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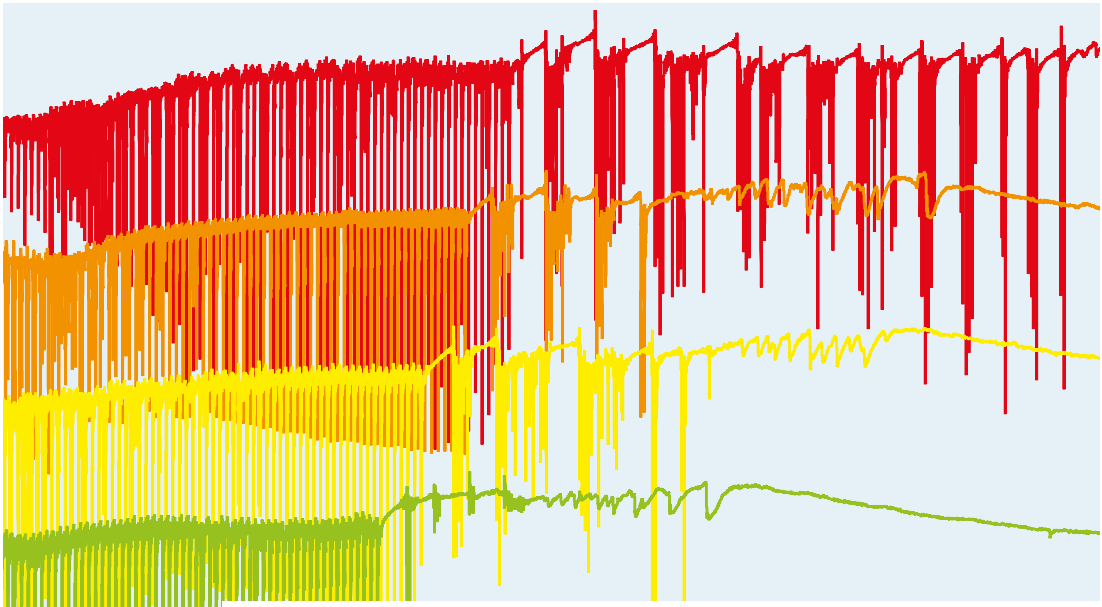

Novel approaches to explore mechanisms of epileptic seizures

Optogenetic and chemogenetic manipulation of hippocampal circuitry

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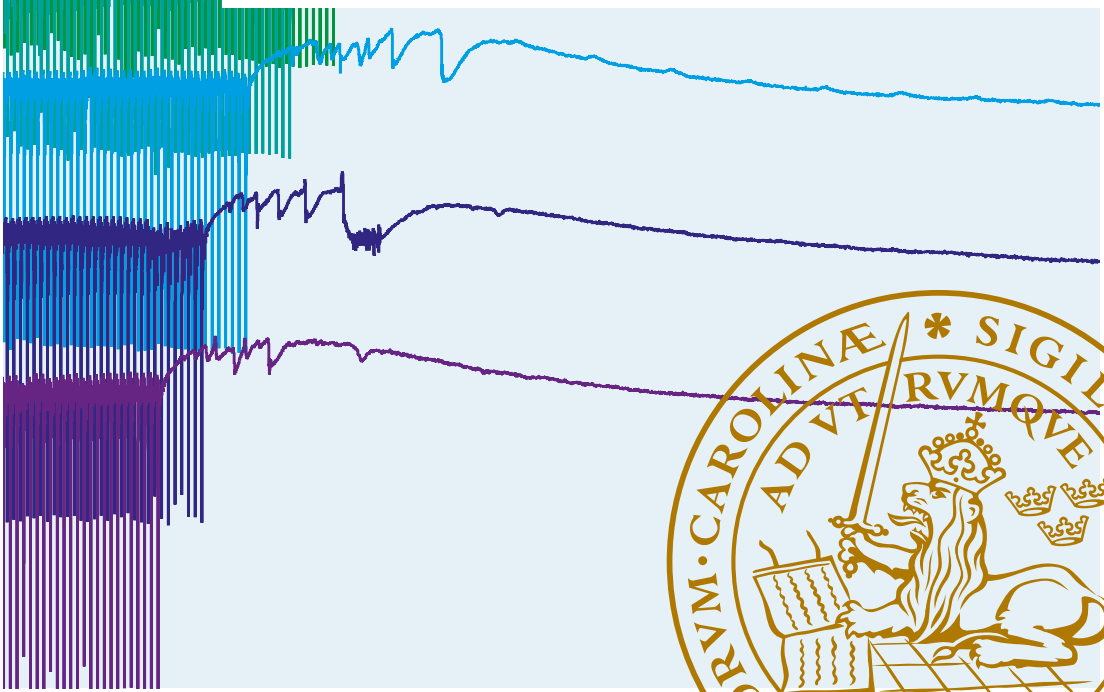

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Optogenetic and chemogenetic manipulation of hippocampal circuitry

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Fredrik Berglind

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DOCTORAL DISSERTATION
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Novel approaches to explore mechanisms of epileptic seizures - Optogenetic and chemogenetic manipulation of hippocampal circuitry

Abstract

Epilepsy comprises a family of neurological disorders characterized by recurrent seizures, which can be highly debilitating. Up to 30% of patients with temporal lobe epilepsy, the most common form of the disorder in adults, arising in the hippocampus, cannot be effectively treated by current pharmaceuticals. Novel treatment strategies are highly needed, as well as increased understanding of the hippocampal components and signaling properties involved in the mechanisms of epileptogenesis and induction of seizures, to guide a rational search for such future treatments.

In the present thesis, we have applied optogenetics to study these questions, a technology based on modified microbial membrane channels or pumps that are introduced into target neurons, which can thereafter be activated by light. This is a powerful means of achieving excitatory or inhibitory control over neurons in a target specific manner.

In paper I, we have explored the use of inhibitory optogenetics to attenuate seizures (epileptiform bursts) in acute chemical models *in vitro* and *in vivo*. GABAergic inhibition was abolished creating a strong excitatory drive among principal neurons in mainly area CA3 of the hippocampus. Under these conditions, we showed that we could inhibit hypersynchronized bursts by activating the inhibitory chloride pump NpHR.

Repeated neuronal discharges gradually creating a hyperexcitable state in the hippocampal circuitry and presenting with seizure-like afterdischarges, known as the kindling process, is traditionally induced by electrical stimulation. In paper II & III, we showed that similarly, afterdischarges could be generated by repeated optogenetic train stimulation in anaesthetized transgenic mice expressing the excitatory cation channel ChR2. Additionally, we corroborated the well known role of the dentate gyrus is this type of progressive seizure model. Finally, using the chemogenetic novel hyperpolarizing Gi-DREADD receptor, activated by CNO, we could show that inhibiting the dentate gyrus and CA3 contralateral to optogenetic stimulation effectively halted the afterdischarge progression.

The work presented in this thesis reinforces the potential of optogenetic and chemogenetic techniques to explore the mechanistic underpinnings and new treatment options for seizures & epilepsy.

Key words

Seizure, epileptogenesis, optogenetics, NpHR, ChR2, DREADD, hippocampus, *in vivo*, mouse

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We know accurately only when we know little;
with knowledge, doubt increases

Johann Wolfgang von Goethe

Content

Original Papers 10

Summary 11

Populärvetenskaplig sammanfattning 13

Abbreviations 15

Introduction 17

- Epilepsy & seizures 17
  - Temporal lobe epilepsy 17
- The hippocampal networks 20
  - Components and basic circuitry 20
  - Connectivity 22
- Models of seizures and epilepsy 25
  - Status epilepticus models 25
  - Kindling models 26
  - The dentate gyrus in seizure generation 26
- Novel techniques in neuroscience research 27
  - Optogenetics 27
  - DREADDs 29

Aims of the thesis 33

Materials and Methods 35

- Animals, Surgery & Chemicals 35
- Status epilepticus model 36
- Viral vector injection 36
- *In vitro* electrophysiology 37
- Setup for *in vivo* optogenetics 37
- Experimental procedures *in vivo* 39
  - Paper I – acute chemical seizures and NpHR-mediated inhibition 39

Paper II – optogenetic train stimulation by ChR2 40
Paper III – optogenetic stimulation and DREADD-based inhibition 41
Histology & Microscopy 41
Data analysis 43
Recording analysis 43
Image analysis 44
Statistics 44
Results 47
Attenuating chemically induced bursting by NpHR-activated hyperpolarization of neurons 47
NpHR expression by AAV vector injection 47
Optogenetic inhibition greatly reduces epileptiform bursting *in vitro* 48
Optogenetic inhibition reduces epileptiform bursting *in vivo* 48
Progressive hippocampal afterdischarge development by activation of ChR2 49
Kindling-like progressive afterdischarges can be induced in transgenic ChR2 mice 49
Bilateral activation of DG granule cells increases AD progression 50
Confirming the presence of contralateral ADs 50
Inhibiting the contralateral hippocampus by Gi-DREADD 51
Discussion 53
Optogenetic silencing in seizure & epilepsy research 53
Investigating hippocampal circuitry involved in seizure generation by optogenetic stimulation 54
Conclusions and future perspectives 59
Acknowledgements 61
References 63

Original Papers

I. **Berglind F**, Ledri M, Sørensen AT, Nikitidou L, Melis M, Bielefeld P, Kirik D, Deisseroth K, Andersson M, Kokaia M (2014) Optogenetic inhibition of chemically induced hypersynchronized bursting in mice. *Neurobiology of Disease* 65:133-141

II. **Berglind F**, Andersson M, Kokaia M (2015) Optogenetic hippocampal stimulation to probe mechanisms of seizure progression. Submitted manuscript, *Journal of Neuroscience*, tracking number JN-RM-4354-15

III. **Berglind F**, Andersson M, Kokaia M (2015) Combination of optogenetics and DREADD reveal remote neuronal network for intensification of epileptiform activity. Manuscript

Summary

Epilepsy comprises a family of neurological disorders characterized by recurrent seizures, which can be highly debilitating. Current treatment options are not sufficient and up to 30% of patients with the most common form of the disorder in adults, temporal lobe epilepsy, cannot be effectively treated by current pharmaceuticals. Temporal lobe epilepsy arises in the hippocampus, a cortical structure specialized in memory formation and spatial navigation.

To combat epilepsy, novel treatment strategies to inhibit seizures are needed. Increased understanding of the hippocampal components and signaling properties involved in the mechanisms of epileptogenesis (the progression of a healthy brain into one with potential for epileptic activity) and induction of seizures is also needed, to guide a rational search for such future treatments.

In the present thesis, we have applied the methodological approach of optogenetics to study these questions. This rather new technology is based on modified microbial membrane channels or pumps which are introduced into target neurons, which can thereafter be activated by light. This is a powerful means of achieving excitatory or inhibitory control over neurons in a target specific manner.

We have explored the use of inhibitory optogenetics, by NpHR, to attenuate seizures (epileptiform bursts) in acute chemical models *in vitro* and *in vivo*. In the models used, GABAergic inhibition is abolished creating a strong excitatory drive among principal neurons in mainly area CA3 of the hippocampus. Under these conditions, we showed that we could inhibit hypersynchronized bursts by activating the inhibitory NpHR chloride pump.

One way of examining epileptogenesis and seizure generation is by repeated electrical stimulations, known as kindling, which gradually creates a hyperexcitable state in the hippocampal circuitry and presents with seizure-like afterdischarges. We posited that with a similar approach based on optogenetics using the excitatory ChR2 cation channel, afterdischarges would

also be generated, while providing new information on the mechanisms of this type of seizure induction. We showed that afterdischarges were indeed produced by repeated optogenetic train stimulation in anaesthetized transgenic mice expressing ChR2. Additionally, we corroborated the well known role of the dentate gyrus is this type of progressive seizure model. Finally, using the chemogenetic novel hyperpolarizing Gi-DREADD receptor, exclusively activated by the inert molecule CNO, we could show that inhibiting the dentate gyrus and CA3 contralateral to optogenetic stimulation effectively halted the afterdischarge progression.

The work presented in this thesis reinforces the great potential of optogenetic and chemogenetic techniques to explore the mechanistic underpinnings and new treatment options for seizures & epilepsy.

