the high number of available chemical compounds, I will only focus on those agents used in this study.

Rimonabant (SR141716A)

SR141716A [N- (piperidin-1-yl)-5-(4-chlorophenyl)- 1-(2,4-dichlorophen- yl)-4-methyl-lH-pyrazole-3-carboxamide hydrochloride] is a potent, competitive and selective CB1R antagonist with restricted activity to the central nervous system (Rinaldi-Carmona et al. 1994; Rinaldi-Carmona et al. 1995; Rinaldi-Carmona et al. 1996). Rimonabant was originally developed for the treatment of obesity (Pi-Sunyer et al. 2006; Scheen et al. 2006) and was launched, approved for this indication, into the European market in 2006 under the brand name Accomplia®. Two years later, rimonabant was withdrawn from the European market due to an increased risk of psychiatric side effects, such as depression, insomnia, anxiety, and aggression (Moreira and Crippa 2009). In addition, case histories suggest a relationship with suicide (Topol et al. 2010). From a clinical perspective, the advantages and the restrictions of this drug have to be seriously interrogated. Nevertheless, for research purposes rimonabant is an excellent compound to study the endocannabinoid system and it is widely used as a tool to reverse cannabinoid induced effects.

JZL184

Termination of endocannabinoid signaling is strictly regulated by the endocannabinoid degrading enzymes FAAH and MAGL (Ahn et al. 2008). By inhibition of these enzymes, the concentration of anandamide and 2-AG is tremendously elevated causing the well-known cannabinoid behavioral effects normally mediated by direct acting agonists. Thus, the enzyme inhibitors act as “indirect-agonists”. Decoupling of anandamide- and 2-AG-dependent signaling pathways by selective and specific pharmacological blockade of the respective degrading enzyme offers the possibility of directly addressing the tissue specific role of endocannabinoids in the brain. Accumulating evidence exists that MAGL is the primary enzyme responsible for the degradation of 2-AG (Dinh et al. 2002; Dinh et al. 2004; Blankman et al. 2007). In 2009 Long and colleagues (2009) synthesized a potent and selective MAGL inhibitor that displays exceptional activity *in vivo* and is superior towards the existing enzyme inhibitors. The “old” and most well-described MAGL inhibitor URB602 is not potent enough for systemic administration in vivo (Mechoulam et al. 1995). The new compound was named after its inventor’s initials: JZL184 (Jonathan Z. Long). When administered to mice, JZL184 causes the classic

CB1R-dependent tetrad effect by an increase of brain 2-AG levels up to eight-fold (Long et al. 2009). Thereby, anandamide is not affected (Long et al. 2009). However, the development of tetrad effects, as well as pharmacological tolerance at higher dosages (40 mg/kg) and chronic administration (Schlosburg et al. 2010), may be a limitation for clinical use. In contrast, low dosage (~8 mg/kg) chronic treatment does not induce tolerance and does not alter memory consolidation (Busquets-Garcia et al. 2011). These physical and chemical conditions highlight MAGL inhibition as a potential therapeutic target for epilepsy.

2.4. The role of the endocannabinoid system in epilepsy

The endocannabinoid system plays an important role in synaptic plasticity (Gerdeman and Lovinger 2003; Chevaleyre et al. 2006; Marsicano and Lutz 2006) and mediates neuronal excitability (Marsicano et al. 2003). Moreover, the endocannabinoid system affects depolarization-induced suppression of excitation and inhibition (DSI and DSE, respectively) at neuronal synapses of several brain regions, including the hippocampus and the amygdala (Wilson and Nicoll 2002; Freund et al. 2003; Zhu and Lovinger 2005; Hofmann et al. 2006). There are some neurological disorders, e.g., multiple sclerosis, post-traumatic stress disorder, traumatic brain injury, Parkinson's disease and also epilepsy, in which the endocannabinoid system may ameliorate the course of the disease by auto-protective up-regulation of endocannabinoid synthesis and release onto subpopulations of its receptors in certain locations (Pertwee 2005). Accordingly, the focus of research is on the therapeutic potential of agents acting on this system. Moreover, the history of cannabis for the treatment of various diseases reaches back about 5000 years (Russo 2007). Systematic research on the potential anticonvulsant potency of cannabinoids began in the early seventies. In 1973 and 1974 different groups started to investigate the anticonvulsant and antiepileptic potency of Δ9-THC in different epilepsy models, e.g., the audiogenic seizure model and the kindling and pentylenetetrazole model (Boggan et al. 1973; Corcoran et al. 1973; Wada et al. 1973; Chesher and Jackson 1974). Carlini and colleagues (1975) analyzed the non-psychoactive cannabinoid *cannabidiol* in the maximal-electroshock-model in mice and were able to show an anticonvulsant effect. In the recent years researchers pharmacologically addressed the role of the endocannabinoid system in the control of neuronal excitability and seizure thresholds in different animal models. Many lines of evidence suggest that the endocannabinoid system has an anticonvulsant potency and is critically involved in the suppression of seizure activity and termination (Marsicano et al. 2002; Marsicano et al. 2003; Naderi et al. 2008; Bhaskaran and Smith 2010a). In line with these findings, blocking CB1R activity by cannabinoid antagonists or genetic deletion is often proconvulsant (Marsicano et al. 2003; Monory et al. 2006; Kow et al. 2014). In our lab, the direct acting CB1R agonist WIN55.212-2 delayed the progression of seizure severity in the kindling model of TLE, but WIN55.212-2 and the FAAH inhibitor URB597 had only minor effects on seizure thresholds and seizure duration (Wendt et al. 2011). In addition, we revealed an impact on cell proliferation and neurogenesis, suggesting that activation of CB1Rs might also promote disease-modifying modalities (Wendt et al. 2011). In strong contrast to our findings, a study from

2009 claimed that the CB1R antagonist rimonabant, a seizure-enhancing drug, paradoxically prevents epileptogenesis when applied within a short time window following traumatic brain injury (Echegoyen et al. 2009). In summary, the main findings from *in vitro* cell culture studies and *in vivo* studies of epilepsy suggest that the endocannabinoid system has anticonvulsant properties under excitatory conditions but is often ineffective or even proconvulsant when inhibitory neurotransmission is blocked. Clearly, the particular importance is the net effect of the endocannabinoid system because the system is active on both excitatory and inhibitory neuronal subpopulations.

Recently, the use of medical marijuana and one of its active ingredients, cannabidiol, is an intensively discussed topic among experts and patients with epilepsy. However, there is only limited knowledge about safety and efficacy of this semilegalized treatment option. In November 2013 the U.S. Food and Drug Administration granted an "Orphan Drug" status to the plant extract cannabidiol from GW Pharmaceuticals (Epidiolex®) for the treatment of Dravet Syndrome and in February 2014 for the treatment of Lennox-Gastaut Syndrome. In June 2014 the efficacy and safety data from 27 patients was published. The company reported an effective reduction of the seizure frequency up to seizure freedom and only mild or moderate side effects, like somnolence, fatigue, diarrhea, decreased appetite, and increased appetite. These first clinical data is very promising and gives first evidence for potential clinical benefit at least for a subgroup of patients with severe epilepsy.

However, one of the major challenges is still to elucidate the anti- or proconvulsant mechanisms of the endocannabinoid system for the prevention and treatment of epilepsy.

3. The endovanilloid system

The transient receptor potential vanilloid type 1 channel (TRPV1; originally vanilloid receptor = VR1) is the main molecular transducer in sensorial systems. It is activated by various stimuli like heat (>42°C), low pH, voltage and the naturally occurring chemicals capsaicin, the active ingredient of red-hot chili peppers, and resiniferatoxin (Szallasi and Blumberg 1990a; Holzer 1991; Caterina et al. 1997; Caterina 2007). Resiniferatoxin is a potent capsaicin analog. Autoradiographic studies with this radiolabeled ligand helped to identify TRPV1 (Szallasi and Blumberg 1990b), which was finally cloned in 1997 and characterized as a subtype of the Transient Receptor Potential family of non-selective cation channels (Caterina et al. 1997). Unlike CB1R, TRPV1 is a six-transmembrane protein with a hydrophobic stretch between segment five and six (**Figure 6**; Caterina et al. 1997). Mezey and colleagues (2000) identified the existence of the receptor in the rat's brain. Further studies confirmed a widespread distribution of TRPV1 in various brain areas, e.g., in the cortex, hippocampus and amygdala to name just a few (Toth et al. 2005; Cristino et al. 2006). When activated, TRPV1 is calcium-permeable and non-selective for cations, leading to increased neuronal excitability (van der Stelt et al. 2005). Next to the "classical" TRPV1 agonists, some endogenous arachidonic acid derivatives like anandamide functionally activate TRPV1, implying a dual role for endocannabinoids

(Al-Hayani et al. 2001; Kofalvi et al. 2007). However, stimulation of CB1Rs and TRPV1 causes opposing effects on intracellular calcium concentrations. As a consequence, excitatory and inhibitory neurotransmission is regulated in an opposing way. Anandamide suppresses GABAergic signaling via presynaptic CB1Rs (Kim and Alger 2010), but stimulates glutamate release via presynaptic TRPV1 (Chavez et al. 2010; Grueter et al. 2010). TRPV1 may thereby also be involved in pathological conditions, like fear, anxiety, excitotoxcity and also epilepsy. Moreover, CB1Rs and TRPV1 are co-expressed in several brain regions, e.g. hippocampus, basal ganglia, thalamus, hypothalamus, cerebellum and others (Cristino et al. 2006; Micale et al. 2009). This phenomenon suggests a cross talk between the two receptors and extends the effects caused by activation (Di Marzo and De Petrocellis 2012), thus leading to an even more complex situation.

The role of the endovanilloid system in epilepsy

Due to its physiological characteristics, TRPV1 is considered to play a role in epilepsy and epileptogenesis (Fu et al. 2009). Beyond this hypothesis, the first evidence exists from different *in vivo* models of epilepsy that TRPV1 may be implicated in the disease. In the majority of the studies pilocarpine or pentylenetetrazole-induced seizure models were used and TRPV1 was modulated pharmacologically by agonists or antagonists. Activation of TRPV1 accelerated epileptogenesis or increased epileptiform activity, whereas antagonism of TRPV1 led to retardation of epileptogenesis or suppression of epileptiform activity (Bhaskaran and Smith 2010b; Gonzalez-Reyes et al. 2013; Kong et al. 2014; Shirazi et al. 2014). In line with these findings is the study of Kong et al. (2014), who modified the endovanilloid system genetically. Deletion of TRPV1 increased hypothermic-induced seizure thresholds (Kong et al. 2014). Moreover, Manna and Umathe (2012) focused on the dual role of anandamide and confirmed an anticonvulsant effect in the low dosage range via TRPV1 and a proconvulsant effect in the high dosage range via CB1Rs in the pentylenetetrazole model. Paradoxically, there is also evidence that the TPRV1 agonist capsaicin prevents kainic acid induced seizures in mice (Lee et al. 2011). Furthermore, in human patients with TLE, Sun et al. (2013) demonstrated an up-regulation of TRPV1 in the cortex and in the hippocampus, further pointing towards a role of the endovanilloid system in epilepsy. However, the exact role of TRPV1 is still ambiguous. All experimental studies focused on the suppression of acute seizures, whereas chronic effects of TRPV1 modulation remain to be evaluated.

To clarify the role of TRPV1 in epileptogenesis and ictogenesis, we combined genetic and pharmacological strategies and analyzed the impact on the generation of a hyperexcitable neuronal network in the kindling model of TLE.

Genetic modulation of the endovanilloid system

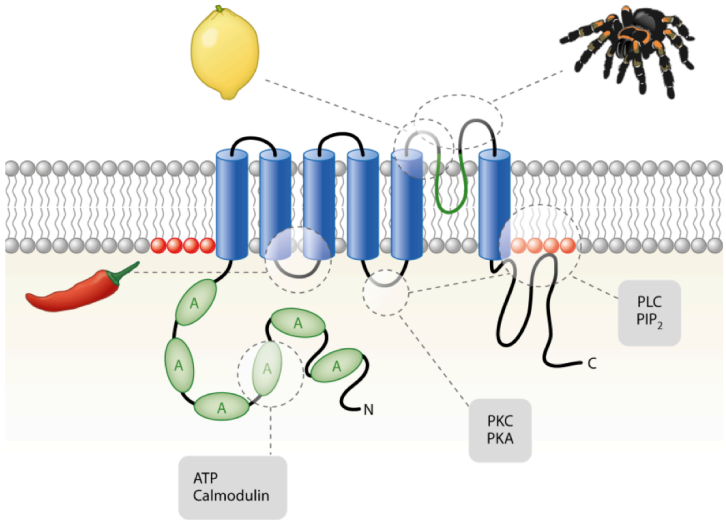
As mentioned above genetic techniques are a very powerful tool to analyze the function of a particular gene and its protein. Caterina et al. (2000) used these techniques and generated TRPV1 knockout mice as follows: A 10-kb HindIII subfragment was used to construct a target vector. The target vector contained the disrupted mouse TRPV1 gene: An exon, encoding a part of the fifth and all of the sixth

transmembrane domain, as well as the pore-loop region, was deleted. The target vector was inserted into JM1 embryonic stem cells by electropolarization. Surviving embryonic stem cells with verified homologues recombination at the VR1 locus were injected at the stage of a blastocyste into foster mothers. Male chimeras were mated with C57/Bl6 females and homozygote offsprings were further backcrossed for at least four generations with C57/Bl6 females to obtain a nearly isogenetic background. TRPV1 knockout mice showed a physiological response towards mechanical stimuli but TRPV1 mediated sensory pain was impaired. Moreover, anxiety-related behavior, conditioned fear and hippocampal long-term potentiation was disrupted in these mice (Marsch et al. 2007).
Homocygotic offsprings along with corresponding littermate controls were used in all experiments.

Pharmacological modulators of the endovanilloid system

Some natural and some endogenous products have powerful pharmacological characteristics to either activate or block TRPV1 receptors. **Figure 6** gives a schematic overview of the different binding sites at the TRPV1 receptor (Julius 2013). The endovanilloid binding site is located within the second and third transmembrane domain and at the pore-loop region (Jordt and Julius 2002). This binding site is sensitive to capsaicin, capsazepine (a synthetic antagonist) and resiniferatoxin (an agonist from *Euphorbia cactus*) as well as to endogenous factors like anandamide and other bioactive lipids that have a high structural similarity to capsaicin. These bioactive lipids are the so-called endovanilloids (Jordt and Julius 2002).

Figure 6.



Julius D. 2013.
Annu. Rev. Cell Dev. Biol. 29:355–84

Schematic structure-function map of TRPV1. *The TRPV1 protein consists of six transmembrane domains and a pore-loop between domain five and six. The different TRPV1 domains confer sensitivity to different ligands. The endovanilloid binding site is located within the second and third transmembrane domain and at the intervening cytoplasmatic pore-loop. The proton binding site is indicated by the lemon and the spider toxin site is indicated by the spider. Furthermore, this schematic drawing gives information about regions being involved in channel modulation by cellular proteins*

and second messengers like ATP. This figure is reproduced with permission from (Julius 2013). C, carboxy terminus; N, amino terminus; PLC, phospholiase C; PIP_2, phosphatidylinositol-2-phasphate; PKC, proteinkinase C; PKA, proteinkinase A.

Pharmaceutical companies invested time, expertise and money to design small, potent and selective molecules which block TRPV1 activity. They focused on the molecular structure of the TRPV1 agonist capsaicin. Based on structure-activity relationships of the three capsaicin subregions (the aromatic ring-, amide bond- and hydrophobic side chain-regions), the classical TRPV1 antagonists have been identified. Some of them are listed in **Table 1**.

Table 1. Classical TRPV1 antagonists

Thiourea Analogs	Urea Analogs	Cinnamide Analogs
Capsazepine	A-425619	SB-366791
JYL-1421	BCTC	AMG-9810
	JNJ-17203212	

The first TRPV1 antagonist, capsazepine, was identified by Walpole and colleagues in 1994 (Walpole et al. 1994). Capsazepine is a competitive TRPV1 antagonist. However, capsazepine has low metabolic stability and poor pharmacokinetic features (Vriens et al. 2009). Moreover, its selectivity for TRPV1 is restricted. Furthermore, capsazepine also inhibits acetylcholine receptors (Liu and Simon 1997), voltage-gated Ca^{2+} channels (Docherty et al. 1997) and TRPM8 (Vadlamudi et al. 2003) and was, for these reasons, never considered for clinical use.

As another strategy, pharmacological companies use large-scale, high-throughput screening of chemical libraries to identify chemically tractable TRPV1 antagonists. More recently, SB366791 was identified and characterized *in vitro* and *in vivo* as a highly selective and more potent TRPV1 antagonist than capsazepine (Gunthorpe et al. 2004; Varga et al. 2005; Lappin et al. 2006). Due to its high selectivity and its good pharmacological features, we used SB366791 to block TRPV1 and to study the impact on the formation of a hyperexcitable epileptic network.

4. Aim and scope of thesis

The overall aim of the current thesis is to determine whether pharmacological and genetic modulation of the endocannabinoid and endovanilloid systems may have preventive antiepileptogenic properties in the kindling mouse model of TLE.

The specific aims of the two manuscripts are as follows:

Research article 1: Analysis in conditional cannabinoid 1 receptor-knockout mice reveals neuronal subpopulation-specific effects on epileptogenesis in the kindling paradigm.

- Evaluation if pharmacological antagonism at CB1R with rimonabant affects epileptogenesis and ictogenesis.
- Evaluation if conditional CB1R knockout in principal neurons of the forebrain affects epileptogenesis and ictogenesis.
- Evaluation if conditional CB1R knockout in principal neurons of the forebrain has an impact on seizure-associated neurogenesis.
- Evaluation if conditional CB1R knockout in GABAergic neurons of the forebrain affects epileptogenesis and ictogenesis.
- Evaluation if pharmacological antagonism at TRPV1 with SB366791 affects epileptogenesis and ictogenesis.
- Evaluation if TRPV1 knockout affects epileptogenesis and ictogenesis.

Research article 2: Inhibition of monoacylglycerol lipase - 2-arachidonoylglycerol mediates cannabinoid1-receptor dependent retardation of kindling progression in a mouse model of temporal lobe epilepsy.

- Evaluation if pharmacological inhibition of the 2-AG degrading enzyme MAGL by JZL184 has an antiepileptogenic and an anticonvulsant effect.
- Investigation if any effects underlying the endocannabinoid signal cascade are CB1R mediated.

RESEARCH ARTICLES

1. Analysis in conditional cannabinoid 1 receptor-knockout mice reveals neuronal subpopulation-specific effects on epileptogenesis in the kindling paradigm

E.L. von Rüden[1, 2], M. Jafari[1, 2], R.M. Bogdanovic[1, 2], C.T. Wotjak[3], H. Potschka[1*]

[1]Institute of Pharmacology, Toxicology and Pharmacy, Ludwig-Maximilians University, Munich, Germany

[2]Graduate School of Systemic Neurosciences, Munich, Germany

[3]Max Planck Institute of Psychiatry, Munich, Germany

Declaration of contribution: Eva-Lotta von Rüden designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with co-authors (for detailed information on author contributions see chapter´Eidesstattliche Versicherung/Affidavit´).

Analysis in conditional cannabinoid 1 receptor-knockout mice reveals neuronal subpopulation-specific effects on epileptogenesis in the kindling paradigm

E.L. von Rüden[1, 2], M. Jafari[1, 2], R.M. Bogdanovic[1, 2], C.T. Wotjak[3], H. Potschka[1*]

[1]Institute of Pharmacology, Toxicology and Pharmacy, Ludwig-Maximilians University, Munich, Germany

[2]Graduate School of Systemic Neurosciences, Munich, Germany

[3]Max Planck Institute of Psychiatry, Munich, Germany

*Correspondence:

Prof. Dr. H. Potschka

Institute of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University, Munich, Koeniginstr. 16, D-80539 Munich, Germany;

Phone: +49-89-2180-2663; Fax: +49-89- 2180- 16556;

E-mail: potschka@pharmtox.vetmed.uni-muenchen.de

Abstract

The endocannabinoid system serves as a retrograde negative feedback mechanism. It is thought to control neuronal activity in an epileptic neuronal network. The purpose of this study was to evaluate the impact of the endocannabinoid and endovanilloid systems on both epileptogenesis and ictogenesis. Therefore, we modulated the endocannabinoid and endovanilloid systems genetically and pharmacologically, and analyzed the subsequent impact on seizure progression in the kindling model of temporal lobe epilepsy in mice. In addition, the impact of seizures on associated cellular alterations was evaluated.

Our principal results revealed that the endocannabinoid system affects seizure and afterdischarge duration dependent on the neuronal subpopulation being modulated. Genetic deletion of CB1-receptors (CB1Rs) from principal neurons of the forebrain and pharmacological antagonism with rimonabant (5 mg/kg) caused longer seizure duration. Deletion of CB1R from GABAergic forebrain neurons resulted in the opposite effect. Along with these findings, the CB1R density was elevated in animals with repetitively induced seizures. However, neither genetic nor pharmacological interventions had any impact on the development of generalized seizures.

Other than CB1, genetic deletion or pharmacological blockade with SB366791 (1 mg/kg) of transient receptor potential vanilloid receptor 1 (TRPV1) had no effect on the duration of behavioral or electrographic seizure activity in the kindling model.

In conclusion, we demonstrate that endocannabinoid, but not endovanilloid, signaling affects termination of seizure activity, without influencing seizure severity over time. These effects are dependent on the neuronal subpopulation. Thus, the data argue that the endocannabinoid system plays an active role in seizure termination but does not regulate epileptogenesis.

