Faculty of Medicine and Health Sciences
Department of Neurology
Laboratory for Clinical and Experimental Neurophysiology, Neurobiology and Neuropsychology

Lisa Thyrion

Investigation of a role for uric acid in experimental seizure models

Promotor: Prof. Dr. Paul Boon

Copromotor: Prof. Dr. Robrecht Raedt

Thesis submitted in fulfillment of the requirements for the degree of doctor (PhD) in Medical Sciences

Ghent - 2016

Onderzoek gefinancierd met een doctoraatsmandaat van het Bijzonder Onderzoeksfonds (BOF) van Universiteit Gent

Research funded by a PhD grant of the 'Bijzonder Onderzoeksfonds' of Ghent University

Members of the Jury

Promotor	**Prof. Dr. Paul Boon** Ghent University, Belgium
Copromotor	**Prof. Dr. Robrecht Raedt** Ghent University, Belgium
Chairman	**Prof. Dr. Ernst Rietzschel** Ghent University, Belgium
Examination Board	**Prof. Dr. Ilse Smolders** Vrije Universiteit Brussel, Belgium
	Prof. Dr. Eleonora Aronica University of Amsterdam, The Netherlands
	Prof. Dr. Geert Van Loo Ghent University, Belgium
	Prof. Dr. Romain Lefebvre Ghent University, Belgium
	Prof. Dr. Veerle De Herdt Ghent University, Belgium
	Dr. Guy Laureys Ghent University, Belgium

“People think that epilepsy is divine simply because they don't have any idea what causes epilepsy. But I believe that someday we will understand what causes epilepsy, and at that moment, we will cease to believe that it's divine. And so it is with everything in the universe”

Hippocrates – On the Sacred Disease

Index table

List of abbreviations 3

INTRODUCTION **5**

Chapter 1 Rationale, aims and thesis outline 7

Chapter 2 General introduction on epilepsy 11

Chapter 3 Manuscript: At the interface of oxidative stress and inflammation: a potential role for uric acid in epilepsy 27

RESULTS **47**

Chapter 4 Manuscript: Induction of the NLRP3 inflammasome after kainic acid induced status epilepticus in freely moving mice: a link with uric acid 49

Chapter 5 Manuscript: Uric acid is released during seizure activity and increases seizure generalization in a mouse model for limbic seizures 67

Chapter 6 Effects of uric acid in a mouse model for acute limbic seizures: expression of inflammatory markers and inflammasome components 89

Chapter 7 Manuscript: Disruption, but not overexpression of urate oxidase alters susceptibility to pentylenetetrazole and pilocarpine induced seizures in mice 103

DISCUSSION **113**

Chapter 8 Conclusions, discussion and future perspectives 115

Summary • Samenvatting 131

Acknowledgements • Dankwoord 139

Scientific resume 143

List of abbreviations

AED	anti-epileptic drug
ALLO	allopurinol
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	one-way analysis of variance
ASC	apoptosis-associated speck-like protein containing a CARD
B2M	bèta-microglobulin
BAS	baseline
BBB	blood-brain-barrier
CA	cornu Ammonis
CaCl2	calcium chloride
CASP	caspase
CI	confidence interval
CNS	central nervous system
COX	cyclo-oxygenase
CSF	cerebrospinal fluid
DAMP	danger associated molecular pattern
DNA	deoxyribonucleic acid
EEG	electro-encephalography
GABA	gamma-aminobutyric acid
GAERS	genetic absence epilepsy rats from Strasbourg
HMBS	hydroxymethylbilane synthase
HMGB1	high-mobility group box 1
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HS	hippocampal sclerosis
i.c.	intracerebellar
i.h.	intrahippocampal
i.p.	intraperitoneal
i.v.	intravenous
IL	interleukin
IL-1Ra	interleukin 1 receptor antagonist
ILAE	International league against epilepsy
KA	kainic acid
KCl	potassium chloride
KO	knock out

LOD	limit of detection
LGI1	leucine rich glioma inactivated protein
LRR	leucine rich repeat
MSU	monosodium urate
NaCl	sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-B
NLRP3	NOD-like receptor family, pyrin domain containing 3
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
O_2^-	superoxide radical
OE	overexpressing
PI3	phophatidylinositol
PTZ	pentylenetetrazole
RIN	Ringer (mofied Ringer's solution)
(m)RNA	(messenger) ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPL13a	ribosomal protein L13a
RT-qPCR	reverse transcriptase - quantitative polymerase chain reaction
SD	standard deviation
SE	status epilepticus
SEM	standard error of mean
SGTCS	secondary generalized tonic-clonic seizures
SOD	superoxide dismutase
SUA	serum uric acid
SUDEP	sudden unexpected death in epilepsy
SYK	spleen tyrosine kinase
TBP	TATA-binding protein
THE	tonic hindlimb extension
(m)TLE	(mesial) temporal lobe epilepsy
TLS	tumor lysis syndrome
TNF	tumor necrosis factor
UA	uric acid
UV	ultraviolet
UOx	urate oxidase
WHO	World health organization
WT	wildtype

Introduction

Chapter 1

Rationale, research aims and thesis outline

Rationale

Epilepsy is a chronic neurological disorder as old as human history, characterized by the occurrence of unprovoked seizures. Seizures were originally considered to be of divine or demonic nature, until Hippocrates, the forefather of neurology, defied this perception and recognized that they were caused by a brain malfunction (Magiorkinis et al., 2010, Breitenfeld et al., 2014). Although a lot of progress has been made since then, major knowledge gaps exist to date and a strong social stigma still surrounds epilepsy. As the disorder affects 65 million people worldwide and has a substantial negative impact on a patient's life (Table 1), it is considered a major public health concern (Thurman et al., 2011, WHO, 2015). In Europe alone 6 million people have epilepsy, and there are 400.000 newly diagnosed cases each year, i.e. one new case every minute (Perucca et al., 2013). Importantly, about 30% of these patients are not adequately aided with the current available treatments (Cross, 2011, Perucca et al., 2013, WHO, 2015), which continuously drives the search for new, improved therapies. To this end, extensive research in animal models is indispensable (Rogawski, 2006).

Increased mortality
• 2-3 times higher
Reduced life expectancy
• 2-10 years
High risk of comorbidities that reduce quality of life
• Learning problems
• Sleeping problems
• Mood disorders
• Depression and anxiety
Reduced human rights
• Reduced access to life and health insurance
• Withholding of driving license
• Barriers to particular occupations
• Denying of right to marry

Table 1 | Several of the negative aspects of epilepsy. (Perucca et al., 2013, WHO, 2015)

Current treatment entails the use of anti-epileptic drugs (AEDs). In most cases AEDs directly target the imbalance in excitatory and inhibitory neurotransmission, that is the hallmark of the epileptic brain (see Chapter 2, Treatment). However, although the pathophysiology of seizures and the development of epilepsy after a precipitating event (i.e. epileptogenesis) has been an active field of research for decades, the proportion of individuals with pharmacoresistant epilepsy largely remained unchanged since the late 1960s (Fattore and Perucca, 2011). It has therefore been suggested recently to focus on novel signaling systems to develop drugs that are not only more efficacious but also have better tolerability and pharmacokinetics. Moreover, a different approach may help in finding disease-modifying drugs that can target the early epileptogenic process (Rogawski, 2006, Devinsky et al., 2013).

Uric acid is a breakdown product of the purine metabolism that has been linked to an increasing number of disorders over the past decades (Kutzing and Firestein, 2008, Alvarez-Lario and Macarron-Vicente, 2011). Increased serum uric acid levels are associated with gout, metabolic syndrome, hypertension, schizophrenia and bipolar disorder. Decreased serum uric acid levels are found in patients with multiple sclerosis, Alzheimer's and Parkinson's disease. For many of these disorders there is a growing body of evidence indicating that uric acid is not only a marker, but also actively influences the underlying pathophysiology. In this regard, uric acid is a therapeutic target in gout (Rock et al., 2013), but a therapeutic agent in multiple sclerosis (Markowitz et al., 2009).

There are indications that uric acid might be involved in epilepsy as well. A number of clinical and preclinical epilepsy studies report elevated uric acid levels and, more importantly, suggest that lowering uric acid levels has anticonvulsant potential (Stover et al., 1997, Hamed et al., 2004, Togha et al., 2007). However, previous literature consistently lacked a uric acid oriented approach and its involvement has never been investigated thoroughly.

Research aims

The main objective of this dissertation was **the investigation of a role for uric acid in experimental seizure models**. More specifically we aimed to:

(1) develop and characterize a seizure model that allows to monitor the changes in local uric acid levels
(2) investigate the effects of pharmacological and genetic uric acid modulations on the seizure characteristics of this model
(3) investigate the effects of these manipulations on the seizure-associated inflammatory response
(4) examine if altering uric acid levels changes seizure characteristics in a different seizure model

Thesis outline

This chapter comprises the rationale, research aims and outline of the thesis.

Chapter 2 provides a general introduction describing the key concepts of epilepsy and its treatment options. A concise overview is given of the different preclinical models used for seizure and epilepsy studies, focusing on the ones used in this thesis.

Chapter 3 is a critical review of the available clinical and preclinical data on uric acid in epilepsy. The link between the molecular actions of uric acid and the pathophysiology of epilepsy is discussed.

Chapter 4 describes the electrographic, behavioral and neuroinflammatory changes as well as changes in local uric acid levels in the intrahippocampal kainic acid model for limbic seizures.

Chapter 5 addresses the effects of complementary pharmacological and genetic uric acid manipulations on the electrobehavioral parameters of the intrahippocampal kainic acid seizure model.

Chapter 6 focuses on the effects of uric acid manipulations on the seizure-associated neuroinflammatory changes in the intrahippocampal kainic acid model.

Chapter 7 details on the effects of genetic uric acid manipulations in the timed pilocarpine and pentylenetetrazole intravenous infusion seizure models.

Chapter 8 is a general discussion in which the insights obtained in the empirical chapters are discussed with respect to the current literature. Future research challenges are marked out.

References

Alvarez-Lario B, Macarron-Vicente J (2011) Is there anything good in uric acid? QJM 104:1015-1024.

Breitenfeld T, Jurasic MJ, Breitenfeld D (2014) Hippocrates: the forefather of neurology. Neurol Sci 35:1349-1352.

Cross JH (2011) Epilepsy in the WHO European region: fostering epilepsy care in Europe. Epilepsia 52:187-188.

Devinsky O, Vezzani A, Najjar S, De Lanerolle NC, Rogawski MA (2013) Glia and epilepsy: excitability and inflammation. Trends Neurosci 36:174-184.

Fattore C, Perucca E (2011) Novel medications for epilepsy. Drugs 71:2151-2178.

Hamed SA, Abdellah MM, El-Melegy N (2004) Blood levels of trace elements, electrolytes, and oxidative stress/antioxidant systems in epileptic patients. J Pharmacol Sci 96:465-473.

Kutzing MK, Firestein BL (2008) Altered uric acid levels and disease states. J Pharmacol Exp Ther 324:1-7.

Magiorkinis E, Sidiropoulou K, Diamantis A (2010) Hallmarks in the history of epilepsy: epilepsy in antiquity. Epilepsy Behav 17:103-108.

Markowitz CE, Spitsin S, Zimmerman V, Jacobs D, Udupa JK, Hooper DC, Koprowski H (2009) The treatment of multiple sclerosis with inosine. J Altern Complement Med 15:619-625.

Perucca E, Glynn M, Baulac M, Boer Hd, C CE, Kälviäinen R, Little A, Mifsud J, Pitkänen A (2013) Epilepsy Research Priorities in Europe: ERF report 2013.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Rogawski MA (2006) Molecular targets versus models for new antiepileptic drug discovery. Epilepsy Res 68:22-28.

Stover JF, Lowitzsch K, Kempski OS (1997) Cerebrospinal fluid hypoxanthine, xanthine and uric acid levels may reflect glutamate-mediated excitotoxicity in different neurological diseases. Neurosci Lett 238:25-28.

Thurman DJ, Beghi E, Begley CE, Berg AT, Buchhalter JR, Ding D, Hesdorffer DC, Hauser WA, Kazis L, Kobau R, Kroner B, Labiner D, Liow K, Logroscino G, Medina MT, Newton CR, Parko K, Paschal A, Preux PM, Sander JW, Selassie A, Theodore W, Tomson T, Wiebe S (2011) Standards for epidemiologic studies and surveillance of epilepsy. Epilepsia 52 Suppl 7:2-26.

Togha M, Akhondzadeh S, Motamedi M, Ahmadi B, Razeghi S (2007) Allopurinol as adjunctive therapy in intractable epilepsy: a double-blind and placebo-controlled trial. Arch Med Res 38:313-316.

WHO (2015) Epilepsy fact sheet.

Chapter 2

General introduction on epilepsy

Definitions

According to the International League Against Epilepsy (ILAE) **epilepsy** is a neurological disease characterized by an enduring predisposition to generate epileptic seizures (Fisher et al., 2005). In practice, a patient was previously diagnosed with epilepsy after having at least two unprovoked seizures that occur more than a day apart. However, the definition of epilepsy was recently extended to include cases that only exhibited one seizure. As such, epilepsy is defined by any of the following conditions (Fisher et al., 2014):

a) at least two unprovoked (or reflex) seizures occurring more than a day apart
b) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years
c) diagnosis of an epilepsy syndrome

An **epileptic seizure** is a transient clinical manifestation of abnormal excessive or synchronous activity of neuronal populations in the brain (Fisher et al., 2005). Depending on the extent of neuronal involvement and site of origin (i.e. **epileptic focus**) the characteristics of the seizure differ, and can include alterations of consciousness, motor, sensory autonomic or psychic events (Engel, 2006). When an epileptic seizure prolongs it is termed **status epilepticus** (SE), and although the exact duration for a seizure to be considered SE is heavily debated, a five-minute definition is accepted for treatment purposes (Cross, 2014). Brain insults such as SE are a risk factor for **epileptogenesis** (Gordon Boyd et al., 2012), the dynamic process that converts a healthy brain into an epileptic one by increasing neuronal excitability via alterations on molecular, cellular and structural level (Pitkanen and Lukasiuk, 2011).

Diagnosis and classification

To diagnose epilepsy, the medical history of a patient and seizure symptoms are reviewed. Moreover, several tests are done to determine the cause and origin of the seizures, the most imperative one being electro-encephalography (EEG) recordings (Smith, 2005). Blood analysis, brain imaging and neuropsychological evaluation can then give additional information necessary to subclassify seizures and epilepsy syndromes and find appropriate treatment. However, **seizure classification** is challenging, as their presentation not only depends on the seizure origin, but also on the maturity of the brain, confounding disease processes, circadian rhythm, medication and triggering factors (Panayiotopoulos, 2007). Moreover, the classification is not absolute, and because knowledge and the variety of analysis tools is continuously expanding, it has been revised several times since the ILAE provided a first recommendation (Engel, 2006, Berg et al., 2010). According to the current ILAE classification, seizures fall broadly into two main groups based on their region of onset in the brain, i.e. focal and generalized seizures (ILAE, 2015).

Focal seizures originate within one hemisphere and can potentially spread to other ipsilateral and contralateral regions. Seizure semiology is highly variable and reflects the ictal onset zone, as well as the pattern of propagation. In this regard, seizures can affect sensory, motoric and autonomic functions, as well as memory, cognition, emotional state and level of awareness or responsiveness. Seizures accompanied by the impairment of awareness or responsiveness are called seizures with a 'dyscognitive' feature, which were earlier termed 'complex partial seizures'. **Generalized seizures** originate and rapidly propagate within both hemispheres. They can be subdivided based on seizure semiology and include absence, tonic, atonic, convulsive and myoclonic seizures. Absence seizures are seizures defined by clinical impairment of consciousness and are associated with typical bursts of bilateral synchronous spike-wave discharges on the EEG. Tonic and atonic seizures are characterized by a sudden increase or loss of muscle tone respectively. Myoclonic seizures are associated with brief muscle contractions. Convulsive seizures include clonic and tonic-clonic seizures. Clonic seizures are repetitive and prolonged myoclonic seizures and tonic-clonic seizures have both a tonic and clonic phase.

The elements that cause epilepsy are multiple and heterogeneous, but can be used to subcategorize it into **epilepsy syndromes**, as this aids in the choice of anti-epileptic treatment (ILAE, 2015). An epilepsy syndrome is a unique epilepsy condition defined by a typical age of onset, seizure type and EEG features (Engel, 2001). Previously, it was subdivided into 'symptomatic, idiopathic and cryptogenic' epilepsy syndromes, but these terms were poorly delineated and led to confusion. With the current understanding it has been possible to categorize the epilepsy syndromes by their specific etiologies, i.e. genetic, structural, metabolic, immune, infectious epilepsy syndromes and epilepsy syndromes that have no known cause (Berg et al., 2010, ILAE, 2015). **Genetic** epilepsy syndromes

are a direct result of a known or presumed genetic defect(s) in which seizures are the core symptom of the disorder (e.g. Angelman syndrome). Seizures in **structural** epilepsy syndromes are attributed to distinct structural brain abnormalities that can be developmental (e.g. tuberous sclerosis) or acquired (e.g. trauma). Metabolic abnormalities are responsible for the increased brain excitability in **metabolic** epilepsy syndromes, such as in mitochondrial disorders. The increased risk of developing epilepsy in **immune** epilepsy syndromes is driven by central nervous system (CNS) inflammation (e.g. Rasmussen syndrome). In **infectious** epilepsy syndromes, infections of the CNS can cause both acute symptomatic seizures and epilepsy (e.g. bacterial meningitis).

Pathophysiology

Information processing in the CNS occurs through neuronal communication, in which neurons encode messages as propagation of action potentials that trigger the release of neurotransmitters at synapses to influence firing of connecting neurons (Figure 1). In the normal brain neuronal firing is strictly regulated and a constant equilibrium between excitation and inhibition exists (Lowenstein, 2001). In epilepsy, however, certain areas of the brain are prone to seizures. It is generally believed that inhibitory actions in these areas cannot overcome sudden excitatory activity and that a trigger is enough to induce uncontrolled hypersynchronous repetitive firing of neurons. The characteristics of the seizure are then the result of the abnormal functioning in specific brain regions (see Diagnosis and classification). As neurotransmission involves multiple steps and is dependent on multiple cellular and molecular players, it is no surprise that the factors that can contribute to hyperexcitability are numerous. As such, it can result from changes in the integrity and molecular architecture of neuronal cells. Neuronal damage and death evidently results in hampering of normal network functioning, but network malfunctions can also be caused by changes in the expression, internalization and conductance of ion channels and receptors, alterations in second messenger systems or changes in the synthesis, release and clearance of neurotransmitters (Lowenstein, 2001). However, as synaptic transmission is also dependent on communication with astrocytes and microglia, changes in gliotransmission, due to dysfunctional activation and profileration of glia, can also promote an increased excitability (Kettenmann, 2007, Parpura et al., 2012, Devinsky et al., 2013). Similarly, the blood-brain barrier (BBB) is also pivotal for neuronal functioning by maintaining brain homeostasis. In that regard BBB damage and subsequent leakage can enhance excitability by stimulating brain inflammation (van Vliet et al., 2015). Moreover, not only inflammation but also oxidative stress is also able to increase the excitability of the network (Vezzani et al., 2012, Puttachary et al., 2015) (see Chapter 3). On a structural level all these epileptogenic changes can result in neurodegeneration, granule cell dispersion, astrogliosis and mossy fiber sprouting. Together, these changes then pathologically rewire the network, as is particularly evident in hippocampal sclerosis (see Temporal lobe epilepsy) (Pitkanen and Lukasiuk, 2011).

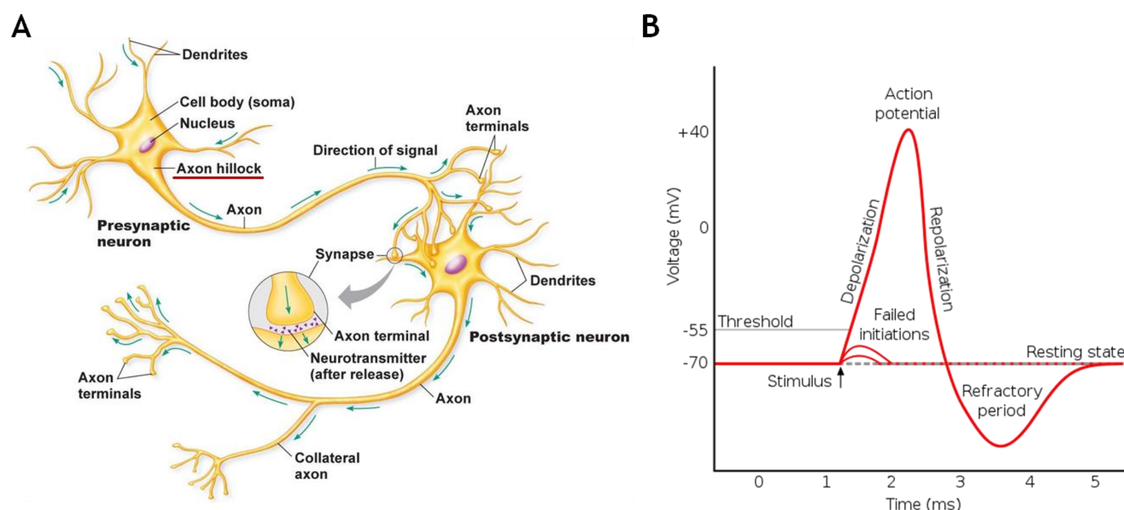


Figure 1 | Basics of neuronal communication. (A) Schematic overview of neurotransmission. An action potential generated at the axon hillock progresses through the axon and results in the release of neurotransmitters in the synaptic cleft. Binding of these neurotransmitters to receptors in the postsynaptic membrane leads to inhibitory or excitatory postsynaptic membrane potentials, which can result in a new action potential at the axon hillock of the subsequent neuron and further propagation of the signal, if the combined membrane potential crosses the -55 mV threshold. Adapted from That Basic Science. **(B)** Overview of an action potential showing the various phases as it passes a point on a cell membrane. It involves the sequential opening and closing of potassium and sodium channels. Adapted from the Tom Kelley gallery.

Treatment

The goal of medical treatment for epilepsy is to render the patient seizure-free without inducing intolerable side effects (Glauser et al., 2006). The standard first-line treatment is chronic administration of **anti-epileptic drugs** (AEDs). These drugs mainly target the disturbed excitation/inhibition equilibrium by blockage of voltage-gated ion channels, stimulation of the inhibitory gamma-aminobutyric acid (GABA)-ergic system or inhibition of the excitatory glutamatergic system (Vajda and Eadie, 2014). Depending on seizure type, epilepsy syndrome, medical history and age of the patient, monotherapy with a specific AED is started. When no optimal response can be achieved, a second monotherapy is commenced. If this too is unsuccessful, a combination therapy of AEDs is administered, although the exact way of substitution or combination is still controversial (Stephen and Brodie, 2012). Around 60% of the patients respond to monotherapy, but only 5% are aided with subsequent regimens if the second AED fails (Brodie and Kwan, 2002, Stephen and Brodie, 2012).

A leading cause for therapy failure are the **adverse effects** associated with AEDs, which can have a considerable negative impact on a patient's life (Perucca and Gilliam, 2012). They can develop acutely or after many years of treatment, and include direct effects on the CNS (fatigue, dizziness, double vision, memory problems, irritability, depression), but also cutaneous, haematological, and hepatic or pancreatic reactions. Moreover, AED intake has been associated with congenital

malformations and with noticeable changes in bodyweight (Perucca and Gilliam, 2012). New AEDs are reported to have a more favorable tolerability and toxicity profile compared to the old ones (Kanner and Balabanov, 2005). However, today more than 30% of the patients with epilepsy remain refractory, even with more than fifteen AEDs available on the market (Arzimanoglou et al., 2010, Lee, 2014).

Refractory epilepsy

The ILAE defines refractoriness when 'adequate trials of two tolerated, appropriately chosen and used AED schedules, whether as monotherapy or in combination, fail to achieve sustained seizure freedom', which refers to the detrimental drop in probability of seizure freedom in those cases (see Treatment) (Kwan et al., 2010). Patients with refractory epilepsy are at high risk of cognitive deterioration and psychosocial dysfunctioning (Quintas et al., 2012). Also, they are more prone to injuries and mortality compared to the normal population, which can be mainly ascribed to the occurrence of sudden unexpected death in epilepsy (SUDEP) (Massey et al., 2014).

Temporal lobe epilepsy

An epilepsy syndrome that is particularly difficult to treat is temporal lobe epilepsy (TLE), in which seizures originate in the temporal lobe. Given that it is the most common form of focal epilepsy and it is resistant in a third of the cases, the share of TLE in refractory epilepsy is considerable (Pascual, 2007). Depending on the seizure focus, two subtypes can be distinguished: **mesial temporal lobe epilepsy** (MTLE) and neocortical TLE. The seizures in most TLE patients have a mesial origin and arise from the first decade of life to early adulthood. They can develop months to years after an initial precipitating insult, such as head trauma, febrile seizures, brain tumor, stroke or SE (Berg, 2008, Tatum, 2012). Moreover, there is a hereditary factor, as MTLE tends to occur more often in some families (Wieser, 2004, Maurer-Morelli et al., 2012). Clinical manifestation is variable and can be associated with the presence of an aura (depending on the region affected e.g. déjà-vu, epigastric rising sensation, panic attack) and/or the loss of consciousness. During the dyscognitive phase of a seizure, most patients have a fixed stare and stereotyped involuntary movements such as oral (lip-smacking) and manual (fumbling of fingers) automatisms (Pascual, 2007).

The inherent potential of the temporal lobe to be predisposed for seizures is based on the unique anatomical-functional organization of the limbic network (Tatum, 2012), a set of interconnected nuclei and cortical structures responsible for emotion formation and processing, learning and memory (Figure 2). An important structure in this system is the hippocampal formation, which consists of three structures each with different histological and functional properties: the dentate gyrus, the hippocampus (regions CA1-4) and the subiculum (Figure 2C). A common feature of most MTLE patients is the presence of **hippocampal sclerosis** (HS), a condition in which the hippocampal formation shows extensive loss in selective subregions of the hippocampus, but also in

its connecting regions (Bonilha et al., 2012, Tatum, 2012). Moreover, HS is associated with extensive astrogliosis and synaptic reorganization (known as mossy fiber sprouting), which are both believed to contribute to the increased excitability of the limbic circuit (Brandt et al., 2003, Bedner et al., 2015). It has also been suggested that seizures themselves can aggravate HS, hence forming a self-reinforcing loop (Sutula and Pitkanen, 2001). As the hippocampus plays a role in higher cognitive functions such as memory and learning, MTLE is associated with related cognitive comorbidities (Zhao et al., 2014).

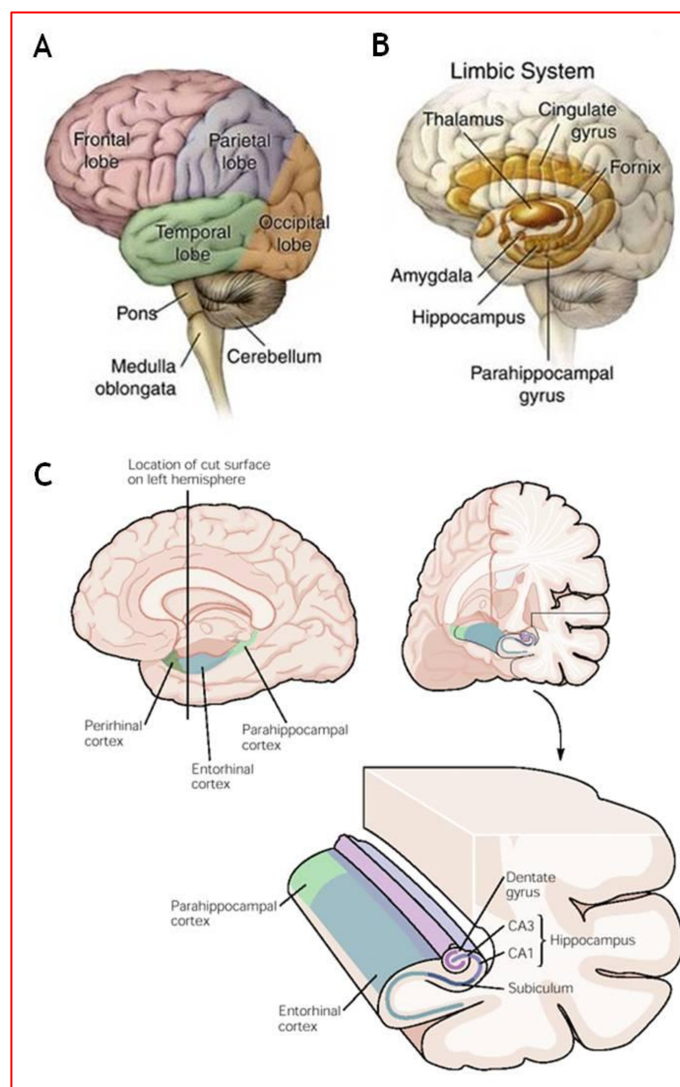


Figure 2 | Schematic overview of the anatomical location of the temporal lobe (A), limbic system (B) and hippocampal formation (C) in the human brain. The detailed view of the hippocampus shows the composing sectors: dendate gyrus, hippocampus and subiculum. Adapted from Kandel et al. (2000) and Brightfocus Foundation.

Alternative treatments

For patients with drug-refractory epilepsy, medicine has taken refuge in alternative treatments, such as epilepsy surgery, neurostimulation, dietary treatments and immune-based therapies.

Epilepsy surgery involves the removal of the seizure focus (resective surgery) or the interruption of the nerve fibers through which seizures spread (disconnective surgery). It is highly curative but only advisable if the seizure onset zone can be clearly identified (Wiebe, 2003, de Tisi et al., 2011). Surgical removal of the hippocampal formation in MTLE patients leads to seizure freedom in 60-70% of the cases, although seizures can reappear in the years following the intervention (Giulioni et al., 2013).

Delivering electrical pulses directly or in the vicinity of nerve tissue can also have a therapeutic effect. There are different types of **neurostimulation** that target different parts of the nervous system, including stimulation of brain structures and the vagus nerve (Boon et al., 2009). In this regard, vagus nerve stimulation, responsive cortical stimulation and stimulation of the anterior nucleus of the thalamus are already approved for epilepsy treatment (Nune et al., 2015). However, the mechanism of action and optimal stimulation parameters are still to be elucidated (Bergey, 2013, DeGiorgio and Krahl, 2013).

Dietary treatments include the ketogenic diet and the modified Atkins' diet. They are both high-fat, low-carbohydrate diets that increase the production of ketones, which become the main fuel source of the body and brain. These diets are associated with a significant seizure reduction in 30-50% of the patients and seizure freedom in up to 15% of the patients (Freeman et al., 2006, Kossoff et al., 2006). Despite the substantial seizure reduction, these diets are often discontinued due to their restrictive nature and high chance of side-effects such as constipation, sluggishness and nausea (Levy et al., 2012). The mechanism of action is still under debate but presumed to be multifactorial (Huffman and Kossoff, 2006).

Immunological treatments target the immune system, which is increasingly recognized to be involved in different epilepsies (Vezzani and Granata, 2005, Vezzani et al., 2012). As such, the use of corticosteroids is currently a primary treatment option in infantile spasms. However, it is associated with hypertension, weight gain, and more importantly a suppression of the immune system, risking overall sepsis (Hancock et al., 2013). However, steroids are also part of the first line treatment of autoimmune epileptic encephalitis, a condition resulting from the erroneous production of antibodies against endogenous synaptic or cell-surface antigens, such as leucine rich glioma inactivated protein 1 (LGI1) and the receptors for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and gamma-aminobutyric acid (GABA) (Davis and Dalmau, 2013). This treatment is complemented with intravenous administration of immunoglobulines and/or plasma exchange, which reduces the effects and the number of circulating antibodies respectively. Although this type of epilepsy has an overall good response to immunotherapy, the process of recovery is slow and can take up many months (Davis and Dalmau, 2013).

Aside from the optimization of the therapies stated above, there is a continuous drive to **search for new AEDs**, to decrease the high number of refractory patients, but also to improve the quality of life in patients with a suboptimal regimen. It has recently been suggested to focus on other, non-neuronal mechanisms that aside from anticonvulsive actions also affect the underlying pathology and epileptogenesis (Devinsky et al., 2013). This requires extensive preclinical research and hence the need for valid animal models (Rogawski, 2006).

Models of seizures and epilepsy

Animal models are indispensable in the elucidation of the fundamental mechanisms of epileptic seizures and epilepsy. They are essential for devising new diagnostic, therapeutic and preventive approaches. As is the case for all disorders, there is no perfect model for epilepsy and different models study different aspects of seizure- and epilepsy-related phenomena. They are each associated with their own advantages and limitations, which should be carefully evaluated bearing the specific topic of research in mind (Engel and Schwartzkroin, 2006).

***In vitro* models** include whole-brain models and more reduced biological systems, such as brain slices and dissociated cell cultures. These simplified, highly controllable models allow a unique insight into cellular and molecular mechanisms of seizures. However, simplification is also their biggest drawback, as they do not take into account important immunological and metabolic contributions to brain function and lack a behavioral component. Moreover, intrinsic and extrinsic neuronal connections are severed, which per definition creates an abnormal situation (Pitkanen et al., 2006).

These problems are circumvented with ***in vivo* models**, where intact animals are used, mostly rats and mice. Depending on the research focus, they fall into two categories: acute seizure models and chronic epilepsy models. In **acute models** focal or generalized seizures are evoked by audiogenic, chemical or electrical stimuli in an otherwise healthy brain. They are ideal to reliably study the development, maintenance and termination of seizures. Together with the fact that the timing of seizure onset can be controlled by the researcher, they are considered good models for the initial screening of potential AEDs. However, as these models are established by an artificial induction of seizures, they do not represent a chronic epileptic condition and hence do not give information on the mechanisms underlying spontaneous seizure generation, i.e. ictogenesis (Pitkanen et al., 2006).

Chronic models are associated with a permanent epileptogenic disturbance, which is present whether or not seizures are occurring. As such, they represent more closely the pathophysiology of human epilepsy. Moreover, they also offer the opportunity to study epileptogenesis and the associated structural and functional changes, which is impossible in acute models. For these reasons, chronic epilepsy models are considered the next step in the preclinical evaluation of potential AEDs.

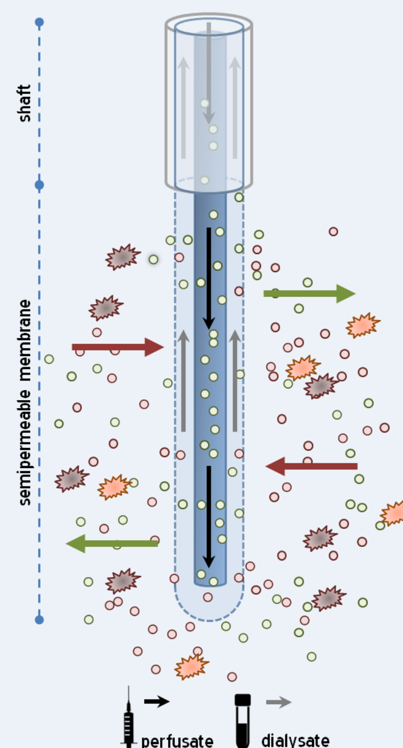
Many models exist that reproduce the traits of specific human focal or generalized epilepsies, in which two different subtypes can be distinguished: genetic models and acquired models. **Genetic** models include models in which specific genes for ion channels, transporters and neurotransmitters pathways are manipulated but also spontaneous models where the underlying gene deficiency has not yet been identified (Burgess, 2006, Noebels, 2006). One example of a spontaneous model is the Genetic Absence Epilepsy in Rats from Strasbourg (GAERS), that is characterized by episodes of behavioral unresponsiveness and symmetrical, generalized electroencephalographic spike and wave discharges typical for human absence epilepsy (Marescaux et al., 1992). **Acquired** models are of particular interest to study MTLE. Here limbic seizures develop in previously healthy rodents through the process of chemical/electrical kindling or after an initial insult, which can be a brain infarction, traumatic brain damage, febrile seizures or SE (Kharlamov et al., 2003, Dube et al., 2007, Pitkanen et al., 2009, Gorter et al., 2015). A drawback of chronic epilepsy models is the necessity of long monitoring periods, because of the unpredictable onset and timing of seizures, making them technically challenging and labor intensive.

Two acute seizure models were used in this thesis and are highlighted in the sections below.

The intrahippocampal kainic acid model of limbic status epilepticus

Kainic acid (KA) (2-carboxy-4-isopropenylpyrrolidin-3-ylacetic acid), is a nondegradable, excitotoxic analogue of glutamate isolated from *Digenea simplex*, a red alga found in tropical and subtropical waters. It induces seizures by increasing excitatory neurotransmission through the AMPA- and KA-subtypes of glutamate receptors, which are most densely located in the limbic system (Bleakman and Lodge, 1998). Simultaneously, KA causes neuronal cell damage, mostly in the hippocampal hilus and CA1 and CA3 cell layers of the hippocampal formation (Wang et al., 2005). The chronic intrahippocampal KA model is a commonly used model for MTLE as it is considered to be highly isomorphic with the human disorder, producing unilateral hippocampal sclerosis and recurrent spontaneous seizures after KA-induced SE (Bragin et al., 1999, Raedt et al., 2009). Aside from that, focal administration of KA is also used widely to study **acute focal seizures and SE with secondary generalizations** (Velisek, 2006). We adapted the intrahippocampal kainic acid mouse model of limbic SE (Bouilleret et al., 1999) by infusing kainic acid in the hippocampus using microdialysis (see Box 1) instead of injecting a bolus, to allow continuous measurement of the changes in the local extracellular environment (Figure 3A). By adding video-EEG monitoring, this set-up has a lot of **advantages**. It allows to quantify electrobehavioral changes, while simultaneously monitoring (and manipulating) local biochemical changes. After the experiment, the brain tissue can be processed to investigate SE-triggered processes, maximizing the information obtained per animal. Finally, the use of mice allows genetic interventions, as these are the main species used for targeted gene manipulation. The main **limitations** are related to the use of microdialysis, specifically the related probe recovery and tissue damage (see Box 1).

Box 1: Principles of microdialysis



Microdialysis is a minimally-invasive **sampling technique** that is used for in vivo continuous measurement of free, unbound analyte concentrations in blood and living tissue. A microdialysis probe is implanted in the tissue of interest which is continuously perfused with an aqueous solution that closely resembles the surrounding extracellular fluid (perfusate). It consists of a shaft and a semi-permeable membrane at the tip, where dialysis takes place. Dialysis is the process of passive diffusion of small molecules (circles in figure) between the extracellular tissue fluid and the perfusate over a semipermeable membrane. The concentration gradient between the extracellular tissue fluid and the perfusate determines the direction of movement of the molecules, allowing to use the probe as local delivery (green circles in figure) as well as sampling tool (red circles in figure). At predefined intervals the fluid (dialysate) is collected for analysis by an adequate technique, such as high-performance liquid chromatography (HPLC) (Fagugli et al., 2002).

The **composition of the perfusate** is highly important in microdialysis. If the perfusate has a lower concentration in any of the present permeable molecules compared to the extracellular environment this will result in their local depletion, thereby disturbing homeostasis. However, it is impossible to have a solution that has the exact same concentration for all solutes of the surrounding tissue. Therefore, most perfusates are made with the ionic composition and pH of plasma or cerebral spinal fluid (CSF) (Chefer et al., 2009).

Another key element is the **relative probe recovery**, which defines the proportional difference in concentration between the highest concentration of substance and the lowest concentration of substance. The concentration of a collected analyte will represent only a fraction of the true concentration in the extracellular fluid and *vice versa* for infusion of a component, as a full equilibrium between both can be never established. Probe recovery is dependent on a number of factors, including flow rate of the perfusate, properties of the analyte, properties of the probe membrane, tissue factors and temperature. The relative recovery of an analyte is inversely proportional with the perfusion **flow rate** as this allows more diffusion to take place. Although it improves the probe recovery, low flow rates are restricted by the minimal sample volume needed for solution analysis. Important **probe membrane** properties include the membrane material used and the size of the membrane surface, of which the latter is directly proportional to the probe recovery. However, the surface area of the probe will be often limited by the size of the structure under study. The molecular weight, the hydrophobicity ('stickyness') and the tertiary structure are the most important **analyte** characteristics that influence probe recovery. A higher molecular weight and greater hydrophobic properties result in lower relative recoveries, because of the resulting decrease in the diffusion coefficient and hindrance by the membrane respectively. Due to a lower fluid volume, higher diffusional path and potential binding of analyte with cell surface proteins, recovery from **tissue** is generally lower than in an *in vitro* set-up. If analytes are cleared rapidly from the extracellular space however (as for neurotransmitters), this lowers the resistance to diffusion and hence increases the recovery. Finally, a higher **temperature** increases the diffusion coefficient and accordingly increases the probe recovery. (Chefer et al., 2009)

To determine the absolute **concentration** of an analyte, it is necessary to have an estimate of the relative probe recovery, that approximates the real probe recovery as close as possible. In this regard, both *in vitro* and *in vivo* (e.g. the flow rate and internal standard) empirical methods exist. Furthermore, implantation of the probe induces a '**trauma layer**' that can also influence analyte concentration. It compromises the BBB, induces gliosis and disturbs the microcirculation (Benveniste,

1989, Georgieva et al., 1993, Clapp-Lilly et al., 1999). To ensure that these processes have stabilized and an equilibrium between the perfusate and tissue fluid can be achieved, a minimal 12 hour interval between probe implantation/perfusion and start of the experiment has to be respected. In that regard, absolute data always has to be interpreted with caution. On a last note, the analyte is sampled from a relatively large region, because of the dimensions of the microdialysis probe. The original site of release can be far away and it is hence not possible to determine the **cellular origin**. As such, the detected analyte can originate from neuronal, neuron-glia and/or neurovascular communication. (Chefer et al., 2009)

A detailed description of the methodology and characterization of this model can be found in the manuscript included in Chapter 4.

The timed intravenous infusion seizure model

The timed intravenous (i.v.) infusion seizure model is an **acute seizure model** and is considered an extremely sensitive screening method to investigate the effects of a compound on seizure susceptibility (Loscher, 2009). In this model, the animal is infused i.v. with a chemoconvulsant, commonly pentylenetetrazole (PTZ) or pilocarpine, but also KA, at a constant flow rate while it can move freely (De Bundel et al., 2011, Loyens et al., 2012, Portelli et al., 2012). PTZ induces seizures by acting predominantly on the $GABA_A$ receptor, antagonizing GABAergic inhibition, while the convulsant effect of pilocarpine is related to its agonistic function on the muscarine cholinergic receptor (Oses et al., 2007, Curia et al., 2008, Portelli et al., 2012, Rambo et al., 2013). To prevent pilocarpine-induced peripheral effects such as salivation, tremor and diarrhea, a muscarine antagonist that is unable to pass the BBB is injected 30 minutes prior to infusion (Curia et al., 2008, Portelli et al., 2012).

The constant flow of these chemoconvulsants elicits an array of rapidly progressing behaviors, which depend on the type of chemoconvulsant used. On the one hand, PTZ infusion sequentially induces ear twitching, myoclonic twitching, clonus of the forelimbs, clonic convulsion with loss of righting reflexes (falling), tonic hindlimb extension and finally death. On the other hand, infusion with pilocarpine sequentially elicits shivering, rearing, falling, tonic hindlimb extension and death. For each mouse, the dose needed to evoke these behavioral endpoints is calculated taking into account the concentration of the solution, the infusion rate, the infusion duration and the weight of the animal (Figure 3B) (Vermoesen et al., 2011). Hence, the **dose represents the threshold** for a specific behavioral endpoint for an individual animal. Anticonvulsant agents will delay the appearance of seizure behavioral endpoints and accordingly increase the dose needed. Compounds with proconvulsant characteristics will do the opposite, resulting in an earlier onset of the endpoints and accordingly a lower dose.