Populärvetenskaplig sammanfattning

Epilepsi innefattar en familj av neurologiska sjukdomar som kännetecknas av återkommande epileptiska anfall, som allvarligt påverkar den drabbade personens möjligheter att leva ett normalt liv. Nuvarande behandlingsalternativ tillmötesgår inte behoven hos alla patienter: omkring 30% av de med den vanligaste formen av sjukdomen hos vuxna, temporallobs-epilepsi, kan inte behandlas effektivt med nuvarande läkemedel. Temporallobs-epilepsi uppstår i hippocampus, en specialiserad hjärnstruktur som är av avgörande betydelse för minnesbildning och rumslig navigering.

För att bättre kunna bekämpa epilepsi, behövs nya behandlingsstrategier som både hämmar anfallen och de processer som leder till epileptogenes (d.v.s utvecklingen från en frisk hjärna till ett patologiskt tillstånd med potential för epileptiska urladdningar). Ökad förståelse av hippocampus funktion och signalering som deltar i mekanismerna för epileptogenes och inducering av epileptiska anfall behövs, för att vägleda en rationell efterforskning av sådana framtida behandlingar.

I denna avhandling har vi tillämpat den innovativa metoden optogenetik för att studera dessa frågor. Denna ganska nya teknik är baserad på modifierade membrankanaler eller pumpar från encelliga mikroorganismer, som med genteknik förs in i nervceller (neuroner), som därefter kan aktiveras av ljus. Detta är en utmärkt väg för att uppnå stimulerande eller hämmande kontroll över neuroner på ett målspecifikt sätt.

Vi har undersökt användningen av hämmande optogenetik, genom kloridpumpen NpHR, för att dämpa artificiellt alstrade anfall (epileptiforma urladdningar) i akuta kemiska modeller i hjärnsnitt från möss samt i levande, sövda möss. I de modeller som används här är GABA-baserad inhibering blockerad för att skapa en stark drivkraft för signalering hos exciterande nervceller, främst i området CA3 i hippocampus. Under dessa betingelser,

visade vi att vi kunde hämma synkroniserade nervurladdningar genom att aktivera NpHR med orange ljus.

Ett sätt att undersöka epileptogenes och inducering av epileptiska anfall är genom upprepade elektriska stimuleringar, så kallad ”kindling”, som gradvis skapar ett hyperexciterbart läge i nervkretsarna i hippocampus, som tar sig uttryck i en speciell form av epileptiforma urladdningar, ”afterdischarges”. Vi föresatte oss att med en likartad metod baserad på optogenetik, genom användning av den exciterande katjonkanalen ChR2, att på liknande sätt generera afterdischarges, samtidigt som nya detaljer om mekanismerna för denna typ av anfall skulle kunna undersökas. Mycket riktigt lyckades vi visa att afterdischarges producerades genom upprepad optogenetisk stimulering med blått ljus i sövda transgena möss som uttrycker ChR2. Dessutom kan vi bekräfta den välkända roll som gyrus dentatus spelar i denna typ av progressiv epileptisk anfallsmodell. Slutligen använde vi s.k. kemogenetik, genom den nya hyperpolariserande Gi-DREADD receptorn som aktiveras av den annars inerta molekylen CNO, och kunde påvisa att hämning av gyrus dentatus och CA3 i hippocampus i hemisfären på motsatta sidan av optogenetisk stimulering, effektivt stoppade utvecklingen av afterdischarges.

Studierna som presenteras i denna avhandling utgör exempel på hur optogenetiska och kemogenetiska metoder möjliggör banbrytande utforskning av mekanismer för epileptisk aktivitet och epilepsi.

Abbreviations

4-AP	4-aminopyridine
AAV	Adeno-associated virus
AD	Afterdischarge
AP	Action potential *or* Anterio-Posterior
BM	Bicuculline Methiodide
BPM	Beats per minute
CA	Cornu Ammonis (hippocampal region)
CaMKIIa	Calcium-Calmodulin Kinase II alpha (promoter)
ChR2	Channelrhodopsin-2
CI	Confidence interval
CL	Coastline
CNO	Clozapine-N-oxide
DG	Dentate Gyrus
DGC	Dentate granule cell
DREADD	Designer receptor exclusively activated by a designer drug
GABA	Gamma-aminobutyric acid
hSyn	Human synapsin (promoter)
IP	Intraperitoneal injection
KA	Kainic acid a.k.a. kainate
LFP	Local field potential
NpHR	*Natronomonas Pharaonis* Halorhodopsin

PB	Phosphate buffer
PBS	Phosphate buffered saline
PTX	Picrotoxin
RMS	Root mean squared
ROI	Region of interest
SD	Standard deviation
SE	Status epilepticus
SQ	Subcutaneous injection
TLE	Temporal lobe epilepsy
TTL	Transistor-transistor logic

Introduction

Epilepsy & seizures

Epilepsy comprises a heterogeneous family of neurological disorders characterized by recurrent seizures (Duncan et al., 2006). It is estimated that more than 65 million people are affected in their lifetime, i.e. about 1% of the world population (Ngugi et al., 2010). Besides the lowered quality of life for people with epilepsy due to the disorder itself or side effects due to drug treatment, there is also a 2-4 times increased mortality in this patient group compared to the general population (Jallon, 2004).

It is important to distinguish between a seizure, which many people may experience in their lifetime as a result of e.g. high fever, severe exhaustion or drug use, and epilepsy, where the latter is a state of permanent change in the balance between excitation and inhibition in networks of the brain, which greatly increases the propensity for spontaneous seizure generation (Fisher et al., 2005; Westbrook, 2013). The process of this transformation from a healthy, balanced state to a hyperexcitable pathological state prone to spontaneous seizures is called epileptogenesis. It should be noted that ”imbalance of excitation and inhibition” of the brain is a quite crude simplification, as interplay between oscillatory networks in the brain, required for normal brain function, is much more complex on both the neural and network level (Jefferys, 2003; Duncan et al., 2006; Goldberg and Coulter, 2013; Strange et al., 2014).

Temporal lobe epilepsy

The most common form of epilepsy in adults is temporal lobe epilepsy (TLE) where seizures originate in the hippocampus and/or adjacent regions in the temporal lobe (Wieser and ILAE, 2004; Duncan et al., 2006). Exact prevalence is hard to estimate due to the heterogeneity of focal epilepsies and

overlap of classifications (Blumcke et al., 2013). Primary generalized epilepsies, such as absence epilepsy involving thalamo-cortical circuits, Dravet syndrome or generalized epilepsy with febrile seizures plus (GEFS+), which all have strong genetic components, often due to monogenic ion channel mutations (Duncan et al., 2006; Westbrook, 2013; Spillane et al., 2015), will not be discussed further in this thesis.

In TLE, seizures originate from a limited area, the seizure focus, but can still affect the whole brain through secondary generalization, leading to e.g. severe motor symptoms and altered consciousness (Wieser and ILAE, 2004; Westbrook, 2013). TLE and other focal epilepsies are especially problematic as they are not effectively treated with current anti-epileptic drugs in more than 30% of cases (Wilby et al., 2005; Duncan et al., 2006). Importantly, current drugs are not curative, merely treating the symptoms of epilepsy (i.e. seizures) and not the underlying cause (Walker et al., 2002). Surgical resection of the seizure focus can be very effective for drug-refractory TLE, even achieving complete seizure freedom (Engel, 2001; Jobst and Cascino, 2015). However, as the hippocampus and the adjacent entorhinal cortex serve a critical role in cognitive functions (e.g. memory formation and spatial awareness) (Siegelbaum and Kandel, 2013; Moser et al., 2015), and is situated in close vicinity of major speech areas, removal of the seizure focus is not always possible due to potential consequences for these functions (Schuele and Luders, 2008). An additional consideration is that advanced surgical treatment is not generally available in developing countries (Dua et al., 2006), where epilepsy burden is higher (Ngugi et al., 2010).

The etiology of the disorder is variable, and includes stroke, brain tumor, cortical dysplasia, head trauma, infectious disease or febrile seizures as well as genetic factors (Duncan et al., 2006). TLE is a progressive disease (Blume, 2006) where it is believed that an initial precipitating insult is followed by a latent period of epileptogenesis, which at a later point manifests as recurrent seizures. The condition may or may not include structural degeneration (reactive gliosis and loss of neurons) of the hippocampus, known as hippocampal sclerosis (HS) (Blumcke et al., 2013), which is present in up to 70% of drug-refractory TLE patients considered for surgery. This large subgroup is defined as having an epilepsy syndrome called Mesial TLE with Hippocampal Sclerosis (MTLE with HS) (Wieser and ILAE, 2004; Berg et al., 2010). In TLE the genetic background is subtle and complex (although there are exceptions), providing susceptibility for TLE that might be triggered by environmental effects, rather than clear monogenetic causes. For example,

the main etiology for MTLE with HS is febrile seizures in childhood (50% of those presenting HS) (Blumcke, 2009), and it was recently found that single nucleotide polymorphisms in the sodium channel SCN1A gene locus constitutes a risk factor for febrile seizures leading to MTLE with HS (Kasperaviciute et al., 2013). It is still debated whether the sclerosis plays a causative role or mainly is an effect of the pathological environment created by the repeated seizures (Sloviter, 2005).

Seizures themselves have been hypothesized to play a major role in development of the chronic state (Ben-Ari et al., 2008). This hypothesis has a long history, with the phrase ”seizures beget seizures” coined by the British neurologist William Gowers already in 1881, and although direct evidence has been less convincingly shown in the clinical setting than in experimental models (Marson et al., 2005; Blume, 2006; Sills, 2007), patients with more frequent seizures after first diagnosis have the poorest prognosis for seizure remission (MacDonald et al., 2000) as well as increased risk with each seizure (Hauser and Lee, 2002). One of the strongest lines of evidence comes from the fact that experimental models with repeated stimulation of hippocampal neurons resulting in seizures, causes axonal sprouting in a distinct pattern (Cavazos et al., 1991), similar to axonal sprouting seen in excised tissue from patients with TLE (Sutula et al., 1989) (see further discussion in Models section below). Either way, the temporal disconnect between a precipitating event and the appearance of seizures means that for an individual patient the root cause of their TLE is often unknown or ambiguous.

In summary, from what is known about the pathophysiology of TLE and the difficulties in treating TLE, it is clear that further research on mechanisms of seizures and epileptogenesis in the hippocampus is highly warranted, as well as development of novel treatment strategies. A number of other important fields of epilepsy research also exist, such as development of biomarkers, mechanisms of drug resistance, the role of inflammation, genetic factors etc. However these topics are outside of the scope of the current thesis and will not be discussed further.

The hippocampal networks

Components and basic circuitry

The hippocampus is essentially a highly specialized cortical structure, having many cell types in common with the neocortex, but organized in a different type of layering (Amaral and Lavenex, 2007). The name is derived from the greek word for seahorse, as the overall morphology has some similarity to the shape of this strange fish. It is located in the temporal lobe, at the bottom of the inferior horn of the lateral ventricle, mirrored in each hemisphere (Duvernoy, 2005). In rodents, the model animals of choice in neuroscience, the hippocampus occupies a proportionally larger volume of the brain compared to humans, and is a curved structure stretching from the dorsal aspect of the brain (septal hippocampus), to a ventrolateral region (temporal hippocampus) (see figure 1), at an oblique angle. This longitudinal orientation needs to be rotated 90° when comparing to the primate or human brain, where the hippocampus is aligned in an anterio-posterior direction (Strange et al., 2014). The hippocampi are interconnected via the dorsal and anterior commissures (see further in Connectivity section).