Highlights:

- CB1R deletion in principal neurons of the forebrain lowers seizure susceptibility
- CB1R affects seizure duration dependent on neuronal subpopulation expression
- The endocannabinoid system is an endogenous mechanism for seizure termination
- TRPV1 do not play a role in the generation of a hyperexcitable network
- The endovanilloid system is not involved in ictogenesis or seizure termination

Keywords:

seizure; hyperexcitable network; ictogenesis; CB1; TRPV1; endovanilloid; neurogenesis; GABA; glutamate; amygdala

Abbreviations:

CB1R- cannabinoid type 1 receptor

TRPV1 - transient receptor potential vanilloid receptor

SS – Seizure severity

SD – Seizure duration

ADD – Afterdischarge duration

ADT – Afterdischarge threshold

Cum ADD - cumulative afterdischarge duration

DCX - doublecortin

Introduction

Temporal lobe epilepsy is the most common type of epilepsy with focal seizure onset (Tellez-Zenteno and Hernandez-Ronquillo 2012). An initial brain-damaging insult can trigger a cascade of molecular and cellular alterations that form a hyperexcitable neuronal network and, eventually, the appearance of recurrent and spontaneous seizures (Pitkänen and Lukasiuk 2009). The generation of this hyperexcitable neuronal epileptic network is called epileptogenesis. In order to develop new prophylactic treatment strategies, it is essential to understand the mechanisms underlying epileptogenesis.

The endocannabinoid system serves a central function as a key regulator of neuronal activity, synaptic transmission and neuronal plasticity in the central nervous system (Katona and Freund 2008). The presynaptically located cannabinoid type 1 receptors (CB1Rs) are G protein-coupled and widely expressed within the central nervous system (Piomelli 2003). When activated on demand by the binding of postsynaptically synthesized endocannabinoid ligands such as anandamide or 2-arachidonoylglycerol, CB1R signaling attenuates both inhibitory neurotransmission and excitatory neurotransmission (Monory et al. 2006).

The endocannabinoid anandamide can also activate transient receptor potential vanilloid (TRPV1, formerly vanilloid receptor VR1) channels (Smart et al. 2000). These channels are nonselective plasma membrane cation channels (Caterina et al. 1997). Stimulation of TRPV1 increases the release of glutamate in an activity-dependent manner (Peters et al. 2010). Thus, the duration of postsynaptic spiking is enhanced at glutamatergic synapses (Shoudai et al. 2010; Mori et al. 2012). It has been confirmed that TRPV1 not only modulate glutamatergic signaling not only in the rodent brain, but also in human cortical tissue (Mori et al. 2012). Therefore, targeting not only the endocannabinoid system but also the endovanilloid system may provide a promising strategy for the regulation of neuronal activity in epilepsies (Lutz 2004).

Implying the cross-system functionality of endocannabinoids, target validation requires a comprehensive understanding of the complex neuromodulatory and protective properties of endocannabinoids against hyperexcitability and acute seizures (Marsicano et al. 2003; Hunt et al. 2012).

The detailed etiology of epileptogenesis remains unknown, and whether the endocannabinoid system is involved in epileptogenesis and promotes anti- or proconvulsant effects is still controversially discussed (Echegoyen et al. 2009; Dudek et al. 2010). This might be partly related to the dichotomous role of endocannabinoid signaling in different neuronal subpopulations. Conditional CB1R knockout mice with deletion in selected neuronal subpopulations offer an excellent opportunity to address the functional role of the endocannabinoid signaling cascade in glutamatergic vs. GABAergic forebrain neurons. Initial studies have already been performed in an acute chemical seizure model with systemic administration of kainate (Marsicano et al. 2003; Monory et al. 2006; Ruehle et al. 2013). The findings indicated that kainic acid-induced seizure severity is modulated by CB1-receptor expression on

cortical glutamatergic neurons. Recently, Kow and colleagues (2014) reported that genetic loss of CB1Rs plays a role in pilocarpine-induced seizures and seizure severity. However, none of the studies provide information about the impact of CB1Rs on seizure thresholds or on epileptogenesis.

Considering CB1Rs as a putative target for anticonvulsant or antiepileptogenic interventions, respective information is urgently needed. In a previous study, we analyzed the impact of pharmacological CB1R agonism on kindling epileptogenesis. In this study we revealed that the direct CB1R agonist WIN55.212-2 delayed kindling acquisition whereas the indirect CB1R agonist URB597 did not affect seizure progression in the kindling paradigm (Wendt et al. 2011). These findings again raise the question about the impact of different neuronal subpopulations. Thus, we aimed to thoroughly assess the consequences of conditional CB1R-knockout in direct comparison with pharmacological blockade of CB1R on epileptogenesis in the kindling paradigm.

In view of the dual function of endocannabinoids as well as the suggested link between the endovanilloid system and epilepsy, there is only limited data from experiments with rather unspecific pharmacological tools available. Therefore, we aim to elucidate the effects of TRPV1 knockout as well as specific TRPV1 antagonism on epileptogenesis in the kindling paradigm.

In the above-mentioned epilepsy model, seizure discharges caused by electrical stimulation of the amygdala via a chronically implanted electrode lead to a progressive and permanent increase in the behavioral and electrographic seizure response (Loscher and Brandt 2010). Thus, kindling assesses the impact of genetic and pharmacological modulation on seizure progression, which reflects the formation of a hyperexcitable network (McIntyre et al. 2002; Morimoto et al. 2004). In addition, one can precisely determine the impact on seizure thresholds before and following kindling acquisition based on application of systematically increasing stimulation intensities.

The aim of this study was to evaluate whether targeting the endocannabinoid and endovanilloid systems might (1) affect epileptogenesis (2) have an impact on ictogenesis and (3) render a basis for disease-modifying approaches.

Materials and Methods

Animals and electrode implantation

Male NMRI mice were purchased at a body weight of 21-25 g (Harlan Netherlands, Horst, Netherlands). Male $CB1^{f/f;CaMKII\alpha Cre}$ mice, (CamK-CB1 KO; (Marsicano et al. 2003)), $CB1^{f/f,Dlx5/6\text{-}Cre}$ mice (Dlx-CB1 KO; (Monory et al. 2006)), CB1R-null mutant mice $CB1^{-/-}$ (CB1–KO; (Marsicano et al. 2002)) as well as $VR1^{-/-}$ mice (Caterina et al. 2000) breeding pairs were obtained from The *Jackson Laboratory,* TRPV1-KO) and their respective wildtype littermate controls (CamK-CB1, Dlx-CB1, CB1- and TRPV1-WT) were bred at the Max-Planck Institute of Biochemistry (Martinsried, Germany) and transferred with a weight of 21-25g to our institute. CamK-CB1 KOs lack CB1Rs in principal forebrain neurons and Dlx-CB1 KOs in GABAergic forebrain neurons. CB1-KOs and TRPV1-KOs do not express the respective receptors at all. Mice were derived from heterozygous breeding pairs (TRPV1-KO and –WT) or from Cre-negative mothers and Cre-positive fathers (CamK-CB1 and Dlx-CB1). CamK-CB1 KO, Dlx-CB1 KO, CB1-KO and TRPV1-KO were genotyped by PCR using a slightly modified protocol as described previously by (Caterina et al. 1997b; Caterina et al. 2000; Casanova et al. 2001; Marsicano et al. 2003; Monory et al. 2006). The animals were kept under controlled environmental conditions (24–25°C; humidity 50–60%; 12 h dark/light cycle, lights on from 7am – 7pm) with free access to water and standard laboratory food. Experimental procedures have been carried out in accordance with the EU directive 2010/63/EU for animal experiments, with the German Animal Welfare act and were approved by the responsible government (license numbers: 55.2-1-54-2532-186-09 and 55.2-1-54-2532-93-11).

To minimize the impact of circadian variations, all experiments were performed within the same time period (8 a.m. to 1 p.m.).

A teflon-isolated bipolar stainless steel electrode with a diameter of 280µm was stereotactically implanted into the right amygdala as described previously (Jafari et al. 2012b). All animals received meloxicam injections (0.2 mg/kg, s.c., Metacam®, Boehringer Ingelheim, Ingelheim, Germany) 30 min prior and 24 h after the surgery to guarantee analgesia. Chloralhydrate (400 mg/kg in 10 ml saline i.p., Merck KGaA, Darmstadt, Germany) in combination with bupivacaine 2% (5 ml/kg s.c., Jenapharm®,Mibe, GmbH, Brehna) was used for anesthesia. The stereotaxic coordinates in millimeter relative to bregma were AP -1.0, L +3.2, DV -5.3 for NMRI mice, AP -1.2, L +3.5, DV -5.2 for CamK-CB1 mice and AP -1.4, L +3.5, DV -5.0 for Dlx-CB1 and TRPV1 mice. Following surgery, all the animals were allowed to recover for at least two weeks.

Kindling procedure

The initial afterdischarge threshold was determined following a stepwise protocol as described previously (Pekcec et al. 2007). The animals were kindled via daily electrical stimulation (1 ms, monophasic square-wave pulses, 50 Hz for 1 s), five times per week, either with a stimulation current 20% above the initial afterdischarge threshold (pharmacological modulation: rimonabant) or with 700

μA (all genetic modulations and pharmacological modulation: SB366791). In the pharmacological modulation with rimonabant the interindividual seizure thresholds varied. To exclude a bias, each mouse was stimulated with individual stimulation currents 20 % above the afterdischarge threshold. For all the other experiments suprathreshold stimulation with 700 μA was used. The stimulation current of 700 μA was chosen based on the range of the initial afterdischarge seizure thresholds. In previous experiments in our lab, using individualized stimulation strength (20 % above initial threshold) did not make a difference as compared to the suprathreshold stimulation with 700 μA, both, regarding the response to the first stimulation following threshold determination and regarding the kindling rate. The only difference was that kindling with 700 μA resulted in a more stable fully-kindled state. The seizure severity was scored according to a slightly modified Racine scale (Racine 1972): 1, immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; 2, head nodding associated with more severe facial clonus; 3, clonus of one forelimb; 4 bilateral forelimb clonus with or without rearing; 5 bilateral fore- and hindlimb clonus and falling with or without rearing. Kindling stages one and two represent focal seizures (limbic seizures), while beginning with seizure stage three, the seizures start evolving into clonic seizures (stages 3-5), a process of secondary generalization. At stages four and five, seizures involve both hemispheres (bilateral clonus) and thus, they are generalized. Motor seizure duration was stopped using an electronic timer and is defined as the visually determined time period of generalized seizure activity plus subsequent focal seizure activity as total motor seizure duration. In addition, electrographic afterdischarge duration was recorded. The sum of afterdischarge durations throughout the experiment was calculated as the cumulative afterdischarge duration.

As we were only interested in kindling progression rates, and did not aim for experiments in fully-kindled mice, we did not continue stimulations once all control mice exhibited at least one generalized stage four or five seizure. Depending on the experiment and on the mouse strain, we had to adjust the 'standard' kindling protocol. We know from several years of kindling experience that mice with different genetic backgrounds (here NMRI or C57/BL6) may kindle faster or slower. The kindling procedure was stopped, when all mice from the control group, either being vehicle-treated or littermate control, experienced at least one generalized stage four or five seizure. This definition has been applied to each single experiment, explaining the different numbers of stimulation. Thus, NMRI mice received 20 and CamK-CB1/Dlx-CB1/TRPV1-mice (bred on a C57/BL6 background) 10 electrical stimulations. In addition, we also made the experience that kindled mice do not reproducibly exhibit the highest seizure score (5) even if stimulations are continued for a long time. In contrast, rats reliably exhibit the highest seizure score once the kindled state is reached. Next, we determined the post-kindling afterdischarge following the stepwise procedure (Pekcec et al. 2007). One Dlx-CB1 KO mouse was statistically identified as significant outlier and was excluded from all further analysis. One vehicle animal received erroneously the TRPV1 antagonist and was therefore excluded from following analysis. Electrode-implanted controls (NMRI vehicle, CamK-CB1/Dlx-CB1/TRPV1-KO/WT mice)

were exposed to identical daily handling procedures except from the electrical stimulation.

For the pharmacological modulation with the TRPV1 antagonist a modified version of the above mentioned protocol was used: following determination of initial afterdischarge thresholds, the animals were grouped into vehicle-treated and TRPV1 antagonist-treated animals. We evaluated a second afterdischarge threshold 30 min after drug or vehicle application. These mice then received 15 daily electrical stimulations.

Six to twelve animals formed an experimental group.

Drugs:

SR141716 and SB366791:

The CB1R antagonist rimonabant (SR141716A – [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochlo-ride] (CAS 158681-13-1, axon medchem BV, Groningen, The Netherlands)) and the TRPV1 antagonist SB366791 ([N-(3-methoxyphenyl)-4-chlorocinnamide], CAS 472981-92-3, biomol GmbH, Hamburg, Germany) were dissolved in a solution of 99 % Ethanol, cremophor EL (Sigma-Aldrich, Taufkirchen, Germany) and saline (1:1:18). The dosages for rimonabant (5 mg/kg) and for SB366791 (1 mg/kg) as well as the pre-treatment time of 30 min were based on previous studies (Micale et al. 2013). Both compounds were injected i.p. at a volume of 10 ml/kg bodyweight always 30 min prior to the kindling stimulation. Control experiments with administration of a vehicle solution (99 % Ethanol, cremophor EL, saline; 1:1:18) were performed in parallel.

BrdU

Dividing cells can be labeled by administration of DNA precursors like the thymidine analogue bromodeoxyuridine (BrdU), which is incorporated into the DNA during the S-phase of the cell cycle. BrdU is available for about 30 min after injection, and thus labels any cells proliferating during this period (Packard et al. 1973). We injected all mice (Kindled and control animals) with BrdU (50 mg/kg i.p.; Sigma-Aldrich, Hamburg, Germany) three times a week, once directly following the kindling stimulation and again in the afternoon (NMRI: total injections: 26; CB1, GABA, and TRPV1 mice: total injections: 14). BrdU administrations did not influence the state or the performance of the animals following seizure activity.

Tissue preparation and immunostaining

The anxiety- and locomotor-associated behavior of all animals was evaluated in the light-dark box and the open field on consecutive days subsequent to kindling experiments. However, we did not intensify the behavioral analysis, because neither kindling nor pharmacological or genetic modulation of both systems exerted any consistent and reproducible behavioral effects.

At a maximum of six days after the last seizure, the mice were deeply anesthetized with pentobarbital

(narcoren® 400 mg/kg i.p., Merial GmbH, Halbergmoos, Germany) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and transferred into 30 % sucrose on the following day. The brains were stored at 4 °C until they were cut into coronal sections, 40 µm thick, collected in five series, on a microtome (Frigomobil, Reichert-Jung; Heidelberg-Nußloch, Deutschland). For long-term storage at -80 °C, we transferred the sections into a cryoprotectant solution (glycerol and 0.1 M phosphate buffer, pH 7.4, 1:1 in volume).

One of the series was stained with thionin for verification of the electrode localization.

For CB1R immunostaining, free-floating sections were washed, pre-incubated in 1.5 % BSA, and then treated for two days at 4 °C with a polyclonal rabbit anti-CB1 antibody (1:500; ABCAM, Cambridge, UK), which recognizes the C-terminus of the receptor. Sections were then washed with TBS + 0.05 % Tween20, incubated with a biotinylated goat anti-rabbit antibody (1:400; DAKO, Eching, Germany) for 1 h at room temperature and afterwards treated with 3 % H2O2 for 20 min. Sections were rinsed in TBS + 0.05 % Tween20 and incubated for 60 min in horseradish peroxidase-labeled streptavidin (1:4000, Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA). To visualize the staining, a nickel-intensified diaminobenzidine reaction was performed using a peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA). For immunolabeling of TRPV1 we used a polyclonal goat antibody recognizing the N-terminus of TRPV1 (1:200; Santa Cruz Biotechnology, CA, USA) and a biotinylated donkey anti-goat antibody (1:400; Vector Laboratories, Peterborough, UK). The protocol was essentially the same as described above. The specificity of the CB1 / TRPV1 antibody binding was verified by means of CB1-KO and TRPV1-KO.

We additionally performed a doublecortin immunostaining and a BrdU/NeuN immunofluorescence double staining as described previously (Jafari et al. 2012a). The following primary antibodies were used: polyclonal goat anti-doublecortin (1:300; Santa Cruz Biotechnology, Santa Cruz, California, USA) and rat anti-BrdU (1:300; AbD Serotec, Düsseldorf, Germany) combined with mouse monoclonal anti-NeuN (1:500; clone A60, Millipore, Temecula, USA).

Secondary antibodies were biotinylated donkey anti-goat (1:550; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) in combination with peroxidase-labeled streptavidin (1:4000, Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) for doublecortin staining and carbocyanine 3-labeled donkey anti-rat (1:1000; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), biotinylated donkey anti-mouse (1:500; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) and carbocyanine 2-labeled streptavidin (1:1000; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) for BrdU/NeuN staining.

All sections were washed, mounted onto glass slides, air-dried, dehydrated, and cover-slipped with Entellan (Merck, Darmstadt, Germany).

Quantification and image analysis

Labeled cells from the doublecortin and BrdU/NeuN stainings were counted across five brain sections per animal in the left and right dentate gyri in an area encompassing the entire dentate granule cell layer (upper and lower blades) and extending two cell body widths deep into the hilus. The sections were spaced 200 μm apart. The experimenter who performed the counting was blinded to the treatment conditions.

We measured the number of doublecortin (DCX) -immunoreactive cells with an unbiased stereological analysis method, the optical fractionator, operated via the computer-assisted imaging system StereoInvestigator 6.0 (Microbrightfield Europe, Magdeburg, Germany). The hardware consisted of a Leica DMLB microscope (Leica, Bensheim, Germany), a Plan-Neofluar lens (Leica, Bensheim, Germany), a single chip charge coupled device (CCD) color camera (CX9000, Microbrightfield Europe, Magdeburg, Germany), and an AMD Athlon (tm) 64 Processor. The thickness of the counting frame was equal to the thickness of the section (minus guard zones from the top and bottom of the section). Only the immunoreactive cells that appeared within the counting frame and came into focus were counted.

We counted BrdU/NeuN immunofluorescent positive cells on a computer monitor to improve visualization using a Hitachi HV-C20A (Hitachi, Japan) digital camera connected to a Zeiss LSM 510 microscope (Carl Zeiss GmbH, Jena, Germany). Double-labeling was verified by careful analysis of the confocal z-series of multiple cells per animal. Counting of the fluorescent signals from BrdU-immunoreactive cells as well as the number of double-labeled BrdU/NeuN cells was performed as described earlier (Pekcec et al. 2007).

We analyzed CB1R and TRPV1 immunohistochemistry based on labeled area intensity using a computer image analysis system. The hardware consisted of an Olympus BH2 microscope with a Plan-Neofluar objective (Zeiss, Göttingen, Germany), a CCD color camera (Axiocam; Zeiss, Göttingen, Germany), and an AMD Athlon™ 64 processor-based computer with an image capture interface card (Axiocam MR Interface Rev.A; Zeiss, Göttingen, Germany). The analyzed images were 1388x1040 pixels in dimension. Images were processed using KS400 image analysis software (Windows Release 3.0; Carl Zeiss Vision, Halbergmoos Germany). The detailed analysis methodology has been reported previously (Volk et al. 2004). Briefly, prior to image analysis, spatial calibration was performed and a signal threshold value was defined for each analyzed brain region (CA1 and CA3 regions of the hippocampus and the inner third of the molecular layer of the dentate gyrus [the dentate gyrus was analyzed only for CB1 receptor immunohistochemistry]) to exclude background signals. Thus, the data obtained render information about the labeled area above the threshold. Due to very weak CB1R immunostaining in the polymorphic layer of the dentate gyrus, this area was analyzed only qualitatively and descriptively in five animals per group.

Statistics

We performed statistical analysis of group differences using GraphPad Prism (Version 5.04, GraphPad, San Diego, CA, USA). Inter-group differences in kindling development were detected with the two-sided Mann Whitney U-test and the unpaired two-sided *t*-test, if necessary with Welch's correction. Two-way ANOVA for repeated measurements (dependent factor: time, independent factor: treatment/genotype) was performed by Statistica, v5.0 (StatSoft (Europe) GmbH, Hamburg, Germany). Immunohistological data were analyzed by two-way ANOVA and was followed by an unpaired T-test to detect group differences.

To detect statistical outliers, the Grubbs' test was used. All data are given as mean values ± SEM, and a $p < 0.05$ was considered significant.

Results

Kindling increases CB1R expression in the hippocampus, whereas TRPV1 expression remains unaltered

Considering CB1Rs and TRPV1 as promising targets for the regulation of neuronal activity in epilepsies we investigated if seizures influence the density of CB1Rs and of TRPV1. Therefore, we analyzed CB1R expression in CamK-CB1 WT mice and TRPV1 expression in NMRI mice associated with or without seizure history in the hippocampal formation six days after the last kindled seizure.