The major **advantages** of this model are its sensitivity and reproducibility in individual animals, vastly limiting the number of animals needed. Moreover, there tends to be a good correspondence between the actions in this model and clinical experience for several drugs (Loscher, 2009). Another advantage is that this model can also be used to identify genotypic differences in seizure

susceptibility of transgenic mice (Loyens et al., 2011, Portelli et al., 2012). However, as for any seizure model, this model also has some **limitations**. Compounds that act on the same receptor as PTZ or pilocarpine or have haemodynamic effects will lead to false results and as mentioned previously, death is eminent within minutes, excluding the animals from further use (Loscher, 2009).

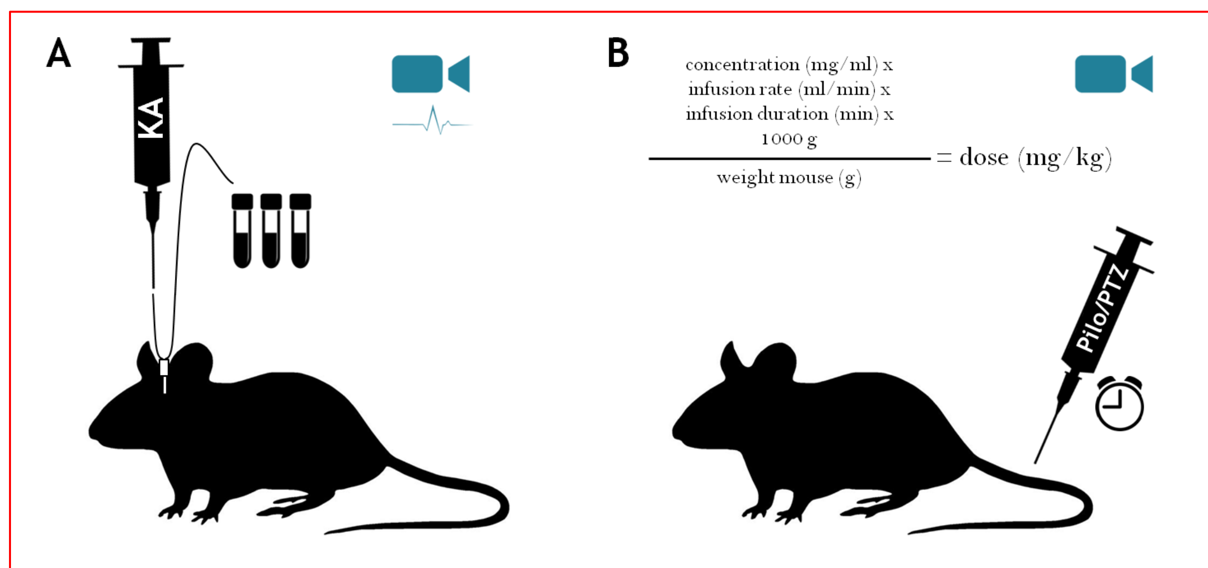


Figure 3 | Overview of the two seizure models used in this thesis. (A) The intrahippocampal kainic acid (KA) model of limbic status epilepticus. KA is infused intrahippocampally through a microdialysis probe to evoke a limbic status epilepticus. Electroclinical changes can be observed using video-EEG, while simultaneously sampling the local extracellular analyte concentration in the dialysates. **(B) The timed intravenous infusion seizure model.** Pilocarpine (Pilo) or pentylenetetrazole (PTZ) is continuously infused in the lateral tail vein at a constant rate. During infusion, the animal is allowed to move freely and the latencies to different behavioral endpoints are timed. The dose needed to evoke an endpoint represents the seizure threshold and is determined according to the formula given in the figure. Proconvulsants lower the dose needed to evoke an endpoint, anticonvulsants increase the dose.

References

Arzimanoglou A, Ben-Menachem E, Cramer J, Glauser T, Seeruthun R, Harrison M (2010) The evolution of antiepileptic drug development and regulation. Epileptic Disord 12:3-15.

Bedner P, Dupper A, Huttmann K, Muller J, Herde MK, Dublin P, Deshpande T, Schramm J, Haussler U, Haas CA, Henneberger C, Theis M, Steinhauser C (2015) Astrocyte uncoupling as a cause of human temporal lobe epilepsy. Brain 138:1208-1222.

Benveniste H (1989) Brain microdialysis. J Neurochem 52:1667-1679.

Berg AT (2008) The natural history of mesial temporal lobe epilepsy. Curr Opin Neurol 21:173-178.

Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, Engel J, French J, Glauser TA, Mathern GW, Moshe SL, Nordli D, Plouin P, Scheffer IE (2010) Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 51:676-685.

Bergey GK (2013) Neurostimulation in the treatment of epilepsy. Exp Neurol 244:87-95.

Bleakman D, Lodge D (1998) Neuropharmacology of AMPA and kainate receptors. Neuropharmacology 37:1187-1204.

Bonilha L, Martz GU, Glazier SS, Edwards JC (2012) Subtypes of medial temporal lobe epilepsy: influence on temporal lobectomy outcomes? Epilepsia 53:1-6.

Boon P, Raedt R, de Herdt V, Wyckhuys T, Vonck K (2009) Electrical stimulation for the treatment of epilepsy. Neurotherapeutics 6:218-227.

Bouilleret V, Ridoux V, Depaulis A, Marescaux C, Nehlig A, Le Gal La Salle G (1999) Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience 89:717-729.

Bragin A, Engel J, Jr., Wilson CL, Vizentin E, Mathern GW (1999) Electrophysiologic analysis of a chronic seizure model after unilateral hippocampal KA injection. Epilepsia 40:1210-1221.

Brandt C, Glien M, Potschka H, Volk H, Loscher W (2003) Epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats. Epilepsy Res 55:83-103.

Burgess D (2006) Transgenic and gene replacement models of epilepsy: targeting ion channel and neurotransmission in mice. In: Models of seizures and epilepsy, pp 199-223: Elsevier.

Chefer VI, Thompson AC, Zapata A, Shippenberg TS (2009) Overview of brain microdialysis. Curr Protoc Neurosci Chapter 7:Unit7 1.

Clapp-Lilly KL, Roberts RC, Duffy LK, Irons KP, Hu Y, Drew KL (1999) An ultrastructural analysis of tissue surrounding a microdialysis probe. J Neurosci Methods 90:129-142.

Cross JH (2014) Setting the scene: definition of prolonged seizures, acute repetitive seizures, and status epilepticus. Do we know why seizures stop? Epileptic Disord 16 Suppl 1:2-5.

Curia G, Longo D, Biagini G, Jones RS, Avoli M (2008) The pilocarpine model of temporal lobe epilepsy. J Neurosci Methods 172:143-157.

Davis, R, Dalmau, J (2013) Autoimmunity, seizures, and status epilepticus. Epilepsia 54 Suppl 6:46-9.

De Bundel D, Schallier A, Loyens E, Fernando R, Miyashita H, Van Liefferinge J, Vermoesen K, Bannai S, Sato H, Michotte Y, Smolders I, Massie A (2011) Loss of system x(c)- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. J Neurosci 31:5792-5803.

de Tisi J, Bell GS, Peacock JL, McEvoy AW, Harkness WF, Sander JW, Duncan JS (2011) The long-term outcome of adult epilepsy surgery, patterns of seizure remission, and relapse: a cohort study. Lancet 378:1388-1395.

DeGiorgio CM, Krahl SE (2013) Neurostimulation for drug-resistant epilepsy. Continuum (Minneap Minn) 19:743-755.

Devinsky O, Vezzani A, Najjar S, De Lanerolle NC, Rogawski MA (2013) Glia and epilepsy: excitability and inflammation. Trends Neurosci 36:174-184.

Dube CM, Brewster AL, Richichi C, Zha Q, Baram TZ (2007) Fever, febrile seizures and epilepsy. Trends Neurosci 30:490-496.

Engel J, Jr. (2001) A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. Epilepsia 42:796-803.

Engel J, Jr. (2006) Report of the ILAE classification core group. Epilepsia 47:1558-1568.

Engel J, Schwartzkroin PA (2006) What should be modeled? In: Models of Seizures and Epilepsy, pp 1-34: Elsevier.

Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339-347.

Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, Engel J, Jr., Forsgren L, French JA, Glynn M, Hesdorffer DC, Lee BI, Mathern GW, Moshe SL, Perucca E, Scheffer IE, Tomson T, Watanabe M, Wiebe S (2014) ILAE official report: a practical clinical definition of epilepsy. Epilepsia 55:475-482.

Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, Lee P, Engel J, Jr. (2005) Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46:470-472.

Freeman J, Veggiotti P, Lanzi G, Tagliabue A, Perucca E (2006) The ketogenic diet: from molecular mechanisms to clinical effects. Epilepsy Res 68:145-180.

Georgieva J, Luthman J, Mohringe B, Magnusson O (1993) Tissue and microdialysate changes after repeated and permanent probe implantation in the striatum of freely moving rats. Brain Res Bull 31:463-470.

Giulioni M, Marucci G, Martinoni M, Volpi L, Riguzzi P, Marliani AF, Bisulli F, Tinuper P, Tassinari CA, Michelucci R, Rubboli G (2013) Seizure outcome in surgically treated drug-resistant mesial temporal lobe epilepsy based on the recent histopathological classifications. J Neurosurg 119:37-47.

Gordon Boyd J, Debicki DB, Young GB (2012) Temporal lobe epilepsy after refractory status epilepticus: an illustrative case and review of the literature. Epilepsy Res Treat 2012:209701.

Gorter JA, van Vliet EA, Lopes da Silva FH (2015) Which insights have we gained from the kindling and post-status epilepticus models? J Neurosci Methods.

Hancock EC, Osborne JP, Edwards SW (2013) Treatment of infantile spasms. Cochrane Database Syst Rev 6:CD001770.

Huffman J, Kossoff EH (2006) State of the ketogenic diet(s) in epilepsy. Curr Neurol Neurosci Rep 6:332-340.

ILAE (2015) Online diagnostic manual of the epilepsies.

Kandel, Kupfermann, Iversen (2000) Learning and memory. In: Principles of neural science (4th edition), pp 1228-1246.

Kanner AM, Balabanov AJ (2005) The use of monotherapy in patients with epilepsy: an appraisal of the new antiepileptic drugs. Curr Neurol Neurosci Rep 5:322-328.

Kettenmann H (2007) Neuroscience: the brain's garbage men. Nature 446:987-989.

Kharlamov EA, Jukkola PI, Schmitt KL, Kelly KM (2003) Electrobehavioral characteristics of epileptic rats following photothrombotic brain infarction. Epilepsy Res 56:185-203.

Kossoff EH, McGrogan JR, Bluml RM, Pillas DJ, Rubenstein JE, Vining EP (2006) A modified Atkins diet is effective for the treatment of intractable pediatric epilepsy. Epilepsia 47:421-424.

Kwan P, Arzimanoglou A, Berg AT, Brodie MJ, Allen Hauser W, Mathern G, Moshe SL, Perucca E, Wiebe S, French J (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia 51:1069-1077.

Lee SK (2014) Old versus New: Why Do We Need New Antiepileptic Drugs? J Epilepsy Res 4:39-44.

Levy RG, Cooper PN, Giri P (2012) Ketogenic diet and other dietary treatments for epilepsy. Cochrane Database Syst Rev 3:CD001903.

Loscher W (2009) Preclinical assessment of proconvulsant drug activity and its relevance for predicting adverse events in humans. Eur J Pharmacol 610:1-11.

Lowenstein D (2001) Neurological disorders - Diseases of the central nervous system: Seizures and epilepsy. In: Harrison's principles of internal medicine (Braunwald, E. et al., eds), pp 2354-2369: McGraw-Hill

Loyens E, Schallier A, Chai SY, De Bundel D, Vanderheyden P, Michotte Y, Smolders I (2011) Deletion of insulin-regulated aminopeptidase in mice decreases susceptibility to pentylenetetrazol-induced generalized seizures. Seizure 20:602-605.

Loyens E, Vermoesen K, Schallier A, Michotte Y, Smolders I (2012) Proconvulsive effects of oxytocin in the generalized pentylenetetrazol mouse model are mediated by vasopressin 1a receptors. Brain Res 1436:43-50.

Marescaux C, Vergnes M, Depaulis A (1992) Genetic absence epilepsy in rats from Strasbourg--a review. J Neural Transm Suppl 35:37-69.

Massey CA, Sowers LP, Dlouhy BJ, Richerson GB (2014) Mechanisms of sudden unexpected death in epilepsy: the pathway to prevention. Nat Rev Neurol 10:271-282.

Maurer-Morelli CV, Secolin R, Morita ME, Domingues RR, Marchesini RB, Santos NF, Kobayashi E, Cendes F, Lopes-Cendes I (2012) A Locus Identified on Chromosome18P11.31 is Associated with Hippocampal Abnormalities in a Family with Mesial Temporal Lobe Epilepsy. Front Neurol 3:124.

Noebels J (2006) Spontaneous epileptic mutations in the mouse. In: Models of seizures and epilepsy, pp 223-249: Elsevier.

Nune G, DeGiorgio C, Heck C (2015) Neuromodulation in the Treatment of Epilepsy. Curr Treat Options Neurol 17:375.

Oses JP, Viola GG, de Paula Cognato G, Junior VH, Hansel G, Bohmer AE, Leke R, Bruno AN, Bonan CD, Bogo MR, Portela LV, Souza DO, Sarkis JJ (2007) Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res 75:104-111.

Panayiotopoulos C (2007) Epileptic seizures and their classification. In: A Clinical Guide to Epileptic Syndromes and their Treatment.

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF, Jr., Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R, Verkhratsky A (2012) Glial cells in (patho)physiology. J Neurochem 121:4-27.

Pascual MR (2007) Temporal lobe epilepsy: clinical semiology and neurophysiological studies. Semin Ultrasound CT MR 28:416-423.

Perucca P, Gilliam FG (2012) Adverse effects of antiepileptic drugs. Lancet Neurol 11:792-802.

Pitkanen A, Immonen RJ, Grohn OH, Kharatishvili I (2009) From traumatic brain injury to posttraumatic epilepsy: what animal models tell us about the process and treatment options. Epilepsia 50 Suppl 2:21-29.

Pitkanen A, Lukasiuk K (2011) Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol 10:173-186.

Pitkanen A, Schwartzkroin PA, Moshe SL (2006) Models of seizures and epilepsy: Elsevier.

Portelli J, Thielemans L, Ver Donck L, Loyens E, Coppens J, Aourz N, Aerssens J, Vermoesen K, Clinckers R, Schallier A, Michotte Y, Moechars D, Collingridge GL, Bortolotto ZA, Smolders I (2012) Inactivation of the constitutively active ghrelin receptor attenuates limbic seizure activity in rodents. Neurotherapeutics 9:658-672.

Puttachary S, Sharma S, Stark S, Thippeswamy T (2015) Seizure-induced oxidative stress in temporal lobe epilepsy. Biomed Res Int 2015:745613.

Quintas R, Raggi A, Giovannetti AM, Pagani M, Sabariego C, Cieza A, Leonardi M (2012) Psychosocial difficulties in people with epilepsy: a systematic review of literature from 2005 until 2010. Epilepsy Behav 25:60-67.

Raedt R, Van Dycke A, Van Melkebeke D, De Smedt T, Claeys P, Wyckhuys T, Vonck K, Wadman W, Boon P (2009) Seizures in the intrahippocampal kainic acid epilepsy model: characterization using long-term video-EEG monitoring in the rat. Acta Neurol Scand 119:293-303.

Rambo LM, Ribeiro LR, Della-Pace ID, Stamm DN, da Rosa Gerbatin R, Prigol M, Pinton S, Nogueira CW, Furian AF, Oliveira MS, Fighera MR, Royes LF (2013) Acute creatine administration improves mitochondrial membrane potential and protects against pentylenetetrazol-induced seizures. Amino Acids 44:857-868.

Rogawski MA (2006) Molecular targets versus models for new antiepileptic drug discovery. Epilepsy Res 68:22-28.

Smith SJ (2005) EEG in the diagnosis, classification, and management of patients with epilepsy. J Neurol Neurosurg Psychiatry 76 Suppl 2:ii2-7.

Sutula TP, Pitkanen A (2001) More evidence for seizure-induced neuron loss: is hippocampal sclerosis both cause and effect of epilepsy? Neurology 57:169-170.

Tatum WOt (2012) Mesial temporal lobe epilepsy. J Clin Neurophysiol 29:356-365.

van Vliet EA, Aronica E, Gorter JA (2015) Blood-brain barrier dysfunction, seizures and epilepsy. Semin Cell Dev Biol 38:26-34.

Velisek L (2006) Models of Chemically-induced acute seizures. In: Models of Seizures and Epilepsy, pp 127-152: Elsevier.

Vermoesen K, Serruys AS, Loyens E, Afrikanova T, Massie A, Schallier A, Michotte Y, Crawford AD, Esguerra CV, de Witte PA, Smolders I, Clinckers R (2011) Assessment of the convulsant liability of antidepressants using zebrafish and mouse seizure models. Epilepsy Behav 22:450-460.

Vezzani A, Auvin S, Ravizza T, Aronica E (2012) Glia-neuronal interactions in ictogenesis and epileptogenesis: role of inflammatory mediators.

Vezzani A, Granata T (2005) Brain inflammation in epilepsy: experimental and clinical evidence. Epilepsia 46:1724-1743.

Wang Q, Yu S, Simonyi A, Sun GY, Sun AY (2005) Kainic acid-mediated excitotoxicity as a model for neurodegeneration. Mol Neurobiol 31:3-16.

Wiebe S (2003) Randomized controlled trials of epilepsy surgery. Epilepsia 44 Suppl 7:38-43.

Wieser HG (2004) ILAE Commission Report. Mesial temporal lobe epilepsy with hippocampal sclerosis. Epilepsia 45:695-714.

Zhao F, Kang H, You L, Rastogi P, Venkatesh D, Chandra M (2014) Neuropsychological deficits in temporal lobe epilepsy: A comprehensive review. Ann Indian Acad Neurol 17:374-382.

Chapter 3

At the interface of oxidative stress and inflammation: a potential role for uric acid in epilepsy

Lisa Thyrion, Robrecht Raedt, Jeanelle Portelli, Kristl Vonck, Paul Boon

Neuropharmacology, Under review

Abstract

As the available anti-epileptic drugs fail to control seizures in one third of the epilepsy patients, there is a need for therapies with a different mechanism of action. Targeting inflammation is considered a promising strategy and recently also oxidative stress has become a field of interest. Identifying new players in these areas is hence becoming an important line of research. In this context, uric acid, a purine breakdown product that is increasingly recognized to act as a pro-oxidans and pro-inflammatory danger signal in a variety of disorders, may be more than an innocent bystander in epilepsy. In light of this, we revise the existing clinical and experimental data on uric acid in epilepsy and discuss the potential mechanisms by which it can contribute to excitability. We end by suggesting possible directions for future research.

Key words: uric acid, excitability, allopurinol, danger signal, oxidant, (pre)clinical data

Introduction

Epilepsy

Epilepsy is a chronic neurological disorder characterized by unprovoked recurrent seizures that affects more than 65 million people worldwide (Moshe et al., 2015). The disease process, known as epileptogenesis, involves a cascade of specific molecular and cellular changes that convert a healthy brain into a hyperexcitable one and is often triggered by a precipitating insult, such as head trauma or status epilepticus (Pitkanen and Lukasiuk, 2011). Although the majority of the patients enter remission after treatment with the available anti-epileptic drugs (AEDs), 20-30% of the patients fail to respond to treatment even after multidrug therapy (Lee, 2014). The patients have refractory epilepsy and are at high risk of cognitive deterioration, psychosocial dysfunction and sudden unexpected death in epilepsy (SUDEP) (Quintas et al., 2012, Massey et al., 2014). Another issue with AEDs on the market is that they only treat seizures, but fail to affect epileptogenesis (Moshe et al., 2015). Findings drugs with a different mechanism of action is hence of an extremely high priority in epilepsy research, not only to find new, improved ways to treat seizures, but also to find disease-modifying drugs that can affect the underlying process (Perucca et al., 2013).

In the last decades, numerous studies have revealed that oxidative stress and inflammation contribute to epileptogenesis and seizure susceptibility in chronic epilepsy. Therefore, there is considerable interest to identify new players in these processes (Vezzani and Viviani, 2014, Puttachary et al., 2015). A potential therapeutic target might be uric acid, a molecule at the interface of oxidative stress and inflammation (Rock et al., 2010, Kang and Ha, 2014).

Uric acid in health and disease

Uric acid is a weak organic acid that is generated from the metabolism of endogenous and dietary purines, which can be found in e.g. RNA and DNA. In the majority of mammals, uric acid is further converted to the more soluble allantoin by the liver-specific enzyme urate oxidase (Figure 1). In humans, however, two independent mutations rendered the urate oxidase gene nonfunctional, which resulted in tenfold higher serum uric acid (SUA) levels compared to other mammals (Oda et al., 2002). High levels are further preserved by the well-described uric acid-specific transport system of the human kidney, that allows net reabsorption of ca. 90% of the filtered uric acid (Mandal and Mount, 2015). In contrast to the peripheral situation, the regulation of uric acid homeostasis in the brain is not fully understood. Uric acid is thought to be actively transported over the BBB since several uric acid transporters are present in cerebral tissue and cerebral uric acid levels are maintained at 10% of the SUA levels (Tomioka et al., 2013). Most likely, uric acid is also produced locally in the brain however, as its synthesizing enzyme, xanthine oxidase, is present in the brain (O'Neill and Lowry, 1995).

Uric acid traditionally was considered an inert waste product, but this has been challenged by numerous reports that link hyperuricemia to disease. On the one hand, hyperuricemia has shown to be associated with a decreased risk of neurodegenerative diseases, such as multiple sclerosis, Parkinson's disease and Alzheimer's disease. Higher uric acid levels have shown to impede disease progression and improve prognosis in these disorders (Guo et al., 1999, Hooper et al., 2000, Alvarez-Lario and Macarron-Vicente, 2011, Chen et al., 2013). On the other hand, hyperuricemia has shown to be associated with an increased risk of a number of diet-related diseases including gout, hypertension, metabolic syndrome and cardiovascular disease (Kutzing and Firestein, 2008, Alvarez-Lario and Macarron-Vicente, 2011) but also with an increased risk of bipolar disorder (Albert et al., 2015). The link between uric acid and disease is well-known to be causative in gout (Rock et al., 2013), but increasing evidence suggests that this is the case for other disorders as well, such as cardiovascular disease (Alvarez-Lario and Macarron-Vicente, 2011, Kang and Ha, 2014).

This review evaluates the preclinical and clinical evidence for a potential causal link between uric acid and epilepsy.

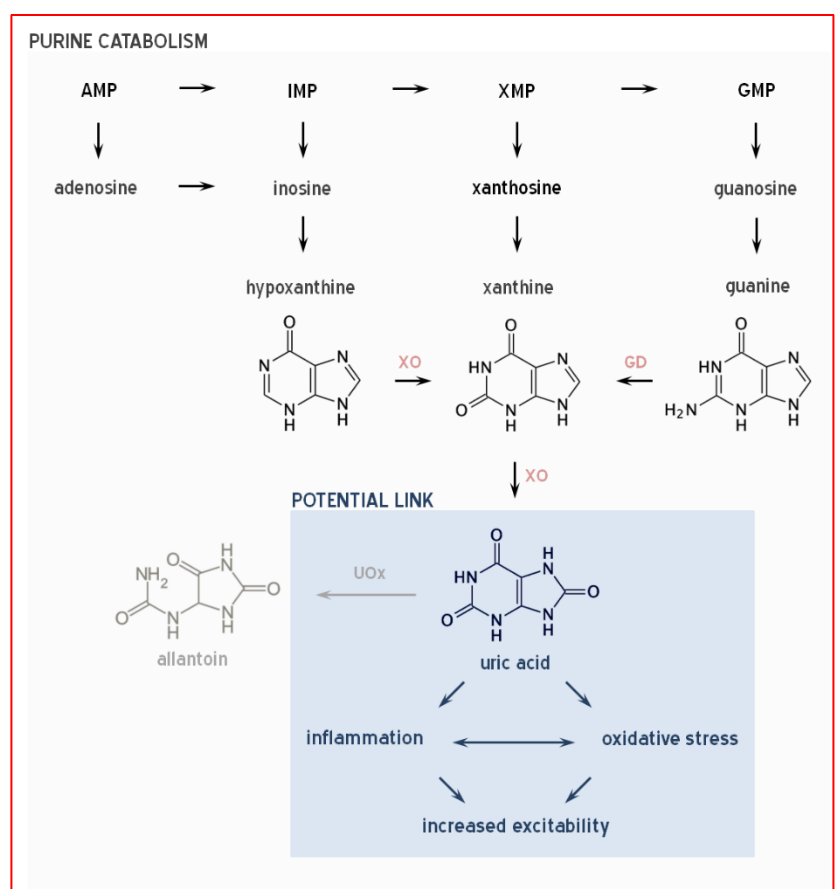


Figure 1 | A simplified schematic of the purine catabolism leading to the production of uric acid and the potential link of uric acid modulated systems to brain excitability. Xanthine oxidase (XO) converts hypoxanthine to xanthine and xanthine to uric acid. Guanine deaminase (GD) converts guanine to xanthine. Urate oxidase (UOx) converts uric acid into the easily excretable allantoin in non-hominid mammals. IMP: inosine monophosphate, AMP: adenosine monophosphate, GMP: guanosine monophosphate, XMP: xanthine monoposphate.

Epilepsy-related increases in uric acid levels in patients and seizure models

Patient studies

Uric acid levels in epilepsy patients have been investigated in a number of studies (Table 1). However, in none of these studies uric acid was causally linked to the pathophysiology of epilepsy. Uric acid was considered in an acute postictal context as a marker of oxidative stress (Stover et al., 1997), increased metabolism (Manzke et al., 1981, Aiyathurai et al., 1989, Rodriguez-Nunez et al., 2000) or because of its role in disorders associated with seizures, such as nephropathy (Warren et al., 1975, Luhdorf et al., 1978, George et al., 2012, Makki et al., 2013). The results of these studies mostly point in the same direction and report an acute increase in uric acid levels after convulsive seizures (Table 1). Moreover, they indicate that this increase occurs both peripheral (serum, urine) and central (cerebrospinal fluid (CSF)). The study of Aiyathurai (1989) showed that uric acid levels decreased again by the third post-seizure day suggesting that seizure-induced increases in uric acid are transient. It needs mentioning that some studies excluded patients with gout, hypertension or abnormal blood chemistry, thereby favoring epilepsy patients with uric acid levels within the normal range (Tan et al., 2009, Menon et al., 2014).

In the chronic interictal state, uric acid changes were analyzed in context of the chronic effects of AEDs on vascular risk factors. Several anti-epileptic treatments have shown to alter SUA levels (Krause et al., 1987, Hamed et al., 2004, Pylvanen et al., 2006, Aycicek and Iscan, 2007, Hamed et al., 2007, Tan et al., 2009, Menon et al., 2014). Carbamazepine and phenytoin, for example, are shown to establish a reduction in SUA while valproate and the ketogenic diet result in an increase (Schwartz et al., 1989, Katsiki et al., 2014). Although this in itself might seem controversial in light of a potential role for uric acid in epilepsy, AED-induced changes in SUA levels are not necessarily related to their anticonvulsive actions, but can be merely due to the numerous the side-effects of these AEDs (Perucca and Gilliam, 2012). SUA levels depend on the dietary intake of purines, endogenous uric acid biosynthesis and the rate of excretion of uric acid through renal and extra-renal pathways (Kutzing and Firestein, 2008, Ichida et al., 2012). Any side-effect that relates to changes in food intake, metabolism or renal and gastrointestional function will hence influence SUA levels, making SUA data in treated patients unreliable to adequately investigate a relationship between uric acid and the chronic epileptic state. Some of the side effects of valproate for example include nausea, gastric irritation, diarrhea, hepatic and pancreatic dysfunction, weight gain and renal tubular dysfunction (Knights and Finlay, 2014). In this regard, four studies determined uric acid levels in an untreated epileptic population in addition to healthy controls, allowing to exclude the effects of AEDs on SUA levels (Hamed et al., 2004, Aycicek and Iscan, 2007, Hamed et al., 2007, Menon et al., 2014). Two of these studies showed an elevation of SUA levels in at least a subset of epileptic patients (Hamed et al., 2004, Hamed et al., 2007). Tan et al. (2009) excluded patients with gout, hypertension or

abnormal blood chemistry, however, thereby favoring epilepsy patients with uric acid levels within the normal range.

Animal studies

Despite the high heterogeneity in the regimens for seizure induction, the majority of the preclinical studies also show that intracerebral uric acid levels are acutely increased in response to seizures (Beal et al., 1991, Layton et al., 1998, Oses et al., 2007, Rambo et al., 2013), although two studies did not find any change in uric acid in response to seizures (Mueller and Kunko, 1990, Waterfall et al., 1995) (Table 1). Elevated uric acid levels were reported in the CNS as late as 7 and 10 days after the chemoconvulsant regimen, suggesting a long-lasting increase (Beal et al., 1991, Oses et al., 2007).

Causes of epilepsy-related uric acid increases

Different factors have been suggested to contribute to the increase in uric acid in response to seizure-activity. While increased muscle breakdown and altered renal secretion (e.g. due to seizure-associated sweating and hyperthermia) are thought to be responsible for systemic increases in uric acid levels after status epilepticus (Warren et al., 1975, Makki et al., 2013), cerebral increases in uric acid levels have been linked to seizure activity and seizure-induced neuronal injury, cell death and BBB leakage (Beal et al., 1991, Stover et al., 1997, Layton et al., 1998, Rambo et al., 2013).

Seizures in conditions associated with uric acid imbalances

Interestingly, the occurrence of epileptic seizures have been described in several disorders that are associated with increased uric acid levels. Tumor lysis syndrome (TLS) is the consequence of cytotoxic tumor treatment resulting in cell lysis and rapid release of intracellular contents into the blood stream. The occurrence of seizures is a diagnosing factor and recently SUA levels have been shown to be highly predictive for the development of TLS (Ejaz et al., 2015). Deficiency of hypoxanthine-guanine phosphoribosyltransferase (HPRT) is an inborn error of the purine metabolism and in its most extreme form the cause of Lesch-Nyhan syndrome. Patients with Lesch-Nyhan show increased uric acid levels and a wide variety of neurological manifestations, including delayed motor development, cognitive and attention deficits, self-injurious behavior but also epileptic seizures in 8% of the cases (Jinnah et al., 2006). This is about ten times higher than the prevalence in the normal population (Thurman et al., 2011). In addition, hyperuricemia observed in pregnant women with preeclampsia has been put forward as an independent predictor for the onset of seizures and the development of eclampsia (Livingston et al., 2014, Taweesuk and Tannirandorn, 2014). Seizures have also been described in other hyperuricemic conditions, such as kidney failure, metabolic syndrome, hypertension and gout although these are less well documented. Conversely, seizures are rarely described in disorders associated with hypouricemia, an exception being molybdenum cofactor deficiency, which is associated with seizures in neonates (Arnold et al., 1993).

	HUMAN STUDIES						
	Study	Patient group	AED treatment	Component analyzed	Timing analysis	UA levels	
Acute postictal	Warren et al., 1975	status epilepticus or recurrent grand-mal seizures male + female adults (n = 7)	not specified	blood serum	immediate	patients vs controls	↑
	Luhdorf et al., 1978	two or more grand mal seizures within 24h male + female adults (n = 17)	not specified	not specified	immediate	-	↑
	Manzke et al., 1981	grand and petit mals male + female children (n = 31)	not specified	CSF	immediate	patients vs controls	↑
	Aiyathurai et al., 1989	febrile seizures male + female children (n = 24)	not specified	blood serum	immediate, 4h, 3 days	immediate and 4 hours vs. 3 days within patients	↑
	Stover et al., 1997	new onset grand mal seizure male + female adults (n = 9)	not specified	CSF	2h	patients vs controls	↑
	Rodriguez-Nunez et al., 200(	febrile seizures male + female children (n = 90)	not specified	CSF	1h	patients vs controls	=
	George et al., 2012	recurrent generalized tonic clonic seizures during 5h 44 yo man (n = 1)	VPA	urine	not specified	crystal formation	↑
	Makki et al., 2013	status epilepticus 35 yo man (n = 1)	not specified	urine blood serum	not specified 24h, 48h	crystal formation	↑ ↑
Chronic interictal	Krause et al., 1987	epilepsy type not specified male + female adults (n = 554)	VPA, CBZ, PHT, PB	blood serum	not specified	treated patients vs controls	↓
	Hamed et al., 2004	idiopathic generalized tonic-clonic epilepsy male + female adults (n = 70)	None, CBZ, VPA, polytherapy	blood serum	> 12h seizure free	untreated patients vs controls untreated vs treated	↑ ↑
	Aycicek and Iscan, 2006	idiopathic and symtomatic epilepsy male + female children (n = 68)	None, CBZ, VPA, PB	blood serum	> 48h seizure free	untreated patients vs controls untreated vs treated	= =
	Pylvanen et al., 2006	generalized and partial epilepsy male + female adults (n = 51)	VPA	blood serum	not specified	treated patients vs controls	↑
	Hamed et al., 2007	primary epilepsy male + female adults (n = 225)	None, CBZ, VPA, polytherapy	blood serum	> 72h seizure free	patients vs controls (in 13,8%) untreated vs treated	↑ =
	Tan et al.., 2009	generalized and partial epilepsy male + female adults (n = 195)	CBZ, VPA, PHT, TPM, PB, LEV VB, GP, polytherapy	blood serum	not specified	treated patients vs controls	=
	Menon et al., 2014	idiopathic generalized epilepsy male + female adults (n = 100)	None, CBZ, PHT, VPA, CLB, PB, LTG TPM, LEV, polytherapy	blood serum	> 72h seizure free	untreated patients vs controls untreated vs treated	= =

ANIMAL STUDIES						
Study	Chemoconvulsant	Regimen	Component analyzed	Timing analysis	UA levels	
Mueller and Kunko, 1990	Pilocarpine	i.p., 3 mg/kg	cortex, hippocampus, caudate nucleus accumbens (extracellular)	100 minutes	-	=
Beal et al., 1991	KA°	uni i.c., 10 nmol, 20 nmol	cortex (lysate)	1 week	only ipsilateral	↑
Waterfall et al., 1995	KA	uni i.c., and i.s. 50 nmol	cortex and striatum (extracellular)	60 minutes	-	=
Layton et al., 1998	KA	i.p., 16 mg/kg	cortex (extracellular)	100 minutes	-	↑
Oses et al., 2007	PTZ	i.p., kindling (35 mg/kg, 10 stimulations)	CSF	10 days	-	↑
Rambo et al., 2013	PTZ	i.p., 30, 45 or 60 mg/kg	cortex (lysate)	20 minutes	only at 60 mg/kg	↑

Table 1 | Clinical and experimental epilepsy studies that included uric acid measurements. °similar results with NMDA, AMPA, quinolonic acid and homocysteic acid. CSF: cerebrospinal fluid; i.c.: intracortical; i.s.: intrastriatal; i.p.: intraperitoneal; KA: kainic acid; PTZ: pentylenetetrazole; VPA: valproate; CBZ: carbamazepine; PB: Phenobarbital; PHT: phenytoine; TPM: topamirate; LEV: leviracetam; VB: vigabatrine; CLB: clobazam; LTG: lamotrigine; VB: vigabatrin; GB: gabapentin. ↑ increased; = unchanged; ↓ decrease

The anticonvulsive potential of allopurinol

A consensus from the studies discussed above is that systemic and intracerebral uric acid levels are increased, sometimes persistent, after epileptic episodes. In addition, clinical conditions associated with high levels of uric acid are associated with an increased risk for seizures. This section will discuss the effects of uric acid lowering drugs on seizure activity, with a main focus on allopurinol, an inhibitor of the enzyme xanthine oxidase/dehydrogenase (Figure 1). Several studies have investigated allopurinol as an add-on therapy in epilepsy and although informational, their results should be interpreted with caution, as it has other effects, besides lowering uric acid, that might affect seizures. As such, allopurinol can decrease the levels of the proconvulsant quinolonic acid (Coppola and Pascotto, 1996) and increase the levels of the endogenous anticonvulsants adenosine and inosine (Togha et al., 2007, Kovacs et al., 2015).

Patient studies

A study by Coleman and colleagues was the first to show that hyperuricemic patients suffering from refractory epilepsy were seizure-free for at least 8 years, after normalizing uric acid biochemistry with allopurinol (Coleman et al., 1986). Since then, numerous studies have shown that allopurinol can be beneficial as add-on therapy in patients with epilepsy (Table 2). Both in children and adults seizure reduction and even seizure freedom was reported when it was added to the AED regimen (De Marco and Zagnoni, 1988, Guzeva et al., 1988, Tada et al., 1991, Zagnoni et al., 1994, Togha et al., 2007) and several groups have suggested that allopurinol was specifically efficient in reducing secondarily generalized tonic-clonic seizures (De Marco and Zagnoni, 1988, Tada et al., 1991, Zagnoni et al., 1994, Togha et al., 2007). Overall, allopurinol was generally well-tolerated and was only associated with mild side-effects, such as nausea, dizziness or abdominal pain in a minority of the patients (Tada et al., 1991, Zagnoni et al., 1994).

In contrast to the abovementioned studies, some studies failed to demonstrate an additional therapeutic benefit of allopurinol add-on treatment (Sussman et al., 1987, Sander and Patsalos, 1988, So and Ptacek, 1988, Coppola and Pascotto, 1996). This might be because the actions of allopurinol are most pronounced in hyperuricemic conditions, which was not a premise in the add-on trials. Arguing against this however is a lack of correlation between uric acid concentration or reduction and seizure reduction after allopurinol add-on treatment (Tada et al., 1991, Zagnoni et al., 1994, Coppola and Pascotto, 1996). This latter observation could indicate that the therapeutic actions of allopurinol is due to other physiological changes than a decrease in uric acid.

Animal studies

The few existing animal studies that have tested the anticonvulsant potential of allopurinol are extremely heterogeneous in their set up and present contradictory findings. Three studies suggest an

anti-epileptic effect of allopurinol (Mikhailov and Gusel, 1983, Wada et al., 1992, Murashima et al., 1998). Two of these studies indicated that allopurinol preferentially targets secondarily generalized seizures, which is in line with clinical findings. In cats, allopurinol reduced the severity of fully kindled, generalized seizures (Wada et al., 1992). In addition, it prevented the induction of seizures during 24 hours in a genetic mouse model for secondarily generalized seizures (EL mice) (Murashima et al., 1998). Two studies, however, could not demonstrate any anti-epileptic effect of allopurinol (Hoppe et al., 1984, Parada-Turska et al., 2004) (Table 2). Parada-Turska and colleagues reported that, besides a lack of effect of allopurinol alone, add-on therapy with allopurinol did not increase the seizure-reducing effects of AEDs in the maximal electroshock seizure (MES) mouse model, a model for primary generalized seizures. Taken together, this might suggest that allopurinol primarily acts on secondarily, but not primary generalized seizures. Only one of these studies determined SUA levels and found that these were slightly, but non-significantly, lower in the allopurinol-treated group compared to the control group (Murashima et al., 1996).

Potential mechanisms of excitability: inflammatory and oxidative actions of uric acid

Epilepsy often develops after an initial precipitating insult, such as head trauma, febrile seizures, brain tumor, infection, stroke or status epilepticus. These damaging insults are associated with increased oxidative stress and inflammation. Both these processes increase brain excitability and contribute to the establishment of chronic epilepsy during epileptogenesis (Vezzani et al., 2012, Puttachary et al., 2015). Since uric acid is released upon cell damage (Shi et al., 2003) and plays a role in oxidative stress and inflammation (Rock et al., 2013, Kang and Ha, 2014), it is likely to play a role in epileptogenesis. This chapter will give a short overview on oxidative stress and inflammation in epileptogenesis and discuss a possible role of uric acid herein.

Oxidative stress

A brain insult, such as a seizure or a traumatic brain injury, leads to the production of reactive oxygen/nitrogen species (ROS/RNS), which causes damage to lipids, proteins and nucleic acids (Cardenas-Rodriguez et al., 2013). Cumulatively, this is known as oxidative stress. The subsequent arrest in mitochondrial function and energy metabolism compromises cell viability, which in turn promotes lipid peroxidation, reactive gliosis and aberrant rearrangements in neural circuitry that promote the establishment of hyperexcitable networks (Puttachary et al., 2015). Moreover, oxidative stress promotes seizure susceptibility in experimental models and patients with chronic epilepsy. This suggests that anti-oxidants could be employed, not only to target epileptogenesis, but also as an add-on therapy in chronic epilepsy (Puttachary et al., 2015).

HUMAN STUDIES						
Study	Study design	Allopurinol regimen	SUA levels		Seizure frequency	
Coleman et al., 1986	6-12 year follow-up case studies hyperuricosuria children (n = 3)	3.4 - 12 mg/kg/day	after vs before (urine)	↓	100%	↓
Sussman et al., 1987*	1-3 month follow-up age or sexe not specified (n = 5)	300 mg/day	after vs before (60%)	↓	100%	=
So and Ptacek, 1988*	4 month follow-up male + female children and adults (n = 13)	> 300 mg/day	not specified	-	8% 92%	↓ =
Guzeva et al., 1988	study design not specified male + female children (n = 38)	4-5 mg/kg/day	not specified	-	36% 64%	↓ =
Sander and Patsalos., 1988	3 month open uncontrolled trial male + female adults (n = 12)	300 mg/day	after vs before	↓	100%	=
De Marco and Zagnoni, 1988	1 year open uncontrolled trial male + female children and adults (n = 64)	150-300 mg/day	not specified	-	67% 25% 8%	↓ = ↑
Tada et al., 1991	1 year open uncontrolled trial male + female children and adults (n = 31)	3-15 mg/kg/day	responders vs nonresponders	=	55% 36% 6%	↓ = ↑
Zagnoni et al., 1994	4 month double-blind, placebo-controlled randomized cross-over trial male + female children and adults (n = 84)	150 mg/day (<20 kg) 300 mg/day (> 20 kg)	allopurinol vs placebo	↓	28% 60% 12%	↓ = ↑
Coppola and Pascotto, 1996	3 month double-blind, placebo-controlled, cross-over trial male + female children (n = 17)	10 mg/kg/day - week 1 15 mg/kg/day after	allopurinol vs placebo responders vs nonresponders	↓ =	47% 30% 23%	↓ = ↑
Togha et al., 2007	6 month double-blind, placebo-controlled trial male + female adults (n = 38)	300 mg/day	not specified	-	66% 34%	↓ =

ANIMAL STUDIES						
Study	Seizure model	Allopurinol regimen	SUA levels		Effect	
Mikhailov and Gusel, 1983	penicillin rats	i.p., 25 or 50 mg/kg acutely	not specified	-	activity penicillin-induced epileptogenic foci	↓
Hoppe et al., 1984	hyperbaric oxygen model for grand mal seizures not specified	not specified	not specified	-	latency oxygen-induced seizures	=
Wada et al., 1992	electrical hippocampal kindling male + female cats	i.p., 5 or 50 mg/kg acutely	not specified	-	severity of induced seizures (50 mg/kg only)	↓
Murashima et al., 1996-8	mutant model for secondary generalized seizures male EL mice	p.o., 75 mg/kg chronic (every other day)	allopurinol (+) vs allopurinol (-)	=	seizures evoked within 24h	↓
Parada-Turska et al., 2004	maximal electroshock-induced convulsions male Swiss mice	i.p. 5, 15 or 45 mg/kg acutely or chronic (5 consecutive days)	not specified	-	electrical seizure threshold	=

Table 2 | Clinical and experimental epilepsy studies on allopurinol efficacy. ↑ increased; = unchanged; ↓ decreased (> 25%); % fraction of patients affected. *abstract. i.p. intraperitoneal; p.o.: per oral; SUA: serum uric acid

Numerous enzymatic (e.g. superoxide dismutase (SOD)) and non-enzymatic (e.g. ascorbate) anti-oxidative defense mechanisms exist (Puttachary et al., 2015). Uric acid can act as a non-enzymatic anti-oxidant, by blocking the formation and actions of peroxynitrite ($ONOO^-$), a RNS that is formed through the reaction of a superoxide radical (O_2^-) and nitric oxide (NO). Peroxynitrite is a powerful oxidant that causes oxidation/nitrosylation of proteins and DNA/RNA, inactivation of enzymes and the peroxidation of lipids (Pacher et al., 2007). Uric acid lowers peroxynitrite formation by preventing the degradation of SOD, the converting enzyme of O_2^- (Kutzing and Firestein, 2008). Furthermore, uric acid can inhibit the iron-dependent oxidation of ascorbate, preventing further increases of free radicals (Sevanian et al., 1991). Aside from these direct scavenging effects, the protective actions of uric acid against oxidative stress are suggested to be dependent on an astroglia-mediated mechanism. (Du et al., 2007). There are several indications that the anti-oxidative actions of uric acid are limited to the hydrophilic environment of biological fluids. In this regard, intracellular uric acid is believed to be pro-oxidative rather than anti-oxidative, as shown in cultured cells and animal models of renal and vascular disease (Sautin and Johnson, 2008, Alvarez-Lario and Macarron-Vicente, 2011, Kang and Ha, 2014). As pro-oxidans, the different radicals of uric acid increase oxidative damage, mainly to lipids, by reacting with other radicals. In addition, uric acid is able to engage an intracellular system that promotes the production of superoxide radicals through NADPH oxidase. The intermediate steps leading to NADPH oxidase activation are still incompletely understood, although it has been put forward that its pro-inflammatory characteristics might be involved (Sautin and Johnson, 2008).