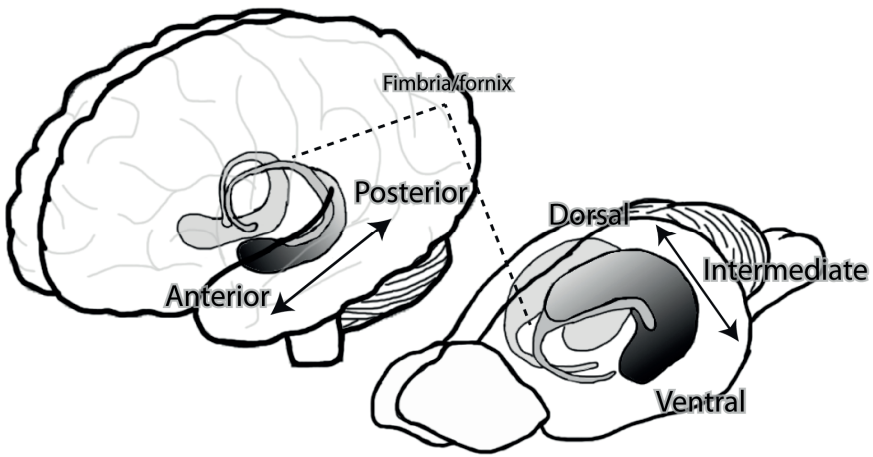

Figure 1. The hippocampus in human and rodent.
Shading and arrows highlight the longitudinal orientation. The fimbria and fornix constitutes the main fiber bundle carrying output to subcortical regions. Dorsal/ventral is often used interchangeably with septal/temporal in rodents. Not to scale.
Adapted from Scholarpedia; Hippocampus © Creative Commons Licence

The terms hippocampus and hippocampal formation are often used interchangeably, though as a functional unit the latter is more correct (Amaral and Lavenex, 2007). Anatomically, the hippocampal formation can be subdivided into dentate gyrus (DG), cornu Ammonis[1] (CA) regions 3, 2 & 1 (a.k.a. hippocampus proper), subiculum, pre- and parasubiculum, and entorhinal cortex (EC) (see figure 2). The region between DG and CA3 is called hilus and is considered part of DG (Amaral, 1978; Amaral and Lavenex, 2007). In humans there is also a region CA4, enclosed by the DG, although functionally this region is identical to CA3, and also referred to as hilus in some contexts (Duvernoy, 2005). CA2 is a transitional region that has been difficult to pin-point, and has not been well studied until recently (Lein et al., 2005; Kohara et al., 2014). In the classical view, the information flow in the hippocampus originates in entorhinal cortex, enters the dentate gyrus via the perforant path, continues through CA3 & CA1 and then out via subiculum back to deep layers of EC.

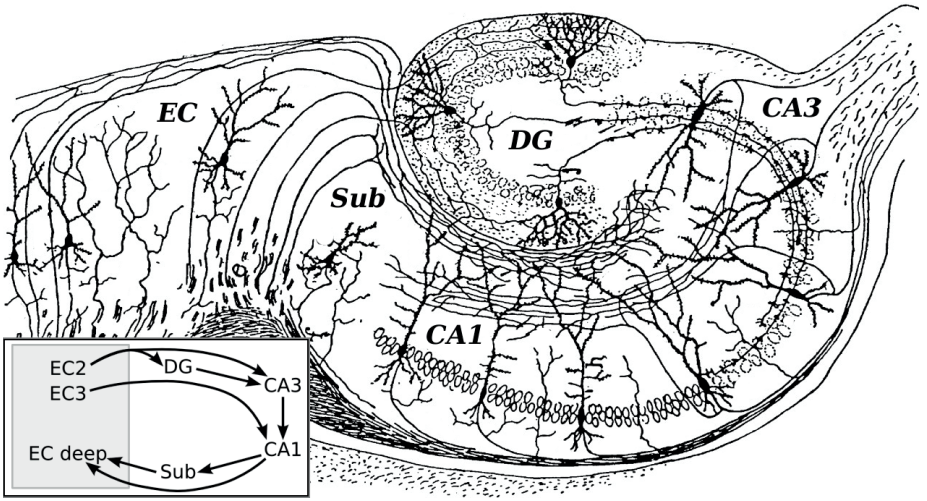

Figure 2. Overview of rodent hippocampus anatomy and basic connectivity.
Examples of excitatory granule cells and pyramidal neurons are highlighted in the granule cell layer of DG and pyramidal layers of CA, respectively. The mossy fiber projections from DG to CA3, and Schaffer collaterals from CA3 and CA1 are also drawn. DG – Dentate Gyrus, Sub – Subiculum, EC – Entorhinal Cortex.
Image adapted from the work of Santiago Ramón y Cajal (1911), © Public Domain, Wiki Commons: https://commons.wikimedia.org/wiki/File:CajalHippocampus_(modified).png

[1] Cornu Ammonis is another reference to the curved shape of hippocampus, likening it to the horn of a ram. The egyptian god Amun was often depicted with a ram's head.

The unidirectional information flow described above was originally known as the tri-synaptic pathway (EC – DG – CA3 – CA1 synapses) (Andersen et al., 1971), however, further research has since extended this to the full loop of the hippocampal formation. Additionally, it has been revealed that many more direct and collateral connections exist between the hippocampal subdivisions, and connectivity also varies in the longitudinal direction (see next section) (Scharfman, 2007; Witter, 2007; van Strien et al., 2009).

The excitatory projection neurons in this network are glutamatergic, in the form of small granule cells in the DG, and larger pyramidal neurons in CA and subiculum (figure 2). In contrast, there are a number of different types of interneurons, which alter the pyramidal neuron outputs by inhibitory action of GABA onto different regions of the excitatory neurons (e.g. dendrites, soma or axonal segments) (Freund and Buzsaki, 1996). Together, the excitatory and inhibitory neurons form loops for feed-forward and feedback inhibition and excitation. Especially, the interneurons are crucial to ”shape” the activity of excitatory cells, creating the oscillatory firing properties of neuronal ensembles. This is a foundation of normal hippocampal function, but also plays a role in synchronizing the neuronal network into epileptiform activity under pathological conditions (Isomura et al., 2008; Mathalon and Sohal, 2015).

Connectivity

The hippocampus is a highly interconnected structure, receiving sensory information from cortical areas and integrating it to form memories, as well as connecting this sensory information to deeper subcortical structures to impart emotional content and behavioural responses to that information. A functional distinction can be made between dorsal and ventral parts of the rodent hippocampus, with the dorsal part being more important for memory and navigation, while ventral hippocampus handles emotional memory contexts, however this view is overly simplified and now partly out-dated, as the hippocampus has more complex regional variations in both connectivity and gene expression (Strange et al., 2014).

As mentioned above, entorhinal cortex constitutes the main in- and output region of the hippocampus. This connectivity occurs on the transverse plane (proximo-distal direction from DG to EC), like in the case of the well-known Schaffer collaterals from CA3 to CA1, forming lamellar layers perpendicular

to the longitudinal axis (septo-temporal i.e. dorso-ventral axis) (see figure 3). But there is also complex connectivity in the longitudinal direction, mostly examined in studies utilizing neuronal tracers. For example, Schaffer collaterals reach layers of CA1 both dorsally and ventrally from the level of origin. Additionally, so called associational CA3 connections (CA3 to CA3) are made both within the same layer (recurrent connections) and in the long axis: collaterals in the dorsal direction are made preferentially by proximal CA3 neurons which target neurons shifted in the distal direction, and vice versa (figure 3c) (Witter, 2007; van Strien et al., 2009).

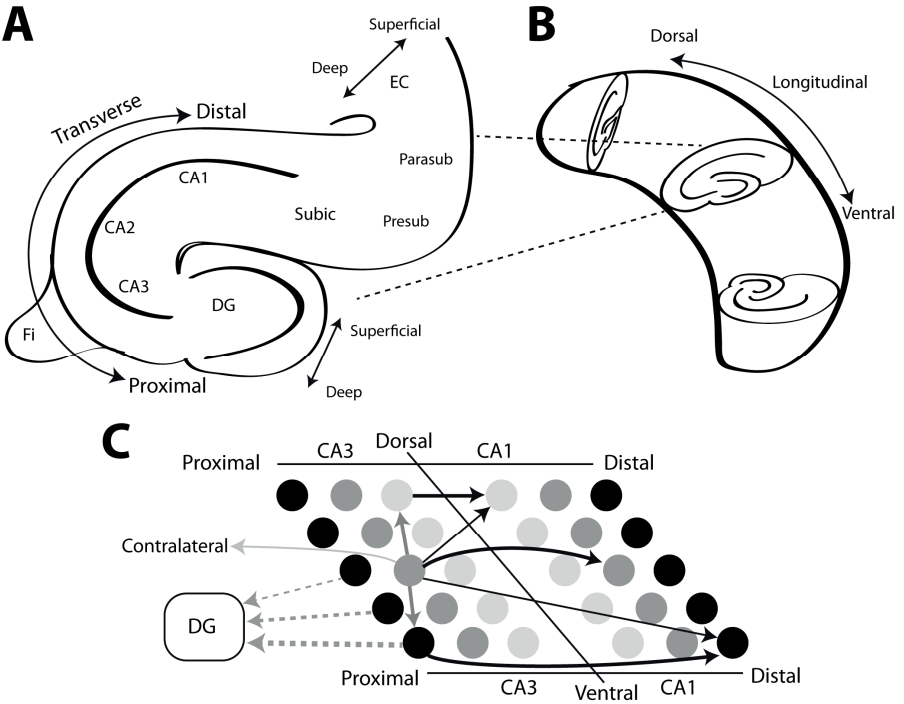

Figure 3. Schematic of the transverse and longitudinal planes and the connectivity of CA3.
A In the transverse plane, you can separate the disto-promixal axis (in relation to DG) and the superficial-deep axis (perpendicular to the outside of the cortical layer). Fi – fimbria. **B** Moving dorso-ventrally in the longitudinal axis, note how the orientation of the transverse plane is rotated. **C** CA3 neurons project Schaffer collaterals in the same plane (thick black arrow) as well as dorso-ventral (thin black arrows). Associational fibers (dark grey arrows) strongly connect CA3 in the dorso-ventral axis. Note that the examplified mid CA3 neuron projects equally to dorsal and ventral segments, while proximal neurons prefers dorsal targets and vice versa. Proximal CA3 neurons have back projections to DG, increasing with ventral level (dotted grey arrows). Lastly, collaterals are also projecting to contralateral hippocampus (light grey arrow). Panel C is adapted from Witter (2007).

Together, this means that activity at one dorso-ventral level of CA3 can be widely dispersed onto the CA1. There are also ”back-projections” from CA3 to DG, mainly from proximal CA3 neurons and increasing from mid to ventral levels, which have been implicated in seizure generation in epileptic rodents (Scharfman, 2007; Witter, 2007). Additionally, the recurrent connectivity of the CA3 area, especially in the ventral part, makes this area particularly prone to induction of epileptiform discharges (Miles and Wong, 1983; Bragdon et al., 1986; Strange et al., 2014).

Importantly, CA3 neurons also project collaterals to the contralateral hippocampus, via the commissures. This has not been as thoroughly examined, but the connectivity seems to mirror the ipsilateral situation, achieving wide signal distribution to both CA3 and CA1 (Witter, 2007). Another set of excitatory projection neurons with importance to bilateral connectivity are the mossy cells of the hilus, which synapse with granule cells in the inner molecular layer. Mossy cells form strong connections to contralateral dentate granule cells on the same dorso-ventral level (homotopic connections) (Ribak et al., 1985; Scharfman and Myers, 2013), although distribution in the long axis has also been reported (Zappone and Sloviter, 2001). Ipsilaterally, they project more strongly in the ventral to dorsal direction, than vice versa. Granule cells of the dentate gyrus, while mainly innervating a transverse section with rather limited extension in the long axis, have also been shown to project along the dorso-ventral axis as their mossy fibers reach the distal end of CA3. However, in the normal situation, DG granule cells are quite resistant to activation. As the entry point to the hippocampus, granule cells perform the task of ”pattern separation”, filtering out weakly transmitted information, but are more prone to discharge when simultaneously assisted by the connections from mossy cells (Hsu, 2007; McHugh et al., 2007). As we shall see, this function might be compromised in pathologic states such as epilepsy (discussed in Models section below). Finally, it should be mentioned that even interneurons, normally perceived as influencing local networks only, have been shown to project contralaterally in a homotopic fashion, although the functional implication is unclear (Zappone and Sloviter, 2001).

The classical electrophysiological *in vitro* methodology, with preparations cut in the transverse plane, has been highly useful to characterize local connections, but as the depth of such a slice is only around 300 μm, longitudinal connections were not as easily studied. *In vivo* electrophysiology is needed to more comprehensively examine connections in the longitudinal

plane, as well as the bilateral information exchange between the hippocampi of both hemispheres, which is completely lacking in the *in vitro* preparation. However, when it comes to bilateral connectivity, it should be noted that there exists clear differences between rodents and primates. Foremost, the dorsal commissure found in rodents is largely absent in primates. Bilateral connectivity does occur from the anterior portion of hippocampus proper and DG, while the main commissural connectivity is supplied by the subiculum (Amaral et al., 1984).

Models of seizures and epilepsy

To be able to study epilepsy and the mechanisms of seizures, modelling of the disorder in experimental animals is needed. As was noted earlier, the presence of a seizure does not equal epilepsy, and likewise models should be divided into acute seizure models as opposed to epilepsy models with a chronic state with spontaneous recurrent seizures. However, even the chronic models might not include all the features of epilepsy in humans.