Non-kindled CamK-CB1 WT mice show the well-described expression pattern of CB1Rs in the analyzed brain areas (Tsou et al. 1997; Egertová and Elphick 2000; Monory et al. 2006). They densely express CB1Rs in the Ammon's horn, especially in the stratum pyramidale in CA1 and CA3 fields. In the stratum oriens, a higher CB1R density was detected than in the stratum radiatum. In the dentate area, the molecular layer intensely expressed CB1Rs with highest density in the inner third, whereas the granule cell layer appears as a translucent band. The polymorphic layer of the dentate gyrus virtually lacked CB1R staining in low-power magnification. In a high resolution only very sparsely immunoreactive fibers were seen. At the junction of the granule cell layer and the polymorphic layer numerous intensely immunoreactive neurons were evident. CB1R immunoreactivity was always associated with the cell membrane and the neuronal somata remaining unstained. (**Fig. 1 B-I, Supl. Fig. 2A, B**). In samples of mice with seizure history, CB1R immunostaining proved to be much stronger throughout the hippocampus. The most striking difference was found in the Ammon's horn. Kindled animals exhibited an increase of CB1R expression by 316% in the CA1-region and by 371% in the CA3-region (CA1 $p = 0.0273$; CA3 $p = 0.0231$; **Fig. 1 A**). In the molecular layer of the dentate gyrus CB1R immunostaining tended to be elevated, with an average increase of 271% ($p = 0.069$; **Fig. 1 A**). In contrast, CB1R expression in the polymorphic layer of the dentate gyrus appeared comparable in both groups.

Non-kindled NMRI mice show homogenous and weak to moderate TRPV1 immunoreactivity in cell bodies and in the cell membrane of hippocampal pyramidal cells (**Supl. Fig. 1B, D-G**). The immunoreactivity in the dentate gyrus was so weak that quantification was impossible (**Supl. Fig. 1H-I**). Only unspecific TRPV1 immunoreactivity was observed in TRPV1-KO mice, confirming specific binding of the antibody (**Supl. Fig. 1C**). In samples of mice with seizure history, TRPV1 immunostaining proved to be comparable in the CA1 ($p=0.1988$) and CA3 ($p=0.4691$) subfields of the hippocampus, when compared to non-kindled controls (**Supl. Fig. 1A**).

The endocannabinoid system affects ictogenesis but not epileptogenesis

CB1R deletion in principal neurons of the forebrain and in GABAergic forebrain neurons exerts opposing effects on seizure duration

The conditional deletion of the CB1R gene in principal neurons of the forebrain but not in GABAergic interneurons (CamK-CB1 KO) caused in naïve animals a lowered afterdischarge threshold when

compared to wildtype controls (p = 0.0281, **Fig. 2A**). We observed the characteristic reduction of seizure thresholds after kindling acquisition in the CamK-CB1 WT and -KO mice (p = 0.0388, **Fig. 2A**).

The progression of the seizure severity was comparable in CamK-CB1 KO mice and in littermate controls. The mean number of stimulations to reach a generalized stage five seizure did not differ between the two groups (CamK-CB1 WT = 6.6 ± 0.82 and CamK-CB1 KO = 6.22 ± 0.66, p = 0.728, **Fig. 2D**). In CamK-CB1 KO the analysis of the cumulative afterdischarge duration exceeded that in littermate wildtype controls (p = 0.0128; **Fig. 2G**). We demonstrated a significant impact of the genotype on seizure duration (p = 0.007, **Fig. 2E**) and afterdischarge duration (p = 0.0051; **Fig. 2F**) by two-way ANOVA. However, after kindling acquisition seizure duration (p=0.2962; **Fig. 2B**) and afterdischarge duration (p=0.2783; **Fig. 2C**) are comparable in CamK-CB1 WT and KO mice.

The deletion of CB1Rs in GABAergic cortical forebrain neurons (Dlx-CB1 KO) had no impact on either initial afterdischarge thresholds or post-kindling thresholds (initial p = 0.1778, post p = 0.1547; **Fig. 2H**). Kindling acquisition caused the characteristic threshold reduction (p = 0.0042, **Fig. 2H**).

The progression of the seizure severity and the mean number of stimulations to reach a generalized stage five seizure was comparable in both groups (Dlx-CB1 WT n = 6.6 ± 0.8 and Dlx-CB1 KO n = 7.2 ± 1.1, p = 0.6908; **Fig. 2K**). The analysis of seizure duration and afterdischarge duration revealed a reduced duration in Dlx-CB1 KO mice as compared to littermate controls (**Fig. 2L, M**). The cumulative afterdischarge duration was decreased in Dlx-CB1 KO mice (p = 0.0147) with a mean duration time of 176.9 ± 5.3 s when compared to Dlx-CB1 WT mice with a mean duration time of 213.9 ± 10.0 s (**Fig. 2N**). Furthermore, we confirmed an impact of the genotype on seizure durations and on afterdischarge duration parameters (seizure duration: p < 0.0001; afterdischarge duration: p < 0.001). Finally, after the kindling process was completed, the seizure duration (p = 0.0705; **Fig. 2I**) and the afterdischarge duration (p = 0.0477; **Fig. 2J**) tended to be lower in Dlx-CB1 KO mice.

The CB1R antagonist rimonabant prolongs seizure duration

Rimonabant treatment affected neither the initial afterdischarge threshold determined prior to the kindling process nor the post-kindling afterdischarge threshold (**Fig. 3A**). However, the seizure duration at post-kindling afterdischarge threshold determination reached higher levels in rimonabant-treated animals as compared to the vehicle-treated group (p = 0.0025, **Fig. 3B**). A comparable trend was visible during pre-kindling threshold stimulation (p = 0.0656, data not shown). Furthermore, during pre- and post-kindling threshold determination, rimonabant treatment resulted in longer afterdischarge duration when compared to vehicle-treated controls (initial: p = 0.0114, data not shown, post p = 0.0027, **Fig. 3C**). We observed the characteristic kindling-associated reduction of afterdischarge thresholds in both groups (p = 0.0469; **Fig. 3A**).

The development of seizure severity during kindling acquisition was comparable in rimonabant- and vehicle-treated mice. The mean number of stimulations to reach a generalized stage five seizure did

not differ between the rimonabant- (n = 11.2 ± 1.5) and vehicle-treated groups (n = 14.3 ± 1.5, p = 0.1599; **Fig. 3D**). However, the relative cumulative afterdischarge duration in rimonabant-treated mice exceeded that in vehicle-treated animals (p = 0.0008; **Fig. 3G**). In addition, the data revealed an impact of the treatment with rimonabant on prolonging motor and electrographic seizure activity (seizure duration: p = 0.0001; afterdischarge duration: p = 0.0001; **Fig. 3E, F**).

The endovanilloid system has no impact on epileptogenesis and on ictogenesis

Genetic knock out of TRPV1 and the TRPV1 antagonist SB366791 has no relevant impact on seizure parameters

The initial and post-kindling afterdischarge threshold was not affected by TRPV1 deficiency and pharmacological antagonism **(Fig. 4A, H)**. Kindling acquisition caused the characteristic kindling-associated reduction of afterdischarge thresholds in TRPV1-WT and –KO (p = 0.0181, **Fig. 4A**) Genetic deletion of TRPV1 had no impact on the development of seizure severity or the duration of motor and electrographic seizure activity (**Fig. 4D, E, F**). The mean number of stimulations to reach a generalized stage five seizure was comparable between WT and KO mice (TRPV1-WT n = 4.5 ± 0.7 and TRPV1-KO n = 4.8 ± 0.5, p = 0.7696; **Fig. 4D**).

Kindling acquisition also proved to be comparable in SB366971-treated and vehicle-treated mice (**Fig. 4K**). The mean number of stimulations to reach a generalized stage five seizure did not differ between SB366791-treated mice (n= 10.5 ± 0.9) and vehicle-treated mice (n = 11.1 ± 1.2, p = 0.689; **Fig. 4K**). There was no statistically specific difference between SB366971-treated and vehicle-treated mice regarding seizure duration and afterdischarge duration (**Fig. 4L, M**).

Surprisingly, seizure duration and afterdischarge duration during threshold determination after the kindling process were significantly longer in TRPV1-KO mice (seizure duration: p = 0.0341, afterdischarge duration: p = 0.0061, **Fig. 4B, C**). In contrast, pharmacological antagonism exerted no effect on seizure duration during post-kindling threshold determination (seizure duration: p = 0.7147 afterdischarge duration: p = 0.8187, **Fig. 4I, J**).

The endocannabinoid system has no impact on neurogenesis during kindling acquisition

Kindling increases hippocampal neurogenesis without interaction of the endocannabinoid system

Conditional deletion of CB1Rs in principal neurons of the forebrain did not affect neuronal progenitor cells or the fate of newborn neurons in the hippocampus of CamK-CB1 KO mice.

However, we identified kindling–associated alterations in hippocampal neuronal progenitor cells and newborn neurons in the hippocampus of knockout mice and their littermate controls. We analyzed the number of neuronal progenitor cells six days after the last kindled seizure and found a kindling-induced increase by 110 and 176% in CamK-CB1 WT and CamK-CB1 KO mice, respectively (**Fig. 5A, B, F**). Kindling also increased the number of BrdU-positive labeled cell nuclei in the dentate gyrus by 118 % in CamK-CB1 WT (p = 0.0057) and by 139 % in CamK-CB1 KO mice (p < 0.001,

data not shown). An increase in the total number of newborn neurons (BrdU/NeuN double-labeled) was confirmed in the two kindled groups when compared to non-kindled controls (CamK-CB1 WT $p = 0.0118$ and CamK-CB1 KO $p = 0.0048$; **Fig. 5C, D, E, G**). In all four groups, 21-23 % of the BrdU-immunoreactive cells co-expressed the neuronal marker NeuN (**Fig. 5H**).

Discussion

Seizures increase CB1R density in the pyramidal cell layer of the hippocampus, whereas TRPV1 expression remains unaltered

In the present study, we demonstrate that kindling stimulations increased the CB1R density in the pyramidal cell layer. No respective response was observed in other sub-regions of the hippocampus, including the polymorphic layer. Similar findings have been reported for different mouse models of epilepsy and in human patients with epilepsy (Magloczky et al. 2010; Karlocai et al. 2011; Bojnik et al. 2012). Repeated electrically-induced seizures form a stable hyperexcitable network, which is associated with permanent enhanced seizure susceptibility and with cellular and molecular alterations. These alterations mimic the pathophysiological findings of temporal lobe epilepsy (Goddard et al. 1969).

The hippocampal up-regulation of CB1Rs may be a compensatory mechanism of excitatory neurons to strengthen the negative feedback loop of the endocannabinoid system and to down-regulate neurotransmitter release. Thus, the endocannabinoid system on these neuronal subpopulations may serve as a protective endogenous mechanism against seizure induced excitotoxicity as proposed in earlier studies (Marsicano et al. 2003; Wallace et al. 2003; Monory et al. 2006). However, our results differ from the studies of Falenski et al. (2009) and Weyeth et al. (2010), who found a reduced expression of CB1Rs or at least a reduction in certain neuronal subpopulations. This divergence could be a result of the differences between the epilepsy models (chemical versus electric models).

We identified weak to moderate TRPV1 expression in pyramidal cells in the CA1 and CA3 subfields of the hippocampal formation, whereas expression levels in the dentate gyrus were extremely low. Accumulating evidences from experiments using the specific radioactive ligand [^{3}H]resiniferatoxin, mRNA probes and immunohistological techniques support the idea of a widespread distribution of TRPV1 in the central nervous system (Mezey et al. 2000; Roberts et al. 2004; Toth et al. 2005; Han et al. 2013). In all these studies TRPV1 protein was detected in the hippocampus, however, quantitative measurements of TRPV1 expression lead to variable outcomes. Toth et al. (2005) identified the hippocampus as the region with highest level of TRPV1 immunoreactivity, whereas Han and colleagues (2013) found the least amount of TRPV1 immunoreactivity in this brain region. The same discrepancies are found for TRPV1 expression in hippocampal subregions. Toth et al. (2005) detected the strongest TRPV1 immunoreactivity in the cellular layer of the dentate gyrus and in the CA1 region. In contrast another study reported densest binding of [^{3}H]resiniferatoxin in CA3 and in the dentate gyrus (Roberts et al. 2004). The analysis of a TRPV1 reporter mouse revealed TRPV1 expression in the hippocampus exclusively in Cajal-Retzius cells (Cavanaugh et al. 2011). Thus, the expression profile of TRPV1 in the brain has yet not been finally clarified.

In contrast to increased CB1R expression due to repetitive seizures, TRPV1 expression is unaltered in the hippocampus of mice with seizure history. There is only one study based on data from human patients with clinical manifestations of temporal lobe epilepsy, which reports an increased TRPV1

expression in the hippocampus of these patients (Sun et al. 2013). Here, we used an animal model of epileptogenesis. Possibly, changes in TRPV1 expression levels may occur later in the course of the disease and are therefore not manifested during epileptogenesis. Further studies in different animal models of epileptogenesis as well as in chronic animal models with spontaneous seizures are necessary to give a definite statement about TRPV1 expression levels and their pathophysiological relevance in epilepsy and epileptogenesis.

The endocannabinoid-system has an impact on seizure susceptibility and on ictogenesis dependent on the neuronal subpopulation being modulated

In naïve CamK-CB1 KO mice we detected lower initial afterdischarge thresholds as compared to littermate controls, but comparable initial afterdischarge thresholds were observed in naïve Dlx-CB1 KO mice. Our findings are in line with a previous study demonstrating increased susceptibility to kainic acid-induced seizures as a consequence of CB1R deletion in glutamatergic forebrain neurons (Monory et al. 2006). The CB1R KO mice used in our study lack CB1Rs in principal neurons of the forebrain, which in the hippocampus are mainly glutamatergic. Therefore, the glutamate release is not endogenously regulated when activated on demand, resulting in increased glutamate levels, enhanced excitation, and decreased seizure susceptibility. Our data show that lack of CB1R, especially in principal forebrain neurons, lowers the afterdischarge threshold in naïve animals, implying that the excitability of the neuronal system in these mice already differs before kindling. In contrast, the progression of seizure severity during kindling was comparable in both groups arguing against any major impact of CB1R on principal forebrain neurons on the formation of the hyperexcitable kindled network. However, the interpretation of our findings is limited by the use of suprathreshold stimulation current during kindling. This might hide possible genotype/ treatment differences during kindling development due to the ceiling effect.

We found that the lack of CB1Rs in CamK-CB1 KO mice as well as antagonism of CB1Rs causes longer seizure duration, whereas a CB1R knock out in GABAergic neurons of the forebrain results in shorter seizure duration. This can be explained by the opposing functions of neuronal subpopulations targeted by the modulation strategies. Due to the negative feedback loop, CB1R knock out in the forebrain results in prolonged excitation of glutamatergic neurons and consecutively in longer seizure duration. In contrast, a lack of CB1Rs in GABAergic neurons increases GABA release and therefore the inhibitory component of neuronal communication, leading to shorter seizure duration. These results provide evidence that GABAergic interneurons play a role in the CB1R dependent control of electrically-induced behavioral seizures by the endocannabinoid system. Taking into consideration that cell-type-unspecific pharmacological blockade of CB1Rs resulted in prolonged seizures during kindling acquisition, we conclude from these experiments that endocannabinoid signaling in hippocampal glutamatergic principal neurons overrules that in GABAergic interneurons during seizure activity. This is probably related to excessive seizure-associated glutamatergic signaling (Blumcke et

al. 2000; Kandratavicius et al. 2013). However, lack of CB1R in GABAergic forebrain neurons caused a decrease in motor and electrographic seizure durations after completing the kindling process. Thus, GABAergic signaling might be responsible for long lasting protective effects in a hyperexcitable neuronal network.

The endocannabinoid system has been suggested to be an important endogenous mechanism for seizure duration and termination (Wallace et al. 2003; Deshpande et al. 2007), but the significance of neuronal subtypes in the context of epileptogenesis and ictogenesis has never been studied in detail in a chronic epilepsy model. In the present study, we not only blocked CB1Rs pharmacologically but also used genetic tools to analyze the cell-type-specific mechanism of seizure termination. To our knowledge, we have shown for the first time that conditional CB1R knockout in GABAergic neurons of the forebrain cause shorter seizure durations. These results confirm that the endocannabinoid signaling cascade plays a major role in seizure termination and duration through activation of CB1Rs.

In a kainic acid-induced seizure model, Monory et al. (2006) revealed that CB1Rs on hippocampal glutamatergic but not GABAergic neurons are required for protection against excitotoxic seizures. In another study CB1R function was reconstituted exclusively in dorsal telencephalic glutamatergic neurons. The rescue of CB1 in this brain area prolonged the time course of depolarization-induced suppression of excitation in the amygdala, indicating that CB1Rs in dorsal telencephalic glutamatergic neurons control neuronal functions (Ruehle et al. 2013). Furthermore, Guggenhuber et al (2010) conditionally overexpressed CB1Rs in pyramidal and mossy cells and demonstrated protective effects against seizures. The authors concluded that conditionally reduced transmitter release can decrease seizure intensity and exert neuroprotective effects (Guggenhuber et al. 2010).

Our data provide further evidence of the functional importance of the localization of CB1Rs and indicate that a specific antagonism at CB1Rs on GABAergic forebrain neurons or selective agonism at CB1Rs on glutamatergic forebrain neurons could be a safeguard against excessive network activity and therefore an antiepileptogenic strategy.

Several new revolutionary techniques, like DREADDs (designer receptors exclusively activated by designer drugs) and optogenetic tools delivered via local administration of viral vectors, open up tremendous future potential to selectively target specific neuronal subpopulations (Conklin et al. 2008; Deisseroth 2011; Farrell and Roth 2013). These techniques are still in their infancy and if they make their way into clinical use and may be applicable for targeting the endocannabinoid system is still a matter of question. So far the only technique to offer event- and site-specific effects is based on inhibition of endocannabinoid degrading enzymes, fatty acid amide hydrolase and monoacylglycerol lipase. The major sites of action of these inhibitors are those synapses with enhanced signaling and excessive on-demand production of endocannabinoids. However, a highly selective targeting of GABAergic or glutamatergic neuronal subpopulations is not possible with this approach. During epileptic seizure activity with excessive activation of glutamatergic synapses, the net effect of CB1R signaling on hippocampal projectory neurons apparently naturally predominates, indicating that a cell-

type-specific treatment might not be necessary. Administration of CB1R agonists might further enhance the negative feedback, thus supporting the endogenous mechanism for seizure termination. In future studies, it might be of interest to assess the efficacy of CB1R agonists in chronic animal models with spontaneous seizures. It is conceivable that targeting the endocannabinoid system could be a new therapeutic strategy, especially for long-lasting refractory status epilepticus and cluster seizures.

The endocannabinoid system has no crucial role in the generation of a hyperexcitable neuronal network

Our data demonstrate that neither genetic nor pharmacological modulation of the endocannabinoid system affects the development of generalized seizures during kindling acquisition when compared to the respective control groups. Previous studies addressing a role of CB1Rs during epileptogenesis have not revealed consistent findings. Echegoyen and colleagues reported a disruption of the epileptogenic process by a single application of rimonabant in a model of post-traumatic brain injury, when applied within a short therapeutic time window (Echegoyen et al. 2009). The antiepileptogenic effect indicated by these findings has not been substantiated yet by follow-up studies. In apparent contrast, Van Riijn et al. (2011) reported that the CB1R antagonist rimonabant can exert a proepileptogenic effect after chronic administration in naïve rats (van Rijn et al. 2011). Another study in the kainate model of temporal lobe epilepsy did not report relevant effects of rimonabant on epileptogenesis (Dudek et al. 2010). Considering these controversial findings, it is obviously necessary to further explore the role of CB1Rs in epileptogenesis in more detail. The use of conditional mutagenesis allowed us to precisely analyze the functional consequences of CB1R loss in different neuronal subpopulations of the forebrain in direct comparison with pharmacological antagonism. Neither genetic nor pharmacological targeting exerted any impact on kindling-associated progression of seizure severity, thus, arguing against a crucial role of CB1Rs during epileptogenesis.

In addition, we observed the characteristic reduction of seizure thresholds after kindling acquisition in all experiments, in which the endocannabinoid system was modulated either genetically or pharmacologically. Since the CB1R antagonist rimonabant was administered continuously during the kindling paradigm and not only to already kindled animals, any changes in the post kindling afterdischarge threshold might be a result of a continuous lack of the involvement of the endocannabinoid system during epileptogenesis. A similar situation occurs logically in CB1R deficient mice, further arguing against a relevant role of the endocannabinoid system in epileptogenesis.

The endocannabinoid system has no effect on seizure-associated alterations in neurogenesis

Evidence exists that the endocannabinoid system is involved in the modulation of cell proliferation, differentiation, maturation and survival of newborn neurons (for review see (Fogaca et al. 2013)). Aguado and colleagues (2005) provided evidence that neuronal progenitor cells possess a functionally endocannabinoid system and that the system remains functional active in adult brain progenitor cells.

The molecular constituents being involved in neuronal development and adult neurogenesis are the endogenous ligands anandamide and 2-AG, CB1R and CB2R as well as the AEA-degrading enzyme fatty acid amide hydrolase (FAAH) and the 2-AG synthesizing enzyme diacylglycerol lipases (Aguado et al. 2005; Goncalves et al. 2008). Our data show that CB1R expression is elevated due to kindling and one would assume temporary elevated endocannabinoid levels due to on demand activation of the endocannabinoid system. We know from the literature that neurogenesis requires CB1Rs and that enhanced endocannabinoid levels increase hippocampal cell proliferation. Thus, we expected endocannabinoid system dependent alterations. Surprisingly, in the present study, proliferation and generation of newborn neurons were induced by repeated seizures regardless of the genetic modulation of the endocannabinoid system. It has also been shown that the kindling process itself leads to increased hippocampal neurogenesis (Parent et al. 1998; Aguado et al. 2005; Pekcec et al. 2011; Jafari et al. 2012b), here pointing toward kindling associated alterations of cell proliferation. These data suggest that the endocannabinoid system plays no role in the disease-associated modulation of neurogenesis in the kindling model of temporal lobe epilepsy.