Inflammation

Communication between neurons and glia is pivotal to maintain homeostasis in the brain and both astrocytes and microglia are essential in monitoring neuronal activity and health (Kettenmann, 2007, Parpura et al., 2012). When neurons are endangered or impaired, glia become activated in an attempt to limit injury and promote healing. Dysfunctional neuroglial communication can contribute to hyperexcitability of neuronal networks (Devinsky et al., 2013). Activated glia release pro-inflammatory cytokines which upregulate excitatory transmission and downregulate inhibitory transmission in various ways (Galic et al., 2012). A central cytokine in this regard is IL-1β. IL-1β not only affects neuronal survival, but also influences neurotransmission and neuroglial communication through rapid effects on receptor-gated ion channels and long-lasting effects on expression levels of transcription molecules, such as NF-kB (Vezzani and Baram, 2007). Similar to anti-oxidant therapies, several preclinical studies show that anti-inflammatory therapies might have potential in targeting epileptogenesis and reduce spontaneous seizures in chronic epilepsy (Vezzani et al., 2013).

Danger associated molecular patterns or DAMPs are endogenous danger signals that alert the immune system in response to damaged and dying cells. DAMPs were recently found to affect excitability as well (Matzinger, 1994, Maroso et al., 2010). Uric acid acts as a pro-inflammatory

DAMP in an increasing number of disorders (Kono et al., 2010, Kool et al., 2011, Rock et al., 2013). The inflammatory pathway through which it acts, is best described in gout (Rock et al., 2013). Uric acid is a weak acid and hence principally exists as sodium salt at physiological pH. It is present at a higher concentration inside the cell (4 mg/ml) compared to the interstitial fluid compartment (6 mg/dl), resulting in a steep increase in the local extracellular concentration after cell damage. When the concentration exceeds the crystallization threshold (7 mg/dl), it crystallizes into monosodium urate (MSU) crystals, which is its presumed pro-inflammatory active state. When an MSU crystal engages the cell membrane and is phagocytosed, the NLRP3 inflammasome is activated through yet an incompletely understood process, that involves at least two different mechanisms (Rock et al., 2013) (Figure 2). Activation of the inflammasome results in the cleavage and activation of caspase-1, which in turn converts pro-IL-1β into its biologically active form IL-1β. Apart from the well-established contribution of IL-1β to excitability, other components of the pathway contribute to epilepsy as well, in particular caspase-1 and more recently NLRP3 (Vezzani et al., 2010, Meng et al., 2014). Importantly, not all inflammatory processes induced by uric acid are NLRP3-dependent, indicating that there are other ways through which it may operate. In an experimental model for allergic asthma the adjuvant effects of uric acid do not require the inflammasome, but act through Syk- and PI3-kinases (Kool et al., 2011). Engagement of MSU crystals to cholesterol in lipid membranes activates same pathway in dendritic cells (Ng et al., 2008).

The two faces of uric acid

Although oxidative stress and inflammation were discussed as two separate topics in the paragraphs above, both processes are highly intertwined. The neurodegeneration induced by increases in ROS/RNS promotes the release of gliotransmitters of neighboring glial cells, which in turn can further increase oxidative stress and neurodegeneration by overstimulating astroglial glutamate release into excitotoxic amounts. It is hence logic to assume that the pathways through which uric acid affects excitability are intertwined as well.

In gout uric acid is solely considered a harmful agent (Rock et al., 2013), while in multiple sclerosis it is considered a therapeutic agent (Markowitz et al., 2009). This has lead to the belief that a molecular switch might exist that changes the molecular feature of uric acid from benefactor to malefactor (Kang and Ha, 2014). Although the exact conditions are unknown, this switch likely depends on the micro-environment: e.g. the extent of the insult, the duration of oxidative stress, the affected organ and tissue location, the timing in the disease process, uric acid concentration and formation of MSU crystals (Kang and Ha, 2014). The anticonvulsive potential of allopurinol indicates that the pro-oxidant/pro-inflammatory actions of uric acid might prevail in epilepsy, suggesting it is a therapeutic target.

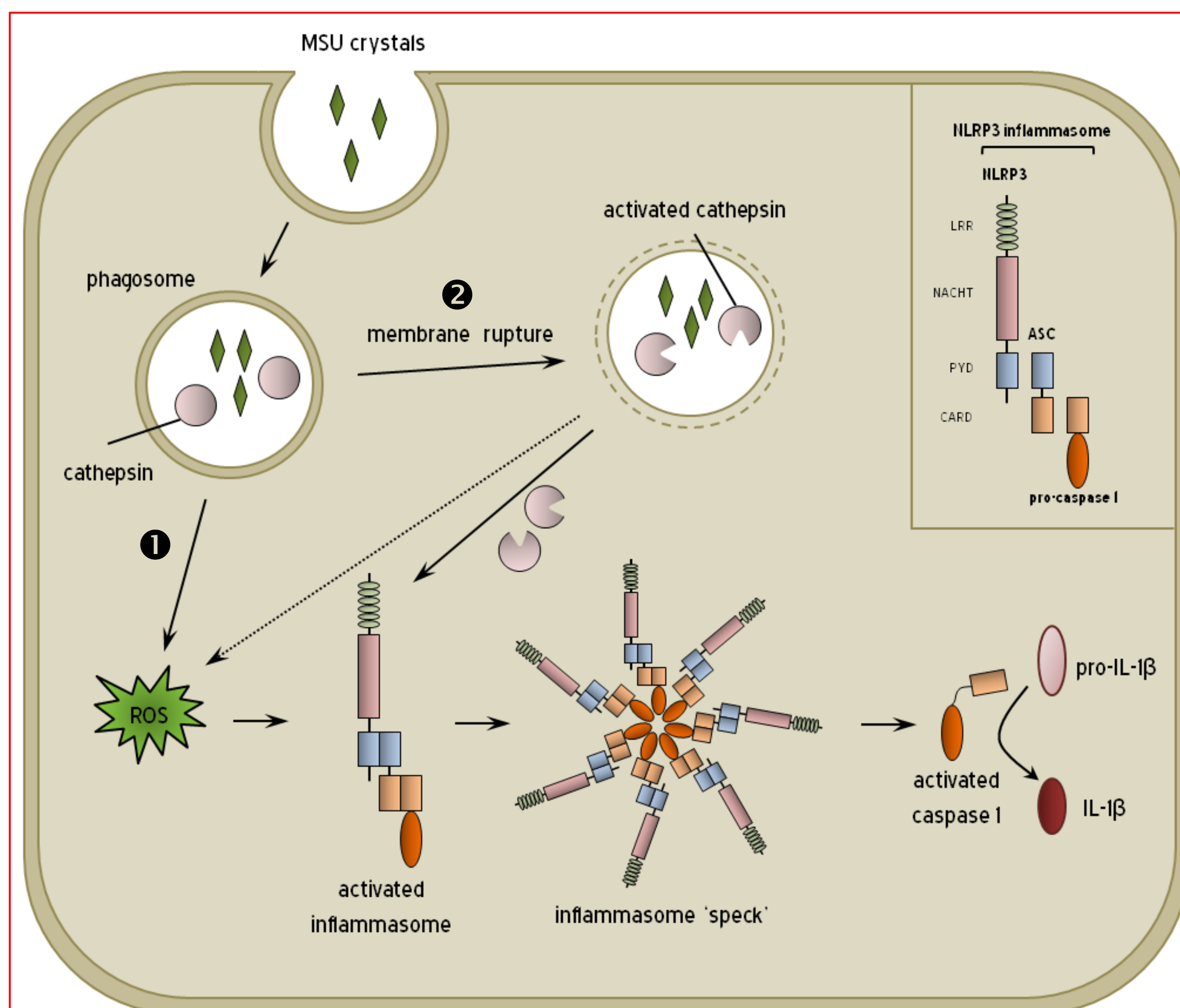


Figure 2 | Monosodium urate (MSU) crystal induced activation of the NLRP3 inflammasome. MSU-containing phagosomes activate the NLRP3-inflammome through at least two different mechanisms. ❶ Production of reactive oxygen species (ROS): internalized particles are believed to stimulate phagocytes to produce ROS, possibly through a mitochondrial-dependent pathway. However, it is unclear how ROS activate the NLRP3-inflammasome. ❷ The release of proteolytic cathepsins: in analogy with other irritant particles, internalization of MSU crystals could result in the rupture of phagosomes, resulting in the release of phagocytic proteases (cathepsins). The resulting internal cell damage is believed to activate the NLRP3-inflammasome, possibly by cleaving it into its active state or by generating a stimulatory ligand (e.g. ROS). Upon activation, the inflammasome oligomerizes into large structures called 'specks' and cleaves pro-caspase-1 into its active form, which cleaves pro-IL-1β into the biologically active form IL-1β. **Small panel:** The NLRP3 inflammasome is made up of three components: the NOD-like receptor protein NLRP3, ASC and pro-caspase 1. The NLR protein is thought to determine the specificity and control the activity of the complex. The leucine-rich repeats (LRR) are thought to be involved in substrate recognition and the pyrin domain (PYD) is responsible for the interaction with the scaffolding protein ASC. The CARD domain of ASC allows the association with the third component of the inflammasome, the inactive zymogen of the enzyme caspase 1: pro-caspase 1. ASC, apoptosis-associated speck-like protein containing a CARD; NLRP3: NACHT, LRR and PYD domains-containing protein. (Rock et al., 2013)

Conclusions and future perspectives

The classic view of uric acid as an inert waste product is increasingly challenged by the growing number of conditions where it is shown to be an active player. Several findings indicate that uric acid might contribute to seizure susceptibility as well. Firstly, uric acid levels increase after seizures, and may remain elevated in chronic epilepsy. Secondly, the uric acid lowering agent allopurinol has anti-seizure potential. Lastly, uric acid is involved in inflammatory and oxidative molecular pathways that are thought to contribute to the pathophysiology of epilepsy. To corroborate the contribution of uric acid to epileptogenesis and the development of seizures however, more in-depth evidence is needed on several levels..

First, a more extensive analysis of the intracerebral uric acid concentration in seizure models and epilepsy patients is needed. Measuring uric acid levels in surgery-obtained brain specimens of drug-resistant epilepsy patients would be of particular interest, as this has strengthened the involvement for other danger signals in the past (Maroso et al., 2010). The presence of MSU crystals could then be considered indicative for the involvement of a uric acid-dependent inflammatory pathway. Secondly, the effect of more selective uric acid manipulations on seizure characteristics should be investigated to confirm the results found with allopurinol. In this regard, urate oxidase is a promising target. Preclinical studies in asthma and liver injury, for example, tested urate oxidase and allopurinol alongside each other and showed they had similar effects (Kool et al., 2011, Kono et al., 2014). Moreover, genetic overexpression and disruption of urate oxidase has shown that uric acid is more than a marker in Parkinson's disease, and that it actively protects against several features of a mouse model for hemiparkinsonism (Chen et al., 2013). In a similar way, uric acid could be targeted in acute seizure models and chronic models for (drug resistant) epilepsies to investigate the effects on seizure characteristics and the development of spontaneous recurrent seizures.

Once the contribution and nature of a uric acid-mediated effect in epilepsy has been established, one could then revisit its potential as therapeutic and disease-modifying target and look into its mechanism of action.

References

Aiyathurai EJ, Low PS, Jacob E (1989) Hyperpolarization and short-circuiting as mechanisms of seizure prevention following febrile convulsions. Brain Dev 11:241-246.

Albert U, De Cori D, Aguglia A, Barbaro F, Bogetto F, Maina G (2015) Increased uric acid levels in bipolar disorder subjects during different phases of illness. J Affect Disord 173:170-175.

Alvarez-Lario B, Macarron-Vicente J (2011) Is there anything good in uric acid? QJM 104:1015-1024.

Arnold GL, Greene CL, Stout JP, Goodman SI (1993) Molybdenum cofactor deficiency. J Pediatr 123:595-598.

Aycicek A, Iscan A (2007) The effects of carbamazepine, valproic acid and phenobarbital on the oxidative and antioxidative balance in epileptic children. Eur Neurol 57:65-69.

Beal MF, Swartz KJ, Finn SF, Mazurek MF, Kowall NW (1991) Neurochemical characterization of excitotoxin lesions in the cerebral cortex. J Neurosci 11:147-158.

Cardenas-Rodriguez N, Huerta-Gertrudis B, Rivera-Espinosa L, Montesinos-Correa H, Bandala C, Carmona-Aparicio L, Coballase-Urrutia E (2013) Role of oxidative stress in refractory epilepsy: evidence in patients and experimental models. Int J Mol Sci 14:1455-1476.

Chen X, Burdett TC, Desjardins CA, Logan R, Cipriani S, Xu Y, Schwarzschild MA (2013) Disrupted and transgenic urate oxidase alter urate and dopaminergic neurodegeneration. Proc Natl Acad Sci U S A 110:300-305.

Coleman M, Landgrebe M, Landgrebe A (1986) Purine seizure disorders. Epilepsia 27:263-269.

Coppola G, Pascotto A (1996) Double-blind, placebo-controlled, cross-over trial of allopurinol as add-on therapy in childhood refractory epilepsy. Brain Dev 18:50-52.

De Marco P, Zagnoni P (1988) Allopurinol in severe epilepsy. A preliminary report. Neuropsychobiology 19:51-53.

Devinsky O, Vezzani A, Najjar S, De Lanerolle NC, Rogawski MA (2013) Glia and epilepsy: excitability and inflammation. Trends Neurosci 36:174-184.

Du Y, Chen CP, Tseng CY, Eisenberg Y, Firestein BL (2007) Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity. Glia 55:463-472.

Ejaz AA, Pourafshar N, Mohandas R, Smallwood BA, Johnson RJ, Hsu JW (2015) Uric acid and the prediction models of tumor lysis syndrome in AML. PLoS One 10:e0119497.

Galic MA, Riazi K, Pittman QJ (2012) Cytokines and brain excitability. Front Neuroendocrinol 33:116-125.

George N, Vivekanandan M, Karnam AH (2012) Reddish-orange discoloration of urine due to uric acid crystalluria after recurrent seizures. Kidney Int 81:1281.

Guo Q, Sebastian L, Sopher BL, Miller MW, Ware CB, Martin GM, Mattson MP (1999) Increased vulnerability of hippocampal neurons from presenilin-1 mutant knock-in mice to amyloid beta-peptide toxicity: central roles of superoxide production and caspase activation. J Neurochem 72:1019-1029.

Guzeva VI, Gusel VA, Mikhailov IB (1988) [Allopurinol in the combined therapy of severe forms of epilepsy in children]. Zh Nevropatol Psikhiatr Im S S Korsakova 88:69-72.

Hamed SA, Abdellah MM, El-Melegy N (2004) Blood levels of trace elements, electrolytes, and oxidative stress/antioxidant systems in epileptic patients. J Pharmacol Sci 96:465-473.

Hamed SA, Hamed EA, Hamdy R, Nabeshima T (2007) Vascular risk factors and oxidative stress as independent predictors of asymptomatic atherosclerosis in adult patients with epilepsy. Epilepsy Res 74:183-192.

Hooper DC, Scott GS, Zborek A, Mikheeva T, Kean RB, Koprowski H, Spitsin SV (2000) Uric acid, a peroxynitrite scavenger, inhibits CNS inflammation, blood-CNS barrier permeability changes, and tissue damage in a mouse model of multiple sclerosis. FASEB J 14:691-698.

Hoppe SA, Terrell DJ, Gottlieb SF (1984) The effect of allopurinol on oxygen-induced seizures in mice. Aviat Space Environ Med 55:927-930.

Ichida K, Matsuo H, Takada T, Nakayama A, Murakami K, Shimizu T, Yamanashi Y, Kasuga H, Nakashima H, Nakamura T, Takada Y, Kawamura Y, Inoue H, Okada C, Utsumi Y, Ikebuchi Y, Ito K, Nakamura M, Shinohara Y, Hosoyamada M, Sakurai Y, Shinomiya N, Hosoya T, Suzuki H (2012) Decreased extra-renal urate excretion is a common cause of hyperuricemia. Nat Commun 3:764.

Jinnah HA, Visser JE, Harris JC, Verdu A, Larovere L, Ceballos-Picot I, Gonzalez-Alegre P, Neychev V, Torres RJ, Dulac O, Desguerre I, Schretlen DJ, Robey KL, Barabas G, Bloem BR, Nyhan W, De Kremer R, Eddey GE, Puig JG, Reich SG (2006) Delineation of the motor disorder of Lesch-Nyhan disease. Brain 129:1201-1217.

Kang DH, Ha SK (2014) Uric Acid Puzzle: Dual Role as Anti-oxidantand Pro-oxidant. Electrolyte Blood Press 12:1-6.

Katsiki N, Mikhailidis DP, Nair DR (2014) The effects of antiepileptic drugs on vascular risk factors: a narrative review. Seizure 23:677-684.

Kettenmann H (2007) Neuroscience: the brain's garbage men. Nature 446:987-989.

Knights MJ, Finlay E (2014) The effects of sodium valproate on the renal function of children with epilepsy. Pediatr Nephrol 29:1131-1138.

Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120:1939-1949.

Kono H, Onda A, Yanagida T (2014) Molecular determinants of sterile inflammation. Curr Opin Immunol 26:147-156.

Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa C, Hammad H, Lambrecht BN (2011) An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. Immunity 34:527-540.

Kovacs Z, Kekesi KA, Juhasz G, Barna J, Heja L, Lakatos R, Dobolyi A (2015) Non-adenosine nucleoside inosine, guanosine and uridine as promising antiepileptic drugs: a summary of current literature. Mini Rev Med Chem 14:1033-1042.

Krause KH, Berlit P, Schmidt-Gayk H, Schellenberg B (1987) Antiepileptic drugs reduce serum uric acid. Epilepsy Res 1:306-307.

Kutzing MK, Firestein BL (2008) Altered uric acid levels and disease states. J Pharmacol Exp Ther 324:1-7.

Layton ME, Samson FE, Pazdernik TL (1998) Kainic acid causes redox changes in cerebral cortex extracellular fluid: NMDA receptor activity increases ascorbic acid whereas seizure activity increases uric acid. Neuropharmacology 37:149-157.

Lee SK (2014) Old versus New: Why Do We Need New Antiepileptic Drugs? J Epilepsy Res 4:39-44.

Livingston JR, Payne B, Brown M, Roberts JM, Cote AM, Magee LA, von Dadelszen P (2014) Uric Acid as a predictor of adverse maternal and perinatal outcomes in women hospitalized with preeclampsia. J Obstet Gynaecol Can 36:870-877.

Luhdorf K, Petersson H, Pedersen K (1978) Grand mal-provoked hyperuricemia. Acta Neurol Scand 58:280-287.

Makki N, Hajj G, Schmidt GA (2013) Seizure-induced acute urate nephropathy: case report and review. Chest 144:666-669.

Mandal AK, Mount DB (2015) The molecular physiology of uric acid homeostasis. Annu Rev Physiol 77:323-345.

Manzke H, Staemmler W, Dorner K (1981) Increased nucleotide catabolism after cerebral convulsions. Neuropediatrics 12:119-131.

Markowitz CE, Spitsin S, Zimmerman V, Jacobs D, Udupa JK, Hooper DC, Koprowski H (2009) The treatment of multiple sclerosis with inosine. J Altern Complement Med 15:619-625.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16:413-419.

Massey CA, Sowers LP, Dlouhy BJ, Richerson GB (2014) Mechanisms of sudden unexpected death in epilepsy: the pathway to prevention. Nat Rev Neurol 10:271-282.

Matzinger P (1994) Tolerance, danger, and the extended family. Annu Rev Immunol 12:991-1045.

Meng XF, Tan L, Tan MS, Jiang T, Tan CC, Li MM, Wang HF, Yu JT (2014) Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. J Neuroinflammation 11:212.

Menon B, Ramalingam K, Kumar RV (2014) Low plasma antioxidant status in patients with epilepsy and the role of antiepileptic drugs on oxidative stress. Ann Indian Acad Neurol 17:398-404.

Mikhailov IB, Gusel VA (1983) [Pharmaco-dynamic mechanisms for reducing the activity of an epileptogenic focus with allopurinol]. Zh Nevropatol Psikhiatr Im S S Korsakova 83:870-873.

Moshe SL, Perucca E, Ryvlin P, Tomson T (2015) Epilepsy: new advances. Lancet 385:884-898.

Mueller K, Kunko PM (1990) The effects of amphetamine and pilocarpine on the release of ascorbic and uric acid in several rat brain areas. Pharmacol Biochem Behav 35:871-876.

Murashima YL, Kasamo K, Suzuki J (1998) Antiepileptic effects of allopurinol on EL mice are associated with changes in SOD isoenzyme activities. Epilepsy Res 32:254-265.

Ng G, Sharma K, Ward SM, Desrosiers MD, Stephens LA, Schoel WM, Li T, Lowell CA, Ling CC, Amrein MW, Shi Y (2008) Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. Immunity 29:807-818.

O'Neill RD, Lowry JP (1995) On the significance of brain extracellular uric acid detected with in-vivo monitoring techniques: a review. Behav Brain Res 71:33-49.

Oda M, Satta Y, Takenaka O, Takahata N (2002) Loss of urate oxidase activity in hominoids and its evolutionary implications. Mol Biol Evol 19:640-653.

Oses JP, Viola GG, de Paula Cognato G, Junior VH, Hansel G, Bohmer AE, Leke R, Bruno AN, Bonan CD, Bogo MR, Portela LV, Souza DO, Sarkis JJ (2007) Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res 75:104-111.

Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315-424.

Parada-Turska J, Czuczwar M, Kis J, Czuczwar P, Cioczek A, Luszczki J, Czuczwar SJ (2004) Allopurinol does not affect the anticonvulsant activity of carbamazepine and valproate in maximal electroshock-induced convulsions in mice. Pol J Pharmacol 56:67-72.

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF, Jr., Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R, Verkhratsky A (2012) Glial cells in (patho)physiology. J Neurochem 121:4-27.

Perucca P, Gilliam FG (2012) Adverse effects of antiepileptic drugs. Lancet Neurol 11:792-802.

Perucca E, Glynn M, Baulac M, Boer Hd, C CE, Kälviäinen R, Little A, Mifsud J, Pitkänen A (2013) Epilepsy Research Priorities in Europe: ERF report 2013.

Pitkanen A, Lukasiuk K (2011) Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol 10:173-186.

Puttachary S, Sharma S, Stark S, Thippeswamy T (2015) Seizure-induced oxidative stress in temporal lobe epilepsy. Biomed Res Int 2015:745613.

Pylvanen V, Pakarinen A, Knip M, Isojarvi J (2006) Insulin-related metabolic changes during treatment with valproate in patients with epilepsy. Epilepsy Behav 8:643-648.

Quintas R, Raggi A, Giovannetti AM, Pagani M, Sabariego C, Cieza A, Leonardi M (2012) Psychosocial difficulties in people with epilepsy: a systematic review of literature from 2005 until 2010. Epilepsy Behav 25:60-67.

Rambo LM, Ribeiro LR, Della-Pace ID, Stamm DN, da Rosa Gerbatin R, Prigol M, Pinton S, Nogueira CW, Furian AF, Oliveira MS, Fighera MR, Royes LF (2013) Acute creatine administration improves mitochondrial membrane potential and protects against pentylenetetrazol-induced seizures. Amino Acids 44:857-868.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Rock KL, Latz E, Ontiveros F, Kono H (2010) The sterile inflammatory response. Annu Rev Immunol 28:321-342.

Rodriguez-Nunez A, Cid E, Rodriguez-Garcia J, Camina F, Rodriguez-Segade S, Castro-Gago M (2000) Cerebrospinal fluid purine metabolite and neuron-specific enolase concentrations after febrile seizures. Brain Dev 22:427-431.

Sander JW, Patsalos PN (1988) Allopurinol as an add-on drug in the management of intractable epilepsy. Epilepsy Res 2:223-225.

Sautin YY, Johnson RJ (2008) Uric acid: the oxidant-antioxidant paradox. Nucleosides Nucleotides Nucleic Acids 27:608-619.

Schwartz RM, Boyes S, Aynsley-Green A (1989) Metabolic effects of three ketogenic diets in the treatment of severe epilepsy. Dev Med Child Neurol 31:152-160.

Sevanian A, Davies KJ, Hochstein P (1991) Serum urate as an antioxidant for ascorbic acid. Am J Clin Nutr 54:1129S-1134S.

So E, Ptacek L (1988) Use of allopurinol as an adjunct therapy in intractable epilepsy. Epilepsia 671.

Stover JF, Lowitzsch K, Kempski OS (1997) Cerebrospinal fluid hypoxanthine, xanthine and uric acid levels may reflect glutamate-mediated excitotoxicity in different neurological diseases. Neurosci Lett 238:25-28.

Sussman M, Callanan M, Jackel R, Harner R (1987) Allopurinol not effective as adjunctive therapy for epilepsy. Epilepsia 28:590.

Tada H, Morooka K, Arimoto K, Matsuo T (1991) Clinical effects of allopurinol on intractable epilepsy. Epilepsia 32:279-283.

Tan TY, Lu CH, Chuang HY, Lin TK, Liou CW, Chang WN, Chuang YC (2009) Long-term antiepileptic drug therapy contributes to the acceleration of atherosclerosis. Epilepsia 50:1579-1586.

Taweesuk P, Tannirandorn Y (2014) Clinical and laboratory parameters associated with eclampsia in Thai pregnant women. J Med Assoc Thai 97:139-146.

Thurman DJ, Beghi E, Begley CE, Berg AT, Buchhalter JR, Ding D, Hesdorffer DC, Hauser WA, Kazis L, Kobau R, Kroner B, Labiner D, Liow K, Logroscino G, Medina MT, Newton CR, Parko K, Paschal A, Preux PM, Sander JW, Selassie A, Theodore W, Tomson T, Wiebe S (2011) Standards for epidemiologic studies and surveillance of epilepsy. Epilepsia 52 Suppl 7:2-26.

Togha M, Akhondzadeh S, Motamedi M, Ahmadi B, Razeghi S (2007) Allopurinol as adjunctive therapy in intractable epilepsy: a double-blind and placebo-controlled trial. Arch Med Res 38:313-316.

Tomioka NH, Nakamura M, Doshi M, Deguchi Y, Ichida K, Morisaki T, Hosoyamada M (2013) Ependymal cells of the mouse brain express urate transporter 1 (URAT1). Fluids Barriers CNS 10:31.

Vezzani A, Auvin S, Ravizza T, Aronica E (2012) Glia-neuronal interactions in ictogenesis and epileptogenesis: role of inflammatory mediators.

Vezzani A, Balosso S, Maroso M, Zardoni D, Noe F, Ravizza T (2010) ICE/caspase 1 inhibitors and IL-1beta receptor antagonists as potential therapeutics in epilepsy. Curr Opin Investig Drugs 11:43-50.

Vezzani A, Baram TZ (2007) New roles for interleukin-1 Beta in the mechanisms of epilepsy. Epilepsy Curr 7:45-50.

Vezzani A, Friedman A, Dingledine RJ (2013) The role of inflammation in epileptogenesis. Neuropharmacology 69:16-24.

Vezzani A, Viviani B (2014) Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. Neuropharmacology.

Wada Y, Hasegawa H, Nakamura M, Yamaguchi N (1992) Anticonvulsant effect of allopurinol on hippocampal-kindled seizures. Pharmacol Biochem Behav 42:899-901.

Warren DJ, Leitch AG, Leggett RJ (1975) Hyperuricaemic acute renal failure after epileptic seizures. Lancet 2:385-387.

Waterfall AH, Singh G, Fry JR, Marsden CA (1995) Detection of the lipid peroxidation product malonaldehyde in rat brain in vivo. Neurosci Lett 200:69-72.

Zagnoni PG, Bianchi A, Zolo P, Canger R, Cornaggia C, D'Alessandro P, DeMarco P, Pisani F, Gianelli M, Verze L, et al. (1994) Allopurinol as add-on therapy in refractory epilepsy: a double-blind placebo-controlled randomized study. Epilepsia 35:107-112.

Results

Research Aims

(1) develop and characterize a seizure model that allows to monitor the changes in local uric acid levels
(2) investigate the effects of pharmacological and genetic uric acid modulations on the seizure characteristics of this model
(3) investigate the effects of these manipulations on the seizure-associated inflammatory response
(4) examine if altering uric acid levels changes seizure characteristics in a different seizure model

Chapter 4

Induction of the NLRP3 inflammasome after intrahippocampal kainic acid induced status epilepticus in freely moving mice: a link with uric acid

Lisa Thyrion, Robrecht Raedt, Griet Glorieux, Wytse Wadman, Sophie Janssens, Bart N. Lambrecht, Lars E. Larsen, Mathieu Sprengers, Wouter Van Lysebettens, Jean Delbeke, Evelien Carrette, Kristl Vonck, Paul Boon

Neuroscience letters, Under review

Abstract

Inflammation is increasingly recognized to contribute to the epileptogenic process that occurs after a precipitating insult, such as status epilepticus. Uric acid has long been considered a waste product, but is increasingly shown to act as a pro-inflammatory danger signal. The objective of our study was to analyze if uric acid is released in a mouse model for limbic status epilepticus and if markers of the NLRP3 inflammasome pathway, the best described inflammatory pathway triggered by uric acid, were induced. Limbic status epilepticus was induced by infusion of kainic acid in the hippocampus of freely moving mice. Microdialysis was used to monitor changes in extracellular levels of uric acid during status epilepticus. One day after kainic acid infusion, expression of inflammatory markers was analyzed using RT-qPCR. Status epilepticus was associated with uric acid release and the induction of the expression of NLRP3 and ASC, two components of the inflammasome, and several downstream inflammatory cytokines. Interestingly, the extent of uric acid release correlated with the amount of epileptic discharges on the EEG and NLRP3 induction. This demonstrates that the release of uric acid depends on the severity of limbic status epilepticus and correlates with the induction of the NLRP3-dependent inflammatory pathway. Our data shed new light on uric acid as a potential player in the epileptogenic process, which may have therapeutic implications.

Key words: uric acid, seizures, inflammation, hippocampus, microdialysis, video-EEG

Introduction

Epilepsy is the second most common neurological disorder after stroke and affects 1% of the world population (Hauser et al., 1993). Temporal lobe epilepsy (TLE) is the most common form of epilepsy and highly pharmacoresistant to the currently available anti-epileptic drugs. TLE often develops after a precipitating brain insult, such as trauma, encephalitis and status epilepticus (SE) (Berg, 2008, Tatum, 2012). These brain insults initiate a cascade of molecular and cellular changes that increase the excitability of the neuronal network, with a growing number of molecular players and pathways being identified (Pitkanen and Lukasiuk, 2011). A large knowledge gap still exists however, and a better understanding of the insult-triggered processes leading to epilepsy is necessary to find improved and possible disease-modifying therapies (Loscher and Brandt, 2010).

Danger signals are endogenous molecules that are released upon a brain insult by stressed or damaged cells (Matzinger, 1994). Upon release in the extracellular environment they induce a pro-inflammatory response that can increase brain excitability and contribute to the development of epilepsy (i.e. epileptogenesis) (Maroso et al., 2010, Vezzani et al., 2013). Although uric acid has long been considered a mere waste product, it is increasingly shown to act as a pro-inflammatory danger signal in multiple disorders (Kono et al., 2010, Kool et al., 2011, Rock et al., 2013). The best described inflammatory pathway triggered by uric acid involves signaling through the NLRP3 inflammasome, a universal detector of danger (Di Virgilio, 2013, Rock et al., 2013). At high concentrations uric acid crystallizes into monosodium urate (MSU) crystals, that triggers the assembly of NLRP3 with the scaffolding protein ASC. This results in the recruitment and activation of caspase-1 and the subsequent cleavage of pro-IL-1β into its biologically active form (Rock et al., 2013). IL-1β plays a major role in the pro-inflammatory response to tissue injury and its involvement in epileptogenesis and the pathogenesis of seizures is well established. It can increase brain excitability by both non-conventional intracellular pathways and the induction of NF-kB-mediated expression of downstream players (Vezzani et al., 2012).

This study investigated whether experimental SE is associated with the release of uric acid and the subsequent induction of the NLRP3 inflammasome. For this purpose, we adapted the intrahippocampal kainic acid mouse model (Bouilleret et al., 1999) to infuse kainic acid in the hippocampus using microdialysis instead of injecting a bolus. This adaptation allowed to continuously measure changes in extracellular uric acid levels during SE. One day after SE induction, expression of the NLRP3 inflammasome and downstream inflammatory markers involved in excitability was evaluated.

Material and methods

Animals

Male C57BL/6 JolaHsd mice (n = 36) (Harlan, Horst, The Netherlands) were housed at controlled temperature (21°-22°C) and relative humidity (40-60%) conditions with a fixed 12-hour light/dark cycle (lights-on period from 7 AM to 7 PM) and food and water available ad libitum. Treatment and care were in compliance with the guidelines of the European Ethical Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University (ECD 09/61). The weight of the animals was 20-30 g at the start of the experiment.

Chemicals and solutions

Modified Ringer's solution, the perfusion solution used for microdialysis, consisted of 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl (all Sigma Aldrich, Schnelldorf, Germany) in ultrapure water. The solution was filtered through a 0.2 µm membrane and stored at 4°C (one week shelf life). Kainic acid (KA; R&D systems, Abingdon, United Kingdom) was dissolved in modified Ringer's solution at a concentration of 4 mg/ml, filtered through a 0.2 µm membrane filter and kept in aliquots at -20°C. On the day of use it was diluted further with modified Ringer's solution to a concentration of 500 µg/ml.

Induction of limbic status epilepticus

Anesthetized mice (isoflurane: 5% induction, 1.5% maintenance) were implanted with a custom-made polyimide-coated bipolar electrode (wire Ø 70 µm) with 0.5 mm tip separation, in which the deepest electrode contact was placed in the upper blade of the left dentate gyrus in the septal hippocampus (coordinates relative to bregma: anterioposterior: -1.8 mm; mediolateral: -1.0 mm, dorsoventral: -2.0 mm). Screw electrodes were placed epidurally over the right frontal and the right parietal cortex, the latter functioning as ground. An anchor screw was placed epidurally over the left frontal cortex and another one over the left parietal cortex. An obturator-enclosed guide cannula was placed in the septotemporal transition zone of the hippocampus (coordinates relative to bregma: anterioposterior: -3.3 mm ; mediolateral: -2.8 mm ; dorsoventral: -2.3 mm). After a recovery period of one week mice were connected to the EEG set-up and the guide cannula obturator was replaced with a microdialysis probe (CMA/7; 2 mm membrane length; theoretical cut-off 6 kDa; CMA, Kista, Sweden). The probe was continuously perfused with modified Ringer's solution at a flow rate of 1.25 µl/min. A minimal 12 hour interval between probe implantation and the start of the experiment was respected to ensure integrity of the blood brain barrier (Benveniste, 1989), absence of excessive reactive gliosis in the tissue surrounding the microdialysis probe (Georgieva et al., 1993) and stable exchange of molecules between the dialysate and the brain parenchyma (Clapp-Lilly et al., 1999). At the inlet (internal

volume: 3.6 µl) and outlet (internal volume: 3.9 µl), the probe was connected to 35 cm of FEP-tubing (internal volume 4.2 µl) (CMA). The next day, at 11 AM, a 500 µg/ml KA solution was infused for 3.2 minutes, after which the solution was switched back to modified Ringer's solution (n = 18). In the non-KA control group, infusion with modified Ringer's solution was not interrupted (n = 15). Taking into consideration an estimated microdialysis probe recovery of 10%, we aimed to deliver 200 ng of KA, which corresponds to the dose that Bouilleret and colleagues administered by bolus injection for the induction of SE in anesthetized mice (Bouilleret et al., 1999). Taking into account the internal volume of pre-inlet FEP tubing and inlet, KA entered the brain 6.3 minutes after the start of its infusion.

Video-EEG monitoring

After probe placement, mice were connected to the video-EEG monitoring set-up. Their head cap was connected to a headstage carrying a unity gain preamplifier. A flexible cable (Product# 363-363 6T (CS), Plastics one, Düsseldorf, Germany) and a 6-channel commutator (Product# SL6C, Plastics one) connected the headstage with a custom-built amplifier. The amplified (500x) analog EEG signals (referenced to ground) were digitized at a sampling rate of 2 kHz using a data acquisition card (NI-USB-6259, National Instruments, Zaventem, Belgium) and data were stored onto a hard-drive for later offline analysis. Video images were captured with an infrared-sensitive camera (CAMCOLBUL5, Velleman, Gavere, Belgium) coupled to a video card (USB Live 2.0, Hauppage, Dartford, United Kingdom) using a freeware program (VirtualDub). Both video and EEG were recorded on the same computer and were time-stamped for later off-line synchronization. Video-EEG was continuously monitored during 400 minutes, from 100 minutes prior to KA infusion until 300 minutes after infusion. EEG was analyzed using a Matlab-based routine.

EEG-analysis

An automated detection algorithm was used to detect epileptic spikes in the hippocampal EEG. According to this algorithm, raw hippocampal EEG-data was digitally filtered with a band pass filter between 1-50 Hz. The threshold for spike detection was set as 3 times the standard deviation of the EEG amplitude during the first 50 minutes of baseline recording, with every upward crossing considered to be a spike. Latency to start of seizure activity was defined as time between entry of KA in the brain and the appearance of the first epileptic spike in the hippocampal EEG. SE duration was defined electrographically as time between the first epileptic spike and the moment spike frequency dropped below 1 Hz for more than one hour (Grimonprez et al., 2015). To assess the progression of electrographic epileptic activity, mean spike frequency was analyzed in 10-minute epochs using automated spike detection.

Video-analysis

Clinical seizures were counted and scored based on a modified scale of Racine (Racine, 1972): (2) head nodding (3) unilateral forelimb clonus (4) bilateral forelimb clonus with rearing (5) rearing and falling, loss of postural control (6) running and bouncing. Clinical seizures with score 1 (immobility, eye closure, staring) were not included because they could not be discriminated from continuous abnormal background behavior.

Measurement of uric acid

Dialysate sampling started simultaneous with video-EEG monitoring at 8.30 AM. The microdialysis probe was continuously perfused with modified Ringer's solution, except during infusion with KA (see higher). Hippocampal dialysates were collected in 50 minute epochs. Two baseline samples were collected before and six samples after KA infusion. Uric acid was quantified in the dialysates by HPLC using a Waters Alliance 2695 HPLC device (Waters, Zellik, Belgium) connected to a Waters 996 photodiode array detector according to an adapted protocol described by Fagugli and colleagues (Fagugli et al., 2002). Data acquisition and processing were performed using Empower software (Waters). Analyses were performed on a reverse-phase XBridge C8 column (3.5 µm, 150 mm x 4.6 mm, Waters) with an Ultrasphere ODS guard column (5 µm, 5 mm x 4.6 mm, Beckman Instruments, Fullerton, CA). The chromatographic separation consisted of a linear gradient of methanol and ammonium formate buffer (50 mM, pH 3.0) from 0 to 100% methanol in 15 min at a flow rate of 1 mL/min. Forty-five microliter of dialysate was automatically injected. Uric acid was analyzed by UV detection at 300 nm. Standard solutions were used to perform calibration curves for uric acid quantification. For concentrations below the limit of detection (LOD; 0.119 µM) the LOD itself was used.

RNA-isolation and RT-qPCR

Twenty-six hours after KA infusion, mice were deeply anesthetized with 5% isoflurane and decapitated. The brains were quickly removed and placed in ice-cold phosphate buffered saline (PBS; 100 mM, pH 7.4). Using cooled microdissection material the left (infused) hippocampus was isolated and immediately frozen in liquid nitrogen. Frozen hippocampal tissue was homogenized in QIAzol (Qiagen) with a homogenizer (IKA) and total RNA was extracted using the RNeasy Lipid Tissue kit (Qiagen). An additional ethanol-precipitation step was performed to increase purity of the samples. RNA concentration and purity was verified using Nanodrop. Genomic DNA was removed using a TURBO DNAse kit (Ambion) and first strand cDNA synthesis was performed using the Superscript III first strand synthesis system for RT-qPCR (Invitrogen), both according to manufacturer's instructions. Real-time quantitative polymerase chain reactions (RT-qPCR) were done using the Fast SYBR master mix kit (Applied Biosystems) or SensiFastTM SYBR No-Rox kit (Bioline). Primers were designed making use of Primerbank (www.pga.mgh.harvard.edu/primerbank) (Table

1). All reactions were performed in triplicate and plates were run on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) or on a Lightcycler 480 (Roche). Relative expression was calculated using an improved version of the ΔΔ-Ct-method (Livak and Schmittgen, 2001). Data were normalized to the geometric average of multiple internal standard genes (Vandesompele et al., 2002). Values represent the average normalized relative expression values, with the expression level of animals infused with modified Ringer's solution set to 1. Two samples were left out because of instability of the internal standard genes.

Histology

Mice (n = 3) were given an overdose of pentobarbital (Nembutal, 180 mg/kg) and were transcardially perfused with cold PBS (100 mM) and a cold 4% paraformaldehyde/0.2% glutaraldehyde solution in PBS next (both pH 7.4). Brains were post-fixed overnight, cryoprotected in an increasingly higher sucrose solution (10 – 20 – 30%), snap-frozen in -30°C isopentane and stored in liquid nitrogen. Coronal frozen sections of 40 μm were made and the location of the depth electrode and cannula were verified on the brain slices (Figure S1).

Statistical analysis

Values are expressed as (geometric) mean and the corresponding 95% confidence interval (CI). Statistical analyses were performed in SPSS Statistics version 22. Uric acid changes were compared using a two-way ANOVA with dialysate collection period as within group factor and treatment as between group factor, with post-hoc Bonferroni-Holm correction. Expression levels were compared using independent t-tests. Correlation analyses were done using Spearman's Rho, excluding one significant outlier as determined by the Grub's test. Differences were considered significant at the 5% level.

Target genes

IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-1α	CTGATGAAGCTCGTCAGGCAG	TGGTGCTGAGATAGTGTTTGTC
IL-1Ra	GCTCATTGCTGGGTACTTACAA	CCAGACTTGGCACAAGACAGG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
COX2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
ASC	CAGCACAGGCAAGCACTCA	GGTGGTCTCTGCACGAACT
CASP1	GGGACCCTCAAGTTTTGCC	GACGTGTACGAGTGGTTGTATT
NLRP3	TACGGCCGTCTACGTCTTCT	CGCAGATCACACTCCTCAAA

Reference genes

HMBS	GAAACTCTGCTTCGCTGCATT	TGCCCATCTTTCATCACTGTATG
TBP	TCTACCGTGAATCTTGGCTGTAAA	TTCTCATGATGACTGCAGCAAA
RPL13a	CCTGCTGCTCTCAAGGTTGTT	TGGTTGTCACTGCCTGGTACTT

Table 1 | Primersequences used for qPCR-amplification.