Status epilepticus models

Both seizure and epilepsy models try to mimic some aspect of seizure generation and/or pathology that is known to exist in the human case. For example, it is known that precipitating events such as febrile seizures can lead to epilepsy, and one class of models mimic this by inducing an acute insult, which after a silent period leads to a chronic epileptic state. This is the basis of status epilepticus (SE) models, where a severe seizure is induced by chemical injection or electrical stimulation (Pitkänen et al., 2006). After this initial event, it is hypothesized that epileptogenesis is taking place, eventually leading to spontaneous seizures. By e.g. studying animals at various timepoints after the initial insult, insight into the development of the disorder might be gained. Common chemical models include kainic acid (KA), which activates glutamatergic kainate receptors, and pilocarpine (Levesque and Avoli, 2013; Gorter et al., 2015). Alternatively, chemicals that block GABAergic transmission can be used to induce temporary hyperexcitability in acute models, such as bicuculline and PTX (Straub et al., 1990; Pitkänen et al., 2006).

Kindling models

Another line of experimental models derive from the observation that repeated seizures can lead to epilepsy. This has been explored in the electrical ”kindling” models (Goddard, 1967; Gorter et al., 2015). Kindling is a process towards permanent increased excitability, whereby the seizure threshold is lowered on subsequent stimulations, and the resultant afterdischarges of epileptiform activity and behavioural manifestations are progressively increased. However, spontaneous seizures are rarely seen unless daily stimulations are given for several months. In kindling, neuropathological damage is not as severe as in SE models. Still, some common features can be seen, foremost that of mossy fiber sprouting (Cavazos et al., 1991; Elmer et al., 1996), granule cell dispersion and cell loss in the DG and hilus. While there is still some controversy on this issue, the increased excitability in kindling can appear prior to mossy fiber spouting and is thus not fully dependent on this process (Lothman et al., 1985; Lothman and Williamson, 1993). SE models have also been criticised because damage to extra-hippocampal areas, not seen in human TLE patients, can be quite severe (Gorter et al., 2015). Newer KA models using intrahippocampal injections have been developed in an attempt to address these issues (Rattka et al., 2013).

The dentate gyrus in seizure generation

As we have seen, the dentate gyrus is one of the main regions of interest in seizure and epilepsy research (together with CA3). The sprouting of aberrant, recurrent mossy fibers, including fibers going back onto granule cells themselves, is one reason. Another is due to the functional role of the dentate gyrus granule cells (DGCs). As mentioned, DGCs are innately resistant to activation and act as a filter in the hippocampal loop. Together with *in vitro* and *in vivo* experimental evidence, this has led to the formulation of the dentate gate hypothesis (Heinemann et al., 1992; Hsu, 2007), which postulates that repeated activation of DGCs eventually lead to a breakdown of the filtering function of DG, and seizures can then be amplified throughout the hippocampus. This is related to the observation of maximal dentate activation (MDA), large synchronous population spikes elicited by DGCs that are required for epileptiform afterdischarges in electrical models of stimulation (Stringer et al., 1989) (but can also be achieved by chemical

stimulation (Stringer and Sowell, 1994)), and bilateral MDA is a prerequisite for the kindling process (Stringer and Lothman, 1992). In other chronic models of TLE, it has been shown that DGCs are subjected to both reduced inhibitory input and increased excitatory input, further implicating the dentate gyrus as a control point for seizure generation (Kobayashi and Buckmaster, 2003; Zhang et al., 2012).

Novel techniques in neuroscience research

Optogenetics

One of the most important methodological innovations for neuroscience research in the last decade is the development of optogenetics. Opsins are microbial membrane ion pumps which, when combined with the chromophore retinal (a vitamin A derivative) into rhodopsin, can be activated by light, and have been known since the 1970s (Zhang et al., 2011; Hegemann and Nagel, 2013). The existence of similar light gated ion channels were suggested around the same time. However, the first definitive characterization of such channelrhodopsins, originally found in the green algae *Chlamydomonas Reihnhardtii*, was done by transgene expression in *Xenopus* oocytes and published in the early 2000s (Nagel et al., 2002; Nagel et al., 2003). While Channelrhodopsin-1 (ChR1) is a proton channel activated by green light, ChR2 is gated by blue light on a very fast timescale, opening a pore selectively conducting smaller cations, most importantly Na^+. Two years later, the seminal paper by the Deisseroth group (in collaboration with Nagel & Bamberg) was published, showing that ChR2, when expressed in mammalian hippocampal neurons, could depolarize the neurons and induce action potentials with millisecond precision (Boyden et al., 2005). A serendipitous discovery was that retinal did not need to be supplemented in mammalian cells. Crucially, the innate electrophysiological properties of neurons were not altered by the expression and membrane integration of the transgene product.

Realizing the enormous potential of optogenetics, they continued exploring available rhodopsins, to complement the excitatory ChR2 with an inhibitory (hyperpolarizing) counterpart. Using the chloride pump Halorhodopsin from the archaebacteria *Natronomonas Pharaonis* (NpHR) they soon showed that

bidirectional control of neurons expressing both ChR2 and NpHR could be achieved, as NpHR was maximally activated by amber light, outside of the activation spectrum peak of ChR2 (see figure 4A) (Zhang et al., 2007b). In the following years, the optogenetics toolbox was improved and expanded by both directed genetic engineering and characterization of rhodopsins from existing cDNA libraries of microbial organisms, or a combination of both (Gradinaru et al., 2008; Zhang et al., 2008; Chow et al., 2010; Gradinaru et al., 2010; Gunaydin et al., 2010), reviewed in (Zhang et al., 2011). This development continues at a steady rate, with notable recent additions such as a fully engineered inhibitory (chloride-conducting) ChR2 (Berndt et al., 2014) and a highly efficient variant of Halorhodopsin that can be non-invasively activated by far-red light (Chuong et al., 2014).

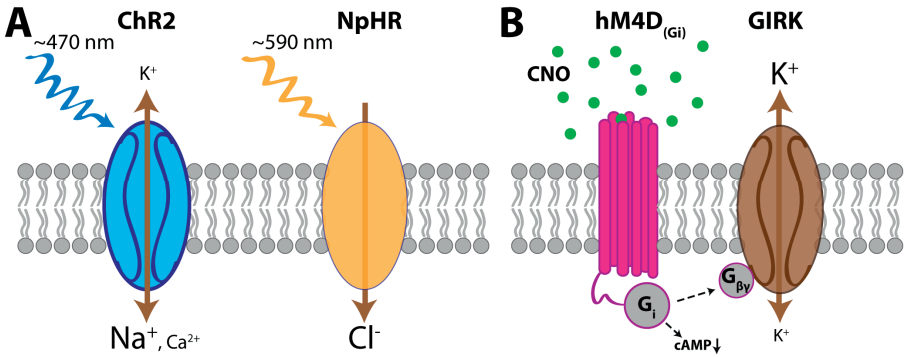

Figure 4. Schematic of basic optogenetic tools and Gi-DREADD
A ChR2 is activated by blue light, allowing passage for smaller cations. Because of the electrochemical gradient, a strong inward sodium current dominates, depolarizing the neuron past the threshold of AP generation. NpHR is activated by amber light, and pumps chloride into the cell, against the electrochemical gradient, hyperpolarizing the neuron. Adapted from (Zhang et al., 2007a). **B** DREADDs are derived from muscarinic ACh receptors, and modified such that only the otherwise inert clozapine-N-oxide (CNO, green) can bind and activate the receptor. The muscarinic receptor family can exert different functions depending on the coupled G-protein. In the case of Gi, activated receptor releases the Gβγ subunit which opens the inward rectifying potassium channel GIRK, causing hyperpolarization as K+ is driven towards equilibrium potential.

A particularly powerful aspect of optogenetics lies in how the excitatory/inhibitory action is combined with modern genetic expression systems. Already at the outset, the genetic code for ChR2 was codon optimized for mammalian use, stripped to the minimal length needed for function and combined with a fluorescent marker to express as a compact fusion protein, suitable for packaging in the limited space of viral vectors

(Boyden et al., 2005). The rhodopsin sequence could then be combined with a promoter of choice, for neuron specific targeting (Zhang et al., 2007b), e.g. CaMK2α for glutamatergic neurons (Liu and Jones, 1996). With careful viral vector injection, it is possible to limit expression to a certain area. This can be extended with the Cre-Lox system (Tsien et al., 1996), where expression is limited to cells co-expressing Cre, allowing stronger ubiquitous promoters to be used. Cre activity introduced by viral vector or through breeding transgenic animals, greatly improves the specificity and reliability of expression that can be achieved (Kuhlman and Huang, 2008; Madisen et al., 2012). There are also transgenic strains not based on Cre, such as the Thy1-ChR2 mouse (Arenkiel et al., 2007; Wang et al., 2007).

Optogenetics has been employed in several mammalian species, including mice, rats and primates, and has been highly useful for behavioural research in rodents (Carter and de Lecea, 2011; Grosenick et al., 2015). Naturally, the possibility of direct and specific excitation and inhibition of neural circuits was very attractive to the field of epilepsy research, and the first paper showing *in vitro* proof-of-concept for optogenetic inhibition of seizures was published in 2009 (Tonnesen et al., 2009), see further in Discussion. At this point, with the recent 10-year anniversary of the Deisseroth ChR2 paper, there is no doubt how important the dissemination and use of optogenetics has been to neuroscience research (Adamantidis et al., 2015).

DREADDs

Another novel and highly dynamic field of method development in neuroscience is termed chemogenetics (Sternson and Roth, 2014). Similarly to optogenetics, transgene systems are used to introduce engineered membrane proteins or enzymes to experimental animals, which can be specifically activated by certain chemicals. While the timescale of activation with chemogenetic tools cannot grant the same temporal control as optogenetics, the addition of the active chemical is potentially much easier compared to employing light sources or fiber guides directed at deep brain structures.

One example of a chemogenetic approach is the so-called designer receptor exclusively activated by a designer drug, DREADD (Armbruster et al., 2007; Urban and Roth, 2015). Originally, this was a group of engineered muscarinic g-protein coupled receptors (GPCR), based on a mutated human

M_3 acetylcholine (ACh) receptor, selected through directed evolution in yeast culture after random mutagenesis. A library of clones were screened in several rounds such that the final modified receptor hM_3D gained high affinity for the pharmacologically inert clozapine-N-oxide (CNO), a metabolite of the antipsychotic drug clozapine (Weiner et al., 2004), while simultaneously losing affinity for the native ligand ACh (Armbruster et al., 2007). Two mutations were identified as required for optimal CNO binding to hM_3D, these were then directly applied to the other four muscarinic receptors of the same family. Thus five subtypes of DREADDs were created, corresponding to the native $M_{1\text{-}5}$ receptors, and acting by either Gq or Gi pathways. The original hM_3D confers Gq activity, increasing intracellular Ca^{2+} leading to burst firing. The most notable other DREADD in this context is hM_4D, which has Gi activity. The Gi pathway decreases cAMP by interaction with adenylate cyclase, but is also known to activate the G-protein regulated inward rectifying K^+ channels (GIRK), which is gated by direct interaction with the $G_{\beta\gamma}$ dimer released from the activated Gi trimer, leading to hyperpolarization in hippocampal neurons (Mark and Herlitze, 2000; Li et al., 2005), and indeed, activation of $hM4D_{(Gi)}$ also leads to hyperpolarization in neurons (Armbruster et al., 2007) (see figure 4B). Importantly, these mutated receptors have no constitutive activity in the absence of CNO.

While the designation of DREADD was conceived as applicable to any modified receptor exclusively interacting with an otherwise inert molecule, use of the term has been limited to the muscarinic receptors described above, together with a few chimeric options with different G-protein activity (Urban and Roth, 2015), and all were using CNO. However, recently a wholly different DREADD was presented, derived from a kappa-opioide GPCR-Gi activated by a different ligand than CNO, thus introducing the possibility of bi-directional DREADD control of neurons (Vardy et al., 2015). Still, similar concepts have been developed under different naming schemes, like the PSAM/PSEM system where engineered ligand gated actuator modules (PSAM) are fused to the transmembrane portion of ion channels of the Cys-loop class (e.g. serotonin and nicotinic receptors) and activated by suitably engineered small effector molecules (PSEM) (Magnus et al., 2011; Sternson and Roth, 2014).