The endovanilloid system has no effects on the different seizure parameters

We found that neither genetic knockout of TRPV1 nor pharmacological antagonism alters seizure susceptibility, seizure progression or seizure duration before and during kindling acquisition. However, afterdischarge duration and motor seizure duration during seizure threshold determination after the kindling process proved to be significantly prolonged in genetically modified animals. Considering that TRPV1 seem to regulate glutamate release in an activity-dependent manner and can strongly potentiate the duration of postsynaptic spiking (Peters et al. 2010; Shoudai et al. 2010), this is a surprising result. TRPV1 are expressed, among others, in the hippocampus (Roberts et al. 2004; Toth et al. 2005; Han et al. 2013) and in contrast to our own results, an increase of TRPV1 protein expression has been described in the hippocampus of mice (Bhaskaran and Smith 2010) as well as in brain tissue from human patients with temporal lobe epilepsy (Sun et al. 2013). We expected an inhibitory effect of TRPV1 antagonism or genetic deficiency on hyperexcitability and acute seizures in the kindling model. Recent studies in chemical animal models of temporal lobe epilepsy have suggested TRPV1 as an interesting target site for epilepsy treatment. Neonatal TRPV1 deficient mice are less susceptible to PTZ-induced seizures following hyperthermia (Kong et al. 2014). Moreover, agonists like capsaicin and OLDA enhanced epileptiform activity, whereas antagonists like capsazepine and AMG9810 abolished these effects (Bhaskaran and Smith 2010; Gonzalez-Reyes et al. 2013; Shirazi et al. 2014). Capsazepine is a competitive antagonist of capsaicin with species-specific activity (McIntyre et al. 2001; Savidge et al. 2002). Furthermore it inhibits acetylcholine receptors (Liu and Simon 1997), voltage-gated Ca^{2+}-channels (Docherty et al. 1997) and hyperpolarization-activated cyclic nucleotide-gated channels (Gill et al. 2004). Thus, it is far from an ideal specific pharmacological tool. Therefore we used SB366791, which is a more selective and potent TRPV1

antagonist as compared to capsazepine (Gunthorpe et al. 2004; Varga et al. 2005). Surprisingly, neither the genetic modulation nor the pharmacological antagonism indicated relevant TRPV1-mediated effects, strongly arguing against a role of the endovanilloid system in ictogenesis. However, the prolonged seizure and afterdischarge duration of TRPV1 KO mice after the kindling process indicates that TRPV1 might contribute to seizure termination once a hyperexcitable neuronal network has been formed in the epileptic brain. In line with this idea is the recent study of Shirazi and colleagues (Shirazi et al. 2014) who reported reduced electrographic seizure duration in fully-kindled mice when treated with a TRPV1 antagonist. However, this assumption needs to be studied in more detail, also considering that pharmacological antagonism did not exert a comparable effect on the post-kindling seizure durations.

Based on earlier studies indicating opposing effects of the endocannabinoid and endovanilloid signaling on ictogenesis, Bhaskaran and Smith (2010) raised safety concerns regarding the application of pharmacological strategies, which non-selectively affect endocannabinoid signaling in epilepsy patients. Our findings rather indicate that effects mediated by TRPV1 can be neglected, as they are unlikely to affect the efficacy and tolerability of respective pharmacological approaches.

Conclusion

The aim of this study was to evaluate whether targeting the endocannabinoid and endovanilloid systems might (1) affect epileptogenesis, (2) have an impact on ictogenesis and (3) exert disease-modifying approaches. Our data indicate that the endocannabinoid system has no crucial role on the generation of a hyperexcitable neuronal network, but affects seizure duration depending on the neuronal subpopulation being modulated. CB1R deficiency in principal neurons of the forebrain causes longer seizure duration, whereas CB1R deletion in GABAergic neurons of the forebrain results in shorter seizure duration. We deduce that the endocannabinoid system is involved in seizure termination as an important endogenous mechanism.

In addition, the data suggest that TRPV1 do not play a major role epileptogenesis, and that the endovanilloid system is not strongly involved in ictogenesis or seizure termination.

These findings suggest that the endocannabinoid system can be considered as a promising target for decreasing seizure duration and supporting the endogenous mechanisms of seizure termination. Respective strategies are of particular interest for the management of long-lasting refractory status epilepticus and cluster seizures.

Figures

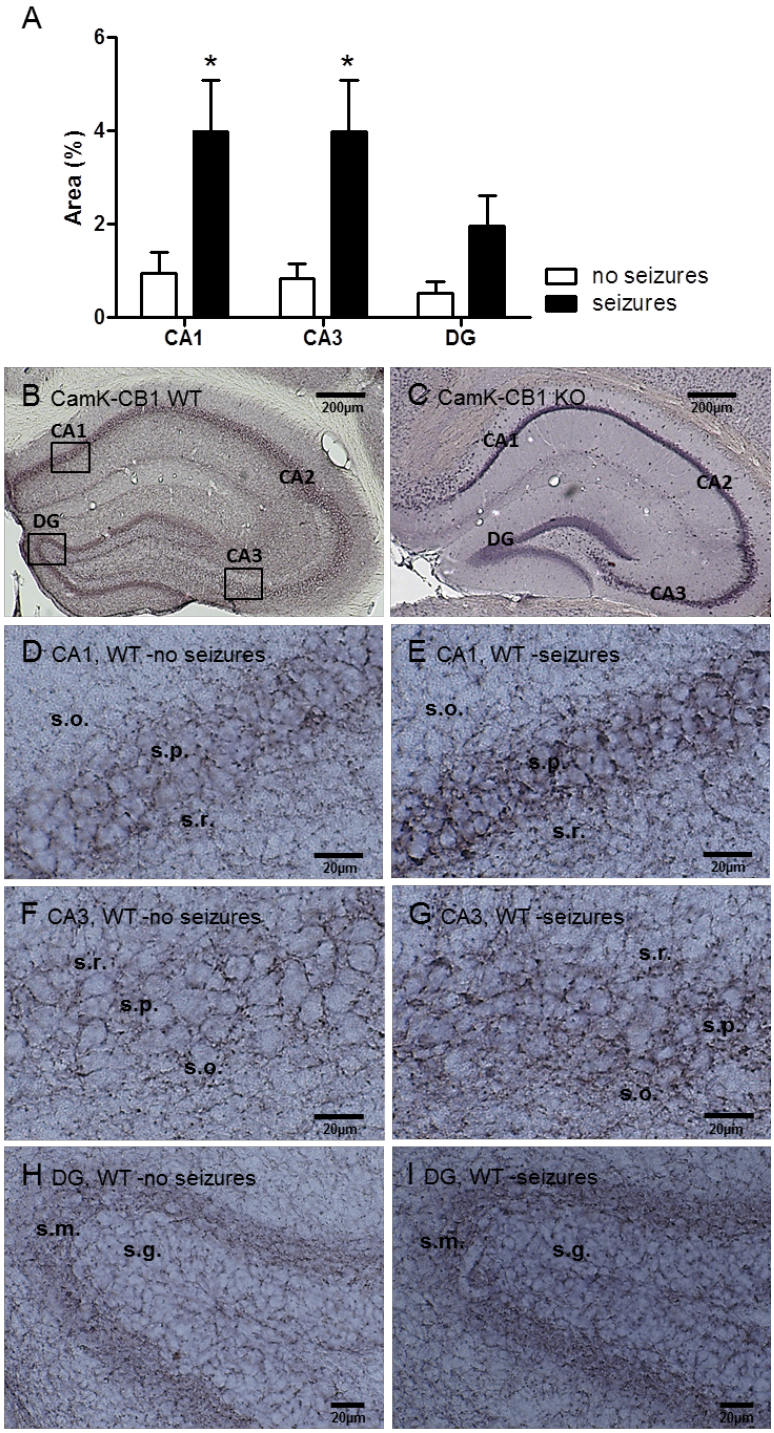
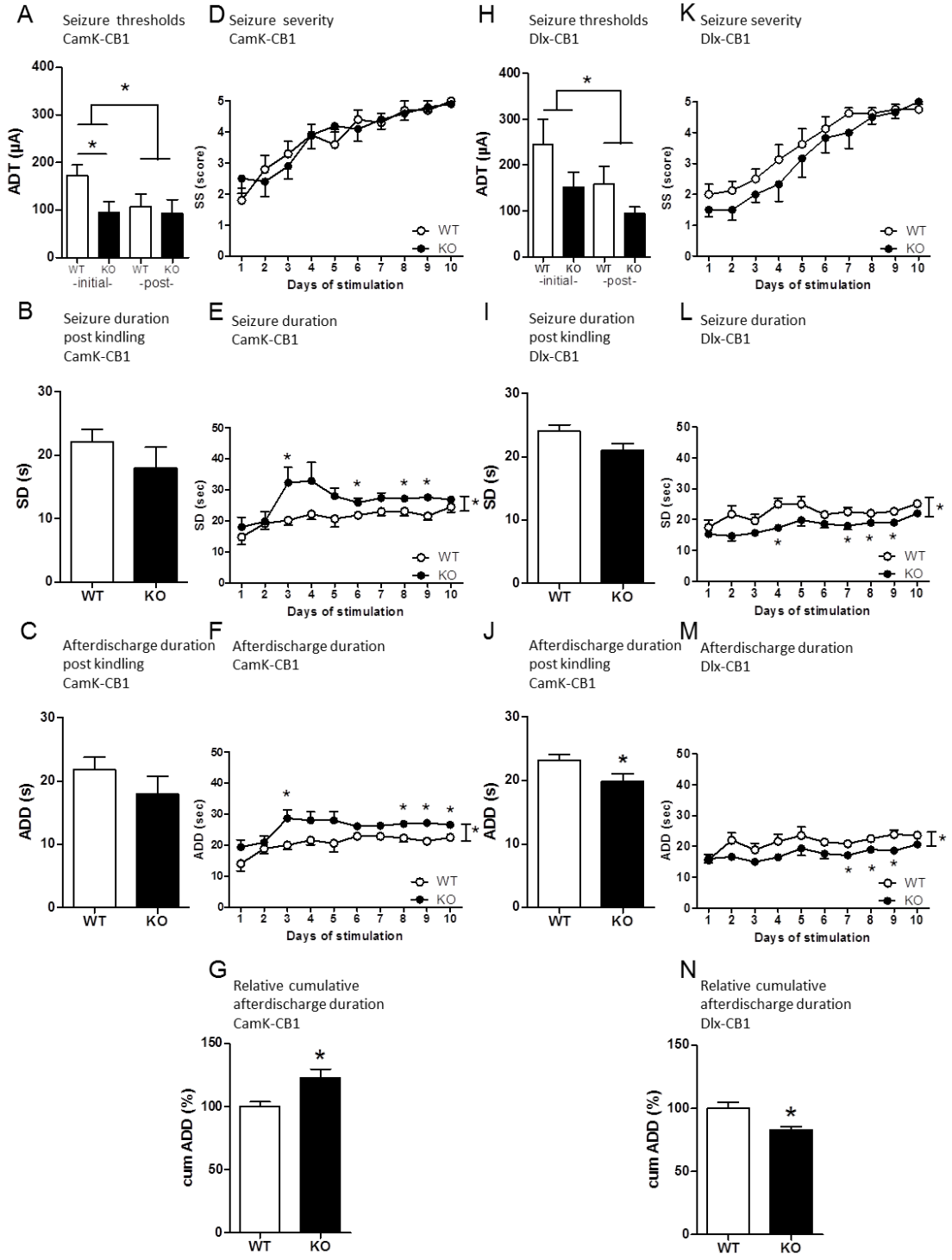


Figure 1:

CB1R expression in the hippocampus. A. Kindling increases the CB1R density in the CA1 and CA3 regions of the hippocampal formation of CamK-CB1 WT mice. The distribution of CB1Rs in the hippocampus of CamK-CB1 WT mice is illustrated in **B** and in comparison a CB1-KO mice as indicated in **C** with the lack of specific staining of CB1Rs (negative control). Magnification of CA1 (**D, E**), CA3 (**F, G**) and dentate gyrus (**H, I**): In non-kindled control animals the specific CB1R expression pattern can be seen in CA1 and CA3 stratum pyramidale and radiatum (**D, F**) and in the molecular layer of the dentate gyrus (**H**). In kindled mice, the CB1R immunostaining is more intense throughout the hippocampus (**E, G, I**). In the dentate gyrus of kindled mice, stained fibers establish a dense meshwork (**I**). Non-kindled CamK-CB1 WT n=8, kindled CamK-CB1 WT n=9; s.o.: str. oriens, s.p. str. pyramidale, s.r.: str. radiatum, s.m.: str. moleculare, s.g.: str. granulosum.

Figure 2:



Effects of genetic modulation of the endocannabinoid system on afterdischarge threshold (ADT), seizure severity (SS), seizure duration (SD), and afterdischarge duration (ADD) during and after kindling acquisition. A-G: CamK-CB1 KO mice versus littermate controls. **A** Pre- and post-kindling thresholds: note the significantly lower seizure threshold in naïve CamK-CB1 KO mice when compared to naïve controls. **B** Seizure duration and **C** afterdischarge duration after kindling acquisition. **D** CB1R knockout in principal neurons of the forebrain fails to have an impact on SS over time. However, motor seizure duration (**E**) and afterdischarge duration (**F**) are increased. The relative

cumulative afterdischarge duration (cum ADD) reflects the overall increase in ADD in mice with CB1R deletion in principal neurons of the forebrain (**G**). **H-N**: Dlx-CB1 KO mice versus littermate controls. **F** Pre- and post-kindling thresholds. **I** Seizure duration and **J** afterdischarge duration are reduced in Dlx-CB1 KO mice after kindling acquisition. **K** CB1R deletion in GABAergic forebrain neurons fails to have an impact on SS, but decreased the seizure duration (**L**) and the afterdischarge duration (**M**) when compared to control animals. **N** A decrease of the relative cumulative afterdischarge duration was observed in mice with CB1R deletion in GABAergic forebrain neurons. All data are given as mean ± SEM, * $p < 0.05$, two-way ANOVA for repeated measurement and unpaired student's *t*-test. CamK-CB1 WT n = 10, CamK-CB1 KO n = 10, Dlx-CB1 WT n = 8, and Dlx-CB1 KO n = 6.

Figure 3:

Effects of pharmacological modulation of the endocannabinoid system with rimonabant on afterdischarge threshold (ADT), seizure severity (SS), seizure duration (SD), and afterdischarge duration (ADD) during and after kindling acquisition. A Rimonabant- versus vehicle-treated animals pre- and post-kindling. **B** Seizure duration and **C** afterdischarge duration after kindling acquisition. **D** pharmacological modulation with rimonabant fails to have an impact on seizure severity over time. **E, F** However, rimonabant treatment increased the duration of epileptic seizures. **G** The relative cumulative afterdischarge duration (cum ADD) reflects the overall increase in ADD in rimonabant-treated mice. All data are given as mean ± SEM, * $p < 0.05$, two-way ANOVA for repeated measurement and unpaired student's *t*-test. Vehicle n = 9 and rimonabant n = 11.

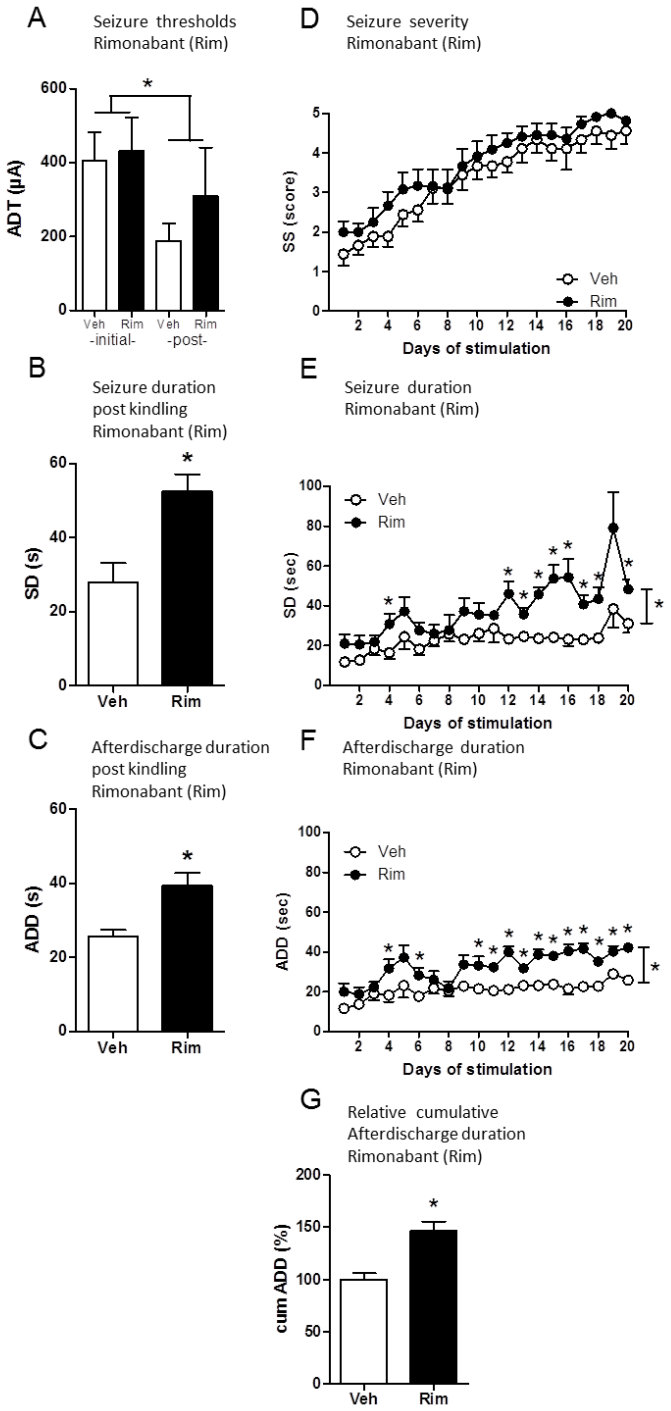
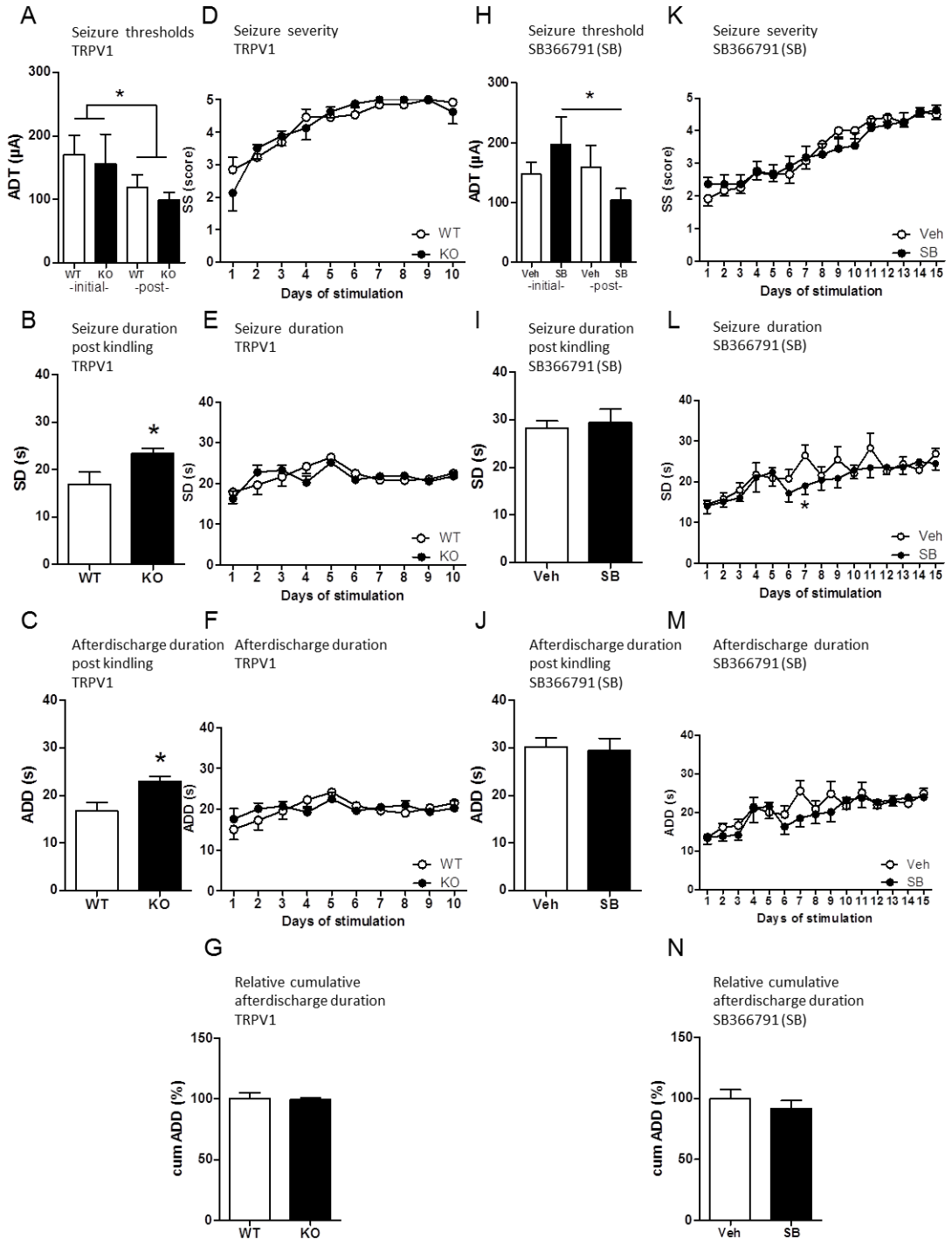


Figure 4:

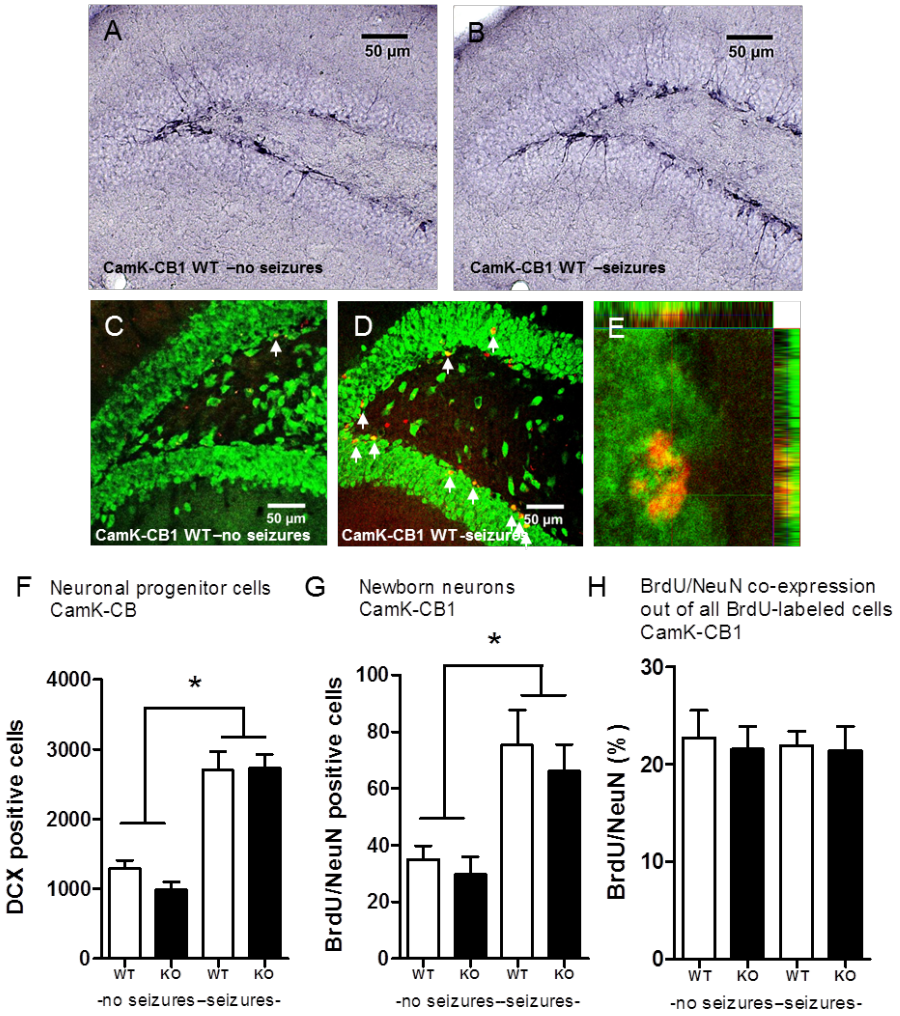


Effects of genetic and pharmacological modulation of the endovanilloid system on afterdischarge threshold (ADT), seizure severity (SS), seizure duration (SD), and afterdischarge duration (ADD) during and after kindling acquisition.