Results

Intrahippocampal kainic acid infusion induces status epilepticus

Intrahippocampal infusion with modified Ringer's solution did not induce any epileptic activity. Intrahippocampal infusion with kainic acid resulted in a status epilepticus in all animals without mortality. Mean latency to SE was 2.9 (2.3-3.5) minutes. The number of epileptic spikes increased steeply initially and reached a maximum between 40 and 50 minutes after kainic acid infusion. Thereafter epileptic spiking decreased slowly until a steep decrease marked the end of the SE. Epileptic spikes kept occurring afterwards, albeit at low frequency (< 1 Hz) (Figure 1). The average SE duration was 166 (143-190) minutes. Qualitative analysis of the EEG revealed that the initial phase of the SE was characterized by the occurrence of discrete electrographic seizures (Figure 1b), after which the frequency and complexity of epileptic spikes increased to become continuous (Figure 1c). Over time, spiking became interspersed with increasingly longer low-voltage periods (Figure 1d).

After the occurrence of the first epileptic spike, all kainic acid infused mice showed increased explorative behavior, extensive scratching and grooming and periods of staring. Against this background of continuous mild behavioral changes more severe clinical seizures occurred, with a variable recurrence between animals. On average, the SE was associated with 11 (8-14) clinical seizures (≥ Racine stage 2), and all animals displayed two or more convulsive (Racine stage 3-6) seizures. The first and last clinical seizure appeared 28 (17-39) and 147 (109-184) minutes after kainic acid infusion respectively.

Hippocampal levels of extracellular uric acid increase during status epilepticus

Mean uric acid concentration in baseline dialysate samples was comparable in control (0.23 µM (0.14 µM – 0.40 µM)) and kainic acid infused animals (0.37 µM (0.25 µM – 0.54 µM)). In contrast to continuous infusion with modified Ringer's solution, kainic acid infusion resulted in an increase in extracellular uric acid levels that largely paralleled seizure activity, with a maximum of 1.38 µM (0.96 µM – 2.00 µM) between 50 and 100 minutes after infusion ($p < 0.001$, $F = 11.548$, two way ANOVA) (Figure 2). This fourfold increase was followed by a steady decline in uric acid concentration over the subsequent collections. The overall change in uric acid levels (area under curve) correlated with the total amount of spikes ($p < 0.05$, $\rho = 0.510$).

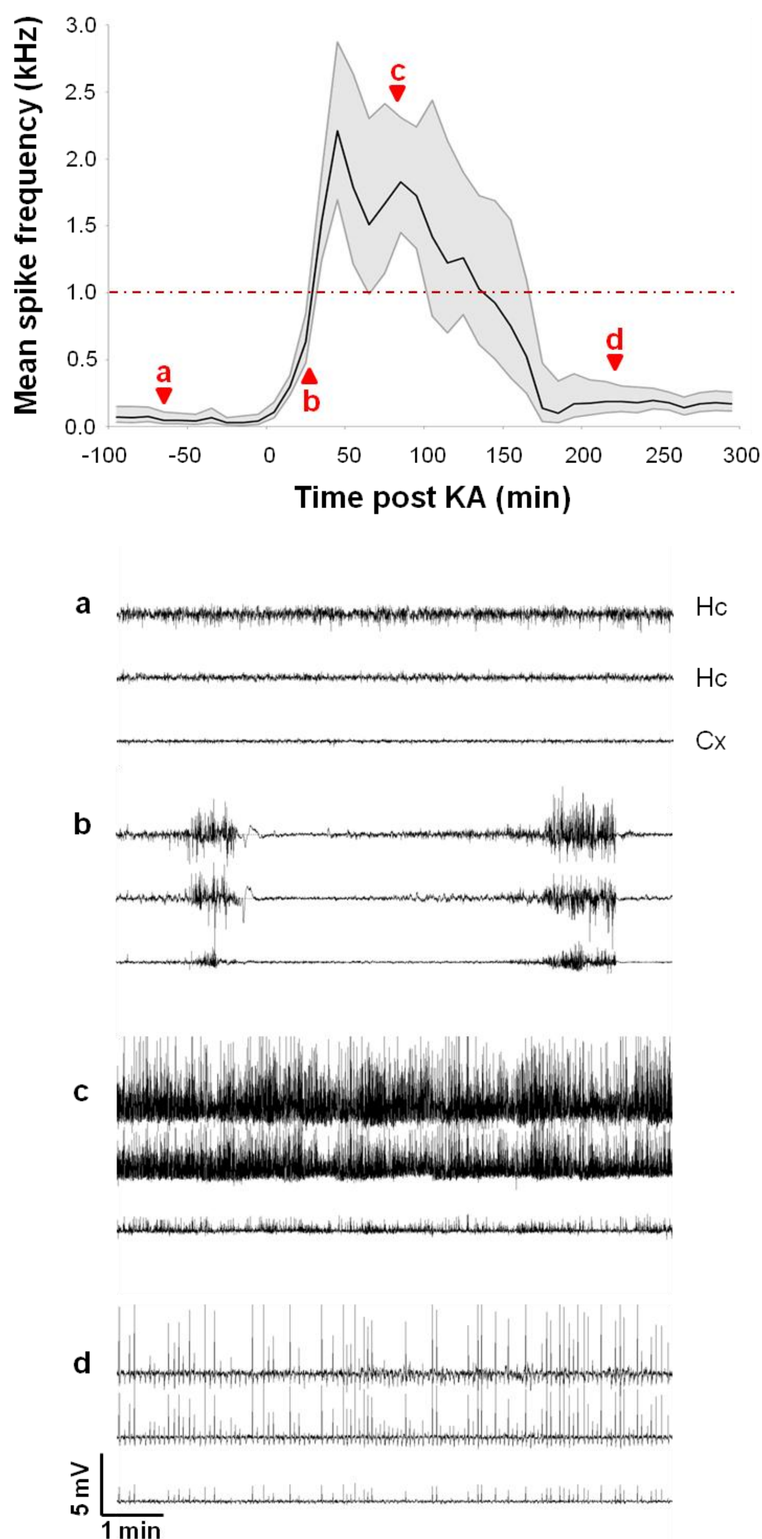


Figure 1 | Progression of KA-induced electrographic status epilepticus. Mean spike frequency per epoch was calculated as the spike count per epoch divided by its time (i.e. 600 seconds). Plot represents the geometric mean spike frequency (black) and 95% CI (grey) (n = 18). Red dotted line represents cut-off of 1 Hz that marks the end of the status epilepticus. **(a-d)** Electroencephalography (EEG) traces of 10 minute epochs illustrating typical EEG-recordings during KA-induced status epilepticus. (a) baseline, normal EEG (b) discrete seizures (c) continuous spiking (d) low-complexity, low-frequency spiking. Hc: hippocampus, Cx: cortex.

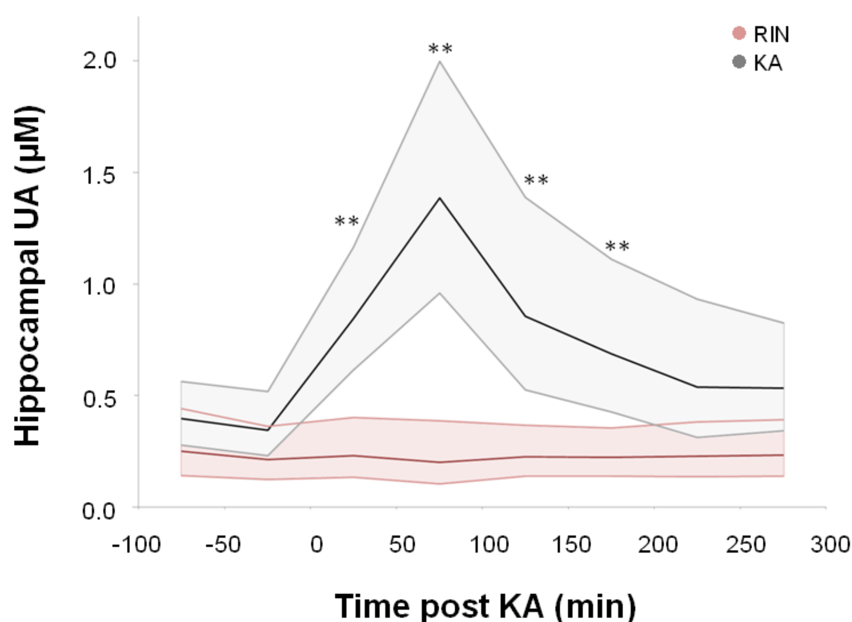


Figure 2| Progression of extracellular uric acid levels in the hippocampal region per 50-minute collection. Plot represents the geometric mean (dark color) and 95% CI (light color) **$p < 0.01$, two-way ANOVA, post-hoc Bonferroni-Holm. KA: kainic acid. UA: uric acid. RIN: modified Ringer's solution ($n_{KA} = 18$, $n_{RIN} = 15$).

Status epilepticus induces expression of the NLRP3 inflammasome and downstream cytokines

In animals infused with kainic acid, expression levels of both NLRP3 and ASC were more than twice as high compared to control animals ($p < 0.05$ and $p < 0.001$ respectively) (Figure 3). The relative expression of NLRP3 correlated with the overall change in uric acid levels (area under curve) ($p = 0.045$, $\rho = 0.543$) and with the number of spikes during the first collection period ($p < 0.05$, $\rho = 0.582$). Levels of CASP1 were increased by a third, although this was only a non-significant trend ($p = 0.073$). Several downstream target genes involved in excitability were also upregulated. mRNA levels of IL-6 and TNF were upregulated fivefold ($p < 0.001$) and sixfold ($p < 0.001$) respectively, while expression of IL-1β, IL-1α and COX2 was induced almost three times ($p < 0.01$, $p < 0.001$ and $p < 0.001$ respectively). Expression of IL-1 receptor antagonist (IL-1Ra) was eightfold increased ($p < 0.001$).

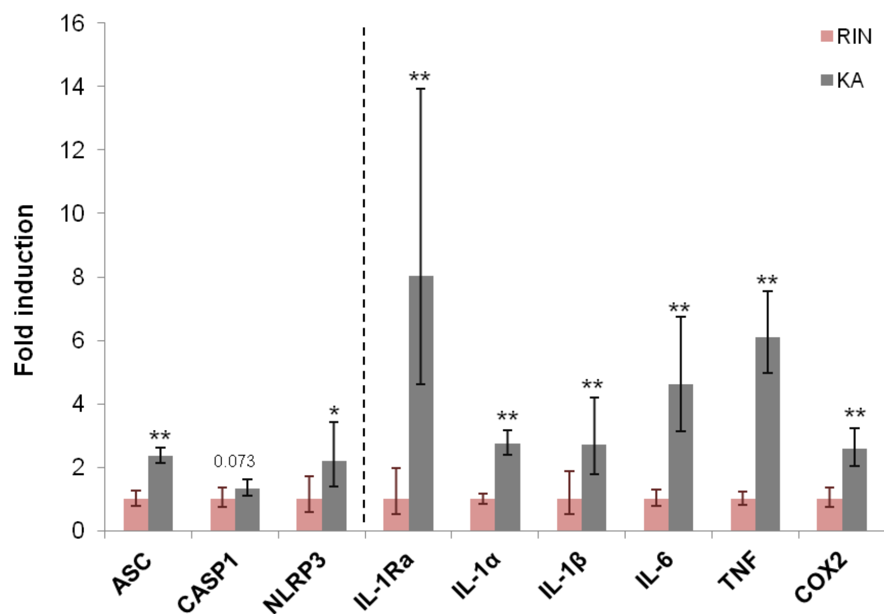


Figure 3 | Changes in expression levels of NLRP3 inflammasome components (left) and downstream inflammatory markers (right) in the ipsilateral hippocampus 26h after KA infusion as determined with RT-qPCR. Analysis was performed on total RNA-extracts of mice infused unilaterally with kainic acid (KA) (n = 15) or infused continuously with modified Ringer's solution (n = 10-12). mRNA expression levels are normalized relative to the geometric mean of the internal standard genes HMBS, RPL13a and TBP. For each cytokine, fold differences were calculated relative to the average cytokine levels in modified Ringer's infused control animals, which were set at 1. One significant outlier was excluded from the IL-1β (RIN) group (Grubb's test, $p < 0.05$). Bars represent geometric mean. Error bars represent 95% CI. * $p < 0.05$, ** $p < 0.01$, independent t-tests.

Discussion

In animal studies, SE is frequently induced as experimental trauma to model the underlying mechanisms leading to acquired epilepsy (Dudek et al., 2006). Intrahippocampal bolus injection of kainic acid is widely appreciated for induction of limbic SE in mice, because the subsequent epileptogenic process, seizure characteristics and histopathology resemble those of human TLE (Bouilleret et al., 1999, Riban et al., 2002, Meier et al., 2007, Pernot et al., 2011). Using a microdialysis probe placed in the hippocampus of freely moving mice, we aimed to deliver a similar amount of kainic acid as previously described for a single bolus injection (Bouilleret et al., 1999, Riban et al., 2002, Pernot et al., 2011). Analogous to our results, they reported the recurrence of isolated spikes and epileptic discharges (Riban et al., 2002, Pernot et al., 2011) and described a similar progression of the SE (Bouilleret et al., 1999). The duration of SE after intrahippocampal bolus injection was longer compared to our study, with the duration ranging from five (Bouilleret et al., 1999) to over ten hours (Riban et al., 2002, Pernot et al., 2011). This is likely due to the different genetic background of the mice and/or the properties of the microdialysis procedure. The pharmacokinetics and dynamics of microdialysis-based kainic acid infusion are highly different compared to bolus injection. In case of microdialysis a smaller volume is administered over a longer time period and a larger surface, which could result in a lower local concentration of kainic acid.
Using this intrahippocampal KA model, we demonstrated that limbic SE is associated with increased extracellular levels of uric acid and increased expression of NLRP3 inflammasome components and inflammatory markers. The overall change in uric acid levels positively correlated with the total amount of epileptic spikes and the relative expression of NLRP3.

Limbic SE is associated with the release of uric acid in the hippocampus

During SE local extracellular uric acid levels transiently increased up to four times the initial concentration. The amount of uric acid release was strongly associated with the amount of spiking and largely paralleled spiking activity. This is similar to a previous study where the extracellular uric acid levels doubled in the piriform cortex after intraperitoneal KA injection in rats (Layton et al., 1998). Analogous to our study, the steady increase aligned with seizure activity in their study. Moreover, when they injected midazolam to block the seizures, uric acid increases were abolished, indicating that it was a seizure related feature and not associated with other excitotoxic effects of kainic acid (Layton et al., 1998). Likely, SE-induced cellular damage and the associated increased turnover of purine-containing molecules, such as DNA and RNA, are responsible for the release of uric acid (Layton et al., 1998). SE-associated opening of the blood brain barrier could also account for this increase, as the concentration in plasma is ten times greater compared to that in the central nervous system (Tomioka et al., 2013). Similarly, the intracellular uric acid concentration is higher than the extracellular concentration, so active release from the intracellular uric acid pool might also contribute (Rock et al., 2013).

NLRP3 inflammasome components are induced after limbic SE and correlate with uric acid release

One day after kainic acid infusion, the hippocampal expression of inflammasome components NLRP3 and ASC was induced. In line with our findings, Meng and colleagues reported increased protein levels of NLRP3 in the hippocampus 24 hours after SE induced by amygdala stimulation. Using a knock-down technique they showed that chronic suppression of the NLRP3-inflammasome results in neuroprotection, decreased inflammation and suppression of the development and severity of spontaneous recurrent seizures (Meng et al., 2014). Our study showed that the induction of inflammasome expression positively correlated with the amount of released uric acid during SE. This suggests that lowering uric acid levels might also affect epileptogenesis in rodent models for post-traumatic epilepsy. Aside from the inflammasome components, expression of downstream inflammatory markers IL-1β, IL-1α, IL-1Ra, IL-6, TNF and COX2 was also increased one day after kainic acid infusion. This is in accordance with previous reports that showed increased levels of these markers after administration of different chemoconvulsants, including kainic acid, both on mRNA and protein level (Pernot et al., 2011; Vezzani et al., 2012; Zhang and Zhu, 2011), which reinforces the reliability of this model.

Conclusions

Taken together, the intrahippocampal KA model can be considered a valuable model for limbic SE which not only avoids anesthesia and restraint, but also permits simultaneous manipulation and sampling of the local environment. In this study we employed this model to show that uric acid is released during SE and that the markers of its inflammatory pathway are induced the day after kainic acid infusion. Together with the fact that the release of uric acid correlates with SE severity and the relative expression of NLRP3, these results hint on a role for uric acid in epileptogenesis.

Funding

Lisa Thyrion is supported by a junior researcher grant from the 'Bijzonder onderzoeksfonds (BOF)' of Ghent University (01D22511). Prof. Dr. R. Raedt and Prof. Dr. K. Vonck are supported by a BOF-grant from Ghent University Hospital. Prof. Dr. P. Boon is supported by grants from the 'Fonds Wetenschappelijk Onderzoek (FWO)', from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital. The funding sources did not have any involvement in the study design; in the collection, analysis and interpretation of the data; in the writing of the report; and in the decision to submit the article for publication.

Acknowledgements

The authors thank Maria Van Landschoot for the technical support in the HPLC analysis and Elke Ydens for the support in the RT-qPCR analyses.

References

Benveniste H (1989) Brain microdialysis. J Neurochem 52:1667-1679.

Berg AT (2008) The natural history of mesial temporal lobe epilepsy. Curr Opin Neurol 21:173-178.

Bouilleret V, Ridoux V, Depaulis A, Marescaux C, Nehlig A, Le Gal La Salle G (1999) Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience 89:717-729.

Clapp-Lilly KL, Roberts RC, Duffy LK, Irons KP, Hu Y, Drew KL (1999) An ultrastructural analysis of tissue surrounding a microdialysis probe. J Neurosci Methods 90:129-142.

CMA Principles of Probe Recovery.

Di Virgilio F (2013) The therapeutic potential of modifying inflammasomes and NOD-like receptors. Pharmacol Rev 65:872-905.

Dudek FE, Clark S, Williams PA, Grabenstatter HL (2006) Kainate-induced status epilepticus: a chronic model of acquired epilepsy. In: Models of Seizures and Epilepsy(Pitkänen, A. et al., eds), pp 415-432: Elsevier.

Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339-347.

Georgieva J, Luthman J, Mohringe B, Magnusson O (1993) Tissue and microdialysate changes after repeated and permanent probe implantation in the striatum of freely moving rats. Brain Res Bull 31:463-470.

Grimonprez A, Raedt R, Dauwe I, Mollet L, Larsen LE, Meurs A, De Herdt V, Wadman W, Delbeke J, Vonck K, Boon P (2015) Vagus Nerve Stimulation has Antidepressant Effects in the Kainic Acid Model for Temporal Lobe Epilepsy. Brain Stimul 8:13-20.

Hauser WA, Annegers JF, Kurland LT (1993) Incidence of epilepsy and unprovoked seizures in Rochester, Minnesota: 1935-1984. Epilepsia 34:453-468.

Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120:1939-1949.

Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa C, Hammad H, Lambrecht BN (2011) An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. Immunity 34:527-540.

Layton ME, Samson FE, Pazdernik TL (1998) Kainic acid causes redox changes in cerebral cortex extracellular fluid: NMDA receptor activity increases ascorbic acid whereas seizure activity increases uric acid. Neuropharmacology 37:149-157.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Loscher W, Brandt C (2010) Prevention or modification of epileptogenesis after brain insults: experimental approaches and translational research. Pharmacol Rev 62:668-700.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16:413-419.

Matzinger P (1994) Tolerance, danger, and the extended family. Annu Rev Immunol 12:991-1045.

Meier R, Haussler U, Aertsen A, Deransart C, Depaulis A, Egert U (2007) Short-term changes in bilateral hippocampal coherence precede epileptiform events. Neuroimage 38:138-149.

Meng XF, Tan L, Tan MS, Jiang T, Tan CC, Li MM, Wang HF, Yu JT (2014) Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. J Neuroinflammation 11:212.

Pernot F, Heinrich C, Barbier L, Peinnequin A, Carpentier P, Dhote F, Baille V, Beaup C, Depaulis A, Dorandeu F (2011) Inflammatory changes during epileptogenesis and spontaneous seizures in a mouse model of mesiotemporal lobe epilepsy. Epilepsia 52:2315-2325.

Pitkanen A, Lukasiuk K (2011) Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol 10:173-186.

Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32:281-294.

Riban V, Bouilleret V, Pham-Le BT, Fritschy JM, Marescaux C, Depaulis A (2002) Evolution of hippocampal epileptic activity during the development of hippocampal sclerosis in a mouse model of temporal lobe epilepsy. Neuroscience 112:101-111.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Tatum WOt (2012) Mesial temporal lobe epilepsy. J Clin Neurophysiol 29:356-365.

Tomioka NH, Nakamura M, Doshi M, Deguchi Y, Ichida K, Morisaki T, Hosoyamada M (2013) Ependymal cells of the mouse brain express urate transporter 1 (URAT1). Fluids Barriers CNS 10:31.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

Vezzani A, Auvin S, Ravizza T, Aronica E (2012) Glia-neuronal interactions in ictogenesis and epileptogenesis: role of inflammatory mediators.

Vezzani A, Friedman A, Dingledine RJ (2013) The role of inflammation in epileptogenesis. Neuropharmacology 69:16-24.

Supporting information

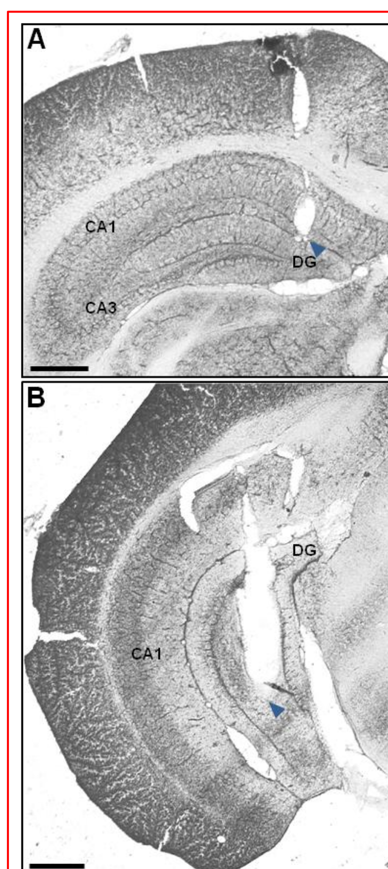


Figure S1 | Histological verification of bipolar depth-electrode (A) and microdialysis probe (B) placement in the left dorsal and septotemporal hippocampus respectively. Bar 500 μm. Blue arrow indicates the lower tip of the electrode/probe. DG: dentate gyrus, CA: cornu Ammonis.

Research Aims

(1) develop and characterize a seizure model that allows to monitor the changes in local uric acid levels:
(2) investigate the effects of pharmacological and genetic uric acid modulations on the seizure characteristics of this model
(3) investigate the effects of these manipulations on the seizure-associated inflammatory response
(4) examine if altering uric acid levels changes seizure characteristics in a different seizure model

Chapter 5

Uric acid is released in the brain during seizure activity and increases severity of seizures in a mouse model for acute limbic seizures

Lisa Thyrion, Robrecht Raedt, Jeanelle Portelli, Pieter Van Loo, Wytse J. Wadman, Griet Glorieux, Bart Lambrecht, Sophie Janssens, Kristl Vonck, Paul Boon

Experimental Neurology, 2016; 277:244-51

Abstract

Recent evidence points at an important role of endogenous cell-damage induced pro-inflammatory molecules in the generation of epileptic seizures. Uric acid, under the form of monosodium urate crystals, has shown to have pro-inflammatory properties in the body, but less is known about its role in seizure generation. This study aimed to unravel the contribution of uric acid to seizure generation in a mouse model for acute limbic seizures. We measured extracellular levels of uric acid in the brain and modulated them using complementary pharmacological and genetic tools. Local extracellular uric acid levels increased three to four times during acute limbic seizures and peaked between 50 and 100 minutes after kainic acid infusion. Manipulating uric acid levels through administration of allopurinol or knock-out of urate oxidase significantly altered the number of generalized seizures, decreasing and increasing them by a twofold respectively. Taken together, our results consistently show that uric acid is released during limbic seizures and suggest that uric acid facilitates seizure generalization.

Key words: acute limbic seizures; kainic acid; video-EEG; uric acid; allopurinol; urate oxidase

Introduction

Epilepsy is a disabling neurological disorder that affects up to 65 million people worldwide and is characterized by spontaneous and recurrent occurrence of seizures (Thurman et al., 2011). Although epileptic seizures are generally thought to be caused by an increased state of brain excitability, it is much less clear what is causing this hyperexcitability (Devinsky et al., 2013). Danger associated molecular patterns (DAMPs) are normal cell constituents that are released into the extracellular milieu during cellular stress or damage which subsequently activate the immune system (Matzinger, 1994). Recently, DAMPs have been shown to be able to increase excitability in the brain (Maroso et al., 2010). Uric acid is a purine degradation product that has multiple functions in the body (Fang et al., 2013). Soluble uric acid is well known for its anti-oxidant capacity, but its crystallized form is known to act as a DAMP in several disorders, such as gout, asthma and liver injury (Kono et al., 2010, Alvarez-Lario and Macarron-Vicente, 2011, Kool et al., 2011, Rock et al., 2013). There are some indications that uric acid might play a role as a DAMP in epilepsy as well. In epilepsy patients, uric acid levels acutely increase upon generalized seizures (Warren et al., 1975, Luhdorf et al., 1978, Stover et al., 1997) and can be chronically elevated when untreated (Hamed et al., 2004, Hamed et al., 2007). Uric acid was also reported to increase in the brain in two animal models, 100 minutes (Layton et al., 1998) and one week (Beal et al., 1991) after kainic acid (KA) administration. Furthermore, several components of the inflammatory signaling pathway downstream of uric acid have been shown to affect seizure characteristics in animal models for epilepsy, i.e. caspase-1 (Vezzani et al., 2010) and NLRP3 (Meng et al., 2014). Notably, this pathway leads to the activation of IL-1β, a central pro-inflammatory cytokine involved in excitability (Vezzani et al., 2011). Finally, a number of studies suggest that allopurinol, an inhibitor of uric acid production, is effective as anticonvulsive therapy (Wada et al., 1992, Zagnoni et al., 1994, Murashima et al., 1998, Togha et al., 2007), indicating that uric acid is not just an epiphenomenon but might actively contribute to the underlying pathophysiology.

In this study we investigated the role of uric acid in the generation of limbic seizures in a intrahippocampal KA mouse model. To do this, hippocampal uric acid levels were monitored during seizures using microdialysis and the effect of pharmacologically and genetically manipulating uric acid levels on their severity was investigated.

Material and methods

Chemicals and solutions

Modified Ringer's solution, the perfusion fluid used for microdialysis, consisted of 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl (all Sigma Aldrich, Schnelldorf, Germany) in ultrapure water and was kept at 4°C (one week shelf life). KA (R&D systems, Abington, United Kingdom) was dissolved in modified Ringer's solution at a concentration of 4 mg/ml and kept in aliquots at -20°C. It was diluted further with modified Ringer's solution to a concentration of 500 µg/ml on the experimental day. Allopurinol (Sigma Aldrich, Schnelldorf, Germany) was suspended in a saline:Tween 20 solution (9:1) at 10 mg/ml and stored at 4°C (one week shelf life). Uric acid (Sigma Aldrich, Schnelldorf, Germany) was prepared in modified Ringer's solution at a concentration of 1.6 mg/ml and was freshly made every day.

Animals

The male C57BL/6 JolaHsd mice that were used for the pharmacological experiments were obtained from Harlan (Horst, The Netherlands). Mice with genetically altered uric acid levels were obtained by targeting urate oxidase (UOx), the enzyme responsible for uric acid breakdown. Hemizygous *UOx* overexpressing (*UOx* OE) mice (C57BL/6 background) were obtained from Kenneth Rock, Department of Immunology, University of Massachusetts, Worcester, USA. The *UOx* transgene in these mice is driven by a strong constitutive (β-actin) promoter (Kono et al., 2010). Hemizygous *UOx* knock-out (*UOx* KO) mice were purchased from The Jackson Laboratory (B6;129S7/J background, Maine, USA) and crossbred. Offspring of these breedings were then genotyped for homozygosity and bred separately. The *UOx* KO mice have a constitutive disruption of the *UOx* gene, by insertion of a neomycin gene into exon 3. Allopurinol (200 mg/l) was provided in the drinking water of *UOx* KO breeding pairs and pups until 8 weeks of age to prevent lethality due to hyperuricemia (Wu et al., 1994). All mice were bred at the animal house of Ghent University Hospital and were housed at controlled temperature (21-22°C) and relative humidity (40-60%) conditions. They had a fixed 12-hour light/dark cycle (lights-on period from 7 AM to 7 PM) and food and water available ad libitum. Treatment and care were in compliance with guidelines from the European Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University (ECD 09/61). All animals were 3-5 months old at the start of the experiments.

Induction of limbic seizures

Anesthetized mice were implanted with a custom-made polyimide-coated bipolar electrode (wire Ø 70 µm) with 0.5 mm tip separation, in which the deepest electrode contact was placed in the upper layer of the left dentate gyrus in the septal hippocampus (coordinates relative to bregma: anterioposterior: -1.8 mm; mediolateral: -1.0 mm, dorsoventral: -2.0 mm). An obturator-enclosed guide cannula was placed in the septotemporal transition zone of the hippocampus (coordinates relative to bregma: anterioposterior: -3.3 mm ; mediolateral: -2.8 mm ; dorsoventral: -2.3 mm). After a recovery period of one week the mice were connected to the EEG set-up and the guide cannula obturator was replaced with a microdialysis probe (CMA/7; 2 mm membrane length; theoretical cut-off 6 kDa; CMA, Kista, Sweden) which was continuously perfused with modified Ringer's solution at a flow rate of 1.25 µl/min. The next day, at 11 AM, a 500 µg/ml KA solution was infused for 3.2 minutes, after which the solution was switched back to modified Ringer's solution. With this protocol 2 µg KA was infused trough the microdialysis probe. Taking into consideration an estimated microdialysis probe recovery of 10%, we aimed to deliver 200 ng of KA, which is the dose frequently used for induction of status epilepticus by bolus injection (Bouilleret et al., 1999). This procedure results in a limbic status epilepticus with secondary generalized seizures and is not associated with any mortality (Fig. S1, Movies S1-5). A control group, in which mice were continuously infused with modified Ringer's solution, but not with KA, was included to control for potential effects of microdialysis on the uric acid level in the region of the probe.

Video-EEG analysis

Latency between the start of intrahippocampal KA infusion and the appearance of the first epileptic spike on the hippocampal EEG was determined as well as the total duration of epileptic activity. The latter parameter was defined as time between the first epileptic spike and the moment that spike activity dropped below a frequency of 1 Hz for more than one hour (Grimonprez et al., 2015). Clinical seizures were counted in this period and scored based on a modified scale of Racine (Racine, 1972) (Movie S1-5): (stage 2) head nodding (stage 3) unilateral forelimb clonus (stage 4) bilateral forelimb clonus with rearing (stage 5) rearing and falling, loss of postural control (stage 6) running and bouncing. Clinical seizures with score 1 (extensive grooming, hyperactivity, eye closure, staring) were not included because they could not be discriminated from continuous abnormal background behavior. Clinical seizures with stage 2 were considered as partial seizures, seizures with stage 3-6 were grouped as generalized seizures (Raedt et al., 2009).

Measurement of uric acid

Uric acid was quantified by HPLC using a Waters Alliance 2695 HPLC device (Waters, Zellik, Belgium) connected to a Waters 996 photodiode array detector according to an adapted protocol described by Fagugli and colleagues (Fagugli et al., 2002). Data acquisition and processing were performed using Empower software (Waters). Analyses were performed on a reverse-phase XBridge C8 column (3.5 µm, 150 mm x 4.6 mm, Waters) with an Ultrasphere ODS guard column (5 µm, 5 mm x 4.6 mm, Beckman Instruments, Fullerton, CA). The chromatographic separation consisted of a linear gradient of methanol and ammonium formate buffer (50 mM, pH 3.0) from 0 to 100% methanol in 15 min at a flow rate of 1 mL/min. Forty-five microliter of dialysate was automatically injected. Uric acid was analyzed by UV detection at 300 nm. Standard solutions were used to perform calibration curves for uric acid quantification. For concentrations below the limit of detection (LOD; 0.112 µM) the LOD itself was used.

Protocol design

A schematic overview of the protocol design is shown in Figure 1. Video-EEG monitoring and dialysate sampling started at 8.30 AM. Hippocampal dialysates were sampled in 50 minute periods. Each experiment started with the collection of two baseline samples in which only modified Ringer's solution was perfused. After KA infusion (or at the equivalent timepoint in the non-KA group) another six dialysates were collected. To test the electroclinical effects of lowering uric acid levels, animals were injected i.p. with either a 100 mg/kg suspension of allopurinol (ALLO) or its dissolving solution (9:1 saline:Tween 20 solution; SAL-T) 30 minutes before KA infusion. To test the electroclinical effects of uric acid increases, animals were infused in the left hippocampus with a 1.6 mg/ml uric acid solution for 200 minutes at a flow rate of 1.25 µl/min, starting 50 minutes before KA infusion. This was compared to animals infused with modified Ringer's solution (RIN). To verify how much uric acid exited the probe, the relative probe recovery was analyzed *in vitro* (CMA, Lee et al., 2003). To do this, a microdialysis probe was placed in a vial containing modified Ringer's solution (62.5 µl) and perfused with the 1.6 mg/ml uric acid solution at a flow rate of 1.25 µl/min for 50 minutes. The uric acid concentration in the vial was determined. This was repeated three times for two independent probes. Based on these experiments the recovery was estimated to be 10.5% (Mean probe recovery for probe 1: 0.18 ± 0.06 mg/ml; for probe 2: 0.21 ± 0.07 mg/ml). Because uric acid infusion interferes with the local uric acid measurement, no dialysates were collected in the mice upon infusion with uric acid since it is not possible to assess the amount of endogenous uric acid release in this case. To test the effects of genetic uric acid alterations, *UOx* OE mice and *UOx* KO mice were compared to their wildtype controls (WT). Sampling and video-EEG monitoring ended five hours after KA infusion (4 PM), unless epileptic activity was still ongoing. In the latter case, monitoring was stopped only after the SE had ended.

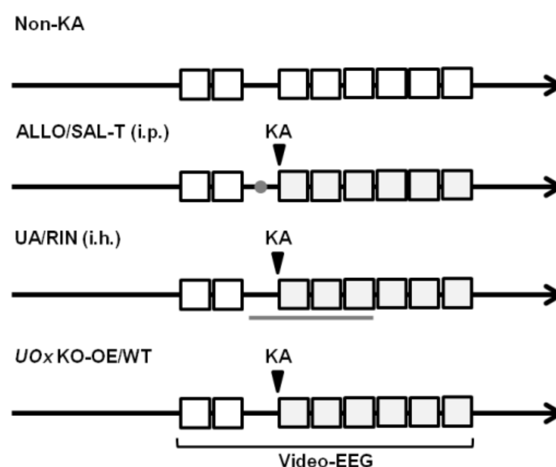


Figure 1 | Schematic overview of experimental protocols. Hippocampal dialysates were collected during 50-minute epochs and analyzed for uric acid (UA) concentration. In the non-KA control group modified Ringer's solution was continuously infused throughout the microdialysis experiment. In all other experimental groups KA was infused in the hippocampus after two baseline dialysate collections. After KA infusion (or at the equivalent timepoint) another six dialysates were collected. The different experimental approaches to alter uric acid levels are indicated in the figure: pharmacologically, uric acid levels were decreased using allopurinol (●, 100 mg/kg i.p., 30 min before KA-infusion, ALLO) and increased by infusion of a 1.6 mg/ml uric acid solution (—) during 200 minutes starting 50 minutes before KA-infusion; genetically, the urate oxidase (UOx) gene, which encodes the enzyme responsible for uric acid breakdown, was targeted. *UOx* knock-out (KO) mice and *UOx* overexpressing (OE) mice were used to study increased and decreased uric acid levels respectively. □: 50 minute dialysate collections; white: baseline; grey: post KA. KA: kainic acid, SAL-T: saline-tween, WT: wildtype

Statistical Analysis

For all experiments, the number of animals (n) analyzed are represented. All data are expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed in SPSS Statistics version 22. Parametric tests were used when data was normally distributed, and non-parametric tests were used otherwise. Comparisons between single groups (mean uric acid concentration, number of seizures, duration and latency) were analyzed with an independent t-test (parametric) or a Mann-Whitney U test (non-parametric). Within group evaluation of changes in uric acid concentration across collection periods was done using a one-way ANOVA for repeated measures (parametric) or Friedman's test (non-parametric). For between group evaluation of differences in uric acid concentration and number of generalized seizures across collection periods a two-way ANOVA with treatment/genotype as between-subject factor and time (i.e. collection) as within-subject factor was used (parametric). In case of non-parametric testing, Mann-Whitney U tests were done at each timepoint (i.e collection period). In case of multiple (post-hoc) comparisons, the p-values were corrected using a Bonferroni-Holm correction (parametric) or Dunn's test (non-parametric). Differences were considered significant at the 5% level.

Results

Control animals, infused with modified Ringer's solution, did not display epileptic activity and had stable levels of uric acid in the hippocampus over the total duration of the experiment (overall average 0.33 ± 0.01 µM, Figure S2). All mice that were intrahippocampally infused with kainic acid developed a status epilepticus without mortality.

Hippocampal uric acid increases during seizures and contributes to the number of subsequent seizures

KA-infused animals (SAL-T) displayed a significant increase in extracellular uric acid levels during the first 200 minutes (i.e. 4 collection periods) after KA infusion with a maximum of 1.40 ± 0.21 µM between 50 and 100 minutes after infusion. This 3.5x increase, relative to baseline uric acid concentration, was followed by a steady decline in uric acid concentration over the subsequent collections (Figure 2A). In order to investigate the effect of pharmacologically blocking uric acid increases on seizure activity, mice were treated with allopurinol (100 mg/kg, i.p.), an inhibitor of xanthine oxidase. As part of the purine degradation pathway this enzyme is responsible for the catalysis of xanthine into uric acid. Mean uric acid concentration in baseline conditions was comparable in both groups (SAL-T: 0.40 ± 0.06 µM; ALLO: 0.41 ± 0.07 µM) (Figure 2A). Allopurinol treatment blocked the transient KA-induced uric acid increase and even reduced its levels below baseline concentrations during the entire follow-up (Figure 2A; $\chi^2 = 23.876$, $p = 0.001$). This treatment did not affect latency and duration of epileptic activity nor the number of partial seizures. However it significantly decreased the number of generalized seizures by half (Figure 2B,C; SAL-T: 8.3 ± 1.6; ALLO: 4.1 ± 0.5; $t = 2.274$, $p = 0.041$). The progression of generalized seizures over time was also different compared to the saline treated animals ($F = 5.563$, $p = 0.008$). In the allopurinol treated group, generalized seizures were restricted to the first 100 minutes, whereas saline treated animals also displayed seizures after this period (Figure 2A).

Infusion of uric acid does not increase seizures

To next investigate the effect of uric acid increases on seizures, mice were infused with uric acid in the hippocampus. Mean uric acid baseline concentrations were comparable between treatment and control groups: 0.52 ± 0.12 µM (RIN) vs. 0.62 ± 0.09 µM (UA) (Figure 3A). Based on the recovery rate of the probe (10.5%), the average local concentration achieved for a 50 minute-collection was 1000 µM during uric acid infusion and 20 µM during peak measurement in the control condition. We hence infused 50 times more uric acid than what was released during peak measurement over a period of 200 minutes (4 collections). However, this increase had no effect on KA induced electroclinical changes (Figure 3A-C).

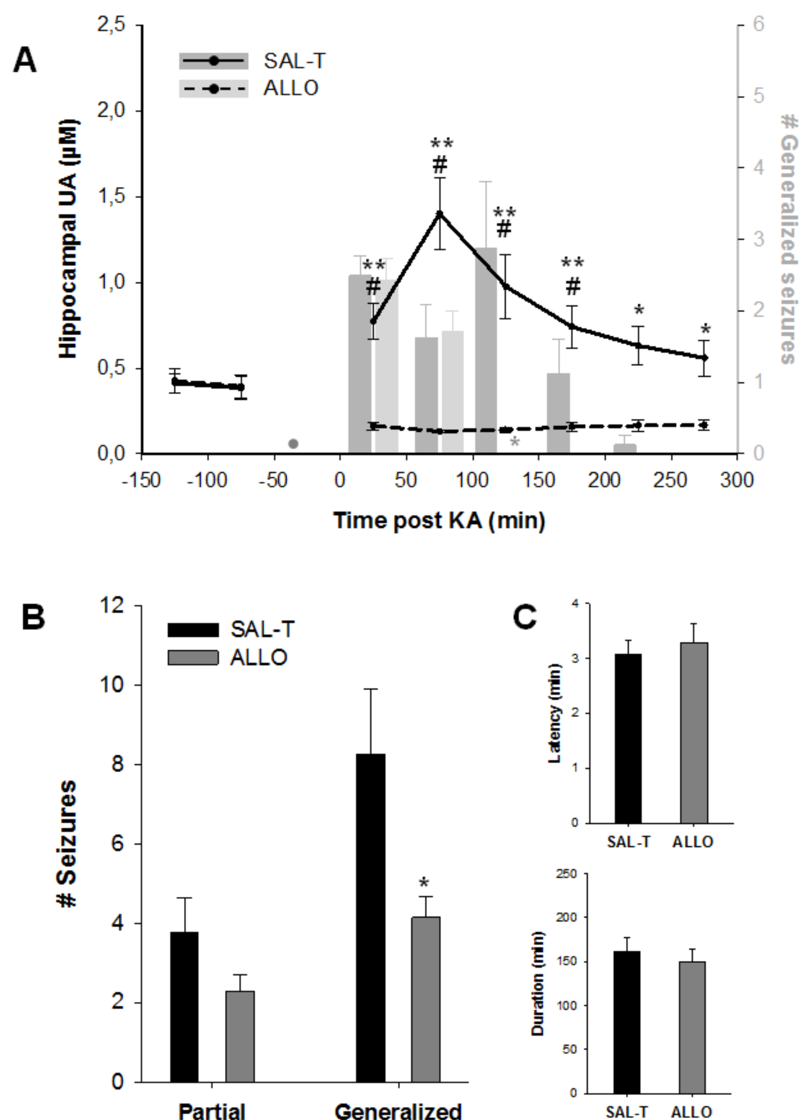


Figure 2 | Blocking the release of uric acid during limbic seizures. (A) Progression of absolute hippocampal uric acid (UA) levels (line plot) and generalized seizures (bar plot) per 50-minute collection. Within group comparisons showed that uric acid levels were significantly higher than baseline during the first 200 minutes (collection 1-4) after kainic acid (KA) infusion in the saline treated (SAL-T) control animals only. Between group comparison showed that for all six dialysates after KA infusion, the level of uric acid was significantly reduced in the allopurinol treated (ALLO) mice compared to SAL-T control mice. Compared to SAL-T there were significantly less generalized seizures during the third collection after KA infusion. • = i.p. injection of 100 mg/kg allopurinol. #significantly different from mean baseline concentration; *significantly different from concentration in SAL-T group for that collection. **(B)** Number of partial and generalized seizures. The number of generalized seizures was significantly lower in the ALLO group (*) compared to the SAL-T control group. **(C)** Latency to first epileptic spike (upper panel) and duration of epileptic activity (lower panel). $n_{SAL-T} = 8$, $n_{ALLO} = 7$. All values represent mean ± SEM. *,# $p < 0.05$, **,## $p < 0.001$.