The inhibitory DREADD hM4D (also called Gi-DREADD) has been validated *in vivo*, e.g. by expression in two different striatal projections, opposing behavioural responses to drug sensitization could be induced in rats (Ferguson et al., 2011). To date, it has been used in more than 20 studies in

both rodents and *Drosophila* (Urban and Roth, 2015), including a study where acute and chronic chemically induced focal cortical seizures were attenuated (Katzel et al., 2014). A nice feature of CNO is that its precursor clozapine has been used for decades as an antipsychotic medication, and thus the metabolism and toxicology of the drug is well known (Voicu et al., 2012), and it can be administered orally, although in animal models IP injection is usually preferred. However, it should also be noted that a small fraction of CNO is interconverted to clozapine in humans (but not in rodents), which would need to be considered for potential future human use (Sternson and Roth, 2014).

Aims of the thesis

The overarching goal of the work presented herein was establishing an *in vivo* electrophysiology platform incorporating optogenetic techniques, to address questions relevant to seizure generation and possible intervention in temporal lobe epilepy. The specific aims of this platform were:

1. To show proof-of-concept of optogenetic inhibition of the hippocampus as a means of seizure control (paper I)
2. To investigate the possibilities of optogenetic seizure generation in transgenic mice (paper II)
3. To determine hippocampal networks involved in optogenetic seizure progression and especially the role of contralateral hippocampus (paper II & III)

Materials and Methods

Animals, Surgery & Chemicals

All procedures were approved by the Malmö/Lund Animal Research Ethics Board. Animals were housed in the local animal housing facility under 12h light/dark cycle and given food and water *ad libitum* in all cases.

Female FVB mice (Charles River) were used in paper I, naïve or subjected to SE (see below). For paper II, breeding pairs of transgenic Thy1.2-ChR2-YFP mice (strain #18, Jackson Laboratory #007612) (Arenkiel et al., 2007; Wang et al., 2007), were homozygously bred in the local facility. Thy1-ChR2 mice are on a BL6 background, and both male and female animals were used for experiments. For paper III, we bred transgenic CaMKII-(Cre)-ChR2-YFP animals using the Cre-Lox system: T29-1 (Camk2a-Cre, Jackson #005359) and Ai32 (RCL-ChR2/eYFP, Jackson #012569) strains were crossed, both are BL6 background, and both males and females were used for experiments. The Ai32 strain carries a lox-STOP-lox sequence, which needs to be excised by Cre before permitting expression of improved ChR2(H132R) under the CAG ubiquitous promoter (Madisen et al., 2012), thus resulting in strong glutamatergic neuron specific expression when crossed with the CaMKIIa-Cre line.

In all surgeries and *in vivo* optogenetic trials animals were anaesthetized by Isoflurane gas inhalation (Baxter), 1.5-2.5 % (4% for induction) in air (viral injection) or 100% oxygen (optogenetic trial), placed in a stereotaxic frame (Kopf Instruments) and local analgesic bupivacain (Marcain, AstraZeneca) was injected SQ to the skull surface. Physiological saline (~0.5 mL) was supplemented by SQ injection to maintain hydration. After surgery, the incision wound was cleaned with Chlorhexidine (Fresenius Kabi) and closed with tissue glue (Histoacryl, B Braun) and resorbing thread (Ethicon). All stock chemicals were obtained from Sigma-Aldrich unless otherwise noted.

During optogenetic trials, body temperature was maintained by generic reusable chemical heat pads (paper I) or a water flow heat pad (Maxi-Therm Lite, Cincinnati Sub-Zero) (paper II & III), whereas for viral injections a small terrarium electric heatpad was used (paper III only). For optogenetic trials in paper II & III, a pressure sensor (AD Instruments) was placed under the ribcage to monitor breathing.

Status epilepticus model

In paper I, a subgroup of FVB mice used for *in vitro* trials were subjected to kainic acid SQ injection inducing SE (30 mg/kg, in sterile PBS, pH ~7.3), monitored for three hours and evaluated on a modified six-level Racine scale (Racine, 1972). KA mice that did not display at least four grade 5 seizures were excluded.

Viral vector injection

AAV vectors were injected into the hippocampus of mice (paper I & III, see table 1). In brief, a glass capillary (Stoelting, # 50613 (paper I), 50811 (paper II)) pulled to a thin tip was fitted to the needle of a 5 µL Hamilton syringe by generic heat-shrink tubing. An incision was made along the skull midline, connective tissue was removed to identify bregma by application of a small amount of 2-3% H_2O_2, and a small hole was drilled over the designated location (see table 1). The dura mater was punctured with a bent 27G hypodermic needle and the injection capillary, prefilled with virus solution, was slowly lowered to the designated depth, where two deposits spaced apart in the DV orientation were made (0.5 µL each, infused at 0.1 µL/minute, total volume 1 µL). After each deposit, the capillary was left in place for 5 minutes to avoid backflow.

Table 1. Viral vectors used and injection sites
Coordinates are given in relation to bregma, midline and dura for AP, ML and DV, respectively.

	Paper I	Paper III
Viral vector	AAV5-hSyn-eNpHR3.0-YFP	AAV8-hSyn-hM4D(Gi)-mCherry
Manufacture and titer	BMC vector core (in-house), 5.4 $x10^{12}$	UNC vector core, 7.4 $x10^{12}$
Injection site, hemisphere & depth	AP-3.2, ML-3.1 (Right) DV-3.6 & -3.2	AP-3.2, ML+3.2 (Left) DV-3.6 & -3.0

In vitro electrophysiology

In paper I, electrophysiology was performed on acute slices from the naïve or KA-treated FVB mice previously injected with AAV-NpHR3.0, as well as non-NpHR-expressing controls. In brief, 300 µm thick horizontal slices containing hippocampus and EC were prepared and incubated in artificial cerebrospinal fluid (aSCF), as previously described (Ledri et al., 2012). For recording, slices were perfused with oxygenated aCSF at 32 °C. Recording glass pipettes were filled with aCSF for field recordings or a modified solution for whole-cell patch clamping (including 3 mg/mL biocytin for cell marking).

Epileptiform activity was induced in the slices by perfusion with a combination of modified zero-Mg2+ aCSF containing 50 µM 4-AP and 100 µM PTX. Field recordings were made from CA3 pyramidal layer, and whole-cell recordings from CA3 pyramidal neurons, in a few cases simultaneously. Experimental data was collected from 9, 8 & 6 slices from 4, 3 & 3 animals (Naïve, KA and Control, respectively), with 2-5 repeat measurements per slice. Data was sampled at 10 kHz with an EPC-10 amplifier (HEKA Elektronik) and offline analysed with PatchMaster software (HEKA). Amber light for optogenetic stimulation was produced by the mercury light bulb in the recording rig microscope illuminating the whole sample via the objective, filtered by a 593±40 nm excitation filter cube. After experiments, slices were postfixed in 4% PFA in 0.1 M PB and stored in ethylene glycol/glycerol antifreeze at -20 °C. Slices were cryoprotected in 20 % sucrose in PB prior to cutting (see Histology below).

Setup for *in vivo* optogenetics

Optrodes (combined optic fibres and recording electrodes) were used for *in vivo* optogenetic stimulation with simultaneous recording (Gradinaru et al., 2007). An optic fiber of 200 µm diameter and numerical aperture of 37 (BFL37-200 or FT200EMT, Thorlabs) was glued to a parylene coated 1 MΩ tungsten electrode (TM21A10, WPI UK) with an exposed tip of 30 µm length and 1 µm diameter. The electrode tip protruded approximately 0.4-0.6 mm from the optic fiber tip, which ensured that the electrode was recording from an area with sufficient optogenetic activation, given the light levels

used. In paper I, the optrode was combined with a glass capillary (1B100F-6, WPI UK) pulled to a thin tip on a Model P-1000 pipette puller (Sutter Instruments), with the tip smoothed on a grinder and microforge (EG-44/MF-830, Narishige). During experiments, the capillary was attached by tubing to an air-pressure microinjector (Picospritzer IID, General Valve Corporation) for chemical injection. The optrodes were fitted to the stereotax holder in various configurations for each study, see figure 5 A, via an MO-10 micromanipulator (Narishige) for increased control of fine movement in the z-axis.

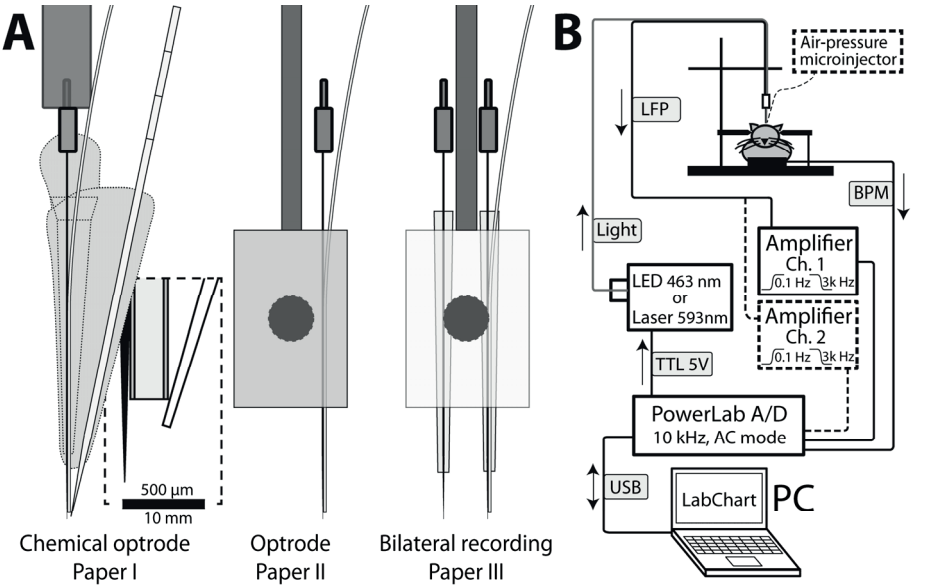

Figure 5. Optrodes and *in vivo* recording setup
A In paper I, the chemical optrode was directly attached to an electrode adapter. Structural support in grey/dotted lines, capillary in white. In paper II & III, optrodes were clamped to the holder with a plate and screw for increased stability and target accuracy. Inset: detail of optrode tip. Scalebar is correct for optrodes, but not holders. **B** Recording setup, see text for details. Dashed line boxes: microinjector only used for paper I, 2nd amplifier only for paper III. 593 nm (amber) laser was used in paper I; 463 nm (blue) LED was used in paper II & III. Pressure sensor for breathing rate (BPM) was not used in paper I.

Local field potentials were recorded in LabChart (AD Instruments) on a Windows PC, via a DP-311 extracellular amplifier (Warner Instruments) (two in parallel for dual-channel recording, paper III) and digitized on a PowerLab 4/35 (AD Instruments) at 10 kHz, see figure 5B. The DP-311 was

set to 10 times amplification, high-pass and low-pass filters at 0.1 Hz & 3 kHz (AC mode). The signal amplification was then corrected on the PowerLab. For recording, a chloride treated silver reference electrode was placed at the skull base and a custom copper-mesh Faraday cage was placed over the setup to reduce noise. Breathing rate was recorded at 0.2 k sample rate and the running average BPM was displayed in LabChart during trials (paper II & III).

For light delivery, a 593 nm amber laser (GCL-025-593, CrystaLaser) was used in paper I and a 463 nm blue LED (Prizmatix) was used in paper II & III. Light delivery was controlled via TTL connection from the PowerLab stimulation output, which was also routed back to a PowerLab input to record pulse deliveries, at 2 k sample rate. Light output was measured prior to experiments using a 1916-C power meter (Newport), and set to approximately 2.3 mW (paper I), 3.0 mW (paper II) or 2.6 mW (paper III). Thus, output levels did not exceed 75 mW mm^{-2} with constant light or 100 mW mm^{-2} with pulsed light, these light levels should not affect the tissue in any way (Cardin et al., 2010).

Experimental procedures *in vivo*

On conclusion of the experiments, all animals were given an IP injected overdose of sodium pentobarbital (40 mg/kg) and were transcardially perfused with ice-cold saline and 4% PFA in 0.1 M PB. Brains were stored in 4% PFA (at least 24 hours) and cryoprotected in 20 % sucrose in PB for another 24 hours before cutting. Total time from the last stimulation to perfusion was approximately 15 minutes. In paper III, mice were given a ”burn-in” (three 1 s, 500 μA pulses) with a DS3 constant-current stimulator (Digitimer) prior to perfusion to aid with post-mortem localization of recording sites.