A-G Genetic modulation: TRPV1-KO mice versus their respective littermate controls. **H-N** Pharmacological modulation: SB366791-treatment versus vehicle-treated animals. The endovanilloid system fails to have an impact on any of the different seizure parameters during and after kindling acquisition. However, TRPV1-KO mice exhibit significantly longer motor and electroencephalographic seizure durations (**B, C**) after the kindling process. All data are given as mean

± SEM, * p < 0.05, unpaired student's *t*-test and two-way ANOVA for repeated measurements. Vehicle n = 9, SB366791 n = 12, TRPV1-WT n = 13, and TRPV1-KO n = 8.

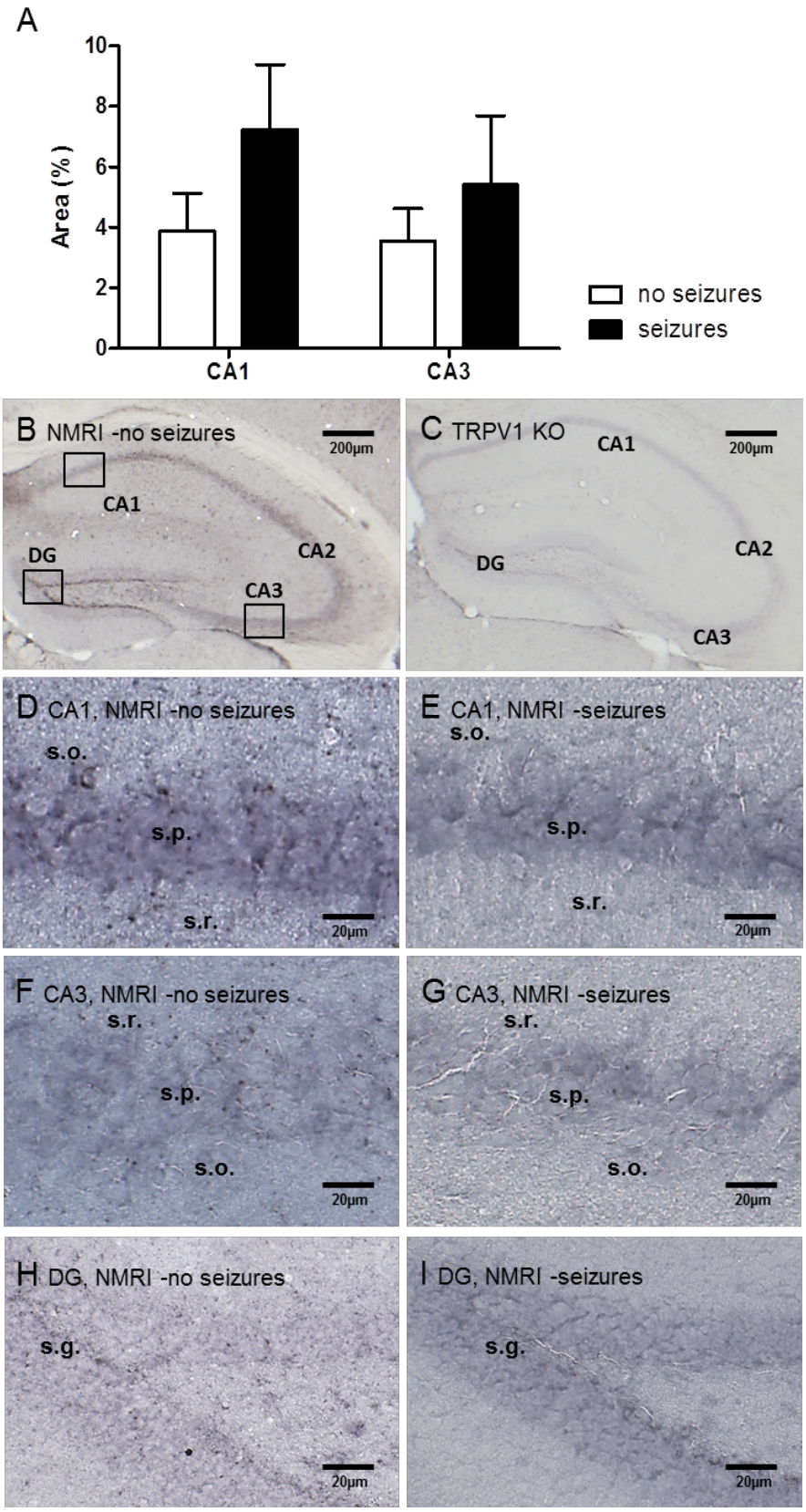
Figure 5:



Neurogenesis in the hippocampus of kindled versus non-kindled mice: **A-B.** Doublecortin (DCX) immunostaining of neuronal progenitor cells and early postmitotic neurons in the dentate gyrus of the hippocampus for representative animals of CamK-CB1 WT control (**A**) and CamK-CB1 WT kindled (**B**) mice. **C-E.** Representative BrdU (red) and neuronal (NeuN - green) nuclei in the dentate gyrus of non-kindled (**C**) and kindled (**D**) animals visualized via immunofluorescent double staining. Cells identified as newborn neurons (white arrows) express NeuN and incorporated BrdU, thus appearing in yellow. **E** The figure illustrates a high magnification of a BrdU/NeuN double-labeled cell of a kindled mouse within the dentate gyrus. Scale bar: 50µm **F-G.** Analysis of DCX-positive cells and BrdU/NeuN-positive cells in the dentate gyrus of the hippocampal formation. Kindling significantly increased the number of neuronal progenitor cells (**F**) and newborn neurons (**G**). **H** BrdU/NeuN co-expression out of all BrdU-labeled cells is comparable in all four groups. All data are given as mean ± SEM, * p < 0.05, two-way ANOVA. CamK-CB1 WT n = 10, and CamK-CB1 KO n = 10.

Supplementary information

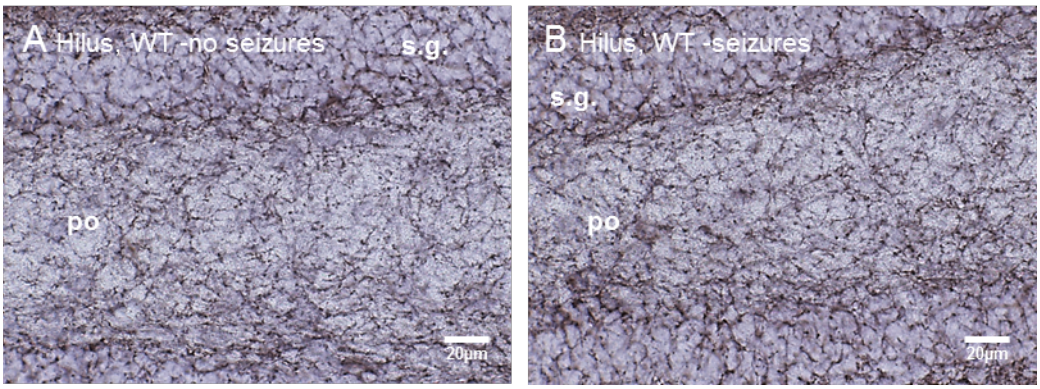
Supplementary Figure 1:



TRPV1 expression in the hippocampus. A. The labeled area of TRPV1 immunoreactivity is not affected by seizures. The interindividual variance was high within the animal groups. The distribution

of TRPV1 in the hippocampus of NMRI mice is illustrated in **B**, whereas no labeling was observed in TRPV1-KO mice (**C**), demonstrating the specificity of the antibody (negative control). Magnification of CA1 (**D, E**), CA3 (**F, G**) and dentate gyrus (**H, I**): Specific TRPV1 expression can be seen in the cytoplasm of CA1 and CA3 pyramidal neurons, which show moderate to weak immunoreactivity (**D-G**). Kindling did not affect TRPV1 expression in CA1 and CA3 subfields of the hippocampus. In the dentate gyrus only weak immunoreactivity was detected (**I**). Non-kindled NMRI n=8, kindled NMRI n=8; s.o.: str. oriens, s.p. str. pyramidale, s.r.: str. radiatum, s.g.: str. granulosum.

Supplementary Figure 2:



CB1R expression in the hilar region of the dentate gyrus. Note the low immunoreactivity in the granule cell layer and in the hilar region. Some more intensely stained neurons are located at the transition zone of the granule cell layer and the polymorphic layer of the dentate gyrus. The CB1R expression pattern and density are comparable in non-kindled mice (**A**) and kindled CamK-CB1 mice (**B**). s.g.: stratum granulosum; po.: polymorphic layer.

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Conflict of interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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2. Inhibition of monoacylglycerol lipase mediates a cannabinoid 1-receptor dependent delay of kindling progression in mice

E.L. von Rüden[1, 2], R.M. Bogdanovic[1, 2], C.T. Wotjak[3], H. Potschka[1*]

[1]Institute of Pharmacology, Toxicology and Pharmacy, Ludwig-Maximilians University, Munich, Germany

[2]Graduate School of Systemic Neurosciences, Munich, Germany

[3]Max Planck Institute of Psychiatry, Munich, Germany

Declaration of contribution: Eva-Lotta von Rüden designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with co-authors (for detailed information on author contributions see chapter´Eidesstattliche Versicherung/Affidavit´).

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Inhibition of monoacylglycerol lipase mediates a cannabinoid 1-receptor dependent delay of kindling progression in mice

E.L. von Rüden1, 2, R.M. Bogdanovic1, 2, C.T. Wotjak3, H. Potschka1*

[1]Institute of Pharmacology, Toxicology and Pharmacy, Ludwig-Maximilians University, Munich, Germany

[2]Graduate School of Systemic Neurosciences, Munich, Germany

[3]Max Planck Institute of Psychiatry, Dept. Stress Neurobiology & Neurogenetics, Munich, Germany

*Correspondence:

Prof. Dr. H. Potschka

Institute of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University, Munich, Koeniginstr. 16, D-80539 Munich, Germany;

Phone: +49-89-2180-2663; Fax: +49-89- 2180- 16556;

E-mail: potschka@pharmtox.vetmed.uni-muenchen.de

Abstract

Endocannabinoids, including 2-arachidonoylglycerol (2-AG), activate presynaptic cannabinoid type 1 receptors (CB1R) on inhibitory and excitatory neurons, resulting in a decreased release of neurotransmitters. Event-specific activation of the endocannabinoid system by inhibition of the endocannabinoid degrading enzymes may offer a promising strategy to selectively activate CB1Rs at the site of excessive neuronal activation with the overall goal to prevent the development epilepsy.

The aim of this study was to investigate the impact of monoacylglycerol lipase (MAGL) inhibition on the development and progression of epileptic seizures in the kindling model of temporal lobe epilepsy. Therefore, we selectively blocked MAGL by JZL184 (8 mg/kg, i.p.) in mice to analyze the effects of increased 2-AG levels on kindling acquisition and to exclude an anticonvulsive potential.

Our results showed that JZL184 treatment significantly delayed the development of generalized seizures ($p=0.0066$) and decreased seizure ($p<0.0001$) and afterdischarge duration ($p<0.001$) in the kindling model of temporal lobe epilepsy, but caused only modest effects in fully-kindled mice. Moreover, we proved that JZL184 treatment had no effects in conditional CB1R knockout mice lacking expression of the receptor in principle neurons of the forebrain.

In conclusion, the data demonstrate that indirect CB1R agonism delays the development of generalized epileptic seizures, but has no relevant acute anticonvulsive effects. Furthermore, we confirmed that the effects of JZL184 on kindling progression are CB1R mediated. Thus, the data indicate that the endocannabinoid 2-AG might be a promising target for an anti-epileptogenic approach.

Keywords

epileptogenesis; endocannabinoid system; fatty acid amide hydrolase; FAAH; anandamide

Highlights

- Event- and side-specific activation of CB1Rs.
- JZL184 delays the development of generalized seizures.
- JZL184 has no anticonvulsive potential.
- Effects of JZL184 are dependent on CB1R expression in principle forebrain neurons.

Abbreviations:

2-AG - 2-arachidonylglycerol

CB1R- cannabinoid type 1 receptor

MAGL - monoacylglycerol lipase

SS – Seizure severity

SD – Seizure duration

ADD – Afterdischarge duration

ADT – Afterdischarge threshold

Cum ADD - cumulative afterdischarge duration

Introduction

Anandamide and 2-arachidonylglycerol (2-AG), the two most studied endocannabinoids in the brain, are small lipid molecules, which retrogradely traverse the synapse and act presynaptically on metabotropic cannabinoid type 1 receptors (CB1Rs) (Kreitzer and Regehr 2001; Maejima et al. 2001; Wilson and Nicoll 2001). Activation of CB1Rs inhibit adenylyl cyclase and voltage-gated Ca^{2+} channels, resulting in a decreased release of neurotransmitters, thus modulating neuronal excitation and inhibition (Bidaut-Russell et al. 1990; Sugiura et al. 1997; Lauckner et al. 2005). As a consequence, activation of the endocannabinoid system may be a safeguard against hyperexcitability, acute seizures and excitotoxity (Ameri and Simmet 2000; Marsicano et al. 2003; Monory et al. 2006), raising the idea to slow down or even to prevent the development or progression of epilepsy. This is of particular interest, when considering that during excessive excitation endocannabinoid signaling has stronger effects on cortical principal neurons than on GABAergic interneurons (von Rüden et al. 2014). Therefore, the endocannabinoid system is discussed as a potential target for the prevention of epilepsy (Hofmann and Frazier 2013). The process of epilepsy development (= epileptogenesis) refers to molecular and cellular alterations which transform a physiological neuronal network into an epileptic state, with an increased risk of recurrent spontaneous seizures (Goldberg and Coulter 2013). The detailed etiology of epileptogenesis and if the endocannabinoid system may be involved in epileptogenesis remains still incompletely understood.

There are only few experimental studies in rodents which indicate anti-epileptogenic properties of direct CB1R activation (Bhaskaran and Smith 2010; Wendt et al. 2011). Recently, we evaluated the impact of the CB1R agonist WIN55.212-2 as well as of the indirect agonist URB597 on kindling progression (Wendt et al. 2011). WIN55.212-2 delayed kindling acquisition whereas URB597, which inhibits the catabolic enzyme fatty acid amide hydrolase and thereby reduces the degradation of anandamide, had no effect on the generation of a hyperexcitable neuronal network. In contrast to anandamide, 2-AG acts as a full agonist on CB1Rs and exhibits higher CB1R efficacy (Sugiura et al. 1999; Sugiura et al. 2000; Sugiura et al. 2006). A molecular and morphological study of mossy cell-granule cell synapses, revealed that 2-AG mediates retrograde signaling at these synapses and may reduce the excitability of granule cells and prevent seizures (Uchigashima et al. 2011).

Although these experimental data are only limited, they strongly suggest a potential therapeutic role for the endocannabinoid 2-AG in epilepsy. However, there is, to our knowledge, no information about the impact of 2-AG on seizure thresholds or on the progression of seizure development in epilepsy. Considering this information, it is of particular interest to evaluate whether increased 2-AG levels affect these parameters in a chronic epilepsy model with excellent predictive validity for temporal lobe epilepsy.

The aim of this study is to evaluate if pharmacological inhibition of monoacylglycerol lipase (MAGL) by JZL184 has an impact on the progression of epileptic seizures in the kindling mouse model of

temporal lobe epilepsy and to demonstrate that JZL184-mediated effects are CB1R dependent. For this purpose the indirect CB1R agonist JZL184 was used. JZL184 potently and selectively inhibits the 2-AG degrading enzyme MAGL and this results in increased 2-AG levels up-to an 8-fold (Long et al. 2009). This strategy offers a clear benefit compared with direct CB1R agonism namely event-specific effects, which are based on inhibition of the endocannabinoid degrading enzyme at CB1Rs localized on excessively activated glutamatergic synapses.

Methods

Animals

Animal experiments have been performed in accordance with the EU directive 2010/63/EU, and with the German Animal Welfare act. They were approved by the responsible government (license numbers 55.2-1-54-2532-93-11 and 55.2-1-54-2532-173-11). Male mice with a body weight of 21-15g were used in all experiments and maintained in standard conditions with food and water ad libitum (24–25°C; humidity 50–60%; lights on from 7am – 7pm). NMRI mice were acquired from Harlan Netherlands (Horst, Netherlands). $CB1^{f/f;CaMKII\alpha Cre}$ mice (CamK-CB1 KO) and their respective wildtype littermate controls (CamK-CB1 WT) were bred at the Max Planck Institute of Biochemistry (Martinsried, Germany). These mice were derived from cre-negative mothers and cre-positive fathers (CamK-CB1) and were genotyped by PCR as described previously (Marsicano et al. 2003). They lack CB1Rs in principal forebrain neurons. All mice were allowed to habituate to the new environmental conditions for at least one week.

Electrode implantation

Stereotactical implantation of the kindling electrode into the right amygdala was performed as described previously (Jafari et al. 2012). The electrode consists of teflon-isolated stainless steel with a diameter of 280 µm. Mice were anesthetized with chloral hydrate (400 mg/kg in 10 ml saline i.p., Merck KGaA, Darmstadt, Germany) and bupivacaine (5 ml/kg s.c., Jenapharm®, Mibe, GmbH, Brehna). For analgesia meloxicam (1mg/kgm, s.c. Metacam®, Boehringer-Ingelheim, Ingelheim, Germany) was administered 30 min prior to and 24 h post-surgery. The stereotaxic coordinates in millimeter relative to bregma according to the atlas of Paxinos and Franklin (2004) were AP -1.0, L +3.2, DV -5.3 (NMRI) and AP -1.2, L +3.5, DV -5.2 (CamK-CB1). Seven mice were euthanized following surgery due to alterations in their general condition.

Kindling

Kindling of mice was initiated following a postsurgical recovery period of 2 weeks. During the experiments body weight varied between 30-50g (NMRI) and 20-35g (CamK-CB1 WT and KO). Mice were housed separately.

The initial afterdischarge threshold (initial ADT) was determined for each animal using an ascending stair-step procedure with an initial current of 8 µA and an increase by 20% of the previous current every minute until afterdischarges were elicited (Pekcec et al. 2007).

The amygdala was electrically stimulated once daily, five times per week with 700 µA (1 ms, monophasic square-wave pulses, 50 Hz for 1 s). The seizure severity was scored according to the Racine scale (Racine 1972): (1) Mouth and facial movements. (2) Head nodding. (3) Forelimb clonus.

(4) Rearing. (5) Rearing and falling. In addition to seizure severity, seizure duration and afterdischarge duration were recorded for each seizure event. The cumulative afterdischarge duration was calculated as the sum of all afterdischarge durations throughout the experiment. Following kindling acquisition, we determined the post-kindling afterdischarge threshold by the stepwise procedure (Pekcec et al. 2007).