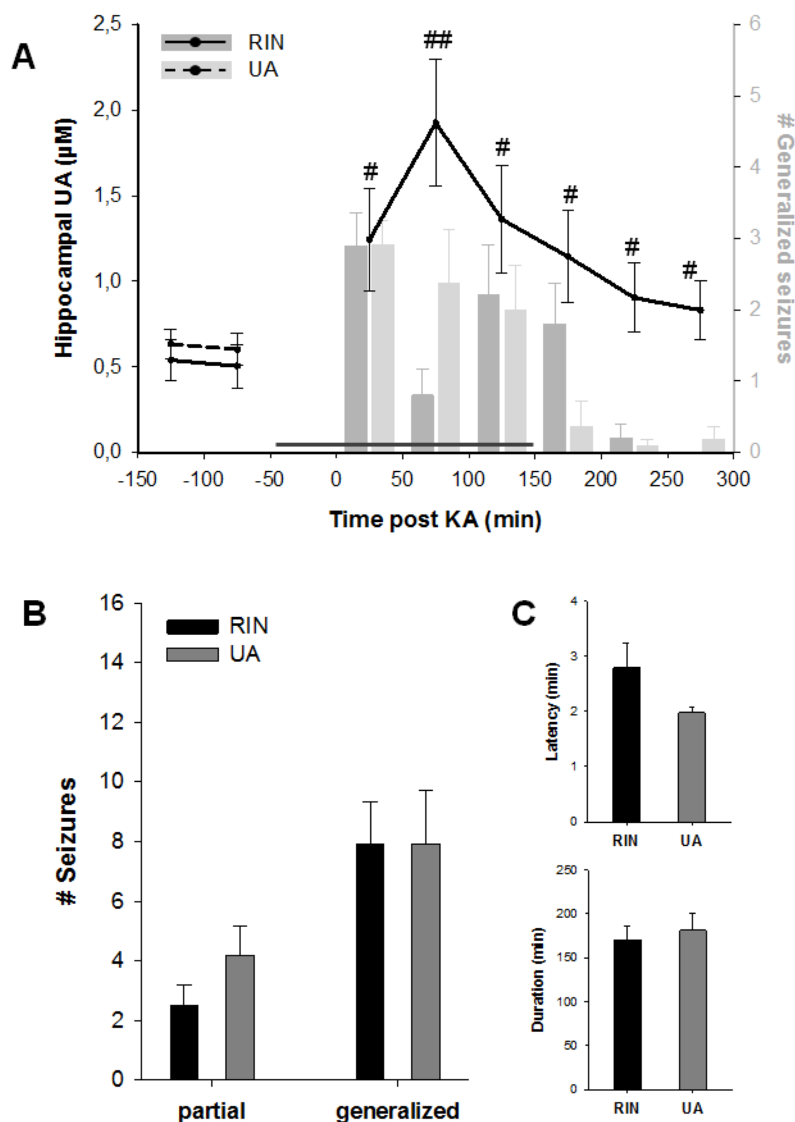


Figure 3 | Infusion of uric acid during limbic seizures. (A) Progression of absolute hippocampal uric acid (UA) levels (line plot) and generalized seizures (bar plot) per 50-minute collection. Uric acid measurements were not possible in the uric acid-infused group from the moment of infusion onwards. Within group comparisons showed that uric acid levels were significantly higher than baseline during entire follow up (collection 1-6) after kainic acid (KA) infusion in the animals infused with modified Ringer's solution (RIN). — i.h. uric acid infusion. #significantly different from mean baseline concentration. **(B)** Number of partial and generalized seizures. **(C)** Latency to first epileptic spike (upper panel) and duration of epileptic activity (lower panel). KA: kainic acid;; $n_{RIN} = 10$, $n_{UA} = 11$. All values represent mean ± SEM. # $p < 0.05$, ## $p < 0.001$

Overexpressing urate oxidase decreases hippocampal uric acid but does not decrease seizures

To test the effect of genetic uric decreases on limbic seizures, *UOx* OE mice were compared to their wildtype controls. Compared to their controls, mean baseline uric acid levels in the hippocampal area of the *UOx* OE mice were twofold decreased (Figure 4A; WT: 0.44 ± 0.07 µM, *UOx* OE: 0.18 ± 0.04 µM; $t = 3.135$, $p = 0.009$). Although *UOx* OE mice still displayed an increase in uric acid levels after KA infusion, the peak was less pronounced and regressed more rapidly (Figure 4A; within group $F = 5.402$, $p = 0.041$; between group, $F = 3.551$, $p = 0.037$). Uric acid levels reached a maximal concentration of 1.63 ± 0.23 µM and 0.64 ± 0.18 µM in the wildtype and the *UOx* OE mice respectively. The decreased uric acid levels in these animals had no significant effect on limbic seizures (Figure 4A-C).

Genetic disruption of uricate oxidase increases hippocampal uric acid and increases seizures

Finally, *UOx* KO mice were compared to their wildtype controls, to assess the effect of genetic uric acid increases on limbic seizures. Compared to their controls, mean baseline uric acid levels in the hippocampal area of the *UOx* KO mice were fivefold increased (Figure 5A; WT: 0.37 ± 0.08 µM, *UOx* KO: 2.06 ± 0.37 µM; $U = 5$, $p = 0.013$). In *UOx* KO animals the KA-induced uric acid peak was higher, delayed and had a slower regression (Figure 5A; within group $F = 9.051$, $p = 0.002$; between group $F = 6.443$, $p = 0.005$). Uric acid levels reached a maximum during the third dialysate collection, between 100 and 150 minutes after start of KA infusion. Peak uric acid concentration was 9.44 ± 1.97 µM, compared to 2.02 ± 0.24 µM in the wildtype animals ($t = 3.209$, $p = 0.007$). The number of generalized seizures in *UOx* KO mice was twice as high compared to wildtype animals (Figure 5B; WT: 6.1 ± 0.7, *UOx* KO: 11.6 ± 1.7; $t = -2.940$, $p = 0.011$) but the number of partial seizures was unaffected. This difference resulted from an overall increase in generalized seizures throughout the entire follow-up period (Figure 5A; $F = 3.186$, $p = 0.034$). Latency to the first epileptic spike after the start of intrahippocampal KA infusion was delayed in *UOx* KO animals compared to their wildtype counterparts (Figure 5C; WT: 1.60 ± 0.23; UOx KO: 2.56 ± 0.32; $t = -2.444$, $p = 0.024$).

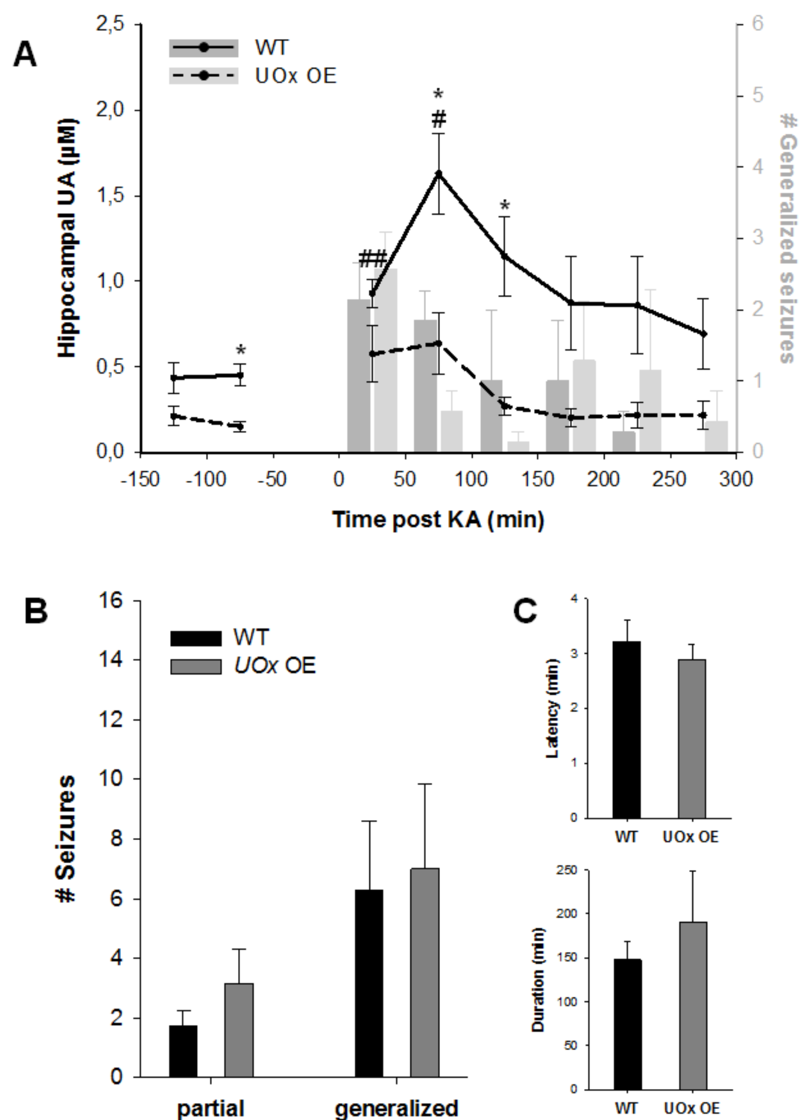


Figure 4 | Hippocampal uric acid levels and limbic seizures in urate oxidase overexpressing mice. (A) Progression of absolute hippocampal uric acid (UA) levels (line plot) and generalized seizures (bar plot) per 50-minute collection. Within group comparisons showed that uric acid levels were significantly higher than baseline during the first 100 minutes (collection 1-2) after kainic acid (KA) infusion in the wildtype (WT) animals only. Between group comparison showed the level of uric acid was significantly reduced in the uricase overpressing (*UOx* OE) mice compared to WT group both during baseline (collection 2) and after KA-infusion (collection 2 and 3). #significantly different from mean baseline concentration; *significantly different from concentration in WT group for that collection. **(B)** Number of partial and generalized seizures. **(C)** Latency to first epileptic spike (upper panel) and duration of epileptic activity (lower panel). $n_{WT} = 7$, $n_{UOx\ OE} = 7$. All values represent mean ± SEM. *,# $p < 0.05$, **,## $p < 0.001$

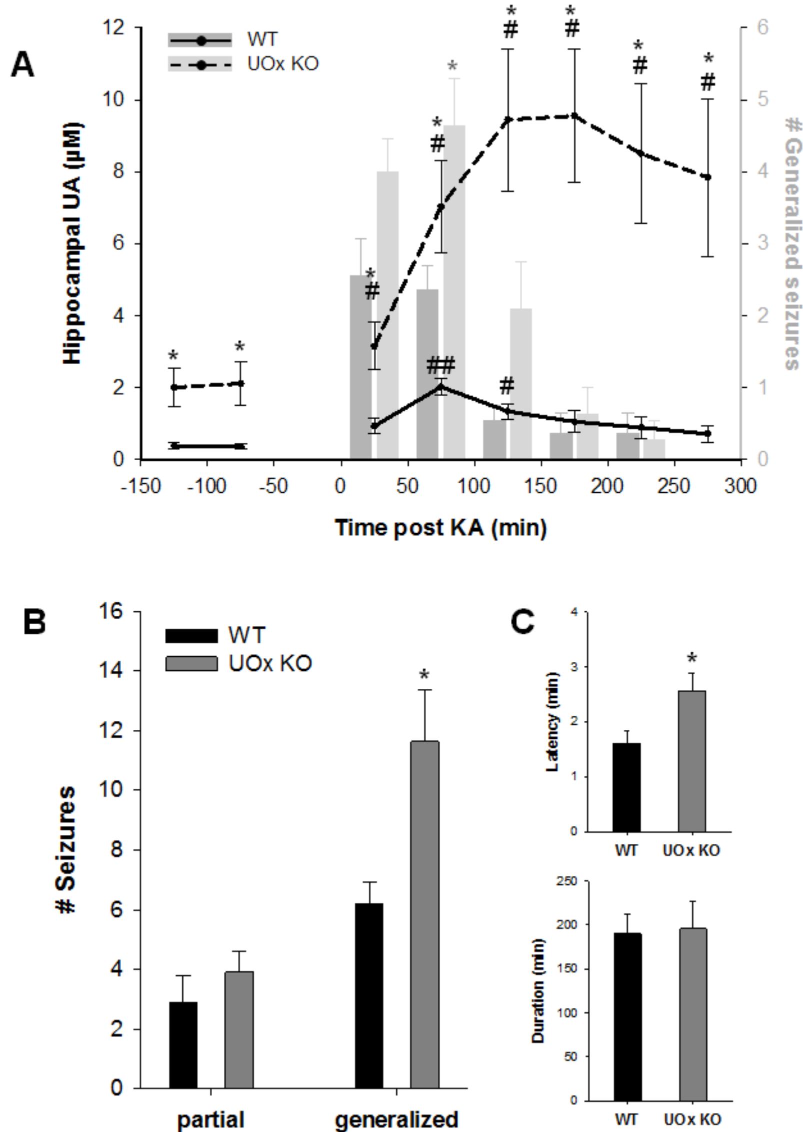


Figure 5 | Hippocampal uric acid levels and limbic seizures in urate oxidase knock-out mice. (A) Progression of absolute hippocampal uric acid (UA) levels (line plot) and generalized seizures (bar plot) per 50-minute collection. Within group comparisons showed that uric acid levels were significantly higher than baseline for both wildtype (WT; collection 2-3) and uricase knock-out (*UOx* KO; collection 1-6) animals after kainic acid (KA) infusion. Between group comparison showed that for all dialysates before and after KA infusion, the level of uric acid was significantly increased in the *UOx* KO mice compared to their WT counterparts. Compared to WT mice there were significantly more generalized seizures during the second collection after KA infusion. #significantly different from mean baseline concentration; *significantly different from concentration in WT group for that collection. **(C)** Latency to first epileptic spike (upper panel) and duration of epileptic activity (lower panel). The latency was significantly (*) longer in *UOx* KO mice compared to the WT mice. n_{WT} = 11, $n_{UOx\ KO}$ = 11. All values represent mean ± SEM. *,# $p < 0.05$, **,## $p < 0.001$

Discussion

In the present study we investigated the role of uric acid in the generation of limbic seizures. Local uric acid changes were monitored during seizure activity and the effect of complementary pharmacological and genetic uric acid manipulations on seizure characteristics were studied.

First, we found that local extracellular uric acid levels transiently increased three- to fourfold during limbic seizures, and peaked between 50 and 100 minutes after KA infusion. A previous study which injected KA intraperitoneally (16 mg/kg) in rats resulted in extracellular uric acid increases in the piriform cortex within 100 minutes (Layton et al., 1998). Importantly, they showed that prevention of seizures using midazolam largely inhibited uric acid release, indicating that seizure activity and not KA was the predominant cause of uric acid release (Layton et al., 1998). It is unclear whether this also holds for the intrahippocampal KA model, however, because the local concentration of KA and extent of the excitotoxic lesion is different compared to the intraperitoneal KA model (Levesque and Avoli, 2013).

Secondly, we found that allopurinol treatment and disruption of the *UOx* gene resulted in a twofold decrease and a twofold increase in the number of generalized seizures respectively. Together, these results suggest that uric acid promotes secondary generalization of seizures induced by intrahippocampal KA administration. Secondarily generalized tonic-clonic seizures (SGTCS) occur in 30% of the patients with refractory focal epilepsy and are a major cause of sudden unexpected death in epilepsy (Hemery et al., 2014). A recent study suggested that targeting SGTCS might present an important parameter for add-on treatment choice in these group of patients (Hemery et al., 2014). In this regard, our report indicates that targeting uric acid can be of particular interest in suppressing these type of seizures. This is in line with studies in patients with intractable epilepsy that found that allopurinol is most effective against SGTCS (De Marco and Zagnoni, 1988, Tada et al., 1991, Zagnoni et al., 1994, Togha et al., 2007). In addition, allopurinol also proved to be efficient in reducing the severity of generalized seizures after hippocampal kindling in cats (Wada et al., 1992) and in blocking secondarily generalized seizures in EL mice (Murashima et al., 1998). Interestingly, the administration of allopurinol and the disruption of the *UOx gene* resulted in a differential time profile in their manipulation of the number of generalized seizures. One hypothesis could be that uric acid only contributes to the secondary generalization of seizures in a later phase. In this scenario, the first phase of the status epilepticus is determined by KA alone, while the increasing levels of uric acid that develop during the status epilepticus stimulate further generalization. This would explain why the actions of allopurinol are only evident after the peak in uric acid concentration and why in *UOx* KO animals – that have chronically increased levels of uric acid basally – the number of generalized seizures is already increased at the start of the status epilepticus.

In contrast to allopurinol administration and disruption of *UOx*, our study could not demonstrate effects of intrahippocampal uric acid administration nor of *UOx* overexpression on the number of generalized seizures in the intrahippocampal KA model. A number of factors could account for this discrepancy. Firstly, intrahippocampal administration of uric acid might not have increased secondary generalization of hippocampal seizures because it did not diffuse far enough to affect brain regions responsible for seizure generalization, such as thalamus and substantia nigra (Gale, 1992, Blumenfeld et al., 2009, Yoo et al., 2014). In contrast, increases in uric acid levels are likely to extend beyond the region of the microdialysis probe in *UOx* KO mice (Chen et al., 2013). Secondly, the *UOx* OE mice displayed only a modest phenotype. The release of uric acid in these animals was not blocked to the same extent as with allopurinol treatment. There was still an induction of uric acid which at peak level was in average 1.5 times higher than baseline levels. Moreover, compared to *UOx* KO mice, the chronic change in uric acid levels was smaller (twofold vs. fivefold) and perhaps too small to evoke any effect. Thirdly, it is possible that the reduction of generalized seizures in response to allopurinol treatment is not directly related to a decrease in uric acid levels but rather to other effects of blocking xanthine oxidase, such as increases in adenosine and inosine. Both compounds have been reported to reduce seizure susceptibility (Boison, 2012, Kovacs et al., 2015). In that scenario the condition in the *UOx* KO mice, where an acute fivefold increase of uric acid is superimposed onto chronically elevated levels, would be the only setting where uric acid affects seizure generalization.

This may be related to the multiple actions of uric acid. On the one hand, the soluble form of uric acid acts as an anti-oxidant, but causes oxidative stress when levels are reduced (Fang et al., 2013). On the other hand, at high concentration, monosodium (MSU) crystals are formed, that can trigger the inflammatory response that can lead to an increased excitability (Vezzani et al., 2012). Not much is known about the formation of MSU crystals *in vivo* and even less outside the context of gouty arthritis, although the concentration of uric acid is considered a major contributing factor. In that regard, crystallization in the joint is assumed to occur above a threshold of 405 µM in humans (Rock et al., 2013), although it is becoming evident that the crystallization threshold is heavily dependent on other factors, such as temperature, pH, local calcium concentration, and mechanical stress. As such, gout is known to develop in patients with serum uric acid levels below 405 µM (Martillo et al., 2014). As the membrane of the microdialysis probe does not allow passage of the MSU crystals it is impossible to measure or administer MSU crystals in our experiments. It is hence possible that the pro-inflammatory MSU crystals are only formed in the UOx KO mice. Future histological studies will be necessary to unequivocally determine the presence of MSU crystals in the brain (Weaver et al., 2009).

In *UOx* KO mice the latency to seizure activity was delayed, which indicates that the fivefold chronical increase in uric acid levels in the brain can, in a seemingly contradictory fashion, increase the threshold for seizure initiation as well. Although difficult to explain at this time, one can

speculate that an increased anti-oxidative potential in these animals could play a role here, although this only seems to be the case in this particular setting. In any case, these results suggest that the different functions attributed to uric acid may occur simultaneously.

Acknowledgements

Lisa Thyrion is supported by a junior researcher grant from the 'Bijzonder onderzoeksfonds (BOF)' of Ghent University (01D22511). Prof. Dr. R. Raedt and Prof. Dr. K. Vonck are supported by a BOF-grant from Ghent University Hospital. Prof. Dr. P. Boon is supported by grants from the 'Fonds Wetenschappelijk Onderzoek (FWO)' and from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital. The authors thank Maria Van Landschoot for the technical support in the HPLC analysis.

References

Alvarez-Lario B, Macarron-Vicente J (2011) Is there anything good in uric acid? QJM 104:1015-1024.

Beal MF, Swartz KJ, Finn SF, Mazurek MF, Kowall NW (1991) Neurochemical characterization of excitotoxin lesions in the cerebral cortex. J Neurosci 11:147-158.

Blumenfeld H, Varghese GI, Purcaro MJ, Motelow JE, Enev M, McNally KA, Levin AR, Hirsch LJ, Tikofsky R, Zubal IG, Paige AL, Spencer SS (2009) Cortical and subcortical networks in human secondarily generalized tonic-clonic seizures. Brain 132:999-1012.

Boison D (2012) Adenosine dysfunction in epilepsy. Glia 60:1234-1243.

Bouilleret V, Ridoux V, Depaulis A, Marescaux C, Nehlig A, Le Gal La Salle G (1999) Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience 89:717-729.

Chen X, Burdett TC, Desjardins CA, Logan R, Cipriani S, Xu Y, Schwarzschild MA (2013) Disrupted and transgenic urate oxidase alter urate and dopaminergic neurodegeneration. Proc Natl Acad Sci U S A 110:300-305.

CMA Principles of Probe Recovery.

De Marco P, Zagnoni P (1988) Allopurinol in severe epilepsy. A preliminary report. Neuropsychobiology 19:51-53.

Devinsky O, Vezzani A, Najjar S, De Lanerolle NC, Rogawski MA (2013) Glia and epilepsy: excitability and inflammation. Trends Neurosci 36:174-184.

Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339-347.

Fang P, Li X, Luo JJ, Wang H, Yang XF (2013) A Double-edged Sword: Uric Acid and Neurological Disorders. Brain Disord Ther 2:109.

Gale K (1992) Subcortical structures and pathways involved in convulsive seizure generation. J Clin Neurophysiol 9:264-277.

Grimonprez A, Raedt R, Dauwe I, Mollet L, Larsen LE, Meurs A, De Herdt V, Wadman W, Delbeke J, Vonck K, Boon P (2015) Vagus Nerve Stimulation has Antidepressant Effects in the Kainic Acid Model for Temporal Lobe Epilepsy. Brain Stimul 8:13-20.

Hamed SA, Abdellah MM, El-Melegy N (2004) Blood levels of trace elements, electrolytes, and oxidative stress/antioxidant systems in epileptic patients. J Pharmacol Sci 96:465-473.

Hamed SA, Hamed EA, Hamdy R, Nabeshima T (2007) Vascular risk factors and oxidative stress as independent predictors of asymptomatic atherosclerosis in adult patients with epilepsy. Epilepsy Res 74:183-192.

Hemery C, Ryvlin P, Rheims S (2014) Prevention of generalized tonic-clonic seizures in refractory focal epilepsy: a meta-analysis. Epilepsia 55:1789-1799.

Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120:1939-1949.

Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa C, Hammad H, Lambrecht BN (2011) An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. Immunity 34:527-540.

Kovacs Z, Kekesi KA, Juhasz G, Barna J, Heja L, Lakatos R, Dobolyi A (2015) Non-adenosine nucleoside inosine, guanosine and uridine as promising antiepileptic drugs: a summary of current literature. Mini Rev Med Chem 14:1033-1042.

Layton ME, Samson FE, Pazdernik TL (1998) Kainic acid causes redox changes in cerebral cortex extracellular fluid: NMDA receptor activity increases ascorbic acid whereas seizure activity increases uric acid. Neuropharmacology 37:149-157.

Lee YS, Kim SJ, Oh JK, Chung JH, Eun HC (2003) Cutaneous microdialysis of uric acid level in the dermis: modification of in vitro recovery. Acta Derm Venereol 83:10-13.

Levesque M, Avoli M (2013) The kainic acid model of temporal lobe epilepsy. Neurosci Biobehav Rev 37:2887-2899.

Luhdorf K, Petersson H, Pedersen K (1978) Grand mal-provoked hyperuricemia. Acta Neurol Scand 58:280-287.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16:413-419.

Martillo MA, Nazzal L, Crittenden DB (2014) The crystallization of monosodium urate. Curr Rheumatol Rep 16:400.

Matzinger P (1994) Tolerance, danger, and the extended family. Annu Rev Immunol 12:991-1045.

Meng XF, Tan L, Tan MS, Jiang T, Tan CC, Li MM, Wang HF, Yu JT (2014) Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. J Neuroinflammation 11:212.

Murashima YL, Kasamo K, Suzuki J (1998) Antiepileptic effects of allopurinol on EL mice are associated with changes in SOD isoenzyme activities. Epilepsy Res 32:254-265.

Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32:281-294.

Raedt R, Van Dycke A, Van Melkebeke D, De Smedt T, Claeys P, Wyckhuys T, Vonck K, Wadman W, Boon P (2009) Seizures in the intrahippocampal kainic acid epilepsy model: characterization using long-term video-EEG monitoring in the rat. Acta Neurol Scand 119:293-303.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Stover JF, Lowitzsch K, Kempski OS (1997) Cerebrospinal fluid hypoxanthine, xanthine and uric acid levels may reflect glutamate-mediated excitotoxicity in different neurological diseases. Neurosci Lett 238:25-28.

Tada H, Morooka K, Arimoto K, Matsuo T (1991) Clinical effects of allopurinol on intractable epilepsy. Epilepsia 32:279-283.

Thurman DJ, Beghi E, Begley CE, Berg AT, Buchhalter JR, Ding D, Hesdorffer DC, Hauser WA, Kazis L, Kobau R, Kroner B, Labiner D, Liow K, Logroscino G, Medina MT, Newton CR, Parko K, Paschal A, Preux PM, Sander JW, Selassie A, Theodore W, Tomson T, Wiebe S (2011) Standards for epidemiologic studies and surveillance of epilepsy. Epilepsia 52 Suppl 7:2-26.

Togha M, Akhondzadeh S, Motamedi M, Ahmadi B, Razeghi S (2007) Allopurinol as adjunctive therapy in intractable epilepsy: a double-blind and placebo-controlled trial. Arch Med Res 38:313-316.

Vezzani A, Auvin S, Ravizza T, Aronica E (2012) Glia-neuronal interactions in ictogenesis and epileptogenesis: role of inflammatory mediators.

Vezzani A, Balosso S, Maroso M, Zardoni D, Noe F, Ravizza T (2010) ICE/caspase 1 inhibitors and IL-1beta receptor antagonists as potential therapeutics in epilepsy. Curr Opin Investig Drugs 11:43-50.

Vezzani A, Maroso M, Balosso S, Sanchez MA, Bartfai T (2011) IL-1 receptor/Toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures. Brain Behav Immun 25:1281-1289.

Wada Y, Hasegawa H, Nakamura M, Yamaguchi N (1992) Anticonvulsant effect of allopurinol on hippocampal-kindled seizures. Pharmacol Biochem Behav 42:899-901.

Warren DJ, Leitch AG, Leggett RJ (1975) Hyperuricaemic acute renal failure after epileptic seizures. Lancet 2:385-387.

Weaver J, Somani N, Bauer TW, Piliang M (2009) Simple non-staining method to demonstrate urate crystals in formalin-fixed, paraffin-embedded skin biopsies. J Cutan Pathol 36:560-564.

Wu X, Wakamiya M, Vaishnav S, Geske R, Montgomery C, Jr., Jones P, Bradley A, Caskey CT (1994) Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. Proc Natl Acad Sci U S A 91:742-746.

Yoo JY, Farooque P, Chen WC, Youngblood MW, Zaveri HP, Gerrard JL, Spencer DD, Hirsch LJ, Blumenfeld H (2014) Ictal spread of medial temporal lobe seizures with and without secondary generalization: an intracranial electroencephalography analysis. Epilepsia 55:289-295.

Zagnoni PG, Bianchi A, Zolo P, Canger R, Cornaggia C, D'Alessandro P, DeMarco P, Pisani F, Gianelli M, Verze L, et al. (1994) Allopurinol as add-on therapy in refractory epilepsy: a double-blind placebo-controlled randomized study. Epilepsia 35:107-112.

Supporting information

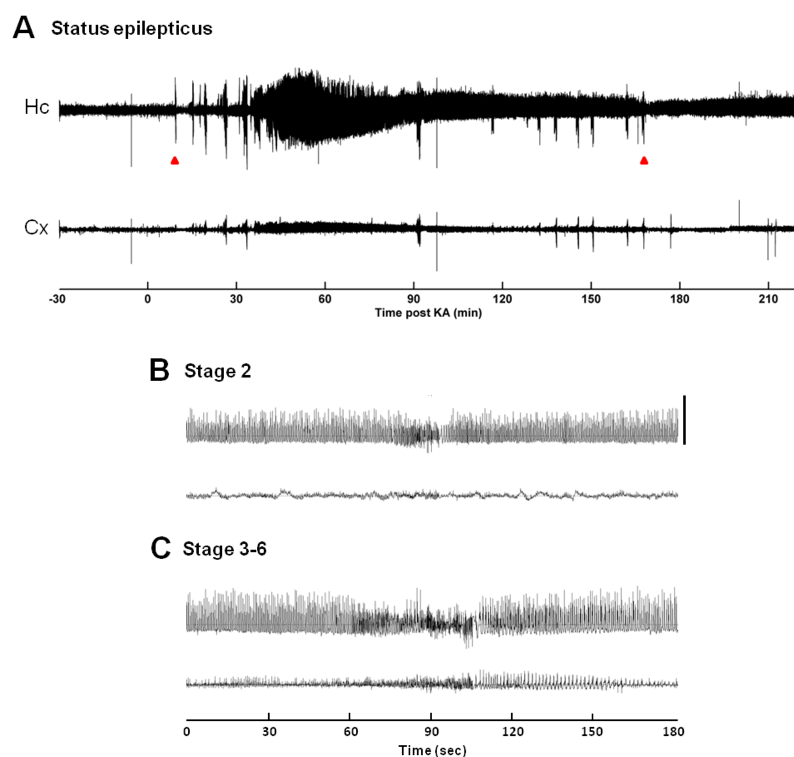


Figure S1 | (A) Example EEG of kainic acid (KA) induced status epilepticus (SE), and example EEGs corresponding (B) partial (stage 2) and (C) generalized (stage 3-6) seizures. The initial phase of the status epilepticus was characterized by the occurrence of discrete electrographic seizures, after which spiking increased in frequency and became more continuous. Over time, spiking became interspersed with increasingly longer low-voltage periods until the EEG only displayed periodic high-voltage epileptiform discharges. Seizures with a Racine score of 3 to 6 were associated with an increased frequency (> 4 Hz) and complexity of epileptic spikes, clearly distinguishable from background epileptiform activity. Head nodding (Racine stage 2) was similarly associated with a less uniform shorter alteration in frequency and/or amplitude. Red arrows indicate the start (first spike) and end (< 1 Hz spike frequency) of hippocampal status epilepticus. Scale bars = 5 mV. Hc: hippocampus, Cx: cortex.

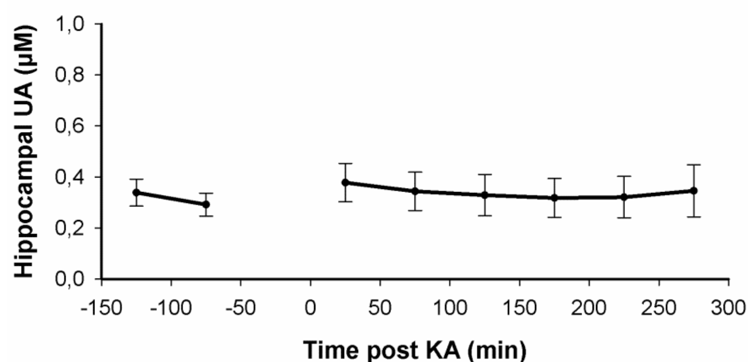
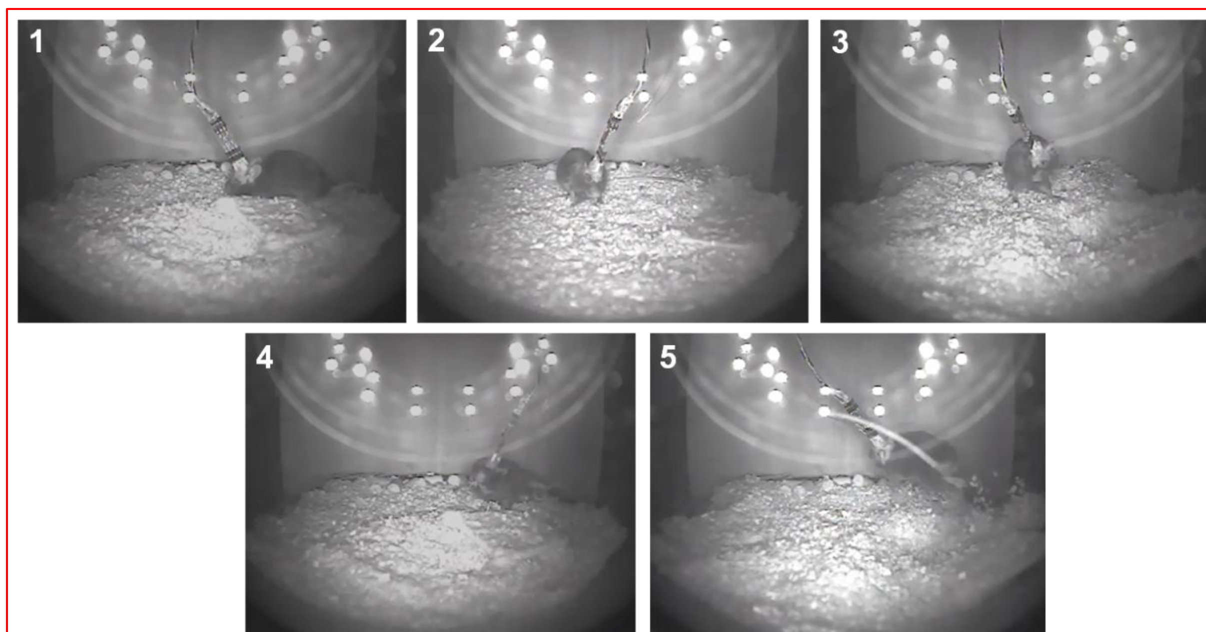


Figure S2 | Extracellular hippocampal uric acid concentration in the non-KA control group. Progression of absolute hippocampal uric acid levels per 50-minute collection in animals continuously infused with modified Ringer's solution. $n_{non\text{-}KA}$= 9. All values represent mean ± SEM.



Movie S1-5 | Examples of seizures associated with KA-induced status epilepticus. (1) Stage 2 - head nodding (2) Stage 3 - unilateral forelimb clonus (3) Stage 4 - bilateral forelimb clonus with rearing (4) Stage 5 - rearing and falling (5) Stage 6 - running and bouncing. KA: kainic acid. *Videos can be found online:* doi: 10.1016/j.expneurol.2016.01.001.

Research Aims

(1) develop and characterize a seizure model that allows to monitor the changes in local uric acid levels:
(2) investigate the effects of pharmacological and genetic uric acid modulations on the seizure characteristics of this model
(3) investigate the effects of these manipulations on the seizure-associated inflammatory response
(4) examine if altering uric acid levels changes seizure characteristics in a different seizure model

Chapter 6

Effects of uric acid in a mouse model for acute limbic seizures: expression of inflammatory markers and inflammasome components

Lisa Thyrion, Robrecht Raedt, Sophie Janssens, Bart Lambrecht, Kristl Vonck, Paul Boon

Abstract

A growing body of evidence points at an active contribution of pro-inflammatory processes to neuronal excitability, with a newly identified involvement of endogenous pro-inflammatory danger signals. We have previously shown that uric acid, a well-known danger signal in the periphery, is released in the brain and is involved in the generation of seizures in a mouse model for acute limbic seizures. In the present study we evaluated whether experimental manipulations of uric acid in the acute limbic seizure model has an impact on the inflammatory state of the brain, by analyzing the hippocampal expression of relevant inflammatory markers one day after seizure induction. In order to gain further insight into the potential inflammatory pathway of uric acid, expression of components of the NLRP3 inflammasome was also examined. Our results show that manipulations of uric acid levels changes the inflammatory state upon induction of acute limbic seizures but only in those conditions where manipulation of uric acid also affected the seizure state.

Key words: uric acid, urate oxidase, allopurinol, inflammation, RT-qPCR, mouse

Introduction

Despite being an active field of research for decades, about one third of the epilepsy patients still does not achieve seizure freedom with the available treatment modalities (Moshe et al., 2015). To conceive more effective medication, novel therapeutic targets need to be identified. This compels to a better understanding of the mechanisms underlying hyperexcitability (Devinsky et al., 2013). In this regard, there is a growing body of evidence that points to a significant contribution of pro-inflammatory danger signals to epilepsy (Vezzani and Viviani, 2014). Recent evidence shows that the pro-inflammatory danger molecule HMGB1 can be targeted to reduce seizures (Maroso et al., 2010). In accordance, we have shown that uric acid, another danger signal with pro-inflammatory actions (Rock et al., 2010), is released in the brain during acute limbic seizures and affects seizure generalization in animals where uric acid levels were pharmacologically and genetically altered (Thyrion et al., 2016). More specifically, we were able to show that allopurinol-induced decrease in uric acid levels reduced seizure generalization, while uric acid increases, resulting from a disruption in the urate oxidase (*UOx*) gene, promoted seizure generalization. In the latter case, the latency to the onset of seizures was, seemingly contradictory, also delayed.

The inflammatory pathway of uric acid involves assembly of the NLRP3 inflammasome with the scaffolding protein ASC, the subsequent recruitment and activation of caspase-1 and the resulting cleavage of pro-IL-1β and pro-IL-18 into their biologically active form (Rock et al., 2013). IL-1β is well-recognized to increase excitability and influences neurotransmission and neuroglial communication through both rapid effects on receptor-gated ion channels and long-lasting effects on expression levels of transcription molecules, such as NF-kB (Vezzani and Baram, 2007). The latter induces the expression of downstream players, including several members of the IL-1 family, IL-6, TNF and COX-2, which all have been shown to affect excitability in various stages of experimental epilepsy (Vezzani et al., 2012).

As seizure-induced inflammation can further increase excitability, this can potentially contribute to seizure precipitation in the future (Vezzani and Viviani, 2014). In order to evaluate if the seizure-induced release of uric acid affects the post-ictal inflammatory state of the brain, we analyzed changes in hippocampal expression of inflammatory markers and inflammasome components, that have been shown to increase in our seizure model (see Chapter 4), upon experimental modulation of uric acid.

Material and methods

Chemicals and solutions

Modified Ringer's solution, the perfusion fluid used for microdialysis, consisted of 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl (all Sigma Aldrich, Schnelldorf, Germany) in ultrapure water and was kept at 4°C (one week shelf life). Kainic acid (KA) (R&D systems, Abington, United Kingdom) was dissolved in modified Ringer's solution at a concentration of 4 mg/ml and kept in aliquots at -20°C. It was diluted further with modified Ringer's solution to a concentration of 500 µg/ml on the experimental day. Allopurinol (Sigma Aldrich, Schnelldorf, Germany) was suspended in a saline:Tween 20 solution (9:1) at 10 mg/ml and stored at 4°C (one week shelf life). Uric acid (Sigma Aldrich, Schnelldorf, Germany) was prepared in modified Ringer's solution at a concentration of 1.6 mg/ml and was freshly made every day.

Animals

The animals used for this study are the same animals used in Chapter 5. The male C57BL/6 JolaHsd mice that were used for the pharmacological experiments were obtained from Harlan (Horst, The Netherlands). Mice with genetically altered uric acid levels were obtained by targeting urate oxidase (UOx), the enzyme responsible for uric acid breakdown. Hemizygous *UOx* overexpressing (*UOx* OE) mice (C57BL/6 background) were obtained from Kenneth Rock, Department of Immunology, University of Massachusetts, Worcester, USA. The *UOx* transgene in these mice is driven by a strong constitutive (β-actin) promoter (Kono et al., 2010). Hemizygous *UOx* knock-out (*UOx* KO) mice were purchased from The Jackson Laboratory (B6;129S7/J background, Maine, USA) and crossbred. Offspring of these breedings were then genotyped for homozygosity and bred separately. The *UOx* KO mice have a constitutive disruption of the *UOx* gene, by insertion of a neomycin gene into exon 3. Allopurinol (200 mg/l) was provided in the drinking water of *UOx* KO breeding pairs and pups until 8 weeks of age to prevent lethality due to hyperuricemia (Wu et al., 1994). All mice were bred at the animal house of Ghent University Hospital and were housed at controlled temperature (21-22°C) and relative humidity (40-60%) conditions. They had a fixed 12-hour light/dark cycle (lights-on period from 7 AM to 7 PM) and food and water available ad libitum. Treatment and care were in compliance with guidelines from the European Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University (ECD 09/61). All animals were 3-5 months old at the start of the experiments.

Induction of limbic seizures

Induction of limbic seizures was done as previously described (see Chapter 5). In brief, an obturator-enclosed guide cannula was placed in the septotemporal transition zone of the hippocampus (coordinates relative to bregma: anterioposterior: -3.3 mm ; mediolateral: -2.8 mm ; dorsoventral: -2.3

mm). After a recovery period of one week the mice were connected to the set-up and the guide cannula obturator was replaced with a microdialysis probe (CMA/7; 2 mm membrane length; theoretical cut-off 6 kDa; CMA, Kista, Sweden) which was continuously perfused with modified Ringer's solution at a flow rate of 1.25 µl/min. The next day, at 11 AM, a 500 µg/ml KA solution was infused for 3.2 minutes, after which the solution was switched back to modified Ringer's solution.

Measurement of uric acid

Hippocampal dialysates were sampled in 50 minute periods. Experiments were started collecting two to three baseline samples in which only modified Ringer's solution was infused. After KA infusion (or at the equivalent timepoint in the non-KA control group) another six dialysates were collected. Uric acid was quantified by HPLC using a Waters Alliance 2695 HPLC device (Waters, Zellik, Belgium) connected to a Waters 996 photodiode array detector according to an adapted protocol described by Fagugli and colleagues (Fagugli et al., 2002). Data acquisition and processing were performed using Empower software (Waters). Analyses were performed on a reverse-phase XBridge C8 column (3.5 µm, 150 mm x 4.6 mm, Waters) with an Ultrasphere ODS guard column (5 µm, 5 mm x 4.6 mm, Beckman Instruments, Fullerton, CA). The chromatographic separation consisted of a linear gradient of methanol and ammonium formate buffer (50 mM, pH 3.0) from 0 to 100% methanol in 15 min at a flow rate of 1 mL/min. Forty-five microliter of dialysate was automatically injected. Uric acid was analyzed by UV detection at 300 nm. Standard solutions were used to perform calibration curves for uric acid quantification. For concentrations below the limit of detection (LOD; 0.112 µM) the LOD itself was used. For the pharmacological studies, values depict the mean uric acid level of six collections during seizure activity (KA) or during the equivalent time (RIN). Due to the low number of animals and lack of consistency in RIN animals in the genetic studies, mean values of the three baseline collections (BAS) of the animals infused with KA were used instead of uric acid levels obtained from dialysates of RIN animals.

Experimental set-up

To pharmacologically test the effects of lowering uric acid levels on seizure-induced inflammation, animals were injected intraperitoneally with either a 100 mg/kg suspension of allopurinol or its dissolving solution (9:1 saline:Tween 20 solution) 30 minutes before KA infusion. To pharmacologically test the effects of increases in uric acid levels, animals were infused in the left hippocampus with a 1.6 mg/ml uric acid solution for 200 minutes at a flow rate of 1.25 µl/min, starting 50 minutes before KA infusion. This was compared to animals continuously infused with modified Ringer's solution. To test the effects of genetic uric acid alterations, *UOx* OE mice and *UOx* KO mice were compared to their wildtype controls (WT). All treatments were shown to alter the local uric acid levels during seizure activity (Thyrion et al., 2016).