Paper I – acute chemical seizures and NpHR-mediated inhibition

Acute seizures were attained by microinjection of Bicuculline Methiodide (Ascent Scientific) to the ventral hippocampus of FVB mice expressing NpHR3.0 by previous AAV vector injection. Briefly, three months after viral injection, animals were anaesthetized, the burr hole reopened and the

chemical optrode, prefilled with 3mM BM & 2 mg/mL biocytin in PBS, was lowered to DV-3.0 (electrode tip, from dura). The optrode was left in place for 5 minutes to allow accommodation of the tissue, after which the optrode was lowered in 50 µm increments, testing the tissue response (inhibition of multi-unit activity, at 300 Hz-3k Hz bandpass real-time digital filtering in the LabChart software) with 5-10 s long periods of amber light at each depth, with at least one minute in between movements.

The final depth ranged between DV -3.05 and -3.3 mm. where 1.5-3.0 µL BM (final total volumes) were injected by short puffs of picospritzer air pressure (50-100 ms) typically moving ~0.5 µL per puff. If BM-induced bursting did not appear in the LFP within 3-4 minutes, another pair of air puffs were given. When bursting appeared stable, the light protocol was given, by 40-second periods of continuous light followed by 80-120 s without light. The control group consisted of NpHR-negative mice, and were subjected to 30 s or 60 s light-on periods (this was normalized in comparisons).

Paper II – optogenetic train stimulation by ChR2

Transgenic Thy1Chr2 mice were subjected to repeated trains of blue light stimulation, to achieve progressive afterdischarge development. Briefly, animals were anaesthetized and a small hole was opened (same procedure as Viral Vector Injection, see above) at AP-2.6, ML-3.1 (right hemisphere), and the isoflurane level was adjusted down to 1.0-1.5%. The optrode was lowered to approximately DV-1.5-2.0 and let to rest for 5 minutes to allow tissue to accomodate. When breathing was stable between 40-70 BPM (without reaction to tail pinch), the optrode was lowered in 50 µm increments, testing the LFP response with 2x 250 ms test pulses at each depth until a suitable response (high population spike) was registered, on average around DV-2.9, targeting the ventral CA3.

Optogenetic train stimulations were then applied, consisting of 10 Hz 5ms pulses for 15 s (i.e. 5% duty cycle, 150 pulses), repeated 40 times with 5-minute inter-train interval. Total recording time was around 3.5 h. Three control animals were stimulated with blue light only for trains #1, #20 & #36-40. The remaining trains were given as amber light pulses with the 593 nm laser, i.e. in total 33 amber light trains and 7 blue light trains. An additional

six animals were given a single blue light train, followed by perfusion 15 minutes later (see below).

Paper III – optogenetic stimulation and DREADD-based inhibition

Transgenic CaMKII-(Cre)-ChR2 mice, either naïve (control) or expressing Gi-DREADD by AAV vector injection 3-4 weeks earlier, were subjected to optogenetic stimulation essentially as described above, but an additional burr hole was made over the left hemisphere, coordinates were AP-3.2, ML±2.6 from bregma, and a dual-electrode optrode was used (see figure 5A). The height of the electrode and optrode was matched by adjusting the mounting pipettes (cut-off 10 µL Eppendorf tip) that housed the electrodes/optrodes. The electrodes were spaced 5.2-5.4 mm apart. The optrode assembly was initially lowered to DV-1.0, let to rest for 5 minutes while breathing stabilized and then lowered in 100 µm increments, with test pulses given as above. Nearer to the target site of approximately DV-2.1, targeting the DG and CA3 of the intermediate hippocampus, increments were reduced to 50 µm. When high amplitude population spikes with test pulses were seen ipsilateral to stimulation, optogenetic stimulation trains were given, as described above.

When the animals displayed ADs containing high frequency bursts for five consecutive stimulations, animals were IP injected with 0.2 mg/mL Clozapine-N-Oxide (CNO) (Tocris) in 1% DMSO in saline, at 10 µL/g bodyweight, or vehicle only (DREADD and control, respectively, CNO dose was 2mg/kg). If mice had not reached the desired ADs with high-frequency bursts within 35 stimulations, the trial was aborted without injection, and the animal was excluded from analysis. After injection, an additional 15 blue light train stimulations were given with the same 5-minute interval, covering 75 minutes.

Histology & Microscopy

Slices from *in vitro* experiments were subcut to 50 µm horizontal sections on a sliding microtome (ThermoScientific). Brains from *in vivo* experiments were cut in 30 µm coronal (paper I) or horizontal sections (paper II & III).

All sections, except those immediately stained, were transferred to anti-freeze solution for storage.

Sections were rinsed three times in potassium-PBS (KPBS) followed by KPBS with 0.25% Triton X-100 (T-KPBS) and then blocked in 5% donkey or goat serum in T-KPBS, as appropriate. Primary antibodies were incubated overnight at room temperature; after rinsing, secondaries were incubated for 1.5-2h each (see table 2). Secondary antibodies (all from Jackson Immunoresearch) were generally applied 1:400, except for Fos staining where 1:300 & 1:200 was used.

Table 2. Antibodies used

To visualize	Primary	Dilution	Manufacturer	Secondary	Secondary (additional)
Biocytin	-	-	-	Cy3-conjugated Streptavidin	-
YFP (NpHR)	Rabbit anti-GFP	1:10 000	Ab290, Abcam	FITC-conjugated goat anti-rabbit	-
Mossy fibers	Rabbit anti-ZnT3	1:500	197003, Synaptic Systems	Cy3-conjugated goat anti-rabbit	-
Fos	Goat anti-cFos polyclonal	1:500	Sc-52-g, Santa Cruz	Biotin-SP-conjugated donkey-anti-goat	Cy5-conjugated Streptavidin

Expression of fluorophore-carrying fusion proteins *in vivo*, i.e. NpHR3.0-YFP (paper I), ChR2-YFP (paper II & III) & Gi-DREADD-mCherry, could be readily appreciated by native fluorescence, unaided by antibody staining. Sections were rinsed in KPBS and mounted on coated glass slides, dried, rinsed in distilled water and coverslipped with 2.5% PVA-DABCO (Polyvinylalcohol with 1,4-Diazabicyclo[2.2.2]octane) mounting medium containing 1:1000 Hoechst-33258 or 33342 solution for nuclear counterstain.

Slides were examined in a BX61 microscope with a DP72 CCD camera (Olympus) using suitable filters for Hoechst/DAPI, YFP/GFP/Cy2, mCherry/Cy3 and Cy5. Images were captured using the CellSens Dimension software (Olympus). For papers II & III, images were taken at dorsal, intermediate and ventral levels, with separate channels as 8-bit monochrome in vsi format (see also Data analysis). Images were stitched in the Fiji distribution of ImageJ2.

Data analysis

Recording analysis

In vitro data (paper I) was analyzed in FitMaster (HEKA) and Igor Pro 6 (Wavemetrics). For evaluation of light-induced inhibition of epileptiform activity, frequency of bursts in field recordings was analyzed in 30 s bins. Additionally, burst durations were analysed in Minianalysis (Synaptosoft) by semi-automatic detection with settings suited for each recording, and visually validated. Chemically invoked bursts from *in vivo* LFP recordings were automatically marked with the cyclic measurements detection tool in LabChart. Bursts were visually validated, and artefacts were discarded. Burst data (including duration) was extracted using the scope function, however only number of bursts was used for analysis. The amount of bursts was counted in the light-on window, as well as corresponding pre- and post periods of equivalent length. Comparing to control group, burst data was normalized to the mean burst count of the pre-light period in the same animal. Period lenghts were normalized to 40 s in the control group. All recording traces are visualized using Igor Pro 6.

For paper II & III, three aspects of AD characteristics were measured: duration, height and coastline (also known as line-length). Coastline (CL) is a measure of the amount of activity in the signal, how ”far” it travels over a given time. CL was calculated in two slightly different ways; in paper II, the hypotenuse was used while in paper III only the absolute voltage difference was counted. This does not have any effect on the outcome of measurements (relative to each paper), but the calculation is simplified in the 2nd case. The integral was taken over the first 5 seconds after each optogenetic stimulation train (i.e. the first 5 seconds of ADs) and baseline subtracted. Prior to calculation, the data channel had been band-pass filtered at 0.5-250 Hz to reduce the effect of high frequency noise. Additionally, interictal coastline was calculated in paper III, by taking the CL of 60-120 s in between stimulations. Duration was calculated as the time from the final light pulse to the last burst exceeding 5-30 times baseline CL RMS, the level was adjusted to avoid false positives. Amplitude was measured on high-pass filtered (0.5 Hz) data to remove very slow waves, by calculating the maximum minus minimum of the first 5 seconds after light cessation.

Population spikes inside the stimulation trains were counted in paper II, by measuring the slope (derivative), and setting a detection threshold in the cyclic measurements tool, 5-7 times RMS of derivative. Post-light spikes were defined as occurring from 3 ms after the light pulse until the end of the synaptic response, spikes detected outside the synaptic response were defined as spontaneous spikes.

In paper III, in ADs containing high frequency bursts, the frequency spectra of 1-4 bursts within such ADs were analysed extracting the maximum power frequency in the 8-90 Hz band. Data had first been suitably decimated and then Fast Fourier Transformed with a 256 sample Hann window to achieve 1 second bins with 1 Hz resolution.

Image analysis

To quantify Fos expression in paper II, ROIs delineating the DGC layer were created by setting a threshold on granule cell nuclei. The ROI were then applied to the Fos Cy5 staining, counting the mean pixel intensities. ROI for background was selected manually from the DG molecular layer, which has very uniform Fos background staining. The DGC layer Fos intensities were then normalized to the mean background of all slices. Animals were then grouped based on whether they displayed bilateral, unilateral (i.e. ipsilateral to stimulation) or no Fos reactivity.

Statistics

Several statistical methods were used depending on the scientific question, grouping and appearance of the data set. Analysis and graphing was done in Prism (GraphPad) with an alpha level of 0.05. All data is presented as mean ± SD, or median with 95% CI, in the included papers. Due to variability between animals, data was normalized to pre-intervention levels (e.g. light application or drug injection) in a few cases. In paper two, within subject modeling was used such that timeseries for AD metrics were transformed to slopes (rate of change) by linear regession, before comparing groups.

For data with normal distribution and two compared groups, either paired or two-sample Student's t-test was used. For more than two groups, repeated measures or regular one-way ANOVA was used. Post-hoc tests for multiple

comparisons were selected as appropriate: Tukey's for comparing all pairs, Bonferroni's or Sidak's for specific pairs, or Dunnett's when comparing to a pre-selected control group. When data was not normal, or could not be assumed to be normal, non-parametric equivalents to the above ANOVAs were used: Friedman or Kruskal-Wallis test, respectively, with Dunn's post-hoc test. Cumulative data was compared by Kolmogorov-Smirnov test, with an alpha level of 0.01. All tests are two-tailed.

Results

Attenuating chemically induced bursting by NpHR-activated hyperpolarization of neurons

Inhibition of neuronal populations by optogenetics raises the possibility of effectively curtailing seizure development. In paper I, we wanted to explore this in a scenario where the native inhibition exerted by interneurons was compromised, and hypersynchronized epileptiform activity was driven by the excitatory system, predominantly by activation of CA3 neurons.

NpHR expression by AAV vector injection

In paper I, inhibition of hippocampal neurons *in vitro* and *in vivo* by amber light activation of NpHR was examined. An enhanced version of NpHR (3.0) was used which is better transported to the cell membrane and has enhanced photo-current activity (Gradinaru et al., 2010). As we wanted to explore inhibition in a context where GABA activity was compromised (see below), the synapsin promoter was chosen so that NpHR was expressed in both excitatory and inhibitory neurons, minimizing GABA release from internerons when activated by light. The expression was strong in the ventral hippocampus, particularly DG and CA3. The intrinsic properties of CA3 neurons expressing NpHR were not altered, as shown by *in vitro* whole-cell patch clamping (e.g. resting membrane potential and input resistance was similar), and activation of NpHR hyperpolarized the neurons. *In vivo*, it was shown that activation of NpHR inhibited spontaneous multi-unit activity. Additionally, onset of NpHR activity can be seen as a deflection in the LFP.