Experimental details: kindling acquisition

To minimize the impact of circadian variations, all experiments were performed within the same time of the day (1 pm to 6 pm). The initial afterdischarge threshold was determined twice on two consecutive days. Following determination of the first initial afterdischarge threshold without JZL-treatment, the animals were distributed into a vehicle-treated and a JZL184-treated group with a comparable mean of the initial afterdischarge threshold and afterdischarge duration (**Fig 2 A, B** and **Fig. 4 A, B**). At the following day the second afterdischarge threshold was evaluated 60 min after drug or vehicle application. Due to the different genetic background NMRI mice received twelve and CamK-CB1 mice seven electrical stimulations until the majority of mice in the control group exhibited generalized stage (4) or (5) seizures (**Fig.1, A**). At stimulation day twelve, eleven out of twelve NMRI mice and at day seven nine out of ten CamK-CB1 WT mice in the control group exhibited generalized stage (4) or (5) seizures. We expected a misplacement of the electrode in the animals not exhibiting generalized seizure activity. Subsequent analysis revealed a correct localization in the right amygdala. Thus, data from these animals were included in further analyses.

JZL184 or vehicle was administered with a volume of 10 ml/kg bodyweight in a dosage of 8 mg/kg once per day 60 minutes prior to the electrical stimulation. Following a washout phase of six days, we determined the post-kindling afterdischarge threshold.

The experimental groups consisted of 12 animals per group for NMRI mice (vehicle-treated and JZL184-treated mice) and of 6-10 animals per group for CamK-CB1 mice (WT vehicle n=10, WT JZL n=7, KO vehicle n= 6 and KO JZL184 n=8).

Experimental details: fully-kindled mice

To minimize the impact of circadian variations, all experiments were performed within the same time period (8 am to 1 pm). Following determination of the initial afterdischarge threshold, NMRI mice (n=10) were electrically stimulated with 700 µA once daily, five times per week until they exhibited at least ten generalized stage (4) or (5) seizures (the number of stimulations to reach this kindling criterion ranged between 10 and 31) and were considered fully-kindled. Please note: known from several years of experience with different mouse strains, mice are kindled to reproducibly exhibit generalized seizures of stage (4) or (5); however, the highest seizure score (5) cannot always be

elicited in fully kindled animals. In contrast, rats reliably exhibit the highest seizure score once the fully kindled state is reached.

Determination of afterdischarge thresholds was repeated in fully-kindled mice until thresholds proved to be stable allowing subsequent drug testing **(Fig.1, B)**. Four days before the drug testing all mice received a re-stimulation with 700 µA. To evaluate the effects of the test compound three different dosages of JZL184 (4 mg/kg, 8 mg/kg and 16 mg/kg; i.p.) were injected in a volume of 10 ml/kg bodyweight 60 min prior to afterdischarge threshold determination. Each drug experiment was preceded by a vehicle control experiment two to three days before the previous drug administration to test whether control thresholds were in the same range as before. In addition to the afterdischarge threshold, the following seizure parameters were determined: generalized afterdischarge threshold, seizure severity, seizure duration, and afterdischarge duration. Each animal was used repeatedly with at least two days before the next vehicle control experiment, thus being its own control. The lowest dosage tested (4 mg/kg), decreased seizure duration and afterdischarge duration in a first experiment. Considering the lack of a dose-dependency of this effect, the experiment was repeated in the same group of animals. The effect was not reproducible. Please note that data of both experiments are combined in the results section.

Two mice of the control group were identified as outliers (Grubb's test) and excluded from further analysis.

Treatment with the MAGL-inhibitor JZL184

JZL184 [4 – nitrophenyl – 4 - [bis (1,3 – benzodioxol – 5 - yl) (hydroxy)methyl] piperidine – 1 - carboxylate] is a potent, highly selective and irreversible inhibitor of MAGL, which induces a rapid and long lasting up-to eight-fold elevation of 2-AG concentrations in the brain. Thereby anandamide levels are not altered (Long et al. 2009). The dosage of 8 mg/kg and the pre-treatment time (60 min) of JZL184 administration were chosen based on previous studies reporting central nervous system effects (Long et al. 2009). JZL184 was dissolved in DMSO (Sigma-Aldrich, Taufkirchen, Germany), cremophor EL (Sigma-Aldrich, Taufkirchen, Germany) and saline in a ratio of 1:1:18. The test compound was injected intraperitoneal with a volume of 10 ml/kg bodyweight. Control experiments with administration of the vehicle solution (DMSO, cremophor EL, saline; 1:1:18) were performed in parallel.

Tissue preparation

One day after the last seizure during the kindling acquisition phase, or eight days after the last drug experiment, mice were deeply anesthetized with pentobarbital (narcoren® 400mg/kg i.p., Merial GmbH, Halbergmoos, Germany) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skull and placed into 4% PFA. On the

following day the brains were transferred into 30% sucrose and were stored at 4°C until further processing. Coronal sections (40 µm, five series) were cut on a microtome (Frigomobil, Reichert-Jung; Heidelberg-Nußloch, Deutschland). The brain sections were stored at -80°C in a cryoprotectant solution (glycerol and 0.1 M phosphate buffer, pH 7.4, 1:1 in volume).
One of the series was stained with thionin for verification of the electrode localization.

Statistics

We performed statistical analysis of group differences using GraphPad Prism (Version 5.04, GraphPad, San Diego, CA, USA). Inter-group differences in kindling acquisition were detected with the Mann Whitney U-test and the unpaired *t*-test, if necessary with Welch's correction, and the Fisher's exact test. Two-way ANOVA for repeated measurements (dependent factor: time, independent factor: treatment/genotype) was performed by Statistica, v5.0 (StatSoft (Europe) GmbH, Hamburg, Germany). Vehicle –control and drug experiments were compared by One-way ANOVA for repeated measures followed by Bonferroni's multiple comparison test or Friedman test followed by Dunn's multiple comparison test using GraphPad Prism (Version 5.04, GraphPad, San Diego, CA, USA). Outliers were detected using the Grubbs' test (http://graphpad.com/quickcalcs/Grubbs1.cfm). All data are given as mean values ± SEM, and $p < 0.05$ was considered significant.

Results

JZL184 administration increased the afterdischarge threshold at the beginning of the kindling process

Afterdischarge thresholds were determined at the beginning of the kindling process (under JZL184-treatment) and again at the end of kindling after a washout time of six days. Treatment with JZL184 at the beginning of kindling significantly increased the afterdischarge threshold by 233% (p=0.0325; **Fig.: 2A**) in NMRI mice. Afterdischarge threshold termination at the end of kindling without JZL184 administration revealed no longtime effect between the two groups (p=0.8939; **Fig.: 2A**). As expected, repeated electrical stimulations throughout the kindling process reduced the afterdischarge threshold in both groups of NMRI mice (NMRI vehicle p=0.0008 and JZL184 p=0.0027; **Fig.: 2A**).

Chronic JZL184-treatment delayed kindling acquisition

The progression of seizure severity over time was attenuated in JZL184-treated NMRI animals (treatment effect p=0.0066; time effect p<0.0001; **Fig.: 2C**). The mean number of stimuli to reach a generalized stage four seizure was increased in these mice when compared to vehicle-treated animals (mean number of stimuli: vehicle n=6.3 ± 0.9 and JZL184 n=9.7 ± 0.9; p=0.0156; data not shown). At the final electrical stimulation with JZL184 administration 92% of vehicle- and 42% of JZL184-treated animals experienced generalized seizures (stage 4 or 5; p=0.0272; **Fig.: 2E**). The analysis of the afterdischarge duration throughout the kindling phase and of its cumulative duration revealed a significant reduction in the JZL184-treated group (afterdischarge duration: treatment effect p=0.0012; time effect p<0.0001; **Fig.: 2D;** cumulative duration p<0.0001; **Fig.: 2F)**.

JZL184 has no effects in fully-kindled mice

The effect of JZL184 on the afterdischarge threshold determined at the beginning of kindling and the lack of a longtime effect after the washout time indicated a putative anticonvulsant effect. Thus, we decided to evaluate the anticonvulsive potency of JZL184 in fully-kindled mice. None of the three different dosages tested (4, 8 and 16 mg/kg), affected the generalized afterdischarge threshold (GST) and associated parameters at threshold stimulation (seizure severity and seizure duration) in comparison to the preceding vehicle control experiment (**Fig.: 3A-C**). However, there was a trend that the afterdischarge duration recorded at the GST stimulation was slightly reduced. This effect, however, failed to reach significance following administration of 8 mg/kg JZL184 when compared to the respective vehicle experiment (p=0.0508; **Fig.: 3C**).

The duration of behavioral and electrographic seizure activity was significantly decreased in response to 4 mg/kg JZL184 when compared to control mice. However, this result was not reproducible in a second experiment. Please note that data from the 4 mg/kg experiment show means calculated from

the two repetitive experiments. In summary, the overall effects of JZL184 are modest at most in fully-kindled mice.

Chronic JZL184-treatment has no effect in CamK-CB1 KO mice

To address the CB1R dependency of JZL184 treatment, we evaluated the impact of JZL184 in CamK-CB1 KO mice. As already reported previously (von Rüden et al. 2014), initial afterdischarge thresholds were reduced in CamK-CB1 KO when compared to littermate controls (p=0.0021). This genotype effect proved to be treatment independent (p=0.3018, 2-way ANOVA) and JZL184-treatment did not affect initial afterdischarge thresholds in CamK-CB1 WT (p=0.1824) nor in KO-mice (p=0.5725; Fig.: **4A**). Repeated electrical stimulations throughout the kindling process reduced the afterdischarge threshold in CamK-CB1 WT vehicle 0.0325; **Fig.: 4A**).

When CamK-CB1 WT mice were treated with the MAGL-inhibitor JZL184 at a dosage of 8 mg/kg, the treatment effects were the same as observed in NMRI mice. The progression of the seizure severity over time was attenuated in JZL184-treated CamK-CB1 WT (treatment effect p=0.002; time effect p<0.001; **Fig.: 4C**). At the final electrical stimulation 90% of vehicle- and only 29% of JZL184-treated CamK-CB1 WT mice experienced generalized seizures (stage 4 or 5; p=0.0345; **Fig.: 4E**). In addition, the analysis of the afterdischarge duration throughout the kindling phase and of its cumulative duration revealed a significant reduction in the JZL184-treated CamK-CB1 WT (afterdischarge duration: treatment effect p=0.0002; time effect p<0.0001; **Fig.: 4D**; cumulative afterdischarge duration p=0.001; **Fig.: 4F**). In comparison, afterdischarge threshold and associated seizure parameters over time were comparable in JZL184-treated and vehicle-treated CamK-CB1 KO mice (afterdischarge threshold p= 0.5725; **Fig.: 4A**; seizure severity: treatment effect p=0.3107; time effect p<0.0001 **Fig.: 4C**; afterdischarge duration: treatment effect p=0.2334; time effect p=0.0733 **Fig.: 4D**; cumulative discharge duration p=0.3269 **Fig.: 4F**) and all CamK-CB1 KO mice exhibited generalized seizures at the final electrical stimulation (seizure stage 4 or 5; **Fig.: 4E**).

Discussion

Elevated 2-AG levels delay the development of generalized epileptic seizures

In the present study, we demonstrate that JZL184 treatment increases seizure thresholds in naïve NMRI mice and delays the development of generalized epileptic seizures in the kindling model. JZL184 treatment strongly increases the concentration of 2-AG in the brain by a selective and effective block of the catabolizing enzyme MAGL (Long et al. 2009; Schlosburg et al. 2010; Wiskerke et al. 2012). Although we did not measure brain 2-AG levels in our experiment, the observed effects confirm that the dosage used was in the efficacious range. Our findings suggest that the indirect CB1R agonist JZL184 can exert protective effects that contributed to the delay of kindling progression and consequently lead to shorter duration of behavioral and electrographic seizure activity. This can be explained by reduced neurotransmitter release mediated by endocannabinoid signaling. Guggenhuber and colleagues (2010) overexpressed CB1Rs in hippocampal pyramidal and mossy cells and demonstrated a protective effect against seizures. In addition, it is known that CB1Rs on glutamatergic neurons can mediate a protection against excitotoxic seizures (Monory et al. 2006; Ruehle et al. 2013; von Rüden et al. 2014) and that endocannabinoid signaling during excessive excitation has stronger effects on hippocampal principal neurons than on GABAergic interneurons (von Rüden et al. 2014). Thus, we expect that during seizure activity the increased 2-AG levels predominantly activate CB1R on principal neurons and therefore suppress glutamate release. This may represent the key mechanism to control neuronal excitability during seizure activity.

The indirect activation of CB1Rs offers a clear benefit compared with direct CB1R agonism, namely event-specific effects based on inhibition of the endocannabinoid degrading enzyme. The major site of action of JZL184 are those synapses with enhanced signaling and excessive postsynaptic on-demand production of 2-AG along with high presynaptic concentration of MAGL. MAGL expression reaches high levels in the principle cell layers of the hippocampal formation (Dinh et al. 2002; Uchigashima et al. 2011). Inhibition of MAGL in these neurons leads to enhanced CB1R activation and consequently to decreased neurotransmitter release. This might render an explanation for the delayed kindling progression in our model. A practical implication of this treatment strategy might be the event-specific elevation of 2-AG by the application of indirect agonists like JZL184. The goal is to increase the negative feedback loop mediated by endocannabinoids and thereby, prevent or at least slow down the generation of a hyperexcitable state.

Accordingly, our findings are supported by recent *in vitro* studies. These studies show that 2-AG reduces excitatory post synaptic currents (EPSCs) in hippocampal slices from mice with temporal lobe epilepsy (Bhaskaran and Smith 2010), that enhanced 2-AG levels ameliorate kainate-induced epileptiform bursting in the CA1, and that 2-AG acting via CB1Rs blocks refractory status epilepticus in a dose-dependent manner (Deshpande et al. 2007; Fezza et al. 2014). These findings confirm an

effective inhibitory role of the endocannabinoid 2-AG *in vitro*. For future perspectives it would be of great interest to analyze local and regional system excitability in hippocampal brain slices of kindled treated and non-treated mice e.g. by electrophysiological methods to confirm treatment associated physiological changes on the cellular and network level.

To our knowledge the only available *in vivo* study reports an increased seizure frequency and duration of spontaneous seizures in response to chronic JZL184 treatment in the pilocarpine model of temporal lobe epilepsy (Ma et al. 2014). In this study, a much higher JZL184 dosage was used (20 mg/kg twice daily versus 8 mg/kg once daily). One has to consider, that CB1R agonists often exert dose-dependent bimodal effects (Moreira et al. 2012). It might be possible that the increase in seizure frequency and seizure duration is related to the relatively high dosage and that low dosages, as we used, might promote protective effects. A plausible explanation might be the development of tolerance mediated by extensive down-regulation and desensitization of CB1Rs following chronic cannabinoid administration (Schlosburg et al. 2010; Lazenka et al. 2013).

Evidence exists that 2-AG is the main endocannabinoid in the central nervous system (Katona et al. 2006; Yoshida et al. 2006; Gao et al. 2010; Tanimura et al. 2010). Our data from this study and a previous study (Wendt et al. 2011) suggest that 2-AG might be the more effective endocannabinoid mediating protection from electrically induced seizures. Here, we confirm that 2-AG indeed promotes a delay in kindling progression along with decreased electrographic and behavioral seizure duration. This finding suggests that 2-AG signaling dominate anandamide signaling in the process of seizure progression. An explanation could be that in contrast to anandamide, 2-AG acts as a full agonist on CB1Rs and that therefore the activation of CB1Rs is more effective (Sugiura et al. 1999; Sugiura et al. 2000; Sugiura et al. 2006).

Elevated 2-AG levels have no anticonvulsive effects

Based on our findings that JZL treatment delayed kindling acquisition, we wanted to exclude an anticonvulsive effect of JZL184 treatment in fully-kindled mice. In line with our idea, that activation of the endocannabinoid system might be a strategy to prevent epilepsy, JZL184 did not exert any robust but rather modest effects in this experiment. In line with these data, modulation of the endocannabinoid system with the direct CB1R agonist WIN55.212-2 and with the indirect CB1R agonist URB597 also lack a robust effect on seizure thresholds in kindled mice (Wendt et al. 2011). Altogether, our data argue against an acute anticonvulsive effect of JZL184 in the kindling model of temporal lobe epilepsy. With these findings, we were able to rule out that a mere anticonvulsant effect renders a bias, when testing the compound during kindling acquisition. This is one of the main effects, which might have biased previous studies in which anti-kindling effects of known antiepileptic drugs did not translate into clinical effects.

Inhibition of MAGL mediates cannabinoid 1-receptor dependent effects

In a previous study, we characterized conditional CamK-CB1 mice, which lack CB1R in principal neurons of the forebrain, in the kindling model of temporal lobe epilepsy (von Rüden et al. 2014). These mice are more susceptible to seizures and have shorter seizure duration when compared to wild type controls, whereas the genotype has no impact on seizure progression. As expected, JZL184 treatment in CamK-CB1 WT mice revealed the same effects as in JZL184-treated NMRI mice. In contrast, all analyzed parameters in vehicle- and JZL184-treated CamK-CB1 KO were comparable. Based on these findings we conclude that the effects of the MAGL-inhibitor JZL184 on kindling acquisition are predominantly CB1R mediated.

However, one has to consider that 2-AG may act as $GABA_A$-receptor agonist at low concentrations of GABA (Sigel et al. 2011). An interaction of cannabinoidergic and GABAergic systems during ictogenesis may be involved in the anticonvulsant action of endocannabinoids (Naderi et al. 2011), adding another level of complexity to endocannabinoid signaling in the central nervous system. However, we are able to exclude a relevant direct interaction, because we did not observe any effects of JZL184 in CamK-CB1 KO mice. If $GABA_A$-receptors would be involved in JZL184 efficacy, then we should have seen a difference between treated and non-treated CamK-CB1 KO mice.

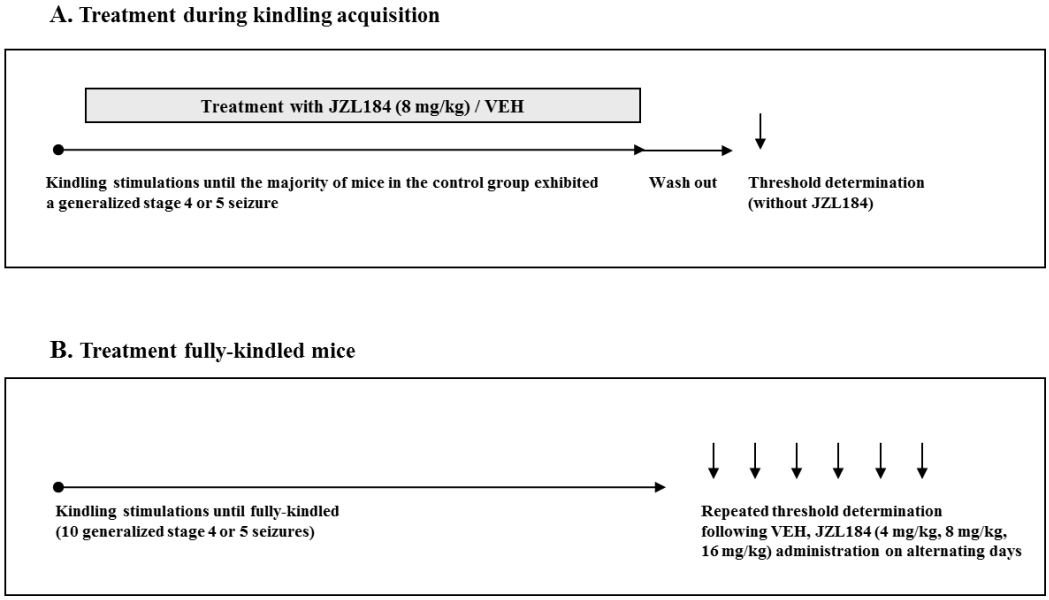
Conclusion

The aim of this study has been to evaluate whether pharmacological inhibition of MAGL by JZL184 affects the development and progression of epileptic seizures in the kindling mouse model of temporal lobe epilepsy and to prove that JZL184-mediated effects are CB1R dependent. Our data demonstrate that indirect CB1R agonism can interfere with the development of generalized epileptic seizures in the kindling model, but has no anticonvulsive effects. Furthermore, we confirmed that the effects of JZL184 on kindling progression are CB1R mediated.

These findings might imply that the endocannabinoid 2-AG may be a promising target for an anti-epileptogenic approach. However, future studies in animal models with spontaneous recurrent seizures (e.g. in a post-status-epilepticus model) are necessary to confirm our hypothesis.