RNA isolation and RT-qPCR

Isolation of RNA and RT-qPCR was done as previously described (see Chapter 4). In brief, hippocampal tissue was isolated and snap-frozen twenty-six hours after KA infusion. Frozen hippocampal tissue was homogenized total RNA was extracted using the RNeasy Lipid Tissue kit (Qiagen). An additional ethanol-precipitation step was performed to increase purity of the samples, which was verified using Nanodrop. Genomic DNA was removed using a TURBO DNAse kit (Ambion) and first strand cDNA synthesis was performed using the Superscript III first strand synthesis system for RT-qPCR (Invitrogen), both according to manufacturer's instructions. Real-time quantitative polymerase chain reactions (RT-qPCR) were done using the Fast SYBR master mix kit (Applied Biosystems) or SensiFastTM SYBR No-Rox kit (Bioline). Primers were designed making use of Primerbank (www.pga.mgh.harvard.edu/primerbank) (see Chapter 4; IL-18: Fw-GACTCTTGCGTCAACTTCAAGG Rv-CAGGCTGTCTTTTGTCAA CGA). All reactions were performed in triplicate and plates were run on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) or on a Lightcycler 480 (Roche). Relative expression was calculated using an improved version of the ΔΔ-Ct-method (Livak and Schmittgen, 2001). Data were normalized to the geometric average of internal standard genes (HMBS, TBP and RPL13a) (Vandesompele et al., 2002). Values represent the average normalized relative expression values, with the expression level of wildtype/nontreated animals infused with modified Ringer's solution. Two samples were left out because of instability of the internal standard genes.

Statistical analysis

For all experiments, the number of animals (n) analyzed are represented. Values are expressed as mean ± standard error of mean (SEM). Statistical analyses were performed in SPSS Statistics version 22. Expression data was analyzed with a two-way analysis of variance (ANOVA) with treatment/genotype and seizures as independent factors. Uric acid data was analyzed using (in)dependent t-tests. Post-hoc testing was corrected using the Bonferroni-Holm method. Differences were considered significant at the 5% level.

Results

Effect of allopurinol treatment on seizure-induced inflammation

The display of seizures was associated with an increased expression of ASC (1.00 ± 0.09 vs. 2.1 ± 0.11), IL-1α (1.00 ± 0.13 vs. 2.79 ± 0.30), IL-1Ra (1.00 ± 0.56 vs. 7.05 ± 2.85), IL-6 (1.00 ± 0.25 vs. 3.59 ± 0.84), TNF (1.00 ± 0.14 vs. 5.95 ± 1.00) and COX2 (1.00 ± 0.14 vs. 2.62 ± 0.35) and a decreased expression of IL-18 (1.00 ± 0.06 vs. 0.79 ± 0.03 (Figure 1). Treatment with allopurinol showed a trend to further decrease the expression of IL-18 (0.79 ± 0.03 vs. 0.61 ± 0.10) and attenuated the induction of the expression of IL-1Ra (7.05 ± 2.85 vs. 0.74 ± 0.14), IL-6 (3.59 ± 0.84 vs. 1.28 ± 0.04), TNF (5.95 ± 1.00 vs. 2.21 ± 0.47) and COX2 (2.62 ± 0.35 vs. 1.39 ± 0.13) in animals that displayed seizures, but did not alter the inflammatory response in animals continuously infused with modified Ringer's solution.

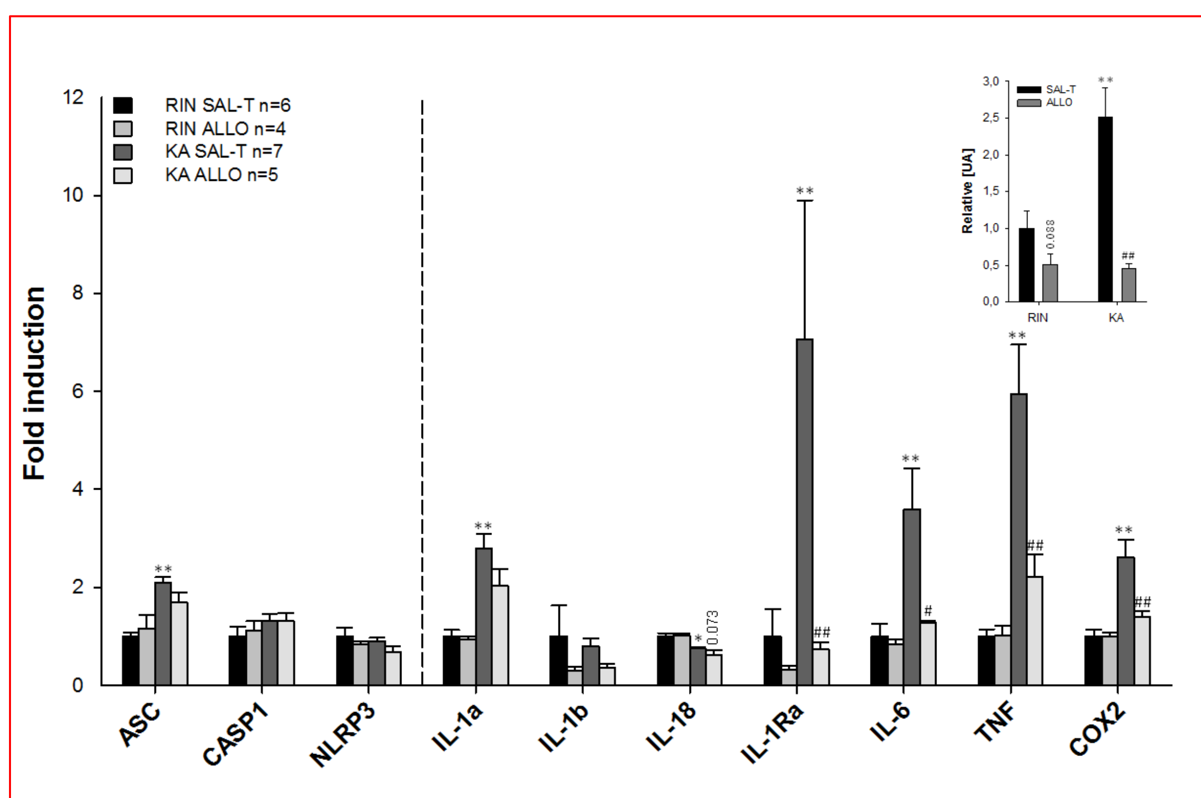


Figure 1 | Allopurinol does not affect the inflammatory state in animals infused with modified Ringer's solution, but decreases seizure-induced inflammation in animals infused with kainic acid. mRNA expression levels are normalized relative to the geometric mean of the internal standard genes. For each gene, fold differences were calculated relative to the average expression levels in modified Ringer's infused control animals injected with saline-Tween, which were set at 1. The results of the two-way ANOVA showed a main effect of seizures on the expression of ASC ($p < 0.01$), IL-1α ($p < 0.01$), IL-1β ($p = 0.086$, trend), IL-18 ($p < 0.01$), IL-1Ra ($p < 0.05$) and IL-6 ($p < 0.01$); a main effect of treatment on the expression of IL-1Ra ($p < 0.01$) and IL-6 ($p = 0.059$, trend) and an interaction between seizures and treatment on the expression of TNF ($p < 0.05$) and COX2 ($p < 0.05$). **Small plot:** Relative extracellular hippocampal uric acid concentration normalized to the average level in the RIN SAL-T group. Bars represent mean ± SEM. RIN: modified Ringer's solution; SAL-T: saline-Tween; ALLO: allopurinol; KA: kainic acid. #(#) and *(*) indicate a statistical significant difference between the RIN SAL-T and KA SAL-T group and the KA SAL-T and KA ALLO group respectively. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$.

Effect of uric acid infusion on seizure-induced inflammation

The display of seizures was associated with an increased expression of ASC (1.00 ± 0.21 vs. 2.42 ± 0.17), NLRP3 (1.00 ± 0.31 vs. 3.65 ± 0.76), IL-1α (1.00 ± 0.11 vs. 2.72 ± 0.21), IL-1β (1.00 ± 0.28 vs. 2.62 ± 0.52), IL-1Ra (1.00 ± 0.40 vs. 6.90 ± 1.39), IL-6 (1.00 ± 0.14 vs. 6.43 ± 0.9), TNF (1.00 ± 0.14 vs.6.40 ± 0.75) and COX2 (1.00 ± 0.24 vs.2.43 ± 0.39) (Figure 2). The increase of local uric acid levels resulted in a trend towards an increase in the expression of IL-1α (1.00 ± 0.11 vs.1.44 ± 0.22) and IL-6 (1.00 ± 0.14 vs. 3.21 ± 1.41) and an increased expression of ASC (1.00 ± 0.21 vs. 1.75 ± 0.45), NLRP3 (1.00 ± 0.31 vs. 2.84 ± 0.33) and IL-18 (1.00 ± 0.11 vs. 1.64 ± 0.29) in Ringer infused animals but did not result in further increases in seizure-induced expression of inflammatory markers, except for a trend towards an upregulation of IL-18 mRNA (0.80 ± 0.07 vs. 1.22 ± 0.13).

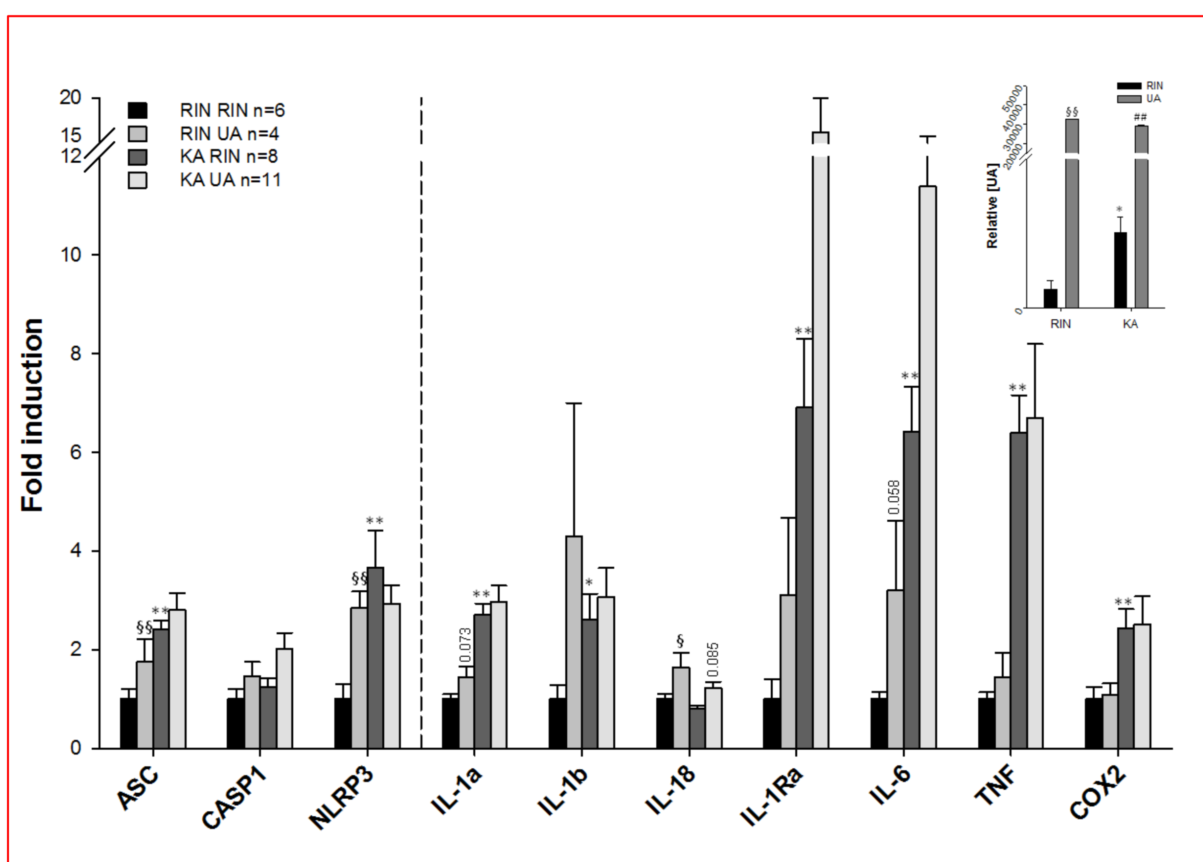


Figure 2 | Uric acid infusion increases the inflammatory state in animals infused with modified Ringer's solution, but fails to further contribute to the seizure-induced inflammatory response in animals infused with kainic acid. mRNA expression levels are normalized relative to the geometric mean of the internal standard genes. For each gene, fold differences were calculated relative to the average expression levels in control animals continuously infused with modified Ringer's solution, which were set at 1. The results of the two-way ANOVA showed a main effect of seizures on the expression of IL-1α ($p < 0.01$), IL-1β ($p < 0.05$), IL-18 ($p = 0.063$; trend), IL-1Ra ($p < 0.01$), IL-6 ($p < 0.01$), TNF ($p < 0.01$) and COX2 ($p < 0.01$); a main effect of treatment on the expression of CASP1 ($p = 0.092$; trend), IL-18 ($p < 0.05$ and IL-6 ($p < 0.05$) and an interaction between seizures and treatment on the expression of ASC ($p < 0.01$) and NLRP3 ($p < 0.05$). **Small plot:** Relative extracellular hippocampal uric acid concentration normalized to the average level in the RIN RIN group. Bars represent mean ± SEM. RIN: modified Ringer's solution; UA: uric acid; KA: kainic acid. §(§), *(*) and #(#) indicate a statistical significant difference between the RIN RIN and RIN UA group; the RIN RIN and KA RIN group and the KA RIN and KA UA group respectively. $^{*,§,\#}p < 0.05$, $^{**,§§,\#\#}p < 0.01$.

Effect of overexpression of urate oxidase on seizure-induced inflammation

Despite a main effect of seizures on the expression of ASC ($p < 0.01$), IL-1Ra ($p < 0.05$), IL-6 ($p < 0.05$) and TNF ($p < 0.01$) and an interaction between seizures and genotype for the expression of CASP 1 ($p < 0.01$), only a significantly decreased expression of CASP1 (1.00 ± 0.26 vs. 0.50 ± 0.09) and increased expression of TNF (1.00 ± 0.32 vs. 2.29 ± 0.59) in wildtype animals infused with KA could be demonstrated (Figure 3). In Ringer infused animals overexpression of the *UOx* gene resulted in a decreased expression of ASC (1.00 ± 0.28 vs. 0.49 ± 0.13) and CASP1 (1.00 ± 0.26 vs. 0.32 ± 0.03). In animals that displayed seizures, no differences between genotypes could be shown.

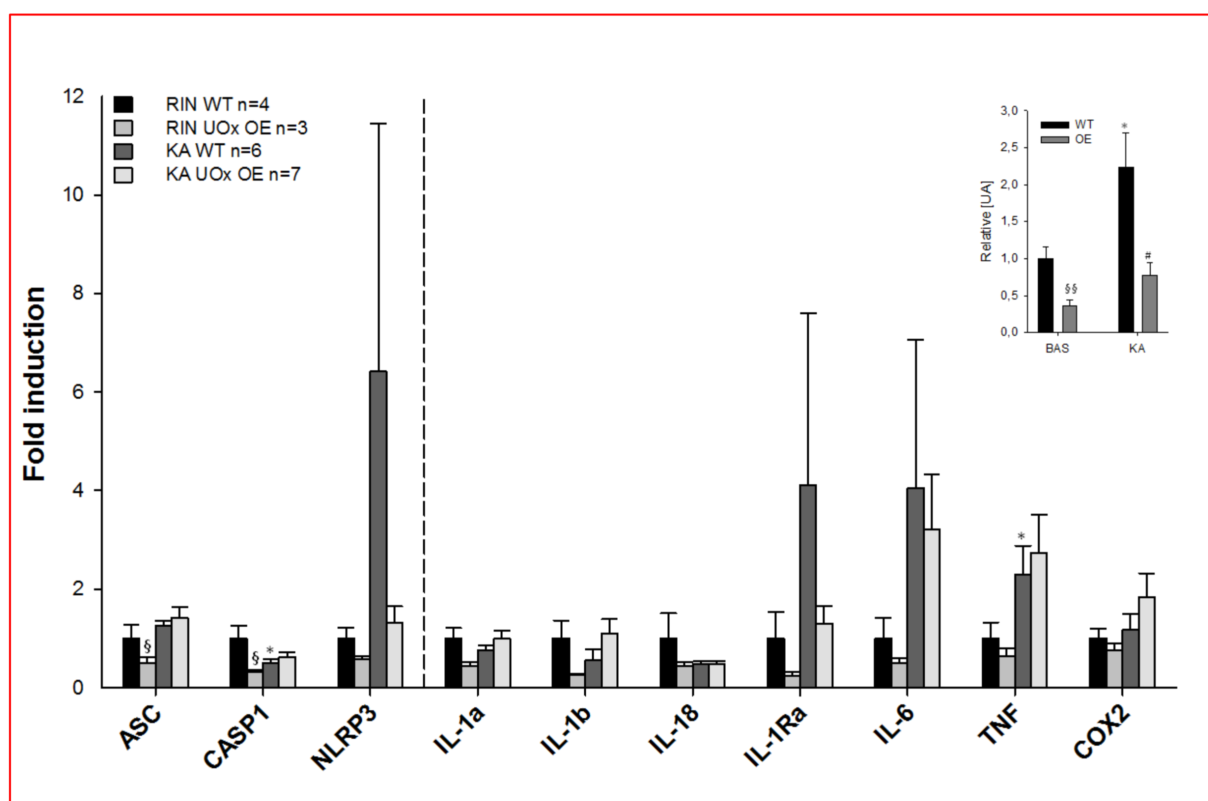


Figure 3 | Overexpression of urate oxidase reduces the expression of inflammatory markers in animals infused with Ringer's solution, but does not seem to alter expression in animals infused with KA, although interpretation should be done with caution because of the high level of variation. mRNA expression levels are normalized relative to the geometric mean of the internal standard genes. For each gene, fold differences were calculated relative to the average expression levels in wildtype animals continuously infused with modified Ringer's solution, which were set at 1. One outlier was removed based on the Grubb's test ($p<0.05$). The results of the two-way ANOVA showed a main effect of seizures on the expression of ASC ($p < 0.01$), IL-1Ra ($p < 0.05$), IL-6 ($p < 0.05$) and TNF ($p < 0.01$); a main effect of treatment on the expression of ASC ($p < 0.099$; trend) and an interaction between seizures and genotype for CASP1 ($p < 0.01$). **Small plot:** Relative extracellular hippocampal uric acid concentration normalized to the average level in the BAS WT group. Bars represent mean ± SEM. BAS: baseline; RIN: modified Ringer's solution; UOx OE: urate oxidase overexpressing; WT: wildtype KA: kainic acid. §(§), *(*) and #(#) indicate a statistical significant difference between the RIN (BAS) WT and RIN (BAS) UOx OE group; the RIN (BAS) WT and KA WT group and the KA WT and KA UOx OE group respectively. $^{*,§,\#}p < 0.05$, $^{**,§§,\#\#}p < 0.01$.

Effect of disruption of urate oxidase on seizure-induced inflammation

The display of seizures was associated with an increased expression of IL-1α (1.00 ± 0.15 vs. 1.73 ± 0.18), IL-1β (1.00 ± 0.39 vs. 2.72 ± 0.34), IL-1Ra (1.00 ± 0.21 vs. 5.46 ± 1.00), IL-6 (1.00 ± 0.32 vs. 3.72 ± 1.08), TNF (1.00 ± 0.14 vs. 2.98 ± 0.46) and COX2 (1.00 ± 0.09 vs. 3.36 ± 1.11) in wildtype animals (Figure 4). In animals infused with Ringer's solution, disruption of the *UOx* gene resulted in an increased expression of IL-1Ra (1.00 ± 0.21 vs. 2.72 ± 0.32) and a trend towards an increased expression of IL-1β (1.00 ± 0.39 vs. 2.11 ± 0.73) compared to wildtype animals. In animals that displayed seizures, disruption of the *UOx* gene resulted in a decreased expression of ASC (0.97 ± 0.12 vs. 0.60 ± 0.08), CASP1 (0.87 ± 0.12 vs. 0.54 ±0.07), IL-1β (2.72 ± 0.34 vs. 1.65 ± 0.36), IL-1Ra (5.46 ± 1.00 vs. 2.68 ± 0.63) and a trend towards a decrease in the expression of TNF (2.98 ± 0.46 vs. 2.28 ± 0.66).

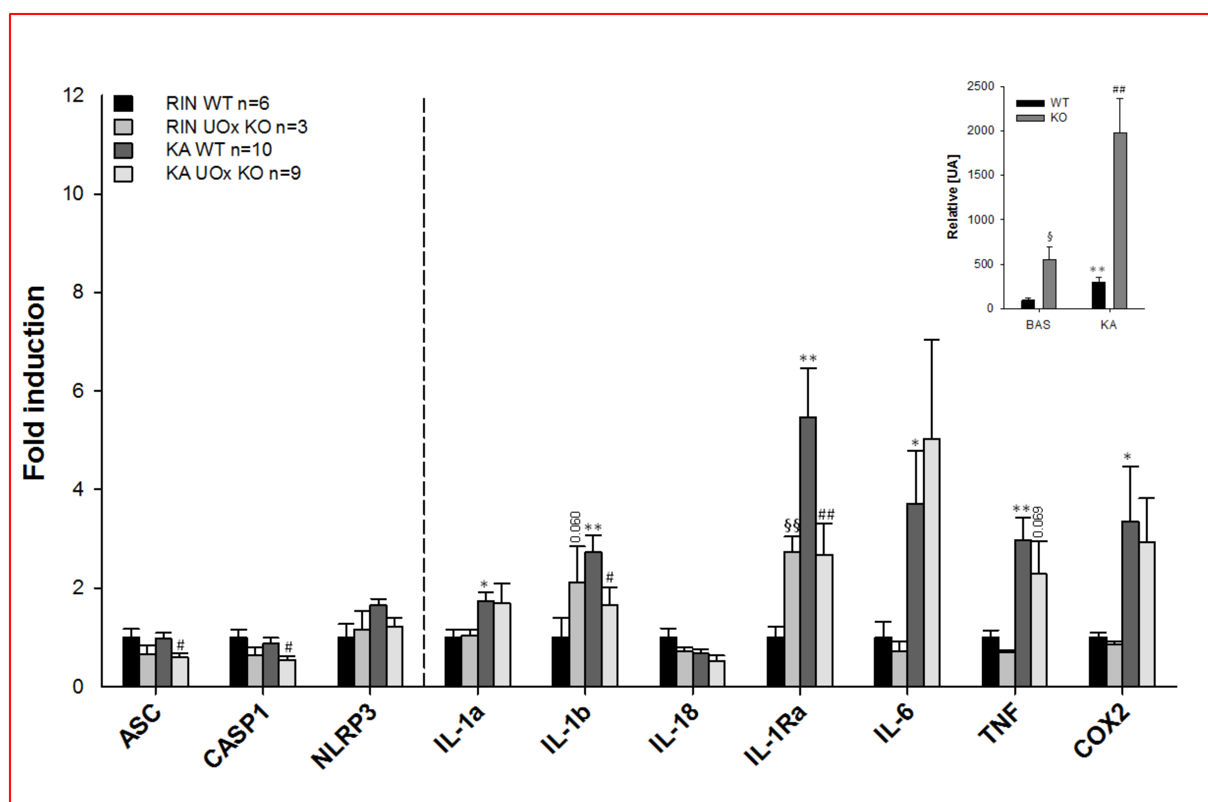


Figure 4 | Disruption of urate oxidase increases inflammatory markers in animals infused with modified Ringer's solution, but decreases the expression of these markers in kainic acid infused animals. mRNA expression levels are normalized relative to the geometric mean of the internal standard genes. For each gene, fold differences were calculated relative to the average expression levels in wildtype animals continuously infused with modified Ringer's solution, which were set at 1. The results of the two-way ANOVA showed a main effect of seizures on the expression of NLRP3 ($p < 0.078$, trend), IL-1α ($p < 0.05$), IL-6 ($p < 0.01$), TNF ($p < 0.01$) and COX2 ($p < 0.01$); a main effect of treatment on the expression of ASC ($p < 0.05$), CASP1 ($p < 0.05$) and TNF ($p = 0.090$, trend) and an interaction between seizures and genotype for IL-1β ($p < 0.01$) and IL-1Ra ($p < 0.01$). **Small plot:** Relative extracellular hippocampal uric acid concentration normalized to the average level in the BAS WT group. Bars represent mean ± SEM. RIN: modified Ringer's solution; WT: wildtype; UOx KO: urate oxidase knock-out; KA: kainic acid. §(§), *(*) and #(#) indicate a statistical significant difference between the RIN (BAS) WT and RIN (BAS) UOx KO group; the RIN (BAS) WT and KA WT group and the KA WT and KA UOx KO group respectively. *,§,#p < 0.05, **,§§,##p < 0.01.

Discussion

In a first study, we described a release of uric acid in the hippcoampus during seizure activity and an increased hippocampal inflammatory state one day after the induction of limbic seizures (see Chapter 4). As uric acid has also been suggested to act as a pro-inflammatory danger signal, we examined whether manipulation of the seizure-induced uric acid release affects the inflammatory state one day after status epilepticus.

A first finding was that allopurinol decreased seizure-induced inflammation. The lack of an effect in control conditions suggests that uric acid decreases in se does not affect inflammation despite the fact that uric acid levels are likely still suppressed up until the moment of RNA isolation, as oxypurinol, the active metabolite of allopurinol, has a half-life of 16-30 hours (Day et al., 2007). These findings seem to support the hypothesis that uric acid only acts on inflammation when there is damage and the associated acute increase in uric acid levels results in the formation of monosodium urate (MSU) crystals (Johnson et al., 2011, Rock et al., 2013). In the previous study, we showed that allopurinol reduced seizure generalization (Thyrion et al., 2016). As seizures are insults known to evoke an inflammatory response (Vezzani and Viviani, 2014), it is possible that the anti-inflammatory characteristics of allopurinol are indirectly due to the reduction of the number of generalized seizures rather than the consequence of a direct effect of reduced levels of uric acid.

A second finding was that uric acid infusion increased the inflammatory state in animals that did not experience seizures, but failed to further contribute to the seizure-induced inflammatory response in animals infused with KA. That local uric acid infusion resulted in increased inflammation, consolidates our hypothesis that uric acid arrived in the brain in sufficient amounts to form MSU crystals (Thyrion et al., 2016). The fact that ASC and NLRP3 expression was similarly affected by uric acid, KA and KA + uric acid treatments indicates that this pathway is possibly saturated in KA infused animals at this point, preventing further uric acid increases from having any effect. These results suggest that an acute increase in uric acid is enough to evoke an inflammatory response, whether it is through infusion of uric acid or KA.

A third finding was that overexpression of the *UOx* gene reduces the expression of ASC and CASP1 in animals that did not experience seizures, which suggests that chronic uric acid decreases can induce a permanent decrease in the expression of inflammasome components. This reduction was however not sufficient to affect seizure susceptibility (Thyrion et al., 2016). Although the observed lack in difference between genotypes in KA-infused animals could suggest that a chronic reduction in uric acid levels does not prevent seizure-induced expression of inflammatory markers either, one needs to be cautious about drawing conclusions on the effect of *UOx* overexpression on KA-induced inflammation. Indeed, only TNF expression was significantly upregulated by KA infusion and there was a high level of variation in the KA groups for most markers.

A fourth finding was that disruption of *UOx* and chronically elevated levels of uric acid increased the expression of IL-1Ra (and possibly also IL-1β) in animals continuously infused with modified Ringer's solution, but decreased the expression of these inflammatory markers in animals that experienced seizures. The latter is contradictory to what one could expect based on the increased number of generalized seizures in *UOx* KO mice, but could alternatively be related to the decrease in seizure susceptibility (i.e. seizure latency) in these animals.

A limitation of this study was the low statiscital power, which resulted from the rather low, unequally distributed number of animals in several experimental groups and the high level of variation, especially in the *UOx* OE experiment. Variation was likely caused by an accumulation of technical and biological differences during the procedures leading up to RNA isolation, including surgery, probe implantation, treatment and seizure induction. Also differences in phenotypic expression of the genotype could contribute to the observed variability. Indeed, both baseline and induced uric acid levels could differ greatly in individual *UOx* OE and *UOx* KO animals. A second limitation of the study was that the KA-infused groups lacked a consistent alteration in inflammasome components, IL-1β and IL-18 expression, even when not taking into account the *UOx* OE data. Although this can be explained by variation in Ringer-infused control group in the allopurinol experiment for IL-1β and the biological significance of the found (statistically significant) reductions in expression is questionable in general for IL-18, this variation is more problematic for the interpretation of the ASC and NLRP3 data, where there was either a strong induction or none at all. This in turn may have been caused by intra- (Portelli et al., 2009) or extrastrain (McKhann et al., 2003) differences, which are known to be able to affect seizure-related changes in neurophysiology. The *UOx* KO mice indeed had a mixed B6;129S7/J background, while the other animals had a pure B6 background. A final, more set-up related drawback of this study was that only one timepoint was included, while the inflammatory response is a highly dynamic process.

Conclusions

Our results show that only in the conditions where manipulation of uric acid levels resulted in the seizure-modifying effect, i.e. allopurinol treatment and *UOx* KO mice, an alteration in the post-seizure inflammatory state was found. This suggests that the effect of uric acid manipulations on seizures rather than changes in uric acid levels itself are responsible for modulation of post-seizure inflammation. That both allopurinol treatment and *UOx* KO are associated with a downregulation of inflammatory markers, despite having opposing effects on seizure generalization, indicates that the mechanisms leading to a specific inflammatory profile are multiple.

Conflict of interest

None of the authors have any actual or potential financial conflict of interest to disclose.

Acknowlegdements

Lisa Thyrion is supported by a junior researcher grant from the 'Bijzonder onderzoeksfonds (BOF)' of Ghent University (01D22511). Prof. Dr. R. Raedt and Prof. Dr. K. Vonck are supported by a BOF-grant from Ghent University Hospital. Prof. Dr. P. Boon is supported by grants from the 'Fonds Wetenschappelijk Onderzoek (FWO)' and from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital. The authors would like to thank Elke Ydens for the support with the RT-qPCR analyses.

References

Day RO, Graham GG, Hicks M, McLachlan AJ, Stocker SL, Williams KM (2007) Clinical pharmacokinetics and pharmacodynamics of allopurinol and oxypurinol. Clin Pharmacokinet 46:623-644.

Devinsky O, Vezzani A, Najjar S, De Lanerolle NC, Rogawski MA (2013) Glia and epilepsy: excitability and inflammation. Trends Neurosci 36:174-184.

Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339-347.

Johnson RJ, Lanaspa MA, Gaucher EA (2011) Uric acid: a danger signal from the RNA world that may have a role in the epidemic of obesity, metabolic syndrome, and cardiorenal disease: evolutionary considerations. Semin Nephrol 31:394-399.

Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120:1939-1949.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16:413-419.

McKhann GM, 2nd, Wenzel HJ, Robbins CA, Sosunov AA, Schwartzkroin PA (2003) Mouse strain differences in kainic acid sensitivity, seizure behavior, mortality, and hippocampal pathology. Neuroscience 122:551-561.

Moshe SL, Perucca E, Ryvlin P, Tomson T (2015) Epilepsy: new advances. Lancet 385:884-898.

Portelli J, Aourz N, De Bundel D, Meurs A, Smolders I, Michotte Y, Clinckers R (2009) Intrastrain differences in seizure susceptibility, pharmacological response and basal neurochemistry of Wistar rats. Epilepsy Res 87:234-246.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Rock KL, Latz E, Ontiveros F, Kono H (2010) The sterile inflammatory response. Annu Rev Immunol 28:321-342.

Thyrion L, Raedt R, Portelli J, Van Loo P, Wadman WJ, Glorieux G, Lambrecht BN, Janssens S, Vonck K, Boon P (2016) Uric acid is released in the brain during seizure activity and increases severity of seizures in a mouse model for acute limbic seizures. Exp Neurol 277:244-251.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

Vezzani A, Auvin S, Ravizza T, Aronica E (2012) Glia-neuronal interactions in ictogenesis and epileptogenesis: role of inflammatory mediators.

Vezzani A, Baram TZ (2007) New roles for interleukin-1 Beta in the mechanisms of epilepsy. Epilepsy Curr 7:45-50.

Vezzani A, Viviani B (2014) Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. Neuropharmacology.

Wu X, Wakamiya M, Vaishnav S, Geske R, Montgomery C, Jr., Jones P, Bradley A, Caskey CT (1994) Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. Proc Natl Acad Sci U S A 91:742-746.

Research Aims

(1) develop and characterize a seizure model that allows to monitor the changes in local uric acid levels:
(2) investigate the effects of pharmacological and genetic uric acid modulations on the seizure characteristics of this model
(3) investigate the effects of these manipulations on the seizure-associated inflammatory response
(4) examine if altering uric acid levels changes seizure characteristics in a different seizure model

Chapter 7

Disruption, but not overexpression of urate oxidase alters susceptibility to pentylenetetrazole and pilocarpine induced seizures in mice

Lisa Thyrion, Jeanelle Portelli, Robrecht Raedt, Griet Glorieux, Lars E. Larsen, Mathieu Sprengers, Wouter Van Lysebettens, Evelien Carrette, Jean Delbeke, Kristl Vonck, Paul Boon

Epilepsia, In Press

Abstract

There is a continuous drive to find new, improved therapies that have a different mechanism of action in order to help diminish the sizable percentage of pharmacoresistant epileptic persons. Uric acid is increasingly recognized to contribute to the pathophysiology of multiple disorders and there are indications that uric acid might play a role in epileptic mechanisms. Nevertheless, studies that directly investigate its involvement are lacking. In this study we assessed the susceptibility to pentylenetetrazole- and pilocarpine-induced seizures in mice with genetically altered uric acid levels by targeting urate oxidase, which is the enzyme responsible for uric acid breakdown. We found that while disruption of urate oxidase resulted in a decreased susceptibility to all behavioral endpoints in both seizure models, overexpression did not result in any alterations when compared to their wildtype littermates. Our results suggest that chronic increase of uric acid levels may result in decreased brain excitability.

Key words: pentylenetetrazole, pilocarpine, uric acid, urate oxidase, mice

Introduction

Although many persons with epilepsy are aided with the existing anti-epileptic drugs (AED), about 30% remain resistant even after multidrug therapy. It has hence been suggested to focus on new AEDs that have a different mechanism of action than the ones currently available (Rogawski, 2006).

Uric acid is a breakdown product of the purine metabolism that has been linked to an increasing number of disorders over the past decades(Alvarez-Lario and Macarron-Vicente, 2011). Increased serum uric acid levels are associated with gout, metabolic syndrome, cardiovascular disease, schizophrenia and bipolar disorder. Decreased serum uric acid levels are found in patients suffering from multiple sclerosis, Alzheimer's and Parkinson's disease. For many of these disorders there is a growing body of evidence that indicates that uric acid is not only a marker, but an active contributor to the underlying pathophysiology. In this regard, uric acid has been shown to be a therapeutic target in gout (Rock et al., 2013) as well as a therapeutic agent in multiple sclerosis (Markowitz et al., 2009).

There are indications that uric acid might be involved in epilepsy as well. A number of clinical and preclinical epilepsy studies report elevated uric acid levels and, more importantly, suggest that lowering uric acid levels has anticonvulsant potential (Stover et al., 1997, Togha et al., 2007). However, its direct involvement has never been investigated thoroughly.

The aim of this study was to assess the effect of uric acid on seizure susceptibility in the timed intravenous infusion seizure model, using genetically altered mice where urate oxidase (UOx), the enzyme responsible for uric acid breakdown, was targeted. Two different chemoconvulsants were infused to verify that potential seizure-modifying effects of uric acid manipulations were independent of the seizure-induction mechanism. In the first model seizures were induced by infusing pentylenetetrazole (PTZ), a $GABA_A$-receptor antagonist. In the second model seizures were induced by pilocarpine, a muscarine receptor agonist (Vermoesen et al., 2011).

Material and methods

Animals

Hemizygous *UOx* overexpressing (*UOx* OE) mice (C57BL/6 background) were obtained from Kenneth Rock, Department of Immunology, University of Massachusetts, Worcester, USA. The *UOx* transgene in these mice is driven by a strong constitutive (β-actin) promoter (Kono et al., 2010). Homozygous *UOx* knock-out (*UOx* KO) mice were purchased from The Jackson Laboratory (B6;129S7/J background, Maine, USA) and crossbred. Offspring of these breedings were then genotyped for homozygosity and bred separately. The *UOx* KO mice have a constitutive disruption of the *UOx* gene, by insertion of a neomycin gene into exon 3. Allopurinol (200 mg/l) was provided in the drinking water of *UOx* KO breeding pairs and pups until 8 weeks of age to prevent lethality due to hyperuricemia (Wu et al., 1994). All mice were bred at the animal house of Ghent University Hospital and were housed at controlled temperature (21-22°C) and relative humidity (40-60%) conditions. They had a fixed 12-hour light/dark cycle (lights-on period from 7h to 19h) and food and water available ad libitum. Treatment and care were in compliance with guidelines from the European Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University (ECD 15/40). All animals were aged 3-5 months when the experiment was performed. Different animals were used for the uric measurements.

Uric acid measurements

Anesthetized mice (n = 28, 6-8 in each genotypic group) were implanted with an obturator-enclosed guide cannula in the hippocampus (coordinates relative to bregma: anterioposterior: -3.3 mm ; mediolateral: -2.8 mm ; dorsoventral: -2.3 mm). A week after surgery, the obturator was replaced with a microdialysis probe (CMA/7; 2 mm membrane length; theoretical cut-off 6 kDa; CMA) which was continuously perfused with modified Ringer's solution (147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl in ultrapure water, all Sigma Aldrich) at a flow rate of 1.25 µl/min. After an infusion period of minimum 12 hours, three dialysates of 50 minutes were collected (62.5 µl). Uric acid concentration was determined with an adaptation of the HPLC-method used by Fagugli and colleagues (Fagugli et al., 2002). In brief, 45 µl of dialysate was chromatographically separated on a reverse-phase C8 column (3.5 µm, 150 mm x 4.6 mm, Waters) with an Ultrasphere ODS guard column (5 µm, 5 mm x 4.6 mm, Beckman Instruments) using a linear gradient of methanol and ammonium formate buffer (50 mM, pH 3.0, 0 to 100% methanol in 15 min, 1 ml/min). Uric acid was analyzed by UV detection at 300 nm and quantified using calibration curves of standard solutions (Empower software, Waters). For concentrations below the limit of detection (LOD; 0.119 µM) the LOD itself was used.

PTZ and pilocarpine seizure threshold assessment

Experiments were performed as described previously (Vermoesen et al., 2011). Mice were restrained to insert a 29 gauge needle into the tail vein, which was secured to the tail by adhesive tape. This was connected to a 2.5 ml Hamilton syringe mounted on an infusion pump (BASi) via polyethylene tubing (Novolab). Mice were infused with PTZ (7.5 mg/ml, Sigma Aldrich, n = 31) or pilocarpine (24 mg/ml, Sigma Aldrich, n = 32) at a flow rate of 0.125 ml/min, during which they were allowed to move freely in a Plexiglas cage (n = 7-9 in each genotypic group). The following behavioral endpoints of seizure activity were assessed: (1) PTZ: ear twitch, myoclonic twitch, forelimb clonus, falling (clonic convulsion with loss of righting reflexes), tonic hindlimb extension (THE) and death. (2) Pilocarpine: shivering, rearing, falling, THE and death. Latency from the start of PTZ or pilocarpine infusion until the onset of each endpoint was determined. Seizure threshold doses were calculated for each animal according to the following equation: dose (mg/kg) = [concentration (mg/ml) x infusion rate (ml/min) x infusion duration (min) x 1000 g]/ weight mouse (g)]. To minimize peripheral effects of pilocarpine, methyl scopolamine (1 mg/kg, Sigma Aldrich) was administered intraperitoneally 30 minutes prior to infusion. The investigator assessing the behavioral endpoints was blinded to the genotype.

Statistical analysis

All data are expressed as mean ± SEM. Mann-Whitney U tests were performed for comparisons of behavioral endpoints between experimental groups and their respective control groups. Differences were considered significant at the 5% level.

Results

Targeting urate oxidase alters uric acid levels in the brain

Extracellular uric acid levels in the brain of *UOx* KO mice and *UOx* OE mice were found to be respectively 5.5 times higher (Figure 1A) and 2.7 times lower (Figure 1B) than their respective wildtypes.

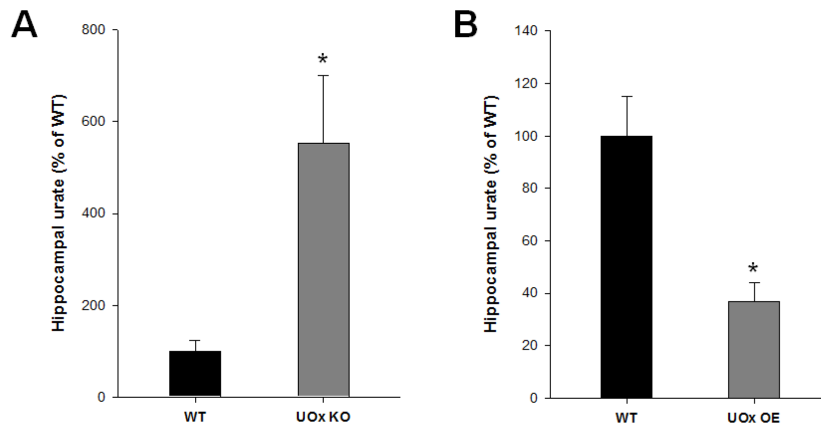


Figure 1 | Extracellular levels of uric acid in the hippocampus of *urate oxidase* (*UOx*) KO and OE mice and their respective wildtype (WT) controls. (A) Hippocampal uric acid levels in male *UOx* KO and WT mice, normalized to the average level in WT mice **(B)** Hippocampal uric acid levels in male *UOx* OE and WT mice, normalized to the average level in WT mice. Bars represent mean ± SEM. n = 6-8 for each group. *$p < 0.05$, Mann-Whitney U test.

Disruption of urate oxidase decreases susceptibility to seizures induced by PTZ and pilocarpine infusion

In *UOx* KO mice, seizure threshold was higher for all behavioral endpoints compared to their wildtype counterparts in both PTZ- and pilocarpine-induced seizure models. There was a significant effect of genotype on the threshold dose of PTZ for inducing ear twitch (WT: 38.6 ± 1.7 mg/kg; *UOx* KO: 62.7 ± 3.8 mg/kg), myoclonic twitch (WT: 47.8 ± 2.6 mg/kg; *UOx* KO: 69.8 ± 3.9 mg/kg), forelimb clonus (WT: 60.3 ± 4.4 mg/kg; *UOx* KO: 83.6 ± 5.9 mg/kg), falling (WT: 73.5 ± 3.0 mg/kg; *UOx* KO: 108.5 ± 4.5 mg/kg), THE (WT: 80.1 ± 4.6 mg/kg; *UOx* KO: 116.1 ± 2.7 mg/kg) and death (WT: 89.5 ± 4.4 mg/kg; *UOx* KO: 126.1 ± 2.9 mg/kg) (Figure 2A). The difference in threshold dose of pilocarpine reached significance for shivering (WT: 86.3 ± 6.3 mg/kg; *UOx* KO: 128.7 ± 10.2 mg/kg), rearing (WT: 410.1 ± 19.0 mg/kg; *UOx* KO: 551.2 ± 17.2 mg/kg), falling (WT: 439.0 ± 19.6 mg/kg; *UOx* KO: 583.8 ± 17.3 mg/kg), THE (WT: 450.2 ± 20.9 mg/kg; *UOx* KO: 595.4 ± 16.9 mg/kg) and death (WT: 465.9 ± 20.8 mg/kg; *UOx* KO: 614.1 ± 17.9 mg/kg) (Figure 2B).

Overexpression of urate oxidase does not affect susceptibility to seizures induced by PTZ and pilocarpine infusion

There were no significant differences between *UOx* OE mice and their wildtype littermates for any of the PTZ or pilocarpine induced behavioral endpoints (Figure 2C,D).