Optogenetic inhibition greatly reduces epileptiform bursting *in vitro*

Epileptiform bursting was induced by $GABA_A$ receptor blockade with PTX, which was potentiated by zero-Mg2+ environment and 4-AP, producing stable bursting around 0.4 Hz. When amber light was applied to slices from naïve animals (i.e. expressing NpHR, but otherwise normal) for 30 or 60 s at a time, the mean number of bursts was greatly reduced, up to 85%. In some slices, bursting was suppressed completely. On light cessation, burst frequencies rose temporarily. To examine whether burst attenuation could be achieved in animals with hyperexcitable CA3 neurons, a group of KA-treated NpHR-expressing animals was used. Having been subjected to such a SE model, these animals display mossy fiber sprouting (confirmed by ZnT3 staining) and will eventually develop chronic epilepsy. Similar to naïve animals, hypersynchronized bursting was greatly reduced (up to 87%) in the KA animals. Additionally, burst durations (in slices where bursts were not completely suppressed) were reduced by 20-30% during light for both groups. However, the duration of the first burst post-light was usually increased.

Optogenetic inhibition reduces epileptiform bursting *in vivo*

In the anaesthetized mice, we induced epileptiform activity by microinjection of the $GABA_A$ receptor antagonist Bicuculline methiodide, as PTX is not as effectively used *in vivo* (Veliskova et al., 1990). Injecting into the CA3 & DG, bursts were stable but not as frequent as in the *in vitro* preparations, therefore slightly longer light periods (40 s) were used. The mean number of bursts was reduced during light exposure, however not as markedly as seen *in vitro*, with a measured reduction of about 20%, compared both to pre- and post periods of NpHR animals (non-normalized data), and to the light-on period of control animals (normalized). Burst durations were not affected. Similarly to *in vitro* data, there was an increased chance of bursts appearing immediately post-light. The relatively low efficacy of burst attenuation, compared to the dramatic effect including complete blockade of hypersynchronized bursts seen *in vitro*, could conceivably be due to diffusion of BM throughout the ventral hippocampus and adjacent cortex, as was indicated by the biocytin present in the BM solution. This could create a much larger population of neurons participating in the epileptiform bursting

than what could conceivably be inhibited by the limited area of light illumination.

Progressive hippocampal afterdischarge development by activation of ChR2

Progressive seizure development has traditionally been induced by electrical stimulation in kindling models, and has contributed to our understanding of epileptogenesis. With optogenetics, more specific and naturalistic ways to stimulate the hippocampal networks towards a seizure generating state could be attained, providing additional insight. We explored this concept in papers II & III.

Kindling-like progressive afterdischarges can be induced in transgenic ChR2 mice

We attempted to induce ADs in anaesthetized transgenic Thy1-ChR2 mice (paper II), mainly expressing ChR2 in excitatory neurons throughout the brain, by activating neurons in the ventral hippocampus. This was achieved by repetitive optogenetic stimulation, with 40 trains of 10 Hz 5ms blue light pulses and 5-minute inter-train interval, targeting predominantly the CA3 region of the ventral hippocampus. Despite the fact that the theoretically activated volume of hippocampal neurons is small (less than 0.5 mm^3) at the level of light power used, with local field potential recording in the area of direct optogenetic activation we could see that train-induced ADs of progressively increasing magnitude were generated, similar to what is known from rapid kindling models. In a subset of mice, strong ADs with high-frequency bursts of high amplitude (~10 mV) population spikes and durations exceeding 10 seconds were generated within about 15-25 stimulations. The remaining mice displayed some ADs, but not of the same amplitude or duration (usually not exceeding 5 s) and appearing in a delayed manner, comparatively. The mice with the strongest ADs also had more dynamic intra-train population spike appearance, with spontaneous population spikes being generated between the light induced spikes.

Bilateral activation of DG granule cells increases AD progression

To evaluate the extent of neuronal activation in the hippocampus, we stained sections for the immediate early gene Fos, known to be expressed after seizure activity (Dragunow and Robertson, 1987; Peng and Houser, 2005). Measuring the Fos antibody reactivity we could deduce that the DG granule cells had been differentially activated between animals. Some animals had intense Fos reactivity in the granule cell layer, including in the DG contralateral to stimulation meaning that the AD activity must have crossed from the initiating side to the other. Other animals had Fos activation only ipsilateral to stimulation, and there were animals that displayed barely any Fos activation. When grouping the animals according to Fos activity levels (Bilateral, Ipsilateral and Non-Activated), and then comparing the metrics of AD for those groups, it was clear that the Bilaterally activated group (BiL) had an AD progression of a magnitude far above the other two. There were some indications that ADs were slightly more progressive for the Ipsilaterally activated group (IpL) compared to the Non-Activated (NA) (especially for AD duration) but these differences did not reach statistical significance.

Looking at the activity within stimulation trains, i.e. post-light spikes and spontaneous spikes, which can be seen as an indication of increased excitability in the local network (in the area of recording), the BiL animals were once again clearly more activated than the other two groups. There was also an increased amount of post-light spikes in the IpL group compared to NA, and when looking at the cumulative distribution of spontaneous spikes, one could see that the spontaneous spikes appeared much later in the NA group, indicating some difference in excitability between those animals.

Confirming the presence of contralateral ADs

In paper II, we had only recorded activity ipsilateral to optogenetic stimulation, and thus had no direct evidence of the ADs presumed to appear in contralateral hippocampus. In paper III, we therefore added an electrode recording from approximately the same depth on the contralateral side. We switched to the transgenic CaMKIIa-ChR2 animals, to increase fidelity of ChR2 expression in excitatory neurons compared to the Thy1-ChR2 stain (see discussion). The CamKIIa-ChR2 strain expresses ChR2 only in neurons where CaMKIIa is active, i.e. excitatory neurons. Looking at expression in horizontal slices, there is clearly stronger expression in mossy fibers of the

DGCs, compared to the Thy1 strain, and generally more uniform expression in CA3 & CA1. Additionally, we shifted the target site for light stimulation slightly. Considering the importance of DG activation to optogenetic AD generation found in paper II, we shifted the target site more towards DG (although activation of parts of CA3 likely also occurred), and to improve targeting accuracy, we shifted it slightly dorsal to intermediate hippocampus.

The same optogenetic stimulation protocol as in paper II was applied to the CaMKIIa-ChR2 mice. They too could respond variably, with some reaching the high frequency, high amplitude (~10 mV), long duration (>10 s) ADs within 15-25 stimulations, similar to the BiL group in paper II, while others had very limited development of ADs. However, the proportion of animals reaching high magnitude ADs was much greater for CaMKII-ChR2: 11/19 compared to 4/21. Importantly, we documented that ADs do indeed occur contralaterally, as the activity spreads from the ipsilateral side. The transition to contralateral hippocampus seems to coincide with the time when AD amplitudes and frequency increases ipsilaterally, i.e. the activity is generated by both hemispheres more or less in synchrony. However, the recorded amplitude of bursts was generally lower on the contralateral side.

Inhibiting the contralateral hippocampus by Gi-DREADD

Given the results in paper II, we had hypothesized that bilateral activation was essential to rapid AD progression of increasing severity. An appealing way to test this would be to selectively inhibit the hippocampus (and particularly DG) contralateral to optogenetic stimulation by using Gi-DREADD. Thus, one group of CaMKII-ChR2 mice were injected with AAV vector carrying $hM4D_{(Gi)}$ under the synapsin promoter, directed to the CA3 & DG of the left hemisphere, contralateral to subsequent optogenetic stimulation. Expression, visualized by mCherry fluorescence, was confirmed to cover DG and CA3 primarily in ventral and intermediate levels along the longitudinal axis. Expression was more variable and generally lower in CA1, as well as in dorsal aspects for all regions. Importantly, DREADD expression did not spread to the side of stimulation. Now, with IP injection of CNO, only the hippocampus contralateral to stimulation would be silenced.

DREADD-injected animals and naïve controls were stimulated until high-frequency bursts were reliably generated over five consecutive optogenetic trains, with ADs exceeding 5 seconds. Then, either 2mg/kg CNO or vehicle

(1% DMSO in saline) was injected in DREADD and controls, respectively. After injection, the progression of ADs was recorded over an additional 15 stimulations. Animals that failed to reach reliable AD induction were not injected, either with CNO or vehicle, and excluded from further analysis. In control animals, the AD progression followed the pattern seen in paper II, and compared to pre injection, AD coastline quadrupled shortly after vehicle injection, while durations were effectively doubled by the end of the experiment. This increase in duration could also be statistically verified with non-normalized data. In contrast, CNO injected Gi-DREADD expressing animals displayed an attenuated progression of ADs, compared to controls. AD durations were reduced both ipsi- and contralaterally, while amplitude and CL was reduced ipsilaterally. This relative lack of efficacy for contralateral reduction of amplitude and CL is, most likely, due in large part to the lower amplitudes measured on the contralateral side. Additionally, interictal CL activity was reduced bilaterally after CNO injection, supporting that the network was inhibited after activating the Gi-DREADD.

It should be noted that the pre-injection level of AD activity in controls and DREADD animals were comparable in at least two ways. First, the average AD duration of the last five trains was not different (around 8 seconds for both groups). Secondly, examining the burst frequencies by fast Fourier transformation, pre-injection AD burst frequencies was not different between groups (around 25 Hz in both). The frequency analysis also revealed that the post-injection contralateral inhibition by CNO did not affect the burst frequency in the DREADD group, indicating that the main effect of contralateral inhibition lies in the duration of afterdischarges, and not their frequency content.

Discussion

Optogenetic silencing in seizure & epilepsy research

The opportunities in epilepsy research for neuronal population specific silencing using optogenetics, by hyperpolarization through NpHR activation, was realised early on (Zhang et al., 2007a). The first paper showing proof-of-concept for attenuation of electrically induced epileptiform bursts by NpHR activation *in vitro* came from the very group I have pursued my PhD thesis work in (Tonnesen et al., 2009). In that paper, electrical stimulation was applied to slices with intact inhibitory drive from interneurons. However, it could be posited that this native inhibition was contributing to the successful NpHR-mediated reduction of epileptiform bursting. Additionally, the first published paper on optogenetic silencing for chronic seizure treatment *in vivo* also used a model where GABA release is only transiently affected (Wykes et al., 2012). Part of the impetus for paper I was therefore to investigate whether hyperpolarization by NpHR activation could also be used to inhibit epileptiform activity when such activity was driven by excitatory network connections of pyramidal neurons released from the native inhibition by application of $GABA_A$ receptor antagonists (Straub et al., 1990; de Curtis and Avanzini, 2001). Indeed, our results indicate that a high level of suppression, and even complete blockade, of hypersynchronized bursting activity can be achieved *in vitro* in such a scenario, and this was supported by the *in vivo* data, although the level of suppression was more modest in the latter experiment.

In parallel with the preparation of our manuscript for paper I, several other *in vivo* studies were published showing efficacy of optogenetic intervention in seizure and epilepsy models, supporting the concept of optogenetics-based approaches in this field. Onset of acute SE was delayed in the pilocarpine model by inhibition of hippocampal pyramidal neurons (Sukhotinsky et al., 2013) and inhibition of thalamocortical neurons could interrupt seizures in a stroke model of chronic cortical absence-like seizures (Paz et al., 2013).

Importantly, direct evidence of treatment efficacy in one of the most relevant models of TLE affecting the hippocampus, chronic epilepsy developed after KA injection, was shown using a closed-loop system in mice (Krook-Magnuson et al., 2013).

One issue with hyperpolarization by NpHR is the rebound activity seen at light cessation both *in vivo* and *in vitro* in our study, and which was also reported previously (Tonnesen et al., 2009). This can be caused by reversed chloride potentials causing $GABA_A$ receptors to operate depolarizing rather than hyperpolarizing (Raimondo et al., 2012). However, as $GABA_A$ receptors were blocked in our study, NpHR-activity induced post-light depolarizations can evidently also be triggered by some additional mechanism, e.g. hyperpolarization-activated membrane channels (Siwek et al., 2012). It could be that simply attenuating the light power rather than using abrupt on-off switching can alleviate this problem, alternatively using shorter pulses to avoid accumulation of chloride intracellularly. Using NpHR expression in only excitatory neurons together with pulsed light was not reported to cause problems in the Krook-Magnusson paper cited above. In any case this issue must be thoroughly investigated and resolved before seriously committing to optogenetic measures (at least if using NpHR) for clinical trials.