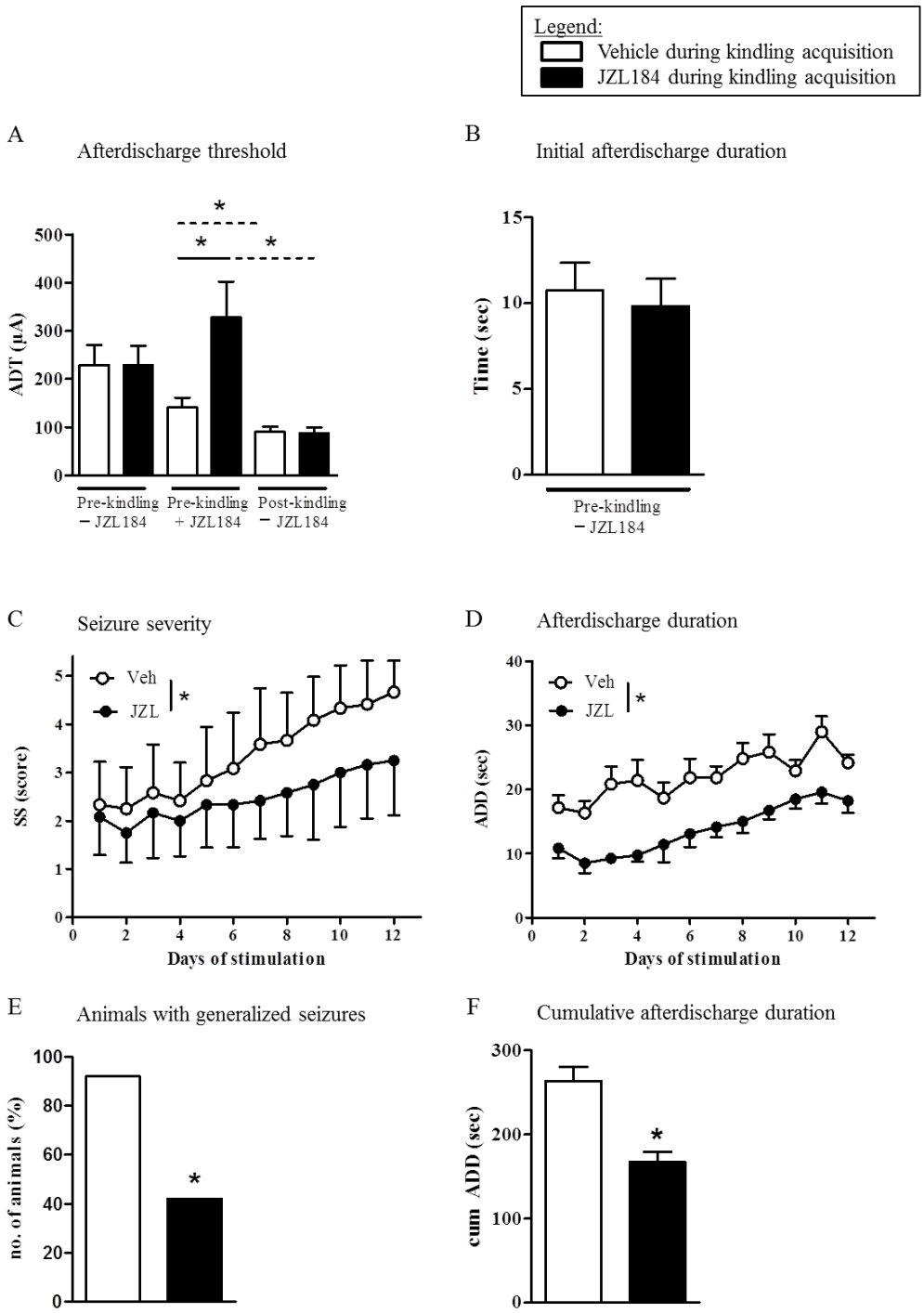
Figures

Figure 1:



Experimental design for (A) experiments analyzing kindling progression under JZL184-treatment and for (B) experiments in fully-kindled mice. (**A)** JZL184 was administered during the kindling stimulation phase. Animals were kindled until the majority of mice in the control group exhibited generalized seizures. Following the kindling procedure five days after the last JZL184/vehicle injection the post-kindling afterdischarge threshold was determined.
(B) Animals were kindled until they exhibited at least 10 generalized stage (4) or (5) seizures and deterrmined to be fully-kindled. Fully-kindled mice were then repeatedly used for drug testing with administration of vehicle or JZL184 before threshold determination. Each drug experiment was preceded by a vehicle control experiment in the same animal.

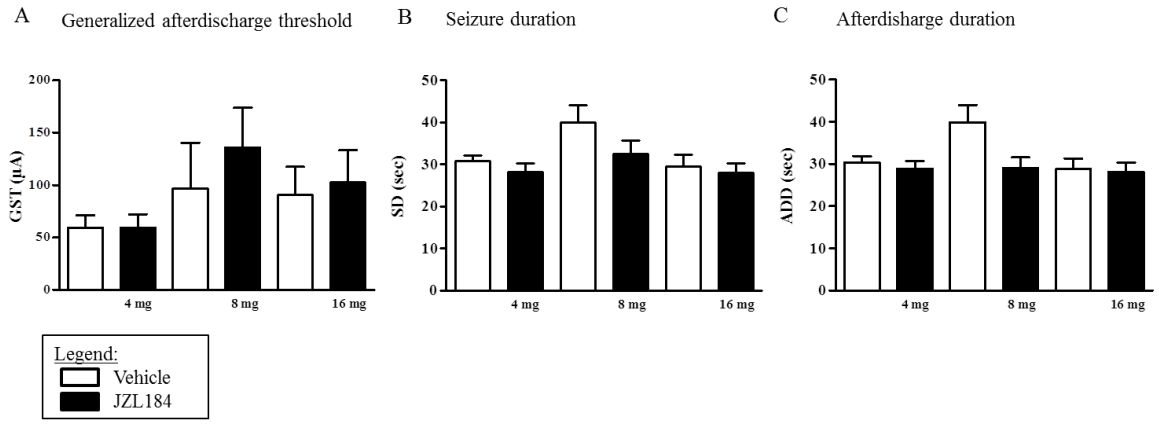
Figure 2:



Impact of JZL184 (8 mg/kg) on kindling progression in NMRI mice. (A) Initial afterdischarge thresholds pre-kindling without JZL184 treatment proved to be comparable in both groups, whereas JZL184 treatment at the beginning of the kindling process increased afterdischarge thresholds when compared to the control group. Kindling reduced the afterdischarge threshold in both groups. **(B)**

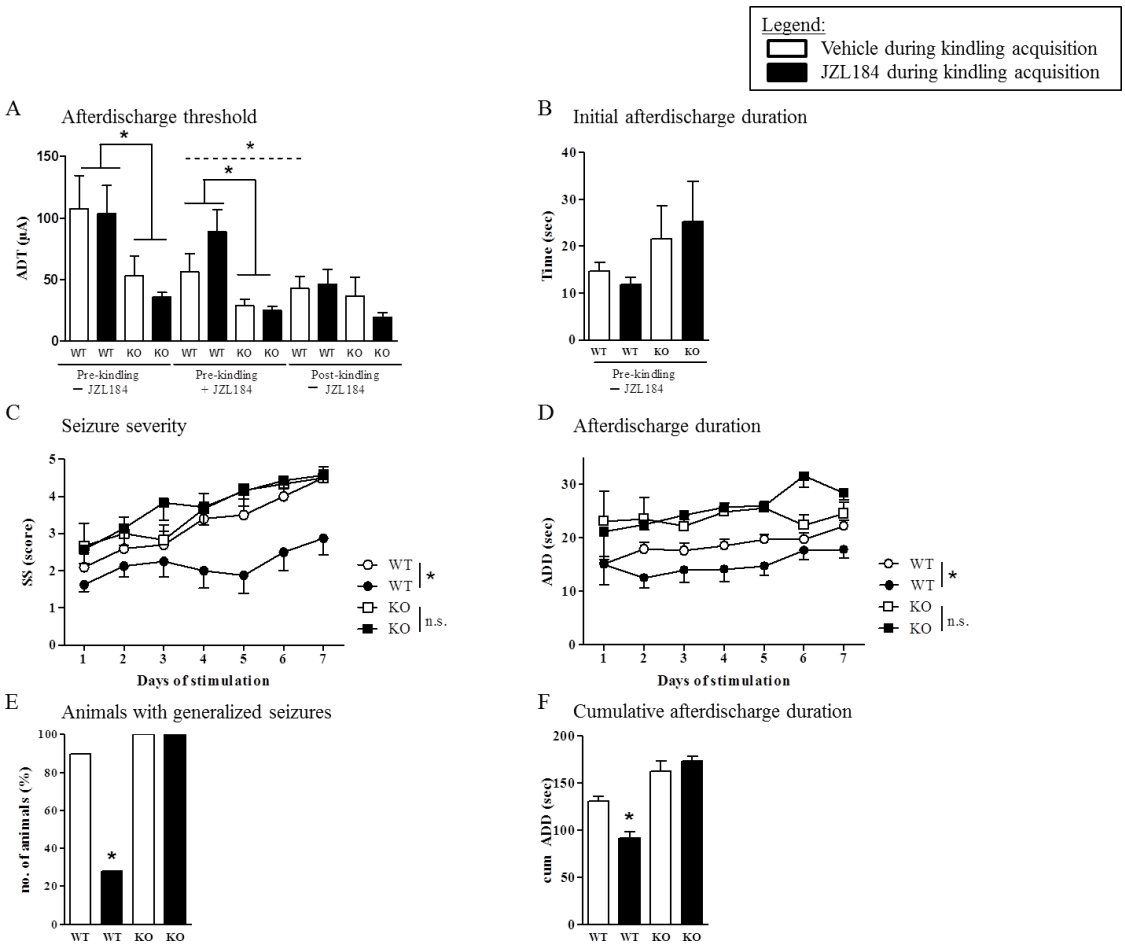
Initial afterdischarge duration pre-kindling in naïve mice proved to be comparable in both groups. **(C)** Seizure severity (SS) and **(D)** afterdischarge duration (ADD) are depicted for each day of stimulation. **(E)** The percentage of JZL184-treated animals with generalized seizures as well as **(F)** the cumulative discharge duration of JZL184-treated mice was significantly reduced when compared to vehicle-treated animals. – JZL184: no drug pretreatment; + JZL-184: with drug pretreatment. All data are given as mean ± SEM, n.s.: no significant difference, * Significant difference $p < 0.05$, analyzed by two-way ANOVA for repeated measurement, Fisher's exact test, unpaired student's t-test, paired t-test, and Mann Whitney test. Vehicle n = 12 and JZL184 n = 12.

Figure 3:



Impact of JZL184 in fully-kindled NMRI mice. (A) Generalized afterdischarge thresholds (GST), **(B)** seizure duration (SD) and **(C)** afterdischarge duration (ADD) recorded at threshold stimulation. There is a trend that JZL184 in a dosage of 8 mg/kg slightly reduced the afterdischarge duration when compared to the respective control experiment. All data are given as mean ± SEM, analyzed by one-way ANOVA for repeated measures followed by Bonferroni's multiple comparison test or Friedman test followed by Dunn's multiple comparison test. Vehicle/JZL184 n = 9 (8 mg/kg experiment vehicle/JZL184 n=9).

Figure 4:



Impact of JZL184 (8 mg/kg) on kindling progression in CamK-CB1 mice. (A) Initial afterdischarge thresholds pre-kindling in non-treated mice proved to be comparable in both groups. Regardless of the treatment status (vehicle or JZL184) initial afterdischarge thresholds proved to be lower in CamK-CB1 KO mice when compared to respective controls. The kindling induced characteristic reduction of the afterdischarge threshold was only noticed in vehicle-treated CamK-CB1 WT mice. **(B)** Initial afterdischarge duration pre-kindling in naïve mice proved to be comparable in both groups. **(C)** Seizure severity (SS) and **(D)** afterdischarge duration (ADD) are depicted for each day of stimulation. **(E)** The percentage of JZL184-treated CamK-CB1 WT mice with generalized seizures is significantly reduced when compared to vehicle-treated WT animals, whereas all CamK-CB1 KO mice experienced generalized seizures. **(F)** The cumulative discharge duration of JZL184-treated CamK-CB1 WT is reduced when compared to vehicle-treated WT animals, whereas treatment has no effects in CamK-CB1 KO mice. – JZL184: no drug pretreatment; + JZL-184: with drug pretreatment. All data are given as mean ± SEM, n.s.: no significant difference, * Significant difference $p < 0.05$, analyzed by two-way ANOVA for repeated measurement, Fisher's exact test,

unpaired student's t-test, paired t test and Mann Whitney test. CamK-CB1 WT: vehicle n = 10 and JZL184 n = 7; CamK-CB1 KO: vehicle n = 6 and JZL184 n = 8.

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Conflict of interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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DISCUSSION AND FUTURE PROSPECTS

Activation of the endocannabinoid system can effectively modulate synaptic signal transmission (Katona and Freund 2008). Its inhibitory feedback mechanisms particularly protect against excessive presynaptic activity. Moreover, it has been described that activation of CB1Rs may affect neuronal plasticity. This includes the formation and fate of newly generated neurons in the dentate gyrus (Gordon and Devinsky 2001; Lutz 2004; Grant and Cahn 2005). The endocannabinoid anandamide can also activate TRPV1 channels (Smart et al. 2000), which leads to the increased release of glutamate (Peters et al. 2010). Therefore, not only targeting of the endocannabinoid system but also of the endovanilloid system is intensively discussed as a possible therapeutic strategy that could affect the formation of a hyperexcitable epileptic network, seizure thresholds and ictogenesis.

In the present thesis we analyzed whether pharmacological and genetic modulation of the endocannabinoid and endovanilloid systems may affect epileptogenesis and ictogenesis in the kindling model of TLE.

Most importantly, we have shown that the endocannabinoid, but not endovanilloid, signaling affects the termination of seizure activity dependent on the neuronal subpopulation. Moreover, we demonstrated that indirect CB1R agonism interferes with the development of a hyperexcitable network, but has only negligible anticonvulsant effects.

Thus, the data argue that the endocannabinoid system plays an active role in seizure termination and is involved in the formation of a hyperexcitable neuronal network. These aspects are discussed in detail in the two research articles. Here I will discuss more general aspects with the focus on the hypothesis that the endocannabinoid system may be a promising target for the prevention and treatment of epilepsy.

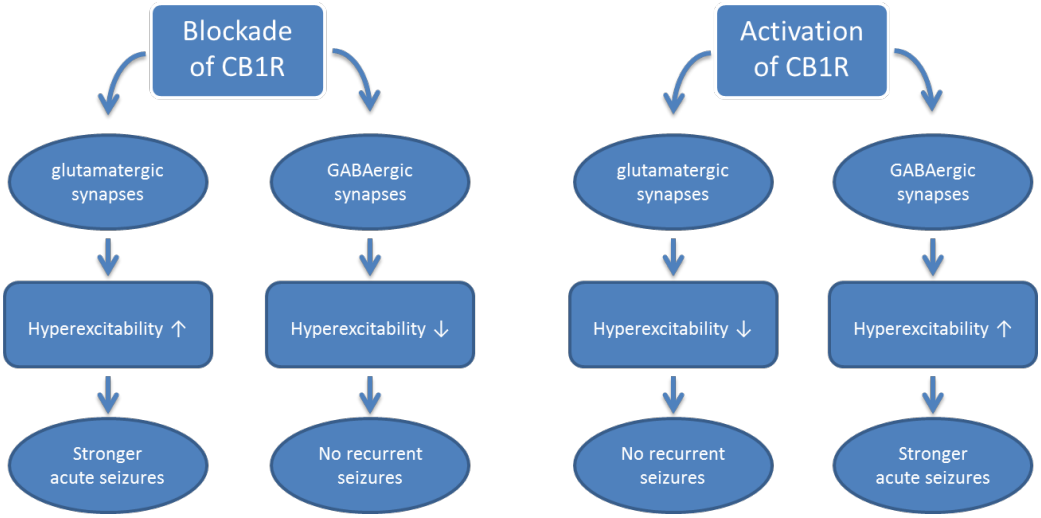
1. Pro- or anticonvulsant effects of CB1R modulation

Among experts there is significant interest in the idea that cannabinoid agonists, as they are found in the marijuana plant *Cannabis sativa*, could have anticonvulsant properties. In the case of epilepsy, a handful of clinical trials and case reports support the anticonvulsant efficacy of cannabinoids (Cunha et al. 1980; Ng et al. 1990; Maa and Figi 2014). However, there are also conflicting reports arguing for no efficacy or even for proconvulsant effects (Gordon and Devinsky 2001; Schneir and Baumbacher 2012). In conclusion, direct scientific data supporting the pro- or anticonvulsant potential is still missing. Recent insights come from basic science experiments which convincingly show that the endocannabinoid system has a central role in the regulation of neuronal excitability. CB1R agonists and antagonists have been tested in various *in vitro* and *in vivo* models of epilepsy, which also show

conflicting results. Cannabinoid agonists predominantly are anticonvulsant (Wallace et al. 2003; Bhaskaran and Smith 2010a; Wendt et al. 2011), whereas cannabinoid antagonists have proconvulsant effects (Wallace et al. 2002; Vinogradova et al. 2011; Arslan et al. 2013). The anticonvulsant property of endocannabinoids is mediated by excitatory glutamatergic neurons, whereas the proconvulsant activity depends on activation of inhibitory GABAergic neurons, leading to a decreased release of GABA, and accordingly to increased seizure susceptibility (Monory et al. 2006).

However, it becomes more complex when looking at different animal models representing different types of epilepsy. Cannabinoid agonists have anticonvulsant properties in the rat pilocarpine model of epilepsy (Wallace et al. 2003; Falenski et al. 2007), in the PTZ-induced seizure model (Shafaroodi et al. 2004; Gholizadeh et al. 2007; Naderi et al. 2011) and in the penicillin-induced epilepsy model (Kozan et al. 2009; Cakil et al. 2011; Arslan et al. 2013). All these models are acute models of TLE induced by chronical enhancement of neuronal excitation. In this situation the activation of CB1Rs by agonists at excitatory synapses decreases glutamate release and is thereby protective (**see Figure 7)**. On the contrary, CB1Rs on GABAergic interneurons have no impact in this context (Marsicano et al. 2003; Monory et al. 2006). However, it would not be accurate to argue that CB1Rs on GABAergic neurons do not play a role at all. The present paper analyzed the modulation of the endocannabinoid system in the kindling model of TLE, another chronic epilepsy model. We were able to show, that CB1R deletion on GABAergic neurons of the forebrain results in shorter seizure duration and causes a decrease in motor and electrographic seizure durations after completing the kindling process. These results provide evidence that GABAergic interneurons play a role in the CB1R dependent control of electrically-induced behavioral seizures by the endocannabinoid system, but are undermined by glutamatergic signaling in the net-outcome during seizure activity. However, once the hyperexcitable neuronal network has developed, GABAergic signaling might be responsible for long-lasting protective effects, as indicated by decreased seizure duration in fully-kindled mice.

Figure 7.



The dichotomous role of CB1R blockade and activation on neuronal excitability dependent on the neuronal subpopulation. *As indicated on the left, CB1R blockade on glutamatergic neurons is proconvulsant, whereas blockade on GABAergic neurons is anticonvulsant. On the other hand, activation of CB1Rs on glutamatergic neurons is anticonvulsant, whereas activation on GABAergic neurons is proconvulsant. This scheme demonstrates that the endocannabinoid system has to be tightly regulated to ensure and maintain synaptic transmission in an optimal physiological range. Modified from (Lutz and Monory 2008).*

In animal models of epilepsy which are induced by disinhibition, the opposite is seen. In these models cannabinoid antagonists may have anticonvulsant properties. At first this idea seems to be intuitively wrong, as it is suggested that endocannabinoids decrease neuronal excitability (Marsicano et al. 2003). Considering that endocannabinoids act on both GABAergic and glutamatergic signal transduction (Chevaleyre et al. 2006), the explanation is obvious that enhanced inhibition may compensate an imbalance between stimulatory glutamate and inhibitory GABA, thus maintaining synaptic transmission in a physiological range. This might be explained by the dichotomous role of cannabinoid signaling at different neuronal subpopulations in the hippocampus (**see Figure 7**).

In the epilepsy model of febrile seizures, long-term alterations in the GABAergic system lead to increased excitability of neurons (Chen et al. 1999) and thereby to uncontrolled neuronal firing. In this situation the endocannabinoid system regulates GABAergic but not glutamatergic signal transduction (Chen et al. 2007). Here, a blockade of CB1Rs by antagonists prevents increased inhibition and thereby elevates seizure thresholds.

Taken together, these examples show that targeting of the endocannabinoid system may be a strategy for the development of drugs with a broad range of effects. However, one has to thoroughly analyze

the etiology of the epilepsy and the associated pathological neuronal alterations. This may help to identify whether CB1R agonists or antagonists will promote anti- or proconvulsant action.

2. Pro- or antiepileptogenic effects of CB1R modulation

In the paragraph above I highlighted the therapeutic potential of modulating the endocannabinoid system and its impact on epileptiform activity. In addition, there is evidence that endocannabinoids may act as antiepileptogenic agents. This aspect is of particular interest because until now, only symptomatic treatment is available and with the existing drugs, cure cannot be obtained.

There are only a limited number of studies which address the hypothesis that the endocannabinoid system may be involved in the process of epileptogenesis (Echegoyen et al. 2009; Bhaskaran and Smith 2010a; Dudek et al. 2010; van Rijn et al. 2011; Wendt et al. 2011; von Rüden et al. 2014). Moreover, these studies revealed inconsistent findings. In a model of post-traumatic brain injury a single application of rimonabant, administered right after the injury, disrupted the process of epileptogenesis (Echegoyen et al. 2009). However, a second study in a different epilepsy model failed to show an antiepileptogenic efficacy of rimonabant (Dudek et al. 2010). Moreover, and in apparent contrast, van Riijn et al. (2011) chronically administered the CB1R antagonist and demonstrated a proepileptogenic effect. Taken together, these inconsistent findings stress the need for detailed analysis of CB1Rs in epileptogenesis. A more elegant and selective tool to address this issue consists of conditional knockout mice, which lack CB1Rs in specific neuronal subpopulation. Monory and colleagues (2006) were able to demonstrate that antiepileptogenic effects of CB1Rs are mediated by glutamatergic rather than GABAergic neurons. We also used the tool of conditional mutagenesis and analyzed the functional consequences of CB1R loss in the kindling model of TLE. However, genetic loss of CB1Rs did not exert any impact on kindling progression in this model, arguing against a crucial role of CB1Rs during epileptogenesis (von Rüden et al. 2014). Nevertheless, when we modified the endocannabinoid system pharmacologically with direct and indirect CB1R agonists in the same model, we detected an impact of CB1R activation on the development of seizure progression (Wendt et al. 2011; von Rüden et al., unpublished data). Although available experimental data are controversial, our studies in the kindling model of TLE, a model with excellent predictive validity, indicate that CB1R activation has an antiepileptogenic potential. These findings stress the fact that the endocannabinoid system is involved in the generation of hyperexcitable neuronal network formation. Moreover, from a therapeutic point of view, indirect agonists, like the enzyme inhibitor JZL184, offer a clear benefit compared with direct CB1R activation. Based on on-demand activation of the endocannabinoid system, this pharmacological intervention is event-specific and the resulting CB1R activation is locally restricted to excessively activated synapses.