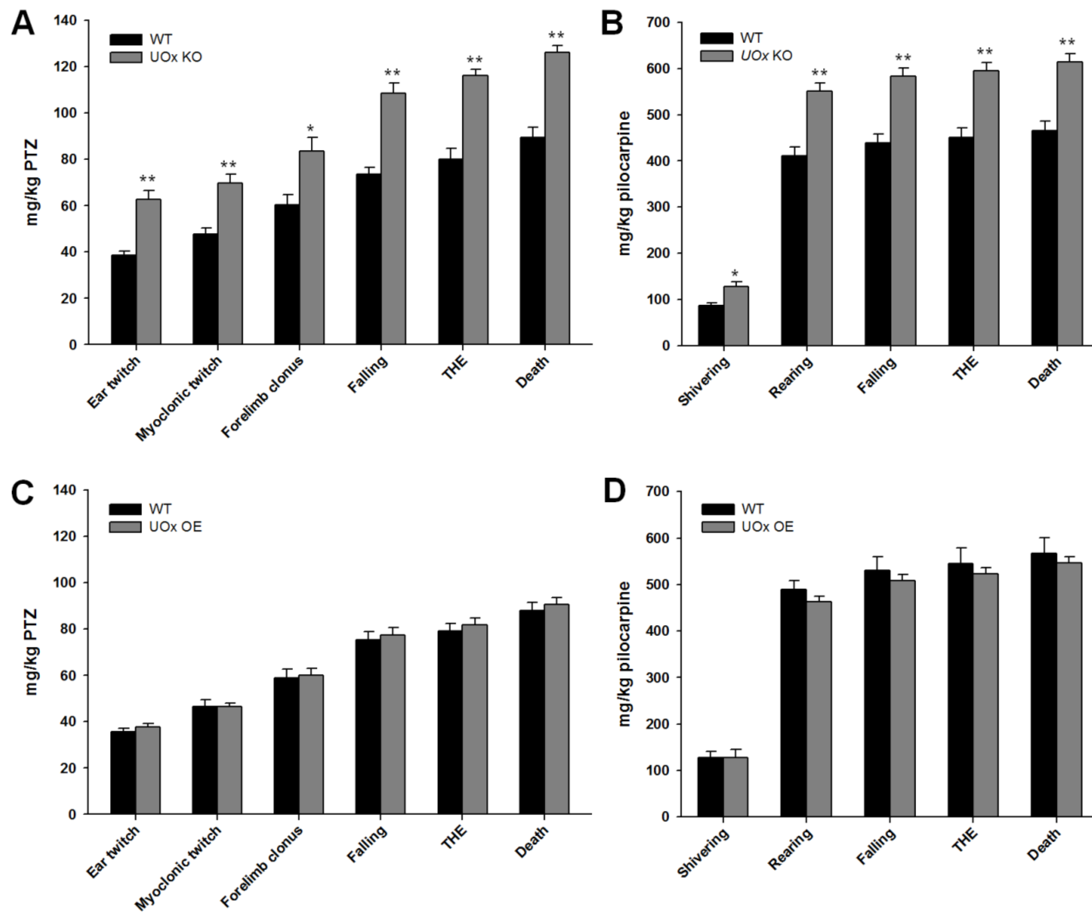


Figure 2 | Behavioral endpoints in *urate oxidase* (*UOx*) KO mice (A,B), *UOx* OE mice (C,D) and their wildtype controls after a timed (A,C) pentylenetetrazole (PTZ) and (B,D) pilocarpine tail infusion experiment. Bars represent mean seizure threshold doses ± SEM. n = 7-9 for each group. Mice are 3-5 months old. *p < 0.05, **p < 0.001, Mann-Whitney U test.

Discussion

Clinical and experimental studies mostly link high uric acid levels to epilepsy (Hamed et al., 2004, Hamed et al., 2007) and indicate that uric acid lowering drugs can be beneficial as add-on therapy (Zagnoni et al., 1994, Togha et al., 2007). Surprisingly, we demonstrated that disruption of the *UOx* gene results in a decreased susceptibility to all PTZ- and pilocarpine-induced behavioral endpoints, which suggest that chronically elevated levels of uric acid decrease brain excitability.

Although the mechanism of action underlying the anticonvulsant-like effect of uric acid has yet to be elucidated, we can hypothesize that the anti-oxidative properties of uric acid are responsible for increasing seizure threshold. The involvement of oxidative stress in seizures, seizure susceptibility and epilepsy are increasingly recognized (Hosseini and Mirazi, 2015, Puttachary et al., 2015, Zhu et al., 2015). It has been shown that different anti-oxidants, including ascorbic acid, decrease seizure susceptibility to various chemoconvulsants, including pilocarpine and PTZ (Gonzalez-Ramirez et al., 2010, Dong et al., 2013). As uric acid is known to inhibit the iron-dependent oxidation of ascorbate, higher uric acid levels might hence promote ascorbate function (Sevanian et al., 1991). In addition, uric acid dependent stabilization of anti-oxidative enzymes such as superoxide dismutase and scavenging of reactive oxygen species such as peroxynitrite could also contribute to the observed decrease in excitability (Kutzing and Firestein, 2008, Puttachary et al., 2015).

Interestingly, in *UOx* OE mice the thresholds to the PTZ- and pilocarpine induced behavioral endpoints were not similarly decreased. This might be due to the fact that a minimum change in uric acid levels is necessary to affect seizure threshold and the phenotype was milder in *UOx* OE mice. Indeed, the reduction in brain uric acid levels was two- to threefold in *UOx* OE mice, compared to a fivefold increase in *UOx* KO mice. However, it is also possible that only a positive change in uric acid levels can evoke an effect on seizures, due to still unknown pharmacological mechanisms.

Conclusion

In summary, our results suggest that chronic elevation of uric acid levels due to disruption of urate oxidase increases seizure threshold in response to two chemoconvulsants. Most likely the anti-oxidant effects of uric acid are responsible for these seizure-suppressing effects. Future studies in complementary (e.g. electrical) seizure models and chronic epilepsy models will be necessary to determine the significance of these results.

Acknowledgements

Lisa Thyrion is supported by a junior researcher grant from the 'Bijzonder onderzoeksfonds (BOF)' of Ghent University. Prof. Dr. R. Raedt and Prof. Dr. K. Vonck are supported by a BOF-grant from Ghent University Hospital. Prof. Dr. P. Boon is supported by grants from the 'Fonds Wetenschappelijk Onderzoek (FWO)' and from the BOF and by the Clinical Epilepsy Grant from Ghent University Hospital. The authors thank the groups of Prof. Bart Lambrecht and Prof. Kenneth Rock for the generous gift of the *UOx* KO and *UOx* WT and the *UOx* OE mice respectively. The authors also thank Maria Van Landschoot for the technical support in the HPLC analysis.

References

Alvarez-Lario B, Macarron-Vicente J (2011) Is there anything good in uric acid? QJM 104:1015-1024.

Dong Y, Wang S, Zhang T, Zhao X, Liu X, Cao L, Chi Z (2013) Ascorbic acid ameliorates seizures and brain damage in rats through inhibiting autophagy. Brain Res 1535:115-123.

Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339-347.

Gonzalez-Ramirez M, Razo-Juarez LI, Sauer-Ramirez JL, Gonzalez-Trujano ME, Salgado-Ceballos H, Orozco-Suarez S (2010) Anticonvulsive effect of vitamin C on pentylenetetrazol-induced seizures in immature rats. Pharmacol Biochem Behav 97:267-272.

Hamed SA, Abdellah MM, El-Melegy N (2004) Blood levels of trace elements, electrolytes, and oxidative stress/antioxidant systems in epileptic patients. J Pharmacol Sci 96:465-473.

Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120:1939-1949.

Markowitz CE, Spitsin S, Zimmerman V, Jacobs D, Udupa JK, Hooper DC, Koprowski H (2009) The treatment of multiple sclerosis with inosine. J Altern Complement Med 15:619-625.

Puttachary S, Sharma S, Stark S, Thippeswamy T (2015) Seizure-induced oxidative stress in temporal lobe epilepsy. Biomed Res Int 2015:745613.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Rogawski MA (2006) Molecular targets versus models for new antiepileptic drug discovery. Epilepsy Res 68:22-28.

Sevanian A, Davies KJ, Hochstein P (1991) Serum urate as an antioxidant for ascorbic acid. Am J Clin Nutr 54:1129S-1134S.

Stover JF, Lowitzsch K, Kempski OS (1997) Cerebrospinal fluid hypoxanthine, xanthine and uric acid levels may reflect glutamate-mediated excitotoxicity in different neurological diseases. Neurosci Lett 238:25-28.

Togha M, Akhondzadeh S, Motamedi M, Ahmadi B, Razeghi S (2007) Allopurinol as adjunctive therapy in intractable epilepsy: a double-blind and placebo-controlled trial. Arch Med Res 38:313-316.

Vermoesen K, Serruys AS, Loyens E, Afrikanova T, Massie A, Schallier A, Michotte Y, Crawford AD, Esguerra CV, de Witte PA, Smolders I, Clinckers R (2011) Assessment of the convulsant liability of antidepressants using zebrafish and mouse seizure models. Epilepsy Behav 22:450-460.

Wu X, Wakamiya M, Vaishnav S, Geske R, Montgomery C, Jr., Jones P, Bradley A, Caskey CT (1994) Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. Proc Natl Acad Sci U S A 91:742-746.

Discussion

Chapter 8

Conclusions, discussion and future perspectives

Conclusions

Due to the high percentage of pharmacoresistance in (temporal lobe) epilepsy, there is a continuous drive to search for therapies with a different mechanism of action and hence the need for a better understanding of the underlying pathophysiology. In this regard, there is an increased interest in inflammatory and (anti-) oxidative pathways to find potential new therapeutic targets. As uric acid is known to affect both processes and there are indications that it could play a role in epilepsy, we set out to **investigate its role in experimental seizure models** for the first time.

In this thesis we have demonstrated the following:

(1) The adapted version of the intrahippocampal KA model using microdialysis to infuse rather than inject KA, resulted in a limbic SE with clearly discernible clinical seizures and an inflammatory response a day later. Uric acid was released during SE and the markers of its inflammatory pathway were induced. The release of uric acid depended on the severity of limbic SE and correlated with the induction of NLRP3 expression.

(2) In this model, blocking uric acid increases with allopurinol and raising uric acid levels through disruption of the *UOx* gene resulted in a twofold decrease and a twofold increase in the number of generalized seizures respectively. Uric acid infusion and overexpression of the *UOx* gene did not induce any electroclinical changes. *UOx* KO mice also displayed a delayed onset of the SE.

(3) The day after limbic SE, only the seizure-modifying uric acid manipulations resulted in an altered inflammatory profile. Both allopurinol treatment and *UOx* KO mice had a reduction in the expression of several inflammatory markers. In the non-KA control animals, uric acid

infusion, overexpression and disruption of *UOx* altered the expression of several markers, while allopurinol did not influence the inflammatory profile.

(4) Disruption of *UOx* resulted in a decreased seizure susceptibility in the PTZ and pilocarpine tail infusion seizure models. *UOx* OE mice did not alter the susceptibility when compared to their wildtype littermates.

Discussion

The **increase of uric acid levels** in the brain in response to a chemoconvulsant is in line with several previous reports (Beal et al., 1991, Layton et al., 1998, Oses et al., 2007, Rambo et al., 2013). Different factors could contribute to local increases in uric acid levels, such as seizure related neuronal injury, cell death and BBB leakage (Beal et al., 1991, Stover et al., 1997, Layton et al., 1998, Rambo et al., 2013). Cell damage and cell death result in the increased turnover of purine-containing molecules, such as DNA and RNA, into uric acid (Layton et al., 1998). BBB leakage distorts the relation between brain and serum uric acid levels, and as the latter has a tenfold higher concentration, this results in an inflow of uric acid in the brain (Tomioka et al., 2013). Importantly, in our study, the amount of uric acid release strongly correlated with the amount of spiking and largely paralleled the spiking activity over time, which might suggest it is a seizure related feature. This would be in line with a previous study where the extracellular uric acid levels doubled in the piriform cortex after intraperitoneal KA injection in rats. Analogous to our study, they found that the steady increase in uric acid aligned with seizure activity (Layton et al., 1998). Moreover, when they injected midazolam to block the seizures, uric acid increases were abolished, indicating it was independent from the direct excitotoxic effects of KA (Layton et al., 1998). It is unclear whether this also holds for our model, however, because the local concentration of KA and extent of the excitotoxic lesion in the intrahippocamal KA model is different compared to the intraperitoneal KA model (Levesque and Avoli, 2013).

As we demonstrated a clear increase in uric acid levels and literature hinted on an anticonvulsive effect of uric acid decreases in clinical and experimental seizure models, we expected that our results would confirm that uric acid is a possible therapeutic target in epilepsy research. Although the results of this thesis indeed suggest that **uric acid is more than an innocent bystander**, they also show that the **story is not that straightforward** (Figure 1). On the one hand, allopurinol treatment and disruption of the *UOx* gene resulted in a twofold decrease and a twofold increase in the number of generalized seizures respectively, which suggest that uric acid can **promote secondary generalization** of seizures. Secondarily generalized tonic-clonic seizures (SGTCS) occur in 30% of the patients with refractory focal epilepsy and are a major cause of SUDEP (Hemery et al., 2014). A recent study suggested that targeting SGTCS might present an important parameter for add-on treatment choice in these group of patients (Hemery et al., 2014). In this regard, our results indicate

that targeting uric acid can be of particular interest in suppressing this type of seizures. This is in line with studies in patients with intractable epilepsy that found that allopurinol is most effective against SGTCS (De Marco and Zagnoni, 1988, Tada et al., 1991, Zagnoni et al., 1994, Togha et al., 2007). On the other hand, we found that despite the promotion of seizure generalization, chronic increases of uric acid in *UOx* KO mice were associated with a delayed onset of the SE in the intrahippocampal KA model and a delayed onset to seizures in the PTZ and pilocarpine infusion seizure models. This indicates that chronically increasing uric acid levels **increase seizure threshold** and might have therapeutic potential as well.

The question then arises how uric acid is able to affect these processes. Inflammation (Vezzani and Viviani, 2014) and recently also oxidative stress (Puttachary et al., 2015) are now recognized to increase neuronal excitability. Although speculative at this point, the effects of uric acid may be hence related to its pro-inflammatory and/or (anti-) oxidative traits (Kutzing and Firestein, 2008, Fang et al., 2013, Kang and Ha, 2014). Its **anti-oxidative traits** are likely the mechanism of action in the increase in seizure threshold observed in our models. As an anti-oxidant, uric acid can block the formation and actions of peroxynitrite, a powerful oxidant that causes oxidation/nitrosylation of proteins and DNA/RNA, inactivation of enzymes and the peroxidation of lipids (Pacher et al., 2007). Uric acid lowers peroxynitrite formation by preventing the degradation of superoxide dismutase, the converting enzyme of O_2^- (Kutzing and Firestein, 2008). Furthermore, uric acid can inhibit the iron-dependent oxidation of the anti-oxidans ascorbate, preventing further increases of free radicals (Sevanian et al., 1991). In this regard, it has been shown that ascorbic acid can decrease seizure susceptibility to PTZ- and pilocarpine-induced seizures (Gonzalez-Ramirez et al., 2010, Dong et al., 2013). Aside from these direct scavenging effects, the protective actions of uric acid against oxidative stress are suggested to be dependent on the upregulation of the glutamate reuptake transporter EAAT-1 by astrocytes, reducing the amount of extracellular levels of this excitatory neurotransmitter (Du et al., 2007). Uric acid has been shown to hamper disease progression due to its anti-oxidative abilities in neurodegenerative disorders such as multiple sclerosis, Parkinson's and Alzheimer's disease (Alvarez-Lario and Macarron-Vicente, 2011).

The effects on seizure generalization might relate to its **pro-inflammatory traits**. Uric acid is believed to act as a pro-inflammatory danger signal in its crystallized form, which can increase brain excitability through activation of the IL-1β pathway (Vezzani et al., 2012). As uric acid is present at a higher concentration inside the cell (4 mg/ml) compared to the interstitial fluid compartment (6 mg/dl), cell damage results in a steep increase in the local extracellular concentration. When the concentration exceeds the crystallization threshold (7 mg/dl), it crystallizes and engages the cell membrane. After phagocytosis it ruptures the endocytic vesicles, a process that activates the NLRP3 inflammasome through yet an incompletely understood process (Rock et al., 2013). Activation of the inflammasome results in the cleavage and activation of caspase-1, which in turn converts pro-IL-1β and pro-IL-18 into their biologically active form. Importantly, uric acid is able to induce an

inflammatory response without inflammasome involvement as well, through Syk- and PI3-kinases (Ng et al., 2008, Kool et al., 2011).

Our experimental set-up did not allow to determine if MSU crystals were present, and hence at this point in time, we **cannot be certain that a crystal-induced inflammatory pathway is involved** in seizure generalization. Not much is known about the formation of MSU crystals *in vivo* and even less outside the context of gouty arthritis, although the concentration of uric acid is considered a major contributing factor. In that regard, crystallization in the joint is assumed to occur above a threshold of 405 μM in humans (Rock et al., 2013), although it is becoming evident that the crystallization threshold is heavily dependent on other factors, such as temperature, pH, local calcium concentration, and mechanical stress. As such, gout is known to develop in patients with serum uric acid levels below 405 μM (Martillo et al., 2014). In a similar way, preclinical studies where increased uric acid levels were detected, crystallization was often assumed without the premise of reaching a certain threshold (Kono et al., 2010, Kool et al., 2011). It is also likely that the situation in *in vitro* studies or even *in vivo* studies in the joints, lungs or liver do not reflect the situation in the brain, as the local environment is completely different. Although crystallization is said to occur at a low pace *in vivo*, it is assumed that a sudden increase in uric acid concentration, as occurs during cell damage or cell death, can vastly promote crystal nucleation (Fiddis et al., 1983, Kono et al., 2010). In addition, *in vitro* studies suggest that growth starts immediately when MSU seeds are added to a supersaturated uric acid solution (Erwin and H., 1981). The fact that we were able to induce an inflammatory response upon infusion with uric acid, does suggest that at least in that condition, MSU crystals were formed in a 24-hour period. Together with the fact that in KA-animals, additional uric acid infusion did not increase NLRP3 expression above that in animals infused with KA alone, shows that the effects of these crystals already reached a maximum after KA-infusion. Assuming that MSU crystals are the driving factor of seizure generalization, the lack of effects after intrahippocampal uric acid administration and in *UOx* overexpression on the number of generalized seizures in the intrahippocampal KA model could be the result of a number of factors. First, intrahippocampal administration of uric acid might not have increased secondary generalization of hippocampal seizures because it did not diffuse far enough to affect brain regions responsible for seizure generalization, such as thalamus and substantia nigra (Gale, 1992, Blumenfeld et al., 2009, Yoo et al., 2014). In contrast, increases in uric acid levels are likely to extend beyond the region of the microdialysis probe in *UOx* KO mice (Chen et al., 2013). Secondly, the *UOx* OE mice displayed only a modest phenotype. Seizure-induced release of uric acid in these animals was not blocked to the same extent as with allopurinol treatment. There was still an induction of uric acid which at peak level was in average 1.5 times higher than baseline levels. Moreover, compared to *UOx* KO mice, the chronic change in uric acid levels was smaller (twofold vs. fivefold) and perhaps too small to evoke any effect. However, it remains to be determined if the steep increase in extracellular levels of uric acid is associated with sufficient crystallization to affect SE characteristics within the range of our

experiment. In this regard, it remains possible that MSU crystals are only formed in the *UOx* KO mice, where an acute increase in uric acid levels is superimposed onto chronically elevated uric acid levels. In that scenario the effects of allopurinol treatment did not directly relate to a decrease in uric acid levels but rather to other effects of blocking xanthine oxidase, such as increases in adenosine and inosine, which both have been reported to reduce seizure susceptibility (Boison, 2012, Kovacs et al., 2015).

A last explanation for our data could be that the acute release in uric acid does affect seizure generalization, but through a MSU-independent **pro-oxidative** pathway. There are several indications that its anti-oxidative actions are limited to the hydrophilic environment of biological fluids, such as plasma. In this regard, intracellular uric acid is believed to mainly contribute to oxidative stress, as shown in cultured cells and animal models of renal and vascular disease (Sautin and Johnson, 2008, Alvarez-Lario and Macarron-Vicente, 2011, Kang and Ha, 2014). As a pro-oxidant, it has been suggested that the different radicals of uric acid can increase oxidative damage, mainly to lipids, by reacting with other radicals. In addition, it has been shown that it is able to engage an intracellular system of superoxide generation by NADPH oxidase. The intermediate steps leading to NADPH oxidase activation are still incompletely understood, although it has been put forward that its pro-inflammatory characteristics again might be involved (Sautin and Johnson, 2008).

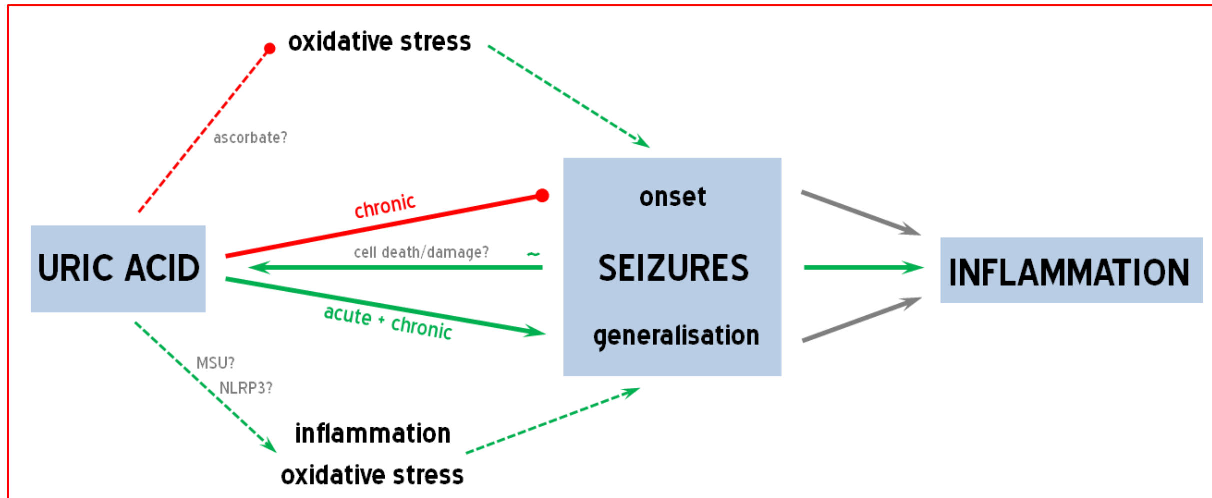


Figure 1 | Summary of our most important results (full lines) and the suggested potential role (dashed lines) of uric acid in seizure generation and seizure-associated inflammation. ~ indicates correlation between seizure severity and uric acid release. Red, green and grey line color indicates inhibitory, stimulatory and complex actions respectively. The complex action concerns the interplay between changes in seizure onset, seizure generalization and other factors in determining inflammatory outcome. MSU: monosodium urate; NLRP3: NOD-like receptor family, pyrin domain containing 3; IH-KA: intrahippocampal kainic acid; TIS: timed intravenous infusion seizure.

Our results suggest that uric acid has a dual role in seizures. In se it is not abnormal for one molecule to have **multiple functions**. It is actually a fairly common phenomenon which exemplifies

how complex and dynamic most disease processes are. TNF for example, has been reported to harbor both ictogenic and neuroprotective effects, through the receptor TNFR1 and TNFR2 respectively. The progressive reduction in TNFR2 expression and concomitant increase of TNFR1 that has been reported in animal models of epilepsy, shifts the balance towards the excitotoxic effects of this cytokine (Iori et al., 2016). However, it is difficult to explain how it is possible that both functions of the same molecule are expressed in the same model simultaneously, as found in the *UOx* KO mice. Perhaps these mice have an *a priori* lower susceptibility for seizures due to the increased anti-oxidant potential associated with elevated uric acid levels, but is the switch turned when an insult abruptly increases uric acid levels above crystallization threshold which then promotes its pro-inflammatory actions. However, the fact that our results suggest that these animals had an increased expression of IL-1Ra – and possibly IL-1β – compared to control animals, does indicate that MSU crystals might be already present in *UOx* KO animals, which could indicate that this turning point would concern a relative increase in the number MSU crystals, rather than crossing the crystallization threshold itself. Nevertheless, this is not necessarily the case, as the induction of IL-1Ra mRNA might also represent an increased anti-inflammatory potential, what could be in concordance with the delayed latency in these animals. Seizure initiation and seizure propagation might then be affected differently because the anti-oxidant and pro-inflammatory pathways engage distinct networks (Pinto et al., 2005), although the level of the independence of these networks is still a matter of debate (Dudek, 2006).

The lack of a facilitating effect of increased uric acid levels on the threshold of generalized seizures in the PTZ and pilocarpine infusion seizure models could probably be explained by the completely **different spatial and temporal dynamics** in the infusion seizure models compared to the intrahippocampal KA model. In the infusion models the proconvulsant substances result in generalized seizures and subsequent death after three (pilocarpine) to five (PTZ) minutes. In the intrahippocampal KA model, KA is only infused in the hippocampus and the secondary generalized seizures only appear after tens of minutes to hours. It is likely that the time window in the infusion models does hence not allow to see the effects of the pro-oxidative/pro-inflammatory actions of seizure-induced uric acid increases, but only the effect of differences in the baseline uric acid concentration. Moreover, PTZ is known to affect the whole brain immediately, resulting in primary, not secondary generalized seizures (Loyens et al., 2012).

Regarding **seizure-associated inflammation** we showed that one day after KA the hippocampal expression of inflammasome components NLRP3 and ASC was induced and that the induction of NLRP3 expression positively correlated with the amount of uric acid released during SE. This suggests that decreasing uric acid levels during SE might hamper the subsequent epileptogenic process. NLRP3 knock-down was recently shown to result in neuroprotection, decreased inflammation and suppression of the development and severity of spontaneous recurrent seizures (Meng et al., 2014). The results of our third study, where only the seizure-modifying uric acid

manipulations resulted in an altered inflammatory profile the day after KA infusion, however, suggest that the effect of uric acid modulation on inflammation may be largely indirect through its effect on seizure activity. Indeed, NLRP3 expression also correlated with epileptic spiking to some extent in our first study.

Future Perspectives

As many questions remain, **more in-depth evidence is needed** to further confirm a role for uric acid and to unravel the conditions and mechanisms involved in its pro- and anticonvulsant effects.

There are some initial points of interest that call for future studies. First, all of our work in the intrahippocampal KA model was done on the ipsilateral side only. It would be interesting to also **investigate the contralateral side**, where seizures occur but direct toxic effects of KA are absent (Pernot et al., 2011). If uric acid levels show a similar profile compared to the ipsilateral side, this would consolidate the hypothesis that uric acid release is a direct consequence of seizure activity and not KA-induced cell death (Stover et al., 1997, Layton et al., 1998, Pernot et al., 2011). The release of other endogenous danger molecules have been shown to be both seizure-dependent (e.g. ATP (Dale and Frenguelli, 2009)) and cell-death dependent (e.g. HMGB1 (Luo et al., 2014)). With regard to seizure-associated inflammation, analyzing the contralateral inflammatory response would also eliminate the confounding KA factor, and would allow for a more direct investigation of the relationship between seizures, uric acid and the expression of cytokines and inflammasome components. It is to be expected that the response will be generally milder here, as the inflammatory and structural changes are most pronounced on the ipsilateral side in the intrahippocampal KA model (Pernot et al., 2011).

Secondly, the **visualization of MSU crystals** could support an immune-based theory of seizure manipulation by uric acid and would be highly interesting for uric acid research in general. There are multiple procedures for the visualization of MSU crystals in the human joint and dermis, including histological and imaging techniques, as means to diagnose and monitor gouty arthritis (Parathithasan et al., 2016). Histologically, the demonstration of negatively birefrigent, needle-shaped MSU crystals in eosin-stained samples using polarized microscopy is the most generally used method (Shidham et al., 2001). Imaging techniques include radiography, ultrasonography, conventional and dual energy CT and MRI (Dalbeth et al., 2012). However, to our knowledge, MSU crystallization has never been actually demonstrated in preclinical models, not for gout, nor for any other condition related to hyperuricemia. The most frequently used model for gout involves the injection of MSU crystals rather than awaiting their natural development (Moilanen et al., 2015), something that has been accepted in other research fields as well, e.g. the fields of asthma and liver injury (Kono et al., 2010, Kool et al., 2011). Conversely, crystallization is presumed when an increase in uric acid is observed (Kono et al., 2010, Kool et al., 2011). It would hence be interesting to optimize staining and/or imaging techniques for use in mice to confirm that MSU crystals are

indeed present *in vivo* and investigate the specific requisites for their development. One could then also examine if MSU crystals are present in pre- and post-KA brain tissue of *UOx* KO animals.

Lastly, it is also worthwhile **investigating further whether allopurinol could be used as add-on therapy for SE**. Similarly to epilepsy, 30% of the SE cases are refractory to the first line of treatment. As it is a life-threatening emergency associated with a high morbidity and mortality, also here the need for new treatments with a different approach is high (Treiman, 2008, Gordon Boyd et al., 2012, Trinka and Shorvon, 2013). In that regard it would be interesting to examine if the SE severity or post-SE neuronal damage could be decreased when allopurinol is administered after SE induction, to probe its therapeutic potential. Subsequently, it could be tested in other chemically or electrically induced convulsive SE models, such as the intrahippocampal pilocarpine model (Portelli et al., 2012) or the perforant path stimulation model (Mazarati et al., 1998), alone or added to a cocktail of established treatments (Loscher, 2015). It would also be interesting to examine if the effectiveness of allopurinol is increased in hyperuricemic conditions, e.g. in *UOx* KO mice. Positive studies in SE models could then be extended to chronic models to re-evaluate its potential as an add-on treatment for standard AEDs. Even if the effects of allopurinol turned out to be only partly dependent on uric acid, this would still be an interesting line to persue.

This brings us to the next step in uncovering a role for uric acid in epilepsy. The seizure models we used in this thesis are relevant for studying the development, maintenance and termination of acute seizures. However, as these models are established in a healthy brain, they do not represent **a chronic epileptic condition** and hence do not give information on the mechanisms underlying epileptogenesis or ictogenesis (Pitkanen et al., 2006). It would hence be of interest to monitor serum/local uric acid levels and analyze the effects of uric acid manipulations at different timepoints after the intrahippocampal induced SE on the characteristics of spontaneous seizures. It might be that the increase in uric acid is limited to the SE which puts into motion a transient/permanent change in the inflammatory and/or oxidative state that alters the neuronal network, but that this is its only point of influence. However, another possibility is that the SE-associated neurodegeneration (Pernot et al., 2011) and spontaneous seizures stimulate additional uric acid release which could result in a sustained higher level of uric acid in the chronic phase, as found in serum of epilepsy patients (Hamed et al., 2004, Hamed et al., 2007). Perhaps the duality of uric acid function has a dynamic profile, and there is a specific time window to target or administer uric acid. Important here is that we will need to determine first if spontaneous recurrent seizures occur in our model, but a previous pilot trial in our group suggests this is indeed the case (Van Loo, 2011). Alternatively, the regular intrahippocampal KA model can be used (Pernot et al., 2011).

Prior to the post-SE model one could consider the less labor-intensive kindling model, in which the hippocampus or amygdala is repeatedly stimulated chemically or electrically with an initially subconvulsive dose/pulse (Pitkanen et al., 2006). Each kindling stimulation evokes an epileptic

seizure, which increases in duration and severity, until the animal is fully kindled. This is a permanent state in which a generalized tonic-clonic seizure can be evoked with only one to a few stimulations. The seizure threshold is considered to reflect seizure susceptibility, the progression to the kindled state the epileptogenic process, and the fully kindled seizures the ictogenic events. Like the post-SE model, the kindling model is a focal model with secondary generalization, and used as a representation of pharmacoresistant TLE, where the need for new therapy development is the strongest (Pitkanen et al., 2006).

To next uncover the **mechanism of action** of uric acid-induced effects on seizures and epilepsy, many techniques can be employed and numerous experiments can be set up. In this regard, it would be ideal to analyze the effects of acute and chronic uric acid changes in a combination of *ex vivo*, *in vitro* and *in vivo* models, while optionally blocking-disrupting/adding-overexpressing relevant receptors/players. In this way a broad range of complementary data can be collected to confirm a role for uric acid and maximize insight into its function. ***Ex vivo*** experiments could entail the comparison of chemoconvulsant-induced epileptiform activity in hippocampal slices of *UOx* KO and their wildtype counterparts (Portelli et al., 2012). To gain insight into its mechanism of action, the presumed pathways could then be blocked, for example by co-infusing *UOx* KO brain slices with inhibitors for NLRP3 (e.g. isoliquiritigenin) or the IL-1β receptor (e.g. anti-mouse IL-1β antibodies) to target the inflammatory pathway, or by adding ascorbate to target the oxidative pathway. *Vice versa* it would also be of interest to analyze if uric acid changes still affect epileptiform activity in mice lacking the *NLRP3* gene. ***In vitro*** set-ups can aid in the determination of the cellular components of the uric acid mediated response, by analyzing the effects of uric acid on neuronal, microglial or astroglial function in pure or mixed cell-cultures. For example, one could analyze the difference in morphology and functionality of cells originating from *UOx* OE and *UOx* KO mice (Panicker et al., 2015) or examine the cytokine profile after uric acid addition in cells of mice lacking the *NLRP3* gene. A dose-response curve of uric acid could then be useful in determining if e.g. cytokine expression is dependent on its concentration and form. ***In vivo,*** it would be of high interest to examine the oxidative and inflammatory state of the brain in naive and in KA-infused *UOx* KO mice at various timepoints. Oxidative parameters could include lipid peroxidation, presence of ROS and glutathione levels (Puttachary et al., 2015). Inflammatory parameters could include the visualization and overlay of potentially involved cellular and molecular markers via fluorescent immunohistochemistry. Using this technique the intramicroglial location and activation of the NLRP3-inflammasome was shown in a preclinical model of Alzheimer's disease (Heneka et al., 2013). In addition, it would be worthwhile to look into the expression and release of cytokines, chemotactic and phagocytotic abilities and overall mobility of microglia in response to the presence of MSU crystals (Krabbe et al., 2012), as these crystals might specifically act as an 'on-signal' for microglia (Biber et al., 2007). Similarly, examining if the anti-oxidant effects are dependent on astrocytic glutamate transport, as was shown for spinal cord injury (Du et al., 2007), could shed light on the

involvement of this cell type. To investigate if the BBB is involved in a uric acid mediated response, BBB leakage between e.g. *UOx* KO/OE animals and their wildtype counterparts could be compared (van Vliet et al., 2016). As age is an important factor in excitability, this should be taken into account when conducting experiments and interpreting results (Kelly, 2010). Together, these and follow-up experiments may uncover the determinants in the effect and nature of uric acid's actions on excitability.

Uric acid is a metabolic product and not a protein resulting from a targetable gene. As a result its levels can mostly be manipulated indirectly only, by targeting the enzymes responsible for its production (xanthine oxidase) and breakdown (urate oxidase – UOx). Although the **uric acid manipulations** used in this dissertation are valuable, they have drawbacks. In general, the BBB is the biggest problem in systemic manipulations, as one does not exactly know the dynamics and extent of the corresponding changes in uric acid levels of the brain. This is an issue when one would want to pharmacologically target the liver-specific UOx enzyme (128 kDa), e.g. when blocking it through administration of potassium oxonate or when administering it directly (Wan et al., 2015). Intravenous uric acid administration has been shown to affect brain insults in a previous study on ischemia, but the local uric acid levels were not investigated (Romanos et al., 2007). Allopurinol, a xanthine oxidase-blocking agent, can cross the BBB due to its small size (0.136 kDa), but has the drawback of being rather nonspecific (Kim et al., 1987). Genetically targeting the *UOx* gene, as we did, has the advantage that it alters uric acid level in a specific way, and also manages to do so locally in the brain. However, a drawback here is that a permanent alteration of UOx activity only allows to investigate chronic uric acid changes and one can never exclude that compensatory mechanisms take place in these animals. Moreover, in the mice we used the entire animal was targeted, rather than only the area of interest. Local uric acid infusion has the advantage of only locally affecting uric acid levels, but has the drawback that it is labor-intensive and cannot be continued for long periods of time. Furthermore, we do not know how far uric acid diffuses in the brain and hence which areas are influenced. For an elaborate and unequivocal investigation of uric acid changes – especially in epileptogenesis and ictogenesis studies – one could consider developing inducible and/or conditional transgenic and knock-out animals for future experiments – if funding and manpower allows it. By manipulating the time- and/or location-dependent expression of *UOx*, one could gain much insight into the 'where and when' of its involvement. This can for example be achieved by using a Cre-Lox system that is transcriptionally controlled by a Tet-On system that itself is under the control of a cell type-specific promotor (e.g. NeuN for neurons) (Gunschmann et al., 2014). However, as the development of these models is expensive, it is likely that complementary pharmacological and genetic models like we used here will be the only option.

Clinically, it would be worthwhile to further investigate whether higher uric acid levels occur more in untreated epilepsy patients than in healthy controls, to see if there is an association. In contrast to what was done in previous studies, it will be important that a clearcut postictal interval is respected

before blood is collected, and that patients are fasted overnight, to avoid dietary-induced variation in purine metabolism. Several subpopulations should also be considered that are clearly delineated in age, sexe, epilepsy syndrome, seizure type, etc. to avoid overlooking possible subcorrelations. Checking whether gout develops more in epilepsy patients or vice versa can give further credence to a uric acid based hypothesis. Measuring uric acid levels in surgery-obtained brain specimens of drug-resistant epilepsy patients would be of particular interest, as this has strengthened the involvement for other danger signals in the past (Maroso et al., 2010). The presence of MSU crystals and increased NLRP3 expression in epileptic brain tissue could also be considered indicative for the involvement of a uric acid-dependent inflammatory pathway.

A last **important note** on the translation of the experimental results on uric acid to the clinic is that there is a genetic difference in uric acid biology between humans and mice. Humans lack the *UOx* gene and hence have higher SUA levels (> 2.0 mg/dL) compared to mice (< 2.0 mg/dL) (Johnson et al., 2011). The expression of *UOx* is liver-specific, but with the existing correlation between uric acid levels in the brain and serum, this cannot be simply ignored (Tomioka et al., 2013). So although studying uric acid in experimental models can give fundamental insights into its involvement, as it did for Parkinson's disease (Chen et al., 2013), multiple sclerosis (Hooper et al., 2000), asthma (Kool et al., 2011) and liver injury (Kono et al., 2010), preclinical data should always be interpreted with caution and should with no means be translated to the human condition without further confirmation. One could consider using *UOx* KO mice as the starting point for all further investigations, but as these animals underwent a forced genetic loss rather than an evolutionary loss of *UOx*, this would likely not represent the human condition either. Indeed, disruption of *UOx* KO is associated with nephropathy-induced death at young age in mice, if not treated with allopurinol, which is not the case in humans (Wu et al., 1994). Lastly, it should be noted that the bias in preclinical studies to solely use male animals also needs to be kept in mind when trying to translate results to humans. There is a difference in SUA levels between men (370 µM) and women (291 µM), which may be present in mice as well (Fang and Alderman, 2000).

References

Alvarez-Lario B, Macarron-Vicente J (2011) Is there anything good in uric acid? QJM 104:1015-1024.

Beal MF, Swartz KJ, Finn SF, Mazurek MF, Kowall NW (1991) Neurochemical characterization of excitotoxin lesions in the cerebral cortex. J Neurosci 11:147-158.

Biber K, Neumann H, Inoue K, Boddeke HW (2007) Neuronal 'On' and 'Off' signals control microglia. Trends Neurosci 30:596-602.

Blumenfeld H, Varghese GI, Purcaro MJ, Motelow JE, Enev M, McNally KA, Levin AR, Hirsch LJ, Tikofsky R, Zubal IG, Paige AL, Spencer SS (2009) Cortical and subcortical networks in human secondarily generalized tonic-clonic seizures. Brain 132:999-1012.

Boison D (2012) Adenosine dysfunction in epilepsy. Glia 60:1234-1243.

Chen X, Burdett TC, Desjardins CA, Logan R, Cipriani S, Xu Y, Schwarzschild MA (2013) Disrupted and transgenic urate oxidase alter urate and dopaminergic neurodegeneration. Proc Natl Acad Sci U S A 110:300-305.

Dalbeth N, Doyle A, McQueen FM (2012) Imaging in gout: insights into the pathological features of disease. Curr Opin Rheumatol 24:132-138.

Dale N, Frenguelli BG (2009) Release of adenosine and ATP during ischemia and epilepsy. Curr Neuropharmacol 7:160-179.

De Marco P, Zagnoni P (1988) Allopurinol in severe epilepsy. A preliminary report. Neuropsychobiology 19:51-53.

Dong Y, Wang S, Zhang T, Zhao X, Liu X, Cao L, Chi Z (2013) Ascorbic acid ameliorates seizures and brain damage in rats through inhibiting autophagy. Brain Res 1535:115-123.

Du Y, Chen CP, Tseng CY, Eisenberg Y, Firestein BL (2007) Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity. Glia 55:463-472.

Dudek FE (2006) Kainate-induced status epilepticus A chronic model of acquired epilepsy

Erwin C-YL, H. N (1981) The crystallization and dissolution of sodium urate. Journal of Crystal Growth 53:215-223.

Fang J, Alderman MH (2000) Serum uric acid and cardiovascular mortality the NHANES I epidemiologic follow-up study, 1971-1992. National Health and Nutrition Examination Survey. JAMA 283:2404-2410.

Fang P, Li X, Luo JJ, Wang H, Yang XF (2013) A Double-edged Sword: Uric Acid and Neurological Disorders. Brain Disord Ther 2:109.

Fiddis RW, Vlachos N, Calvert PD (1983) Studies of urate crystallisation in relation to gout. Ann Rheum Dis 42 Suppl 1:12-15.

Gale K (1992) Subcortical structures and pathways involved in convulsive seizure generation. J Clin Neurophysiol 9:264-277.

Gonzalez-Ramirez M, Razo-Juarez LI, Sauer-Ramirez JL, Gonzalez-Trujano ME, Salgado-Ceballos H, Orozco-Suarez S (2010) Anticonvulsive effect of vitamin C on pentylenetetrazol-induced seizures in immature rats. Pharmacol Biochem Behav 97:267-272.

Gordon Boyd J, Debicki DB, Young GB (2012) Temporal lobe epilepsy after refractory status epilepticus: an illustrative case and review of the literature. Epilepsy Res Treat 2012:209701.

Gunschmann C, Chiticariu E, Garg B, Hiz MM, Mostmans Y, Wehner M, Scharfenberger L (2014) Transgenic mouse technology in skin biology: inducible gene knockout in mice. J Invest Dermatol 134:e22.

Hamed SA, Abdellah MM, El-Melegy N (2004) Blood levels of trace elements, electrolytes, and oxidative stress/antioxidant systems in epileptic patients. J Pharmacol Sci 96:465-473.

Hamed SA, Hamed EA, Hamdy R, Nabeshima T (2007) Vascular risk factors and oxidative stress as independent predictors of asymptomatic atherosclerosis in adult patients with epilepsy. Epilepsy Res 74:183-192.

Hemery C, Ryvlin P, Rheims S (2014) Prevention of generalized tonic-clonic seizures in refractory focal epilepsy: a meta-analysis. Epilepsia 55:1789-1799.

Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A, Axt D, Remus A, Tzeng TC, Gelpi E, Halle A, Korte M, Latz E, Golenbock DT (2013) NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature 493:674-678.

Hooper DC, Scott GS, Zborek A, Mikheeva T, Kean RB, Koprowski H, Spitsin SV (2000) Uric acid, a peroxynitrite scavenger, inhibits CNS inflammation, blood-CNS barrier permeability changes, and tissue damage in a mouse model of multiple sclerosis. FASEB J 14:691-698.

Iori V, Frigerio F, Vezzani A (2016) Modulation of neuronal excitability by immune mediators in epilepsy. Curr Opin Pharmacol 26:118-123.

Johnson RJ, Lanaspa MA, Gaucher EA (2011) Uric acid: a danger signal from the RNA world that may have a role in the epidemic of obesity, metabolic syndrome, and cardiorenal disease: evolutionary considerations. Semin Nephrol 31:394-399.

Kang DH, Ha SK (2014) Uric Acid Puzzle: Dual Role as Anti-oxidantand Pro-oxidant. Electrolyte Blood Press 12:1-6.

Kelly KM (2010) Aging models of acute seizures and epilepsy. Epilepsy Curr 10:15-20.

Kim P, Yaksh TL, Burnett PC, Blum MR, Sundt TM, Jr. (1987) Cerebrospinal fluid levels of uric acid in dogs and the effect of allopurinol. Brain Res 402:87-92.

Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120:1939-1949.

Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa C, Hammad H, Lambrecht BN (2011) An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. Immunity 34:527-540.

Kovacs Z, Kekesi KA, Juhasz G, Barna J, Heja L, Lakatos R, Dobolyi A (2015) Non-adenosine nucleoside inosine, guanosine and uridine as promising antiepileptic drugs: a summary of current literature. Mini Rev Med Chem 14:1033-1042.

Krabbe G, Matyash V, Pannasch U, Mamer L, Boddeke HW, Kettenmann H (2012) Activation of serotonin receptors promotes microglial injury-induced motility but attenuates phagocytic activity. Brain Behav Immun 26:419-428.

Kutzing MK, Firestein BL (2008) Altered uric acid levels and disease states. J Pharmacol Exp Ther 324:1-7.

Layton ME, Samson FE, Pazdernik TL (1998) Kainic acid causes redox changes in cerebral cortex extracellular fluid: NMDA receptor activity increases ascorbic acid whereas seizure activity increases uric acid. Neuropharmacology 37:149-157.

Levesque M, Avoli M (2013) The kainic acid model of temporal lobe epilepsy. Neurosci Biobehav Rev 37:2887-2899.

Li M, Hou W, Zhang X, Hu L, Tang Z (2014) Hyperuricemia and risk of stroke: a systematic review and meta-analysis of prospective studies. Atherosclerosis 232:265-270.

Loscher W (2015) Single versus combinatorial therapies in status epilepticus: Novel data from preclinical models. Epilepsy Behav 49:20-25.

Loyens E, Vermoesen K, Schallier A, Michotte Y, Smolders I (2012) Proconvulsive effects of oxytocin in the generalized pentylenetetrazol mouse model are mediated by vasopressin 1a receptors. Brain Res 1436:43-50.

Luo L, Jin Y, Kim ID, Lee JK (2014) Glycyrrhizin suppresses HMGB1 inductions in the hippocampus and subsequent accumulation in serum of a kainic acid-induced seizure mouse model. Cell Mol Neurobiol 34:987-997.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16:413-419.

Martillo MA, Nazzal L, Crittenden DB (2014) The crystallization of monosodium urate. Curr Rheumatol Rep 16:400.

Mazarati AM, Wasterlain CG, Sankar R, Shin D (1998) Self-sustaining status epilepticus after brief electrical stimulation of the perforant path. Brain Res 801:251-253.

Meng XF, Tan L, Tan MS, Jiang T, Tan CC, Li MM, Wang HF, Yu JT (2014) Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. J Neuroinflammation 11:212.

Moilanen LJ, Hamalainen M, Lehtimaki L, Nieminen RM, Moilanen E (2015) Urate crystal induced inflammation and joint pain are reduced in transient receptor potential ankyrin 1 deficient mice--potential role for transient receptor potential ankyrin 1 in gout. PLoS One 10:e0117770.

Ng G, Sharma K, Ward SM, Desrosiers MD, Stephens LA, Schoel WM, Li T, Lowell CA, Ling CC, Amrein MW, Shi Y (2008) Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. Immunity 29:807-818.

Oses JP, Viola GG, de Paula Cognato G, Junior VH, Hansel G, Bohmer AE, Leke R, Bruno AN, Bonan CD, Bogo MR, Portela LV, Souza DO, Sarkis JJ (2007) Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res 75:104-111.

Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315-424.

Panicker N, Saminathan H, Jin H, Neal M, Harischandra DS, Gordon R, Kanthasamy K, Lawana V, Sarkar S, Luo J, Anantharam V, Kanthasamy AG, Kanthasamy A (2015) Fyn Kinase Regulates Microglial Neuroinflammatory Responses in Cell Culture and Animal Models of Parkinson's Disease. J Neurosci 35:10058-10077.

Parathithasan N, Lee WK, Pianta M, Oon S, Perera W (2016) Gouty arthropathy: Review of clinico-pathologic and imaging features. J Med Imaging Radiat Oncol 60:9-20.

Pernot F, Heinrich C, Barbier L, Peinnequin A, Carpentier P, Dhote F, Baille V, Beaup C, Depaulis A, Dorandeu F (2011) Inflammatory changes during epileptogenesis and spontaneous seizures in a mouse model of mesiotemporal lobe epilepsy. Epilepsia 52:2315-2325.

Pinto DJ, Patrick SL, Huang WC, Connors BW (2005) Initiation, propagation, and termination of epileptiform activity in rodent neocortex in vitro involve distinct mechanisms. J Neurosci 25:8131-8140.

Pitkanen A, Schwartzkroin PA, Moshe SL (2006) Models of seizures and epilepsy: Elsevier.

Portelli J, Thielemans L, Ver Donck L, Loyens E, Coppens J, Aourz N, Aerssens J, Vermoesen K, Clinckers R, Schallier A, Michotte Y, Moechars D, Collingridge GL, Bortolotto ZA, Smolders I (2012) Inactivation of the constitutively active ghrelin receptor attenuates limbic seizure activity in rodents. Neurotherapeutics 9:658-672.

Puttachary S, Sharma S, Stark S, Thippeswamy T (2015) Seizure-induced oxidative stress in temporal lobe epilepsy. Biomed Res Int 2015:745613.

Rambo LM, Ribeiro LR, Della-Pace ID, Stamm DN, da Rosa Gerbatin R, Prigol M, Pinton S, Nogueira CW, Furian AF, Oliveira MS, Fighera MR, Royes LF (2013) Acute creatine

administration improves mitochondrial membrane potential and protects against pentylenetetrazol-induced seizures. Amino Acids 44:857-868.

Rock KL, Kataoka H, Lai JJ (2013) Uric acid as a danger signal in gout and its comorbidities. Nat Rev Rheumatol 9:13-23.

Romanos E, Planas AM, Amaro S, Chamorro A (2007) Uric acid reduces brain damage and improves the benefits of rt-PA in a rat model of thromboembolic stroke. J Cereb Blood Flow Metab 27:14-20.

Sautin YY, Johnson RJ (2008) Uric acid: the oxidant-antioxidant paradox. Nucleosides Nucleotides Nucleic Acids 27:608-619.

Sevanian A, Davies KJ, Hochstein P (1991) Serum urate as an antioxidant for ascorbic acid. Am J Clin Nutr 54:1129S-1134S.

Shidham V, Chivukula M, Basir Z, Shidham G (2001) Evaluation of crystals in formalin-fixed, paraffin-embedded tissue sections for the differential diagnosis of pseudogout, gout, and tumoral calcinosis. Mod Pathol 14:806-810.

Stover JF, Lowitzsch K, Kempski OS (1997) Cerebrospinal fluid hypoxanthine, xanthine and uric acid levels may reflect glutamate-mediated excitotoxicity in different neurological diseases. Neurosci Lett 238:25-28.

Tada H, Morooka K, Arimoto K, Matsuo T (1991) Clinical effects of allopurinol on intractable epilepsy. Epilepsia 32:279-283.

Togha M, Akhondzadeh S, Motamedi M, Ahmadi B, Razeghi S (2007) Allopurinol as adjunctive therapy in intractable epilepsy: a double-blind and placebo-controlled trial. Arch Med Res 38:313-316.

Tomioka NH, Nakamura M, Doshi M, Deguchi Y, Ichida K, Morisaki T, Hosoyamada M (2013) Ependymal cells of the mouse brain express urate transporter 1 (URAT1). Fluids Barriers CNS 10:31.

Treiman DM (2008) Importance of early recognition and treatment of generalised convulsive status epilepticus. Lancet Neurol 7:667-668.

Trinka E, Shorvon S (2013) Status epilepticus--where are we in 2013? Epilepsia 54 Suppl 6:1-2.

Van Loo P (2011) The role of uric acid in epilepsy. Master thesis Biomedical Sciences.

van Vliet EA, Otte WM, Wadman WJ, Aronica E, Kooij G, de Vries HE, Dijkhuizen RM, Gorter JA (2016) Blood-brain barrier leakage after status epilepticus in rapamycin-treated rats I: Magnetic resonance imaging. Epilepsia 57:59-69.

Vezzani A, Auvin S, Ravizza T, Aronica E (2012) Glia-neuronal interactions in ictogenesis and epileptogenesis: role of inflammatory mediators.

Vezzani A, Viviani B (2014) Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. Neuropharmacology.

Wan X, Xu C, Lin Y, Lu C, Li D, Sang J, He H, Liu X, Li Y, Yu C (2015) Uric acid regulates hepatic steatosis and insulin resistance through the NLRP3 inflammasome-dependent mechanism. J Hepatol.

Wu X, Wakamiya M, Vaishnav S, Geske R, Montgomery C, Jr., Jones P, Bradley A, Caskey CT (1994) Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. Proc Natl Acad Sci U S A 91:742-746.

Yoo JY, Farooque P, Chen WC, Youngblood MW, Zaveri HP, Gerrard JL, Spencer DD, Hirsch LJ, Blumenfeld H (2014) Ictal spread of medial temporal lobe seizures with and without secondary generalization: an intracranial electroencephalography analysis. Epilepsia 55:289-295.

Zagnoni PG, Bianchi A, Zolo P, Canger R, Cornaggia C, D'Alessandro P, DeMarco P, Pisani F, Gianelli M, Verze L, et al. (1994) Allopurinol as add-on therapy in refractory epilepsy: a double-blind placebo-controlled randomized study. Epilepsia 35:107-112.

Summary

Epilepsy is a disabling chronic neurological disorder characterized by the occurrence of unprovoked seizures, which result from abnormal excessive or synchronous activity of neuronal populations in the brain. As it affects 65 million people worldwide and has a substantial negative impact on the patient's life, it is considered a major public health concern. The first line of treatment entails the administration of anti-epileptic drugs (AEDs), and although they alleviate the symptoms in many patients, one third remains refractory, even after multi-drug therapy. A particularly refractory type of epilepsy is temporal lobe (limbic) epilepsy (TLE). TLE often develops after an initial precipitating event such as head trauma, encephalitis or status epilepticus (SE), which causes a set of molecular and cellular changes in the neuronal network that increase excitability. As the existing AEDs act purely symptomatic and mostly target the imbalance in excitatory and inhibitory neurotransmission, finding effective therapies with a different mechanism of action is of high priority in epilepsy research. This prompt the need for a better understanding of the underlying pathophysiology and hence requires extensive research in animal models.

Targeting the increased inflammatory state associated with epilepsy is considered a promising strategy in epilepsy therapy development and recently also oxidative stress has become a field of interest. Uric acid is a purine breakdown product that has been shown to influence the pathophysiology of an increasing number of disorders, both through inflammatory and (anti-) oxidative pathways. Several clinical and experimental studies in epilepsy have reported elevated uric acid levels and suggest that lowering uric acid has anticonvulsive potential. Together, this implies that uric acid could be an active player in epilepsy, but its direct involvement was never examined. The aim of this thesis was hence to investigate **a role for uric acid in experimental seizure models**.

We developed and characterized an adapted version of the intrahippocampal kainic acid model for limbic SE, that allowed us to monitor extracellular changes in local uric acid levels during seizure activity, using microdialysis. Infusion of kainic acid resulted in a limbic SE with clinical seizures, and was associated with hippocampal inflammation a day later. The objective of **our first study** in this model was to analyze the seizure-induced changes of uric acid levels and the expression of markers of the NLRP3 inflammasome pathway, the best described inflammatory pathway triggered by uric acid. We showed that uric acid is released during SE and that the markers of its inflammatory pathway are induced the day after kainic acid infusion. Moreover, the release of uric acid depended on the severity of limbic SE and correlated with the induction of NLRP3 expression.

In **a second study** we aimed to unravel the contribution of uric acid to seizure generation, by analyzing the effects of pharmacological and genetic uric acid modulations on the seizure characteristics of the abovementioned model. Pharmacologically, we decreased uric acid levels using

allopurinol, an inhibitor of xanthine oxidase, the enzyme responsible for conversion of hypoxanthine into uric acid. We increased uric acid levels by infusing uric acid into the hippocampus. Genetically, we targeted urate oxidase (*UOx*), the enzyme responsible for the breakdown of uric acid. We used *UOx* overexpressing (OE) and *UOx* knock-out (KO) mice to respectively decrease and increase uric acid levels. We found that allopurinol treatment and disruption of the *UOx* gene resulted in a twofold decrease and a twofold increase in the number of generalized seizures respectively. Together, these results suggest that uric acid promotes secondary generalization of seizures. However, as the reasons for the lack of effect in animals infused with uric acid and mice overexpressing *UOx* can be multiple and are unknown at this point, the exact conditions necessary to evoke a uric acid-mediated effect remain elusive. Moreover, uric acid likely exerts both proconvulsive and anti-convulsive effects, as enhanced pre-seizure levels of uric acid in the *UOx* KO mice delayed the onset of the SE.

The brain tissue of these animals was subject of **a third study**, which investigated the effects of these four uric acid manipulations on seizure-associated inflammatory response in the hippocampus one day after seizure induction. Relevant inflammatory markers and components of the NLRP3 inflammasome were analyzed. Our results suggest that only the seizure-modifying conditions, i.e. allopurinol treatment and *UOx* KO mice, are associated with an alteration in the post-seizure inflammatory state. This would indicate that effects of uric acid manipulation on seizure-associated inflammation are mainly modulated by indirect effects on seizure activity. That both conditions are associated with a decreased expression of inflammatory markers, despite having opposing effects on seizure generalization, indicates that the mechanisms leading to a specific inflammatory profile are multiple.

In **a fourth study**, we analyzed if altering uric acid levels could change seizure characteristics in a second model, the timed intravenous infusion seizure model. More specifically we assessed the susceptibility of *UOx* OE and *UOx* KO mice to pentylenetetrazole- and pilocarpine-induced seizures. We found that while disruption of *UOx* resulted in a decreased seizure susceptibility in both models, overexpression did not result in any alterations compared to the wildtype littermates. This suggests that a chronic increase of uric acid levels may result in an increased seizure threshold.

Taken together, our results indicate that uric acid is **more than an innocent bystander** in seizure generation and its associated inflammatory response, but also suggest that the **nature of its involvement is complex and possibly dual**. On the one hand, the suppression of seizure generalization and reduction in seizure-induced inflammation when uric acid is decreased implies that it can be considered a therapeutic target. On the other hand, the increase in the latency to the first seizure and reduced inflammatory response to increases in uric acid, suggest exactly the opposite. More in-depth evidence will be needed to unravel the conditions and mechanisms in which

uric acid exerts pro- and anticonvulsive effects, but as it would potentially offer novel insights and therapy options, we believe it to be of great interest to conduct further research.

Samenvatting

Epilepsie is een chronisch neurologische aandoening die gekarakteriseerd wordt door het voorkomen van spontane epileptische aanvallen, die ontstaan door het excessief of hypersynchroon vuren van neuronale populaties in de hersenen. Omdat wereldwijd meer dan 65 miljoen mensen lijden aan epilepsie en de ziekte een uiterst negatieve impact op het leven van de patiënt heeft, wordt epilepsie beschouwd als een groot gezondheidprobleem. De eerstelijnsbehandeling is het toedienen van anti-epileptische geneesmiddelen (AEGs), en ondanks het feit dat veel patiënten hiermee geholpen zijn, blijft één derde refractair, zelfs wanneer meerdere AEGs gecombineerd worden. Een bijzonder refractaire vorm van epilepsie is hierbij temporale kwab (limbische) epilepsie (TKE). TKE ontwikkelt zich vaak na een initieel precipiterend insult, zoals een hoofdletsel, encefalitis of status epilepticus (SE). Dit brengt een reeks moleculaire en cellulaire veranderingen in het neurale netwerk tot stand die leiden tot een verhoogde exciteerbaarheid. Omdat de bestaande AEGs louter symptomatisch werken en zich meestal rechtstreeks richten op het onevenwicht tussen exciterende en inhiberende neurotransmissie, is de zoektocht naar nieuwe doeltreffende therapieën met een ander werkingsmechanisme van hoge prioriteit binnen epilepsie onderzoek. Hierbij is het nodig de onderliggende pathofysiologie beter te begrijpen, wat uitgebreid preklinisch onderzoek vereist.

Een veelbelovende strategie in de ontwikkeling van nieuwe AEGs is het beïnvloeden van de verhoogde inflammatoire staat die gepaard gaat met epilepsie en ook oxidatieve stress krijgt recent steeds meer aandacht als potentieel therapeutisch doelwit. Urinezuur, een afbraakproduct van het purine katabolisme, wordt in groeiende mate erkend als spelende factor in the pathofysiologie van verschillende aandoeningen, via zowel inflammatoire als (anti-)oxidatieve mechanismen. Verschillende klinische en preklinische studies rond epilepsie hebben verhoogde urinezuurniveaus aangetoond en suggereren dat het verlagen ervan een anticonvulsief potentieel heeft. Dit alles geeft aan dat urinezuur een actieve rol kan spelen in epilepsie, maar de directe betrokkenheid ervan werd nooit onderzocht. Het doel van deze thesis was dan ook om **de rol van urinezuur in experimentele aanvalsmodellen** na te gaan.

We hebben een aangepaste versie van het intrahippocampaal kainaat model voor limbische SE ontwikkeld en gekarakteriseerd, dat ons toeliet om de extracellulaire veranderingen in de lokale urinezuurconcentratie te monitoren tijdens de aanvalsactiviteit, door middel van microdialyse. Infusie van kainaat resulteerde in een limbische SE met klinische aanvallen, en was geassocieerd met hippocampale inflammatie een dag later. Het doel van **onze eerste studie** in dit model was om aanvalsgeïnduceerde veranderingen in urinezuurniveaus na te gaan, alsook de expressie van merkers van de NLRP3 inflammasoom pathway, wat de best beschreven inflammatoire pathway is die geactiveerd wordt door urinezuur. We toonden aan dat urinezuur vrijgesteld wordt tijdens SE en dat de expressie van de merkers van zijn inflammatoire pathway geïnduceerd worden de dag na

kainaatinfusie. Bovendien was de vrijstelling van urinezuur afhankelijk van de ernst van de SE en correleerde dit met de inductie van NLRP3 expressie.

In **een tweede studie** hebben we getracht de betrokkenheid van urinezuur in aanvalsontwikkeling te bepalen, door het effect van farmacologische en genetische urinezuurmodulaties op de aanvalseigenschappen na te gaan. Farmacologisch hebben we de urinezuurniveaus verlaagd door gebruik te maken van allopurinol, een inhibitor van xanthine oxidase, het enzym verantwoordelijk voor omzetting van hypoxanthine in urinezuur. Een stijging in urinezuurniveaus werd bekomen door urinezuur via de microdialyse probe in de hippocampus te infuseren. Genetisch hebben we uraat oxidase (*UOx*) gemanipuleerd, het enzym verantwoordelijk voor de afbraak van urinezuur. Hierbij hebben we *UOx* overexpresserende (OE) en *UOx* knock-out (KO) muizen gebruikt om de urinezuurniveaus respectievelijk te verlagen en te verhogen. We vonden dat een behandeling met allopurinol en de uitschakeling van *UOx* resulteerde in respectievelijk een tweevoudige daling en een tweevoudige stijging in het aantal gegeneraliseerde aanvallen. Samen suggereren deze resultaten dat urinezuur secundaire generalisatie promoot. Het gebrek aan effect in dieren geïnfuseerd met urinezuur en dieren die *UOx* overexpresseren kan meerdere oorzaken kennen, waardoor de exacte condities waarin urinezuur dit effect veroorzaakt, evenwel onopgehelderd blijven. Bovendien heeft urinezuur waarschijnlijk zowel pro- als anticonvulsieve effecten, aangezien de verhoogde urinezuurniveaus in de *UOx* KO dieren resulteerden in een latere start van de SE.

Het hersenweefsel van deze dieren werd gebruikt **in een derde studie**, waarin onderzocht werd wat het effect is van deze vier urinezuurmanipulaties op de aanvalsgeassocieerde inflammatoire respons in de hippocampus een dag na aanvalsinductie. Relevante inflammatoire merkers en componenten van het NLRP3 inflammasoom werden geanalyseerd. Onze resultaten suggereren dat enkel die condities die een effect hadden op aanvallen, i.e. de behandeling met allopurinol en de *UOx* KO dieren, de aanvalsgeassocieerde inflammatoire staat beïnvloedden. Dit doet vermoeden dat het effect van urinezuur op manipulatie van aanvalsgeassocieerde inflammatie voornamelijk indirect gemoduleerd wordt, via indirecte effecten op aanvalsactiviteit. Het feit dat beide condities geassocieerd zijn met een verlaagde expressie van inflammatoire merkers, ondanks het feit dat ze een tegengesteld effect hadden op de generalisatie van aanvallen, toont aan dat er meerdere mechanismen spelen in het vormen van een specifiek inflammatoir profiel.

In **een vierde studie** gingen we na of de modulatie van urinezuurniveaus de aanvalseigenschappen in een tweede model, het intraveneuze infusie aanvalsmodel, kon veranderen. Meer specifiek onderzochten we de gevoeligheid van *UOx* OE en *UOx* KO muizen voor pentylenetetrazol- en pilocarpine-geïnduceerde aanvallen. We vonden dat de uitschakeling van *UOx* resulteerde in een verlaagde gevoeligheid voor aanvallen in beide modellen, terwijl overexpressie van *UOx* geen enkele verandering ten opzichte van zijn wildtype tegenhanger met zich meebracht. Dit suggereert dat een chronische stijging in urinezuur kan leiden tot een verhoogde aanvalsdrempel.

Samengenomen suggereren deze resultaten dat urinezuur **meer is dan een onschuldige bijstaander** in de ontwikkeling van aanvallen en aanvalsgeassocieerde inflammatoire respons, maar dat **de aard van de betrokkenheid complex en mogelijk tweezijdig** is. Aan de ene kant duidt de onderdrukking van aanvalsgeneralisatie en de reductie in aanvalsgeassocieerde inflammatie bij urinezuurdalingen erop dat urinezuur kan gezien worden als een therapeutisch doelwit. Aan de andere kant suggereren de toegenomen latentie tot de eerste aanval en de gereduceerde inflammatoire respons op de toegenomen urinezuurniveaus, exact het tegenovergestelde. Doortastend onderzoek zal nodig zijn om de precieze condities en mechanismen te ontrafelen waarin urinezuur pro- dan wel anticonvulsieve effecten uitoefent. Aangezien het mogelijk nieuwe inzichten en therapeutische opties met zich mee kan brengen, geloven we er echter sterk in dat verder onderzoek waardevol zou zijn.

Dankwoord | Acknowledgements

Ondanks het feit dat er zich tijdens dit doctoraat meer dan genoeg emotionele momenten voor hebben gedaan, valt dat in dit boekje nergens te bespeuren. Voor iedereen die alles heeft overgeslagen en onmiddellijk naar deze pagina is gesprongen: neem het maar van mij aan dat alle voorgaande pagina's enkel droge, wetenschappelijke koek bevatten. Het is zo droog, dat je haast zou denken dat de woorden zichzelf spontaan in deze volgorde hebben gestructureerd om een punt duidelijk te maken aan de wereld. Maar niets is minder waar, natuurlijk, en zonder de hulp van talloze mensen zou er gewoonweg geen boekje geweest zijn om van te spreken. Want verdomme, het was lastig.

Eerst en vooral wil ik mijn promotor **Prof. Paul Boon** bedanken om mij de kans te geven om in een stimulerend kader aan onderzoek te kunnen doen en mij, ondanks de drukke agenda, te ondersteunen op een aantal kritische momenten in mijn doctoraat.

Verder had ik graag mijn copromotor **Prof. Robrecht Raedt** bedankt voor de begeleiding in het uitbouwen van mijn kennis over neurowetenschappen in het algemeen en epilepsie in het bijzonder, wat voor mij veelal onbekende terreinen waren aan de start van dit project. Je immer kritische blik en wetenschappelijk inzicht waren onontbeerlijk voor het tot stand brengen van dit doctoraatswerk.

Ook de voorzitter **Prof. Dr. Ernst Rietzschel** en de leden van de lees- en examencommissie, **Prof. Dr. Ilse Smolders, Prof. Dr. Eleonora Aronica, Prof. Dr. Geert Van Loo, Prof. Dr. Romain Lefebvre, Prof. Dr. Veerle De Herdt, Dr. Guy Laureys,** wil ik graag bedanken voor het nalezen van mijn thesis en de nuttige feedback. Ook veel dank aan de administratief secretaris, **Lynn De Poorter,** en de decaan van onze faculteit, **Prof. Guy Vanderstraeten**, voor hun bijdrage.

Ook **Prof. Kristl Vonck** en **Dr. Jeanelle Portelli** ben ik bijzonder dankbaar voor hun waardevolle input bij elke stap van het denk- en schrijfproces. Jeanelle, you really pulled me through at the end. Thank you so much!

Andere leermeesters waar ik veel van heb opgestoken waren **Prof. Wytse Wadman** en **Prof. Jean Delbeke**.

Geen doctoraat zonder de nodige administratie natuurlijk, en in dat kader had ik graag alle medewerkers van het secretariaat bedankt, in het bijzonder **Corine** en **Elsie,** voor al hun hulp over de jaren heen.

Ook de andere leden van de dienst Neurologie die op een of andere manier hebben bijgedragen aan het tot stand komen van dit werk wil ik bedanken: **Alfred**, **Evelien**, **Veerle**, **Marijke**, **Dimitri**,...

Een woord van dank is er zeker ook voor de mensen buiten het departement Neurologie. Graag had ik **Ingrid**, **Marit**, en **alle dierenverzorgers** bedankt voor de goede begeleiding bij de bestelling, de kweek en de verzorging van de proefdieren.

Daarnaast moet ik ook zeker de groep van **Prof. Raymond Van Holder** bedanken voor het analyseren van de honderden microdialysestalen. **Griet** en **Mieke**: bedankt voor het steeds spoedig inplannen van de analyses op jullie drukbezet HPLC-toestel en al jullie tijd!

Twee andere departementen waar ik veel mensen te danken heb, zijn het IRC in Gent en het VIB in Antwerpen. **Prof. Bart Lambrecht**, **Prof. Sophie Janssens** en **Prof. Vincent Timmerman** wil ik graag bedanken voor de mogelijkheid tot het gebruik van de faciliteiten van hun onderzoeksgroep. **Sophie**, je hebt het nooit nagelaten om tijd te maken en ik heb enorm veel gehad aan onze meetings. **Elke**, zelfs op het einde van je doctoraat heb je de tijd genomen om mij veelvuldig te helpen met de qPCRs. Je bemoedigende woorden hebben me dat nodige duwtje in de rug gegeven. Verder bedankt aan alle andere mensen die mij op een of andere manier geholpen hebben met praktische vragen. Speciale dank ook aan **Lana**, als jaargenoot Biotechnologie was jij mijn aanknopingspunt binnen het labo, ik kon je alles vragen. **Niels**, eeuwige dank voor het gebruik van de robot en voor alle hulp met de qPCR-analyses. Tot slot, **Silvie**, merci voor de lunchdates wanneer ik op het VIB moest zijn, maar ook voor alle toffe momenten daarbuiten.

Als het op mijn directe collega's aankomt, is het zo eenvoudig als dit: zonder jullie ambiance, input en steun had ik er hoogstwaarschijnlijk op een bepaald moment de brui aan gegeven. De werksfeer was geweldig en ik weet nu al dat het moeilijk zal zijn om dit in de toekomst te evenaren. **Bregt**, ik heb de humor rond 'boobsman' nooit begrepen, maar dat is een bijzaak aan het feit dat je mij enorm veel hebt geholpen, ook als je het zelf druk had. **Mathieu**, Meester der Droge Humor, je hebt mij meermaals overtuigd van absolute onzin, maar mij even vaak inzicht gegeven in verschillende zaken (wat ook zo hoort, natuurlijk, als dokter). Je doorzettingsvermogen is werkelijk bewonderenswaardig en ik weet zeker dat je een topneuroloog wordt. **Lars**, I am happy to have had you as a colleague. When struck with doubt or fear or when I had practical problems, you were always prepared to help. And although you like Denmark (and Germany and probably all of the other Scandinavian countries) better than Belgium, I am delighted that Kim has found you, and that you make her happy. **Ine B**, een belangrijke les die ik van jou heb ik geleerd is hoe belangrijk het was om je onderzoek te blijven balanceren met je privéleven om je energiepeil op orde te houden. Verder ben je mij, ook nadat je al een andere job had, blijven helpen en steunen tijdens die lastige laatste loodjes. **Ine D**, jij hebt mij, toen ik nog een nieuweling was, ingewijd in het labo en mij quasi alle technieken aangeleerd. Enorm bedankt voor het nalezen van al die teksten die ik voor je neus geschoven heb. **Lies**, je zit al eventjes op een andere job, maar toen je mijn bureaubuur was, heb ik ook erg veel aan je gehad. Je hebt mij ook de 'hoe en wat' getoond van de microdialyse, een techniek die ik daarna continu heb gebruikt. **Diego** en **Jeroen**, zonder jullie hulp zouden we nu nog in het stenen tijdperk zitten, zonder internet

en zonder monitoring- en stimulatieopstellingen. De nu-al-niet-meer-zo newbies, **Wouter**, **Sofie C** en **Sofie D**, kunnen hier natuurlijk ook niet ontbreken. Vanaf jullie komst hebben jullie mee de sfeer gemaakt, veel succes gewenst de komende jaren! En als het over sfeer gaat, wil ik zeker ook de doctoraatsstudenten en medewerkers Neurologie in de kliniek (**Eline**!), de leden van Medisip (**Nathalie**!) en onze ganggenoten van Centrum Medische Genetica bedanken.

Annelies en Leen, jullie waren collega's van een nog geheel ander kaliber, hoe moet ik dat gaan verwoorden? **Annelies**, onze interesses liggen behoorlijk ver uiteen, maar op een of andere manier klikt het supergoed tussen ons en voelen we elkaar perfect aan. Een blik was vaak genoeg om te weten wat er scheelde en je wist me telkens te helpen focussen als ik het weer eens niet meer zag zitten (ook als dat meerdere keren per dag was). Talloze keren hebben we 'The Destress Tour' gewandeld op het UZ en ik kan je zeggen, het hielp. Zoals ook je gevoel voor humor en grappige insteek hielp (je geitimitatie is nog steeds ongeëvenaard). **Leen,** ik weet niet hoe je het doet, maar ik word altijd vrolijk en enthousiast als ik jou zie. Je bent het voorbeeld van iemand die weet hoe je van het leven moet genieten en je ondernemingszin is aanstekelijk. Het is super om met jou op te trekken en onze uitjes kikkerden me steevast op als het even tegenzat. Ik kon ook steeds bij je terecht voor de behandeling van opgelopen trauma's die hoorden bij het doctoraat. Jij gaat vast een superspannend leven tegemoet. Jullie liggen mij allebei nauw aan het hart, jullie zijn mijn maatjes, mijn vriendinnen. Tot vaak!

Zonder de nodige vrije tijd zou het boekje uiteraard ook niet tot stand gekomen zijn. De boog kan niet altijd gespannen staan. Bedankt daarom aan **al mijn vrienden** (ook degenen die hierboven reeds vermeld zijn) voor al de leuke aangelegenheden de voorbije jaren en de luistersessies als ik weer eens een klaagzang afstak. Jullie hebben mijn doctoraat lichter, luchtiger en daardoor haalbaarder gemaakt. Speciale dank aan **Barbara, Evelien**, **Kim**, **Elia en Mieke** omdat jullie de volledige rit van zeer dichtbij hebben gevolgd en mij toch niet halverwege hebben gedumpt door de overdaad aan PhD-nonsense.

Vanzelfsprekend kan het thuisfront hier ook niet ontbreken. Bedankt **mama** en **papa** voor alle kansen die jullie me hebben gegeven en de onvoorwaardelijke steun de voorbije jaren. De kritische blik, die ik hier zo vaak nodig had, heb ik zeker ook te danken aan jullie. **Sanne** en **Bethje**, mijn siblings, ook aan jullie een dikke merci. Het feit dat we op dezelfde manier prettig gestoord zijn, maakt het een waar plezier om met jullie op te trekken. Het was vaak de ideale afleiding. Zeker ook bedankt aan alle **plusfamilie**: **Nathalie**, you're the best! **Marianne**, **Janne** en **Tom**, bedankt voor alle gezellige momenten in het verre Diksmuide en dichterbij in Gent.

Mijn laatste woorden van dank gaan uit naar **Bram**, mijn toeverlaat, mijn liefde. Dank je voor je eindeloze geduld en ondersteuning de voorbije jaren en voor alle warme momenten samen. Ik ben helemaal klaar voor het vervolg.

Lisa

Gent 2016

Dit werk werd mogelijk gemaakt met de financiële steun van het Bijzonder Onderzoeksfonds van de Universiteit Gent.

Scientific resume

LISA THYRION

°29/10/1987, Ghent

Lindenlei 38, B-9000 Ghent, Belgium

+32 496 62 62 76

lisa.thyrion@gmail.com

Work experience

2011-2016 **Doctoral researcher in Medical Sciences** | BOF aspirant scholarship

Faculty of Medicine and Health Sciences, Ghent University
Topic: 'Investigation of a role for uric acid in experimental seizure models'
Promotors: Prof. Paul Boon and Prof. Robrecht Raedt

Education

2009 – 2011 **Master in Biochemistry and Biotechnology** | Major Biomedical Biotechnology

Faculty of Sciences, Ghent University
Thesis topic: 'TLR9 ligands promote strong cellular immune responses to particulate antigen formulations: role of inflammatory dendritic cells'. Promotors: Prof. Johan Grooten and Dr. Stefaan De Koker
Masterprojects: 'Cloning of potential RIPK1 interaction partners' and 'Maize leaf development'
Graduation: Summa cum laude

2006-2009 **Bachelor in Biochemistry and Biotechnology**

Faculty of Sciences, Ghent University
Bachelorprojects: 'Protein crystallization', 'Molecular analysis of the immune response to TNF' and 'Regulation of the DNA stress checkpoint during cell cycle in plants'
Graduation: Summa cum laude

Scientific honors and awards

01/07/2011 **Ablynx award for best student in Biochemistry and Biotechnology**
Ghent University - Ghent, Belgium

01/07/2011 **KVCV award for best student in Biochemistry and Biotechnology**
Ghent University - Ghent, Belgium

25/06/2012 **EPHAR Fellowship**
11th EPHAR Summerschool of Neuroscience - Catania, Sicily – Italy

11/03/2014 **ILAE Travel Bursary – Platform Session**
11th European Congress on Epileptology - Stockholm, Sweden

28/03/2014 **Best poster prize**
'Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis.'
16th Annual international clinical symposium Kempenhaeghe - Heeze, The Netherlands

12/05/2015 **UGent Mobility Fund**
9th World Congress of the International Brain Research Organization - Rio De Janeiro, Brazil

Publications in international peer-reviewed journals

A1 **Thyrion L**, Raedt R, Portelli J, Van Loo P, Wadman W., Glorieux G, Lambrecht BN, Janssens S, Vonck K, Boon P. Uric acid is released in the brain during seizure activity and increases severity of seizures in a mouse model for acute limbic seizures. Experimental Neurology, IF: 4.696, Q1 Neurosciences.

Thyrion L, Raedt R, Glorieux G, Wadman WJ, Delbeke J, Larsen LE, Sprengers M, Van Lysebettens W, Carrette E, Janssens S, Lambrecht BN, Vonck K, Boon P (Under review). Disruption, but not overexpression of urate oxidase alters susceptibility to pentylenetetrazole and pilocarpine induced seizures in mice. Epilepsia, IF: 4.571, Q1 Clinical Neurology.

Thyrion L, Portelli J, Raedt R, Delbeke J, Larsen LE, Sprengers M, Van Lysebettens W, Carrette E, Vonck K, Boon P (Under review). Induction of the

NLRP3 inflammasome after intrahippocampal kainic acid induced status epilepticus in freely moving mice: a link with uric acid. Neuroscience Letters, IF: 2.030, Q3 Neurosciences.

Thyrion L, Raedt R, Portelli J, Vonck K, Boon P (To be submitted). At the interface of oxidative stress and inflammation: a potential role for uric acid in epilepsy. Neuropharmacology, IF: 5.106, Q1 Neurosciences, Pharmacology and Pharmacy.

Larsen LE, Wadman WJ, Van Mierlo P, Delbeke J, Daelemans D, Sprengers M, **Thyrion L**, Van Lysebettens W, Carrette E, Boon P, Vonck K, Raedt R (In preparation). Modulation of hippocampal activity by vagus nerve stimulation – comparison of stimulation paradigms.

C3 **Thyrion L**, Raedt R, Ydens E, Janssens S, van Loo P, Kips J, Portelli J, Lambrecht BN, Meurs A, Vonck K, Boon P. Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis. 11th European Congress on Epileptology. Epilepsia, IF: 4.571, Q1 Clinical Neurology

Participation (inter)national conferences

2011 **Stimulating (the) brain: neuromodulation and cognitive neuroscience**
12/12/2011 – Institute for Neuroscience – Ghent, Belgium

IUAP Meeting: Molecular and Cellular Mechanisms of Electrical Excitability
16/12/2011 – Interuniversitaire Attractiepolen – Ghent, Belgium

2012 **SWO midwinter meeting**
"New therapies in pharmacoresistant epilepsy"
10/02/2012 – Nederlandse Liga tegen Epilepsie – Amsterdam, The Netherlands

Spring meeting of the Belgian Society of Physiology and Pharmacology
"Microglia – active sensor and versatile effector cells in the normal and pathologic brain" 16/03/2012 – National Committee of Physiology and Pharmacology – Brussels, Belgium

14th Annual international clinical symposium Kempenhaeghe
23/03/2012 – Kempenhaeghe Centre – Heeze, The Netherlands

2nd Ghent International Epilepsy Workshop
26-27/10/2012 – LCEN3 – Ghent, Belgium

Belgian Brain and Cognition Congress
27/10/2012 – Belgian Brain Council – Liège, Belgium

2nd Ghent Institute for Neuroscience Symposium
12/12/2012 – Institute for Neuroscience – Ghent, Belgium

2013

SWO midwinter meeting
"Temporal Lobe Epilepsy with Hippocampal Sclerosis"
01/02/2013 – Nederlandse Liga tegen Epilepsie – Amsterdam, The Netherlands

15th Annual international clinical symposium Kempenhaeghe
22/03/2013 – Kempenhaeghe Centre – Heeze, The Netherlands

Knowledge for Growth Life Sciences Convention
30/05/2013 – FlandersBio – Ghent, Belgium

PhD Day Institute for Neuroscience: Connecting the regions of Interest
13/12/2013 – Institute for Neuroscience, Ghent University – Ghent, Belgium

2014

SWO midwinter meeting
"Status Epilepticus: basic mechanisms and therapy"
14/02/2014 – Nederlandse Liga tegen Epilepsie – Amsterdam, The Netherlands
Oral presentation/Poster presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis

16th Annual international clinical symposium Kempenhaeghe
28/03/2014 – Kempenhaeghe Centre – Heeze, The Netherlands
Poster presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis. Awarded "Best poster" prize.

Students research symposium | Studenten Onderzoekssymposium
29/04/2014 – Ghent University – Ghent, Belgium
Poster presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis

Knowledge for Growth Life Sciences Convention

08/05/2014 – FlandersBio – Ghent, Belgium
Poster presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis

Neuroscience Forum
19/06/2014 – Institute for Neuroscience – Ghent, Belgium
Oral presentation: The role of uric acid in status epilepticus and its associated inflammatory response

11th European Congress on Epileptology
29/06-03/07/2014 – International League Against Epilepsy – Stockholm, Sweden
Oral presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis

Autumn meeting of the Belgian Society of Physiology and Pharmacology
17/10/2014 – National Committee of Physiology and Pharmacology – Brussels, Belgium
Poster presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis

2015

LCEN3 International Peer Review
13/02/2015 – LCEN3 – Ghent, Belgium
Oral presentation: The role of uric acid in status epilepticus and its associated inflammatory response

Science Day of the Faculty of Health sciences and Medicine
05/03/2015 – Ghent University – Ghent, Belgium
Oral presentation: Allopurinol reduces seizure severity and the inflammatory response in a mouse model for status epilepticus: a potential role for uric acid in ictogenesis

SWO midwinter meeting
"Mechanisms of epileptogenesis and potential treatment targets"
11/03/2015 - Nederlandse Liga tegen Epilepsie – Amsterdam, The Netherlands
Oral presentation/Poster presentation: Characterization of status epilepticus induced by intrahippocampal kainic acid infusion in freely moving mice

17th Annual international clinical symposium Kempenhaeghe
27/03/2014 – Kempenhaeghe Centre – Heeze, The Netherlands
Poster presentation: Characterization of status epilepticus induced by intrahippocampal kainic acid infusion in freely moving mice

2nd PhD Day Institute for Neuroscience: Connecting the regions of Interest
03/04/2015 - Institute for Neuroscience, Ghent University – Ghent, Belgium

9th World Congress on Neuroscience
07-11/07/2015 – International Brain Research Organization – Rio De Janeiro, Brazil
Poster presentation: Characterization of status epilepticus induced by intrahippocampal kainic acid infusion in freely moving mice

Courses and trainings

2010 **FELASA-C Laboratory Animal Science**
Ghent University – Ghent, Belgium

2012 **Advanced Epilepsy course**
Vlaamse Liga tegen epilepsie – UZ Jette – Brussels, Belgium

Biosensor Workshop
Sarissa Biomedical – Warwick University – Coventry, UK

Isolation of microglia using flow cytometry
Department of Neuroscience – UMCG – Groningen, The Netherlands

The 10th Summerschool of Neuroscience
"Neuroinflammation in CNS disorders: priming a target for new therapies"
EPHAR – University of Catania – Catania, Sicily

Statistical analysis using SPSS
Ghent University – Ghent, Belgium

qPCR experiment design and data-analysis
Biogazelle – Ghent, Belgium

Concepts of Neurology | Begrippen van Neurologie
Regular Course Bachelor Audiology and Logopedy sciences
Ghent University – Ghent, Belgium

2013 **Advanced course on Epilepsy**
Kempenhaeghe Academic Centre – Heeze, The Netherlands

Advanced Academic English Writing Skills: Life science and medicine
UCT – Ghent University – Ghent, Belgium

2014 **Species-specific welfare in a laboratory environment**
"Rat and mouse"
Ghent University, ILVO, UFAW – Ghent, Belgium

Workshop on general anesthesia and monitoring in rat and mouse
Carfil Quality – Ghent, Belgium

2015 **Popular scientific writing | Schrijven voor niet-vakgenoten en pers**
Ghent University – Ghent, Belgium

Teaching, supervision and other activities

2011-2012 **Master thesis supervision Tineke Desmet**
"Neurotransplantatie in een diermodel voor partiële epilepsie"
Biomedical Sciences

2012-2014 **Research internship | Master thesis supervision Isabel Follebout**
"De rol van urinezuur in epileptogenese"
Biomedical Sciences

Hands-on epilepsy workshop on EEG and design of electrodes
1° Master Students Biomedical Sciences (# 5, three times)

2014-2016 **Research Internship |Master thesis supervision Lien Delmulle**
"De rol van urinezuur in epilepsie"
Biomedical Sciences

Lecture on epilepsy research
1° Master Students Biochemistry and Biotechnology (# 30)

Lab representative of animal welfare cell
Attendance of meetings and implementation of guidelines