The optogenetic tools are continuously being developed, and additions such as far-red activated NpHR and inhibitory ChR2 could all find potential use in future studies on seizure control. Additionally, it should be mentioned that inhibition of principal neuronal populations is not the only way to supress seizures. Taking the opposite approach, activation of inhibitory neuronal populations by optogenetic excitation with ChR2, has also shown promise both *in vivo* and *in vitro* (Krook-Magnuson et al., 2013; Krook-Magnuson et al., 2014; Ledri et al., 2014).

Investigating hippocampal circuitry involved in seizure generation by optogenetic stimulation

The processes and minimal neuronal circuits involved in focal epileptogenesis are still not completely understood, which is a limiting factor in devising novel treatment strategies. The epileptogenic process of plastic changes increasing excitability in a network have traditionally been studied using electrical stimulation models such as kindling and rapid kindling

(Goddard, 1967; Lothman et al., 1985; Pitkänen et al., 2006), but electrical stimulation inherently activates all neurons and supporting cells in the area. With optogenetic stimulation, one can envision much more specific and controlled types of stimulation. Additionally, with optogenetics it is possible to study what is happening in the area directly subjected to stimulation by light, without electrical artefacts. Thus, we posited that optogenetic stimulation could achieve progressive afterdischarges, similar to the kindling phenomenon, while allowing for delineation of circuitry needed for epileptogenic plasticity, and in paper II & III we pursued this using transgenic mice expressing ChR2, targeting the hippocampus.

In paper II the main finding was that the appearance of post-light and spontaneous spikes within the optogenetic trains, indicating an increased local recurrent excitability, seemed to be required for effective recruitment of the contralateral hippocampus, specifically the DGC layer, and only in animals with this bilateral activity did the progression of ADs become potentiated towards a massive state of strong hypersynchronized activity (figure 6A). There has been a study published on optogenetically induced afterdischarges in the rat, but there they did not report this type of progressive phenomenology (Osawa et al., 2013). We propose that this is due to the much higher light power levels used in that study, stimulating a substantially larger portion of the hippocampus and thus effectively bypassing the potentiating step of local recurrent activity seen in our study. The mechanism of AD activation in our study is also in agreement with findings from electrical stimulation models *in vivo*, determining that maximal dentate activation was a prerequisite for kindling, and the hypothesis of dentate gating as a control point for seizure generation (as discussed in the introduction). This was also recently corroborated using optogenetic stimulation in freely moving mice, receiving stimulation only to DG neurons (Krook-Magnuson et al., 2015).

We can not conclusively say what caused the large variations in response between animals, although targeting of particular cell layers is one likely reason. Additionally, while working with the Thy1-ChR2 mice, it became clear that expression was not totally stable over successive breeding generations, and expression in interneurons could also be an issue. In fact, a study utilizing Thy1-ChR2 mice to investigate the mechanisms of deep brain stimulation proposed that the inhibitory action they could see was due to optogenetically induced release of GABA (Chiang et al., 2014). It can be mentioned that we have unpublished data indicating that PV interneurons in Thy1-ChR2 mice can both express ChR2, and be activated by optogenetic

stimulation, as indicated by Fos expression, which reflects results from the initial characterization of another Thy1-ChR2 substrain (Wang et al., 2007). It is not beyond reason that, with the small activation volume achieved in our stimulation protocol, local variations in interneuron to principal cell ratios could cause large variation in excitable response.

To substantiate our findings from paper II, and avoid possible confounders seen with Thy1-ChR2, we switched over to transgenic mice exclusively expressing ChR2 in excitatory neurons (expressing CaMKIIa) by control of Cre-lox. Additionally, we made direct recordings of LFP activity in the hippocampus contralateral to stimulation. Finally, to test if bilateral dentate activation was crucial to AD development as proposed, we injected Gi-DREADD to the contralateral hippocampus (figure 6B). The recordings from contralateral hippocampus confirmed that ADs were indeed occurring there, and that the spread of activity coincided with the increased amplitude and higher frequency bursts type of ADs ipsilaterally. The generation of these intense ADs was also much more reliable in CaMKII-ChR2 mice than what we had seen in Thy1-ChR2 mice. In line with our hypothesis, when inhibiting the contralateral DG and CA3 by activation of Gi-DREADD with CNO, the progressive AD intensification was halted (figure 6C).

Our data adds to the recent evidence of the chemogenetic approach as a viable treatment strategy for cortical seizures (Katzel et al., 2014), but in addition shows that DREADD inhibition can be effective also when applied to distant network components, such as the hippocampus contralateral to the seizure focus. However, in the optogenetic dentate gate study mentioned above, they did not find that contralateral DG inhibition had any effect on spontaneous seizures in chronically epileptic KA mice (Krook-Magnuson et al., 2015), and perhaps this type of intervention is not applicable to advanced stages of chronic epilepsy where the network is wholly transformed, including massive neuronal losses such as seen in hippocampal sclerosis. Our results still show an intriguing possibility of preventing chronic TLE from developing, when applying the inhibitory intervention at an early enough stage in the epileptogenic process. For this to be feasible clinically however, prediction of epileptic outcomes after precipitating insults such as febrile seizures would have to be dramatically improved such that those with very high risk of developing epilepsy can be approached for preventive measures.

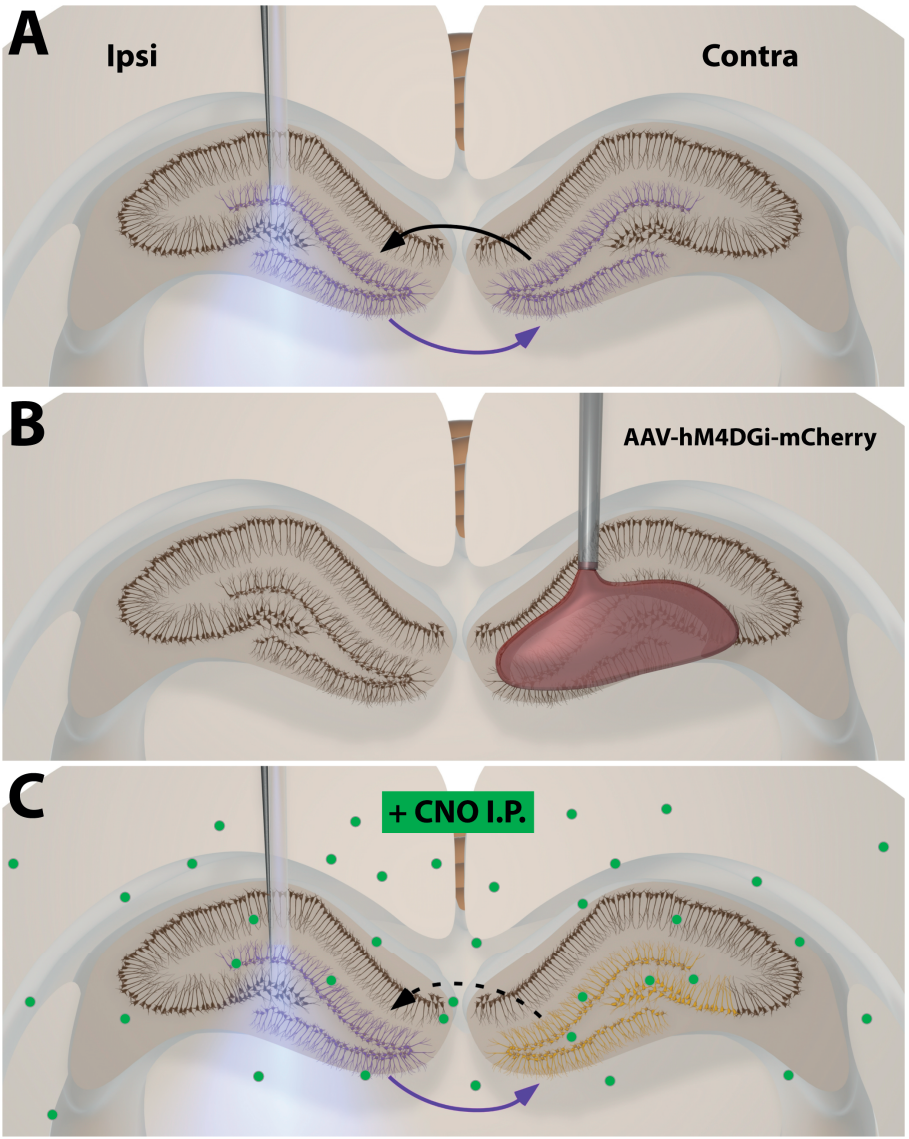

Figure 6. Mechanism of bilateral dentate activation in optogenetic AD generation
Note that the actual stimulations/injections are done in intermediate/ventral hippocampus. Dorsal hippocampus is shown for schematic ease. **A** When stimulating DG & CA3 neurons in the hippocampus unilaterally, at some point the activity transfers to the contralateral hemisphere, activating DGCs (purple), and this activation greatly increases the AD magnitude. **B** In paper III, the contralateral hippocampus was first injected with AAV vector to express Gi-DREADD. **C** Upon subsequent stimulation, CNO (green) is injected after the first ADs have been generated. Only the contralateral hippocampal areas expressing Gi-Dreadd are inhibited (yellow neurons), limiting the inter-hippocampal excitability. In this case, AD progression is halted.

Going forward, the optogenetic stimulation models can surely only be improved. Selective expression of ChR2 to various subregions of hippocampus could reveal additional mechanisms and the relative importance of the different network components to the total excitability required for epileptogenesis. Combining this with selective inhibition or activation of subsets of interneurons would also be of very high interest, and has already proven useful to elucidate interneuron to pyramidal neuron interactions in vitro (Ledri et al., 2014). We ourselves did not yet move on to awake freely moving animals, which would also be a crucial step to start looking at long term effects of optogenetic stimulations and whether chronic epileptic states can be reached by this method.

Conclusions and future perspectives

Devising effective novel epilepsy treatments is a considerable challenge, not the least because of the complex etiologies and intricate mechanisms leading to the epileptogenic state. In addition, knowledge on the function of the brain and the hippocampus is obviously far from complete. Development of optogenetic tools and their applications continues to be a highly dynamic and exciting research area. The question is, are optogenetic approaches feasible as future treatment options for epilepsy? How soon can optogenetics be applied in clinical trials? Certainly, there are technical issues concerning implantation of optic fibers or light sources into the brain, as well as using them in conjunction with seizure detection systems (Chow and Boyden, 2013; Ramgopal et al., 2014). Also the safety and efficacy of transgene delivery by viral vectors needs to be further assessed, although data from clinical trials are now starting to accumulate (Simonato et al., 2013). An upside in the case of epilepsy research is actually that there is a patient population where optogenetic trials could be attempted without too great a risk: TLE patients selected for surgery. If the treatment is beneficial, patients avoid having to have a part of their brain removed, while if there is an averse reaction, or no treatment effect, the surgery can go ahead as planned (Kullmann et al., 2014).

Even if optogenetic methods eventually might fail to deliver on the promise of future therapeutic use, undoubtedly the optogenetic methodologies will continue to help uncover the underlying mechanisms of seizure generation and the epilepsies.

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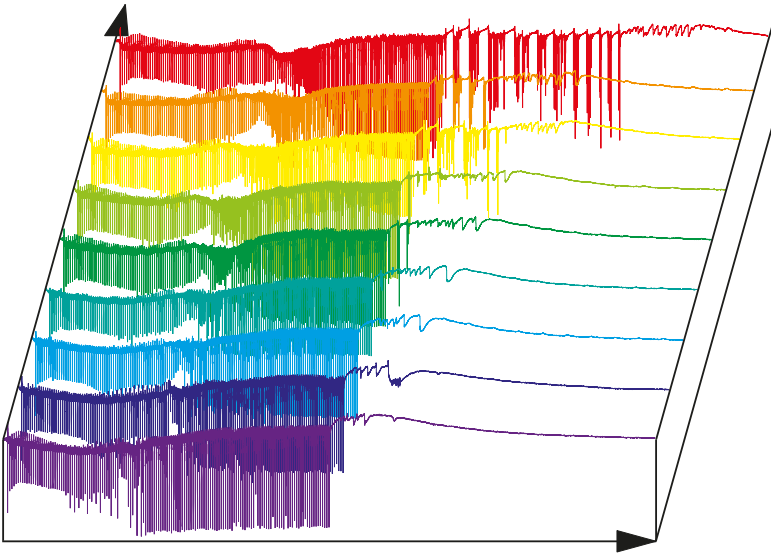

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