Taken together our studies help to increase the understanding of the importance of the endocannabinoid system in epileptogenesis even if a final conclusion is not yet possible.

3. Monoacylglycerol (MAGL) is a promising target for the prevention of epilepsy

Anandamide and 2-AG are the two primary ligands for CB1Rs in the central nervous system. 2-AG is thought to be the major endocannabinoid in the brain (Sugiura et al. 1995; Stella et al. 1997), which, in contrast to anandamide, acts as a full agonist on CB1Rs with high efficacy (Sugiura et al. 1999; Sugiura et al. 2000; Sugiura et al. 2006). Although the concentration of 2-AG is regulated by MAGL (Dinh et al. 2002), the data for a supporting role of MAGL in modulating the endocannabinoid system was initially questionable. The first experimental studies, which demonstrated potentiated endocannabinoid action at inhibitory (Makara et al. 2005; Szabo et al. 2006; Hashimotodani et al. 2007) and excitatory (Hashimotodani et al. 2007) synapses by MAGL inhibition, were discredited because of nonspecific binding and low binding affinity of the test compounds.

Recently, a new and very selective MAGL-inhibitor, JZL184, has been developed and characterized *in vitro* and *in vivo* (Long et al. 2009; Schlosburg et al. 2010; Wiskerke et al. 2012).

The findings of a previous study of our lab (Wendt et al. 2011) and data from this study, where we characterized the auspicious compound JZL184 *in vivo* in the kindling paradigm, suggest that 2-AG has an antiepileptogenic effect whereas anandamide is ineffective. In contrast to our findings, there is one recent contradictory *in vivo* study that reports proepileptogenic effects of JZL184 (Ma et al. 2014). A possible explanation might be the different dosages that were used in the two studies, which might promote opposing effects. These characteristics of endocannabinoids are known as dose-dependent bimodal effects (Moreira et al. 2012) and have to be seriously considered when it comes to therapeutic application.

Anandamide and 2-AG regulate neuronal activity in two different ways. 2-AG preferentially acts on GABAergic synapses, whereas anandamide selectively reduces glutamate release (Ameri et al. 1999). Therefore, blocking the 2-AG degrading enzyme MAGL by specific inhibitors elevates 2-AG levels. This may improve GABAergic inhibition, which might in the following prevent or at least delay the generation of a hyperexcitable neuronal network as we have demonstrated in the kindling model of TLE.

Considering all these aspects, MAGL-inhibition could be a promising new target for the prevention of epilepsy.

4. The endovanilloid system has no crucial role in temporal lobe epilepsy

The endocannabinoid anandamide activates not only CB1Rs but also TRPV1 channels (Smart et al. 2000) by facilitating the release of glutamate in an activity-dependent manner (Peters et al. 2010). It has been confirmed that TRPV1 modulates glutamatergic signaling in the rodent and in the human brain (Mori et al. 2012) and that TRPV1 protein expression is altered in the hippocampus of mice

(Bhaskaran and Smith 2010b) and human patients with TLE (Sun et al. 2013). Therefore, targeting the endovanilloid system is suggested as a promising strategy for the regulation of neuronal activity in epilepsies (Lutz 2004). Recently, the non-psychoactive component cannabidiol of *Cannabis sativa* has been discussed among epilepsy researchers and granted orphan drug status under the name Epidiolex™ for the treatment of Dravet Syndrome, a rare and severe syndrome of infantile-onset, genetic, drug-resistant epilepsy in children. However, the molecular mechanism of the antiepileptic effects has not been well defined yet, and likely includes multiple mechanisms. Cannabidiol is supposed to act on various targets in the central nervous system, including CB1Rs and CB2Rs, 5-HT1a receptors, FAAH/anandamide transporter inhibition, NF-κB, PPARγ, and also TRPV1 (Bisogno et al. 2001; Russo et al. 2005; Thomas et al. 2007; O'Sullivan et al. 2009; Leweke et al. 2012), mediating neuroprotective, antioxidative and antiinflammatory effects. It is possible that the modulation of all these different targets supplement each other in promoting antiepileptic effects. Thus, cannabidiol might be one of the "dirty" but effective drugs. However, our findings argue that targeting TRPV1 alone is insufficient to suppress seizures or to prevent the generation of a hyperexcitable neuronal network, as we did not find any impact of genetic or pharmacological modulation on epileptogenesis or ictogenesis. This statement might be valid only in the case for TLE and cannot be transferred to other epilepsy models, e.g., genetic epilepsy models of Dravet Syndrome.

5. Future directions

5.1. Considerations for cannabinoid therapeutics

Cannabinoids as adjunctive anticonvulsant therapies

Three main facts argue for the endocannabinoid system as a future antiepileptic or anticonvulsant therapeutic target: (1) the endocannabinoid system is involved in the regulation of neuronal excitability (Wilson and Nicoll 2002; Freund et al. 2003; Zhu and Lovinger 2005; Hofmann et al. 2006); (2) the endocannabinoid system is altered in epilepsy or epileptic seizures (Ludanyi et al. 2008; Magloczky et al. 2010; Romigi et al. 2010; Zurolo et al. 2010; Gesell et al. 2013; von Rüden et al. 2014); and (3) genetic and pharmacological modulation of the endocannabinoid system alters seizure susceptibility or seizure activity (Wallace et al. 2003; Monory et al. 2006; Wendt et al. 2011; von Rüden et al. 2014). There is strong evidence from experimental studies that some CB1R agonists exert anticonvulsant effects and that these effects are predominantly mediated through the activation of CB1Rs (Wallace et al. 2001; Wallace et al. 2002; Kow et al. 2014; von Rüden et al. 2014). Surprisingly, the effects of the non-psychoactive plant-derived cannabinoid cannabidiol are not mediated through a CB1R-dependent mechanism (Hill et al. 2013; Devinsky et al. 2014).

Clinical reports of the medical use of marijuana with high-ratio cannabidiol:Δ9-THC for the treatment of epilepsy claimed efficacy, but double-blind randomized and controlled studies and the mechanism of action are still missing (Devinsky et al. 2014). Nevertheless, for some special types of epileptic syndromes, like intractable childhood epilepsy (e.g., Dravet Syndrome and Lennox-Gastaut Syndrome), cannabidiol may help to reduce seizure frequency up to seizure freedom (Porter and Jacobson 2013; Maa and Figi 2014). Very recently, a pharmaceutical company, GW Pharmaceuticals, got the "orphan drug" status for Epidiolex™, a drug containing cannabidiol for the treatment of Dravet Syndrome (GW Pharmaceuticals. GW Pharmaceuticals provides update on orphan program in childhood epilepsy for Epidiolex. November 14, 2013. http://www.gwpharm.com/GW Pharmaceuticals Provides Update on Orphan Program in Childhood Epilepsy for Epidiolex.aspx Accessed March 3, 2014). The first clinical treatment outcomes show promising results and demonstrate a therapeutic potential of cannabidiol in the treatment of epilepsy.

A strategy to bring new agents into clinics is to test them as adjunctive therapy in clinical trials. This strategy also has clinical relevance because polypharmacy with more than one antiepileptic drug is often required in the treatment of intractable epilepsy (Deckers et al. 2000). Indeed, the possibility of cannabinoids in adjunctive therapy is under investigation in experimental models of epilepsy. The Luszczki lab analyzed the combination of low dose arachidonyl-2'-chloroethylamide (ACAE), a cannabinoid agonist, and several classic antiepileptic drugs (clonazepam, ethosuximide, phenobarbital, valproate, carbamazepine, oxcarbazepine, lamotrigine, phenytoin and topiramate) in two different epilepsy mouse models (Luszczki et al. 2006; Luszczki et al. 2010; Andres-Mach et al. 2012). They were able to demonstrate that co-administration with phenobarbital, ethosuximide and valproate enhanced the anticonvulsant potential of these drugs without having negative adverse effects in behavioral tests of motor performance and long-term memory (Luszczki et al. 2006; Andres-Mach et al. 2012). However, the observed pharmacological effects were additive and increased the bioavailability of valproate and ethosuximide in the brain, whereas the free plasma and total brain concentration of phenobarbital was unaltered (Luszczki et al. 2006; Andres-Mach et al. 2012). In contrast, the anticonvulsant potential of the co-administration of ACEA and carbamazepine, oxcarbazepine, lamotrigine, phenytoin and topiramate was not affected (Luszczki et al. 2010). In a different study, Naderi et al. (2008) found a synergistic interaction of WIN55212-2, a direct cannabinoid agonist, and the benzodiazepine diazepam in a ratio of 1:3 (WIN55212-2:diazepam), whereas 1:1 and 3:1 ratios caused additive effects (Naderi et al. 2008). Surprisingly, the FAAH inhibitor URB597, the CB1R antagonist rimonabant and the endocannabinoid reuptake blocker AM404 in co-administration with diazepam revealed no interaction of the different compounds in anticonvulsion (Naderi et al. 2008). However, in this study total brain and free plasma levels have not been analyzed and therefore pharmacokinetic alterations cannot be excluded. It is also possible that CB1R mediated GABA-inhibition may counteract diazepam-mediated activation of GABA-receptors.

Alternatively, seizure dependent alterations of the endocannabinoid system may distract the balance of endocannabinoid signaling or even the effects caused by AEDs on neuronal inhibition and excitation, resulting in a modified net outcome of inhibition and excitation. Thus, the interaction of exogenous cannabinoids and AEDs is not that easy to explain.

In summary, the findings from these research groups support the idea that administration of cannabinoid agonists may be promising for the treatment of intractable epilepsy when they are combined with AEDs that act on GABA receptors. In this context, it might be of interest to characterize the combination of the MAGL-inhibitor JZL184 with the classical AEDs and to analyze the impact on seizures to see if the action of these AEDs can be improved.

5.2. Safety pharmacology of cannabinoid agents

A widely held belief is that the acute toxicity of cannabinoids is low. Classical adverse effects in the medical use of marijuana are nausea, increased weakness, behavioral or mood changes, hallucination, dizziness, and fatigue (Koppel et al. 2014). These are more or less the same adverse effects that are observed with the classical AEDs and do not argue against clinical use. However, safety concerns like tolerance, withdrawal and dependence have to be seriously considered when cannabinoids are used for the treatment of epilepsy.

Psychosis, dysphoria, and anxiety are adverse effects which occur at high concentrations of Δ9-THC (Koppel et al. 2014). Unfortunately, it is not reasonable to directly administer marijuana to epileptic patients because of its addictive and psychoactive side-effects, which are discussed in detail in the paragraphs below.

Psychoactive side-effects

The plant *Cannabis sativa* contains multiple components of which up to 60 are known to be active cannabinoids (Pertwee 2006). Its primary psychoactive component is Δ9-THC, acting as a partial agonist at CB1Rs (Pertwee 2006). A high CB1R density is found in brain areas which are strongly involved in the modulation of mood and motivation (Tsou et al. 1997), being a first explanation for the psychoactive effects of marijuana. Depending on the dose, on the cannabis strain and on the consumption frequency, the following psychoactive effects are described by users: mild euphoria, relaxation, sedation, loss of concentration but also enhanced sensory input, anxiety, paranoia, disorientation, and psychosis (Green et al. 2003). Moreover, the use of cannabis is associated with a higher risk of depression, schizophrenia and suicide (Serra and Fratta 2007; Foti et al. 2010; Nussbaum et al. 2011). Furthermore, the development of psychiatric complications is increased in adolescents with an early onset of cannabis consumption (Cuenca-Royo et al. 2013).

The composition of different phytocannabinoids determines the grade of psychotic effects. Plant strains with high rates of cannabidiol and low rates of Δ9-THC cause less severe psychotic symptoms (Schubart et al. 2011). Therefore, cannabidiol is one of the most promising phytocannabinoids for therapeutical use because it lacks psychoactive effects.

However, when applied in the formulation of medical marijuana, great caution is needed. Medical cannabis contains hundreds of different cannabinoids and there is no quality control of the diverse cannabinoid ratios, resulting in a non-standardized medical mixture. In particular, patients with cardiovascular diseases, mental disorders or adolescents should use medical marijuana with utmost caution (Borgelt et al. 2013). This potential risk has to be kept in mind especially when talking about the use of medical marijuana for the treatment of Dravet Syndrome or Lennox Gastaut Syndrome of children because children have per se a higher risk of psychiatric side effects.

Human and animal studies provide evidence that not only phytocannabinoids but also synthetic cannabinoids constitute the risk of psychotic side-effects (Roohbakhsh et al. 2007; Ugur et al. 2008; Moreira and Crippa 2009; Moreira and Wotjak 2010; Moreira et al. 2012). However, a synthetic cannabinoid which completely lacks psychoactive side-effects has not been developed so far. But the search for a safe and efficacious compound continues and new synthetic cannabinoids with better pharmacodynamics and pharmacokinetics as well as fewest possible side-effects are constantly developed.

Dependence and withdrawal

For a long time cannabis was not considered to be a drug of abuse or dependence. It was considered as a harmless drug. Dependence is defined by the National Institute on Drug Abuse as "*a state in which an organism functions normally only in the presence of a drug*" and is "*manifested as a physical disturbance when the drug is removed (withdrawal)*."

Today, the abuse potential of cannabis is recognized and up to 10% of regular users are defined as substance dependent (Iversen 2003). Increasing evidence from animal and clinical studies support the claim that dependence and withdrawal exists and specific withdrawal symptoms like anxiety, irritability, depressed mood, restlessness, sleep disturbances, gastrointestinal symptoms, and decreased appetite have been identified (Iversen 2003; Budney et al. 2004). Studies in rodents demonstrate that they self-administer the cannabinoid agonist WIN55212 (Ledent et al. 1999) and severe withdrawal symptoms can be provoked by administration of the CB1R antagonist rimonabant after chronic administration of cannabinoid agonists (Aceto et al. 1998; Aceto et al. 2001; Moranta et al. 2009). Moreover, the phenomenon of rebound hyperexcitability during the withdrawal phase is a consequence that has to be considered in the context of epilepsy. In the pilocarpine model of epilepsy the seizure frequency and seizure duration was increased when rimonabant was administered following chronic administration of WIN55212 (Wallace et al. 2003). These findings clearly prove

that dependence and withdrawal exist. However, dependence, withdrawal and rebound hyperexcitability should not exclude cannabinoids as anticonvulsants. It is more a question of how to handle these difficulties. In addition, many of the current AEDs used in clinical practice also cause the above mentioned phenomena. Moreover, it is state-of-the-art to gradually taper down medications over weeks rather than ending the application abruptly. Therefore, dependence and withdrawal have to be taken seriously but should not limit the use of cannabinoids as anticonvulsants in the therapy of epilepsy.

5.3. Tolerance issues of cannabinoid agents

Another important issue that one has to bear in mind is the development of tolerance when considering cannabinoids for therapeutic use.

By now, it is well known that the pharmacological effects of Δ9-THC or synthetic cannabinoid agonists experience tolerance as well as cross-tolerance to their classic effects, like ataxia, hypolocomotion, hypothermia, and analgesia following chronic administration (Wiley et al. 1993; Fan et al. 1994; Lamb et al. 2000). Tolerance or cross-tolerance occurs along with prolonged exposure of Δ9-THC or synthetic cannabinoid agonists simultaneously with CB1R desensitization via uncoupling of the G-protein signaling cascade and downstream effectors, as well as receptor downregulation via receptor internalization. The extent of CB1R desensitization and downregulation vary by brain region. For example, the hippocampus is a highly privileged region of rapid CB1R alterations (Sim-Selley 2003; McKinney et al. 2008). This pattern is comparable in the human and rodent brains (Villares 2007; Hirvonen et al. 2012).

Experimental *in vitro* and *in vivo* studies in epilepsy research suggest a dose- and duration-dependent manner of tolerance development. In a hippocampal neuronal cell culture model, Blair and colleagues (2009) observed after prolonged exposure to WIN55212 CB1R downregulation along with proconvulsant activity. Early *in vivo* studies are in line with these findings showing, reduced Δ9-THC efficacy after multiple applications in electrically kindled rats (Fried and McIntyre 1973; Corcoran et al. 1978) and in gerbils with spontaneous seizures (Ten Ham et al. 1975). Although the aspect of tolerance may be the main hurdle for the use of cannabinoids for the treatment of epilepsy, newer studies did not investigate this issue any further.

As mentioned above, indirect CB1R agonists offer the advantage of site-specific activity, with low CB1R activation elsewhere. The indirect acting CB1R agonist JZL184, which we used in our experiments, covers this advantage. However, it is known that chronic administration of high dosages of JZL184 (40 mg/kg) induces tolerance, CB1R desensitization, and alterations in synaptic plasticity as well as cross-tolerance to WIN55212 (Schlosburg et al. 2010). Recently, Kinsey and colleagues (Kinsey et al. 2013) demonstrated that tolerance and associated alterations as well as cross-tolerance

can be avoided by using low doses of JZL184, even when repeatedly administered. This is in line with our findings. Low doses (we used 8 mg/kg) repeatedly administered delayed kindling progression without losing efficacy or being proconvulsant at any time. Thus, we have not seen any evidence for tolerance development. However, we did not analyze CB1R density or cross-tolerance to other cannabinoid agonists. Nevertheless, our data in combination with those of the Kinsey lab indicate that partial inhibition of MAGL may be a potential targeting strategy for the treatment of epilepsy, which can avoid the development of tolerance as well as cannabinoid mimetic side effects.

Collectively, it is very important to further analyze the molecular mechanisms underlying tolerance development to find strategies which overcome this serious problem.

CONCLUSION

This thesis sought to determine if pharmacological and genetic modulation of the endocannabinoid and endovanilloid systems may have preventive antiepileptogenic properties in the kindling mouse model of TLE.

We found that CB1Rs expressed in the forebrain increase or decrease seizure duration dependent on the neuronal subpopulation, but that cell-type-unspecific pharmacological blockade of CB1Rs prolongs seizures. These findings allow the conclusion that the endocannabinoid system is involved in seizure termination as an important endogenous mechanism and that endocannabinoid signaling in hippocampal glutamatergic principal neurons overrules endocannabinoid signaling in GABAergic interneurons during seizure activity.

Furthermore, genetic and pharmacological modulation of the endovanilloid system had no impact on the generation of a hyperexcitable neuronal network or on ictogenesis, proposing that targeting TRPV1 alone is not a sufficient strategy to prevent epileptogenesis or suppress seizure activity in TLE.

In addition, the investigation of the role of the indirect CB1R agonist JZL184 in the development of a hyperexcitable network and in ictogenesis shows that JZL184, by blocking MAGL, delays kindling acquisition but has no relevant effects in fully-kindled mice. Further, we were able to confirm that the effects of JZL184 on kindling progression are CB1R mediated.

Altogether, these findings demonstrate that the endocannabinoid system can be considered as a target for decreasing seizure duration and supporting the endogenous mechanisms of seizure termination. Moreover, targeting MAGL may be a promising strategy for an antiepileptogenic approach.

Respective strategies are of particular interest on the one hand for the management of long-lasting refractory status epilepticus and cluster seizures, and, on the other hand, for a prophylactic treatment to prevent the development of symptomatic epilepsies after an initial insult.

This thesis helps to reveal new details of the role of the endocannabinoid and endovanilliod systems in ictogenesis and epileptogenesis, which may help to develop new options for the treatment and prevention of TLE.

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EIDESSTATTLICHE VERSICHERUNG/AFFIDAVIT

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation *Pharmacological and genetic modulation of the endocannabinoid system: Evaluation of preventive strategies in the amygdala kindling model of temporal lobe epilepsy* selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum oder annähernd übernommen worden sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation *Pharmacological and genetic modulation of the endocannabinoidsystem: Evaluation of preventive strategies in the amygdala kindling model of temporal lobe epilepsy* is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

Zu den Manuskripten habe ich wie folgt beigetragen:

Analysis in conditional cannabinoid 1 receptor-knockout mice reveals neuronal subpopulation-specific effects on epileptogenesis in the kindling paradigm. von Rüden E.L., Jafari M., Bogdanovic R.M., Wotjak C.T., and Potschka H.; Neurobiol Dis. 2014 Aug 11. pii: S0969-9961(14)00230-7. doi: 10.1016/j.nbd.2014.08.001; Epub ahead of print

- Studiendesign und Studienplanung: in Zusammenarbeit mit MJ, CTW und HP
- Durchführung der *in vivo* Experimente: in Zusammenarbeit mit MJ (1 Teilversuch)
- Durchführung der immunhistologischen Analysen: in Zusammenarbeit mit RMB (DCX-Auswertung, BrdU-NeuN Färbung); TRPV1 und CB1 Immunhistologie, Immunhistochemie und entsprechende Auswertungen: alleinige Durchführung
- Datenanalyse: alleinige Analyse, Diskussion mit HP